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Fluorescent probes for monitoring virus fusion kinetics: comparative evaluation of reliability

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Abstract

Fluorescence assays for viral membrane fusion employ lipidic probes whose kinetics of fluorescence dequenching should mimic the actual kinetics of membrane merging. We examined the fusion of influenza virus with CEM cells, erythrocyte ghosts or liposomes by monitoring the fluorescence dequenching of each one of the three probes, octadecylrhodamine B chloride (R18), *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE), or rac-2,3-dioleoylglycerol ester of rhodamine B (DORh-B), inserted into the virus membrane. Experimental conditions were designed to allow a clear distinction between membrane mixing and non-specific probe transfer. Fluorescence dequenching observed with Rh-PE was much slower than with R18, unless a particular experimental procedure was used. Using liposomes as a target membrane, the kinetics and extent of the decrease in resonance energy transfer between *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and Rh-PE, initially embedded in the liposome membrane, were matched by that of the dequenching of viral R18, but not of viral Rh-PE. DORh-B was found not to be appropriate to follow membrane merging. Our results indicate that on a time scale of several minutes R18 more accurately reflects the kinetics of membrane fusion. Nevertheless, control experiments should be performed to evaluate non-specific probe transfer of R18 molecules, whose contribution to fluorescence dequenching can become significant after long incubation times.

Keywords: Influenza virus; CEM cell; Erythrocyte ghost; Liposome; Membrane fusion; Fluorescent probe; Nonspecific probe transfer

Abbreviations: $C_{12}E_8$, octaethylene glycol dodecyl ether; LUVs, large unilamellar vesicles; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; R18, octadecylrhodamine B chloride; RET, resonance energy transfer; DORh-B, rac-2,3-dioleoylglycerol ester of rhodamine B; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine

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1. Introduction

The development of a number of assays to measure the merging of lipid bilayers has greatly facilitated research on the molecular mechanisms of membrane fusion. Lipid-conjugated rhodamines have been widely used in lipid mixing assays that are based either on resonance energy transfer (RET) between *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) [1], or on the self-quenching properties of rhodamine [2]. The first of these methods is known to monitor accurately membrane fusion, but Rh-PE and NBD-PE cannot be introduced into pre-existing membranes at the appropriate concentrations. Therefore, they are typically used to label artificial membranes (such as liposomes), upon their addition to the purified lipid mixture prior to vesicle preparation. Fusion between these labeled liposomes and another type of (unlabeled) membrane can thus be followed. To label pre-existing membranes it is much simpler to use only one kind of fluorescent probe, most commonly octadecylrhodamine B chloride (R18), introduced into the bilayer at self-quenching concentrations. Upon fusion with unlabeled target membranes, dilution of the probe takes place, with a concomitant increase in fluorescence that allows for the measurement of lipid mixing. This is the most widely used method to monitor membrane fusion, especially in virus–cell fusion studies, due to the aforementioned advantage of allowing intact biological membranes to be easily labeled by the exogenous addition of the probe [2–5].

Nevertheless, significant non-specific probe transfer from labeled membranes to unlabeled target membranes upon prolonged incubation [6–9], gradual inactivation of influenza virus with increasing concentrations of R18 [7] and non-homogeneous distribution of R18 molecules in the labeled membranes have been reported [10,11]. Since the R18 assay has found widespread application in the field of membrane fusion, a careful investigation is necessary to avoid erroneous conclusions. Although non-homogeneous probe distribution is not likely to affect the kinetics of membrane fusion, and significant R18-induced viral inactivation only occurs at probe concentrations that are higher than the ones normally used, non-specific R18 transfer remains a major issue. This is especially evident in studies that require long incubation periods, such as the internalization of lipid-enveloped viruses by endocytosis [5], and/or in systems where fusion activity is very low where even minute probe transfer can greatly influence the results.

Since it is possible that R18 transfer results from its sole bilayer-anchoring chain, attempts to overcome this problem have involved the use of fluorescent lipid probes that have two long fatty acid chains. Among these probes is Rh-PE, which has been shown to be a non-exchangeable lipid analogue when incorporated into liposomes or biological membranes [1,12,13]. Another probe that might be useful in these studies is the rac-2,3-dioleoylglycerol ester of rhodamine B (DORh-B). DORh-B has two unsaturated aliphatic chains, each with eighteen carbon atoms, and therefore it is expected to be incorporated into membranes more efficiently than its saturated analogues, and to have a low propensity to be transferred non-specifically [14].

To follow precisely membrane merging it is imperative to use a fluorescent probe whose kinetics of fluorescence dequenching follow the actual kinetics of the membrane fusion processes under investigation. To identify the best probe to monitor membrane fusion of pre-existing bilayers we have compared systematically the behaviour of R18, Rh-PE and DORh-B as probes to monitor membrane fusion, with a special focus on the possible artifacts that may occur after long periods of incubation.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, proteinase K, Sephadex G-25, streptomycin and penicillin were purchased from Sigma Chemical (St. Louis, MO, USA). $C_{12}E_8$ and ganglioside GD1a were from Calbiochem (San Diego, CA, USA). NBD-PE, PC, PE and Rh-PE were obtained from Avanti Polar Lipids (Alabaster, AL, USA). R18 was purchased from Molecular Probes (Eugene, OR, USA), and fetal calf serum from Biochrom (Berlin, Germany). The probe

DORh-B was a gift from Dr. V. Slepushkin (VIRXSYS, Gaithersburg, MD).

2.2. Cells

CEM cells, a human T lymphocytic leukemia cell line, were obtained from the UCSF Cell Culture Facility, San Francisco, USA. Cells were incubated in RPMI 1640 medium containing 25 mM HEPES buffer, supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml of streptomycin and 100 unit/ml of penicillin, in T-75 flasks at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown up to a cell density of 1×10^6–1.5×10^6/ml, and were harvested by centrifugation at $180\times g$ for 8 min at room temperature and washed twice in phenol red-free RPMI 1640 containing 25 mM HEPES buffer (pH 7.4) (Medium A). The cells were resuspended in the latter buffer at a stock density of 10^8/ml and kept on ice in polypropylene centrifuge tubes until use in the same day. Cell viability was determined by Trypan blue exclusion and was routinely above 95%.

2.3. Preparation of erythrocyte ghosts

Erythrocyte ghosts were prepared from freshly drawn human blood by hypotonic lysis of the erythrocytes in 5 mM sodium phosphate, 0.1 mM EGTA, pH 8.0 at 4°C, essentially as described previously [15], and stored at 4°C. The protein concentration of the erythrocyte ghosts was determined by the Sedmak assay [16].

2.4. Liposome preparation

Large unilamellar lipid vesicles (LUVs) composed of PC and PE at a 6:3.5 molar ratio, and containing 5 mol% of the ganglioside GD1a, were prepared by mixing the lipids and the ganglioside and drying them from chloroform solution under vacuum using a rotatory evaporator. The dried lipid film was rehydrated with 145 mM NaCl, 10 mM HEPES, pH 7.4. The resulting liposomes were then extruded five times through two stacked polycarbonate membranes (100 nm pore diameter) and stored at 4°C, under nitrogen, until use. The phospholipid concentration of the liposome preparations was determined by a phosphate assay [17].

LUVs labeling with R18 followed the same procedure described for viral labeling with this probe (see next section). LUVs labeled with Rh-PE were prepared with 5 mol% of this probe incorporated in the lipid membrane. For the RET assay [1,18], LUVs were prepared with 0.6 mol% of both NBD-PE and Rh-PE incorporated in the lipid membrane.

2.5. Virus

Influenza virus, A/PR/8/34 (H1N1) strain was obtained from SPAFAS (Preston, CT, USA). The virus was grown for 48 h at 37°C in the allantoic cavity of 11-day-old specific pathogen-free embryonated eggs, purified by discontinuous sucrose density gradient centrifugation and stored at −70°C in phosphate-buffered saline.

2.5.1. Viral labeling

Influenza virus was labeled with R18 as described previously [2,3,5,19,20]. The labeling with the fluorescent probe DORh-B followed the same procedure. Influenza virus labeling with the fluorescent probe Rh-PE was performed as previously described [21]. The final self-quenching concentration of added probes corresponded to approximately 5 mol% of the total viral lipid, and that of ethanol was less than 1% (v/v). It was assumed that 1 mg of viral protein corresponds to 362 nmol of viral lipid [20]. The mixtures were incubated in the dark for 30–45 min at room temperature. The labeled virus particles were separated from non-inserted fluorophore by passage through a column (Bio-Rad bio-spin) of Sephadex G-25 and collected by centrifugation at $850\times g$ for 4 min at 4°C. The protein concentration of the labeled virus was determined by the Sedmak assay [15].

2.5.2. Enzymatic treatment of the virus

Influenza virus (4 μg of viral protein) was pre-incubated with 5 μg of proteinase K in a final volume of 100 μl (145 mM NaCl, 10 mM HEPES) at pH 5.0 and 37°C for 30 min. Subsequently the pH was raised to 7.4 with Tris buffer and the treatment continued for 15 min. Following this incubation, the virus was added to the fluorometer cuvette containing the target membrane, at 20°C or 37°C. In the fusion experiments, the proteinase K concentration was reduced 20-fold.

2.6. Lipid mixing experiments

2.6.1. Lipid mixing experiments performed with labeled virus

Lipid mixing between influenza virus and CEM cells, erythrocyte ghosts or liposomes was evaluated by monitoring the increase of fluorescence of the probes, R18, Rh-PE or DORh-B, incorporated in the viral membrane at a self-quenching concentration. Excitation was set at 560 nm and emission at 590 nm.

2.6.2. Lipid mixing experiments performed with labeled liposomes

Fusion between LUVs labeled with R18 or Rh-PE and unlabeled influenza virus was monitored by the dequenching of R18 or Rh-PE fluorescence, as in the case of the experiments using labeled virus. With the RET assay, fusion between unlabeled influenza virus and NBD/Rh-PE labeled (0.6 mol% each) lipid vesicles was followed by an increase of NBD fluorescence with the excitation and emission monochromators set to 465 nm and 530 nm, respectively [1,18].

In all experiments the extent of lipid mixing was determined according to the following equation:

$$\%\ \text{lipid mixing} = \frac{F_t - F_0}{F_{\max} - F_0} \times 100 \qquad (1)$$

where F_0 is the value of the initial fluorescence of the virus and target membranes, F_t is the value of fluorescence after t minutes of incubation, and $F_{\max}$ (100% fluorescence) is the value of fluorescence after addition of $C_{12}E_8$ (2 mM) to dissolve the viral (or liposome) membrane and to disperse maximally the probe.

All fluorescence measurements were performed in a Spex Fluorolog 2 fluorometer. The sample chamber was equipped with a magnetic stirrer and the temperature was controlled with a thermostatic circulating water bath.

3. Results

3.1. Fusion of labeled influenza virus with CEM cells

To compare the behavior of the fluorescent probes R18 and Rh-PE we first examined the fusion activity of labeled influenza virus towards a physiologically relevant target membrane, namely CEM cells. This cell line constitutes a target membrane with which influenza virus exhibits high fusion activity [3,20]. Physiologically, the virus binds to a target cell at neutral pH and 37°C, and fusion is only triggered by acidification at a later stage. Therefore, we used a short virus–cell preincubation at pH 7.4, to allow viral binding. Fig. 1 shows that, upon triggering membrane fusion by lowering the medium pH, the dequenching of Rh-PE was very low as compared to that of R18. To exclude the possibility that the fluorescence increase observed at acidic pH may arise from events other than membrane fusion, parallel experiments were carried out with influenza virus that had been inactivated by enzymatic treatment (see Section 2). No significant fluorescence increase was noted upon incubation of the inactivated virus with the cells at pH 5.0, especially for the R18 labeling (Fig. 1), suggesting that non-specific exchange of either probe does not occur under these conditions, when short (5 min) periods of incubation are used to monitor membrane merging. It should be noted that as observed for other cell lines [19,20], enzymatic treatment did not result in any significant reduction in the ability of influenza virus to bind to CEM cells (data not shown). Under our experimental conditions the virus/cell ratio was kept relatively low to enable most of the virions to bind to the target membrane.

In contrast to what happens at acidic pH (data not shown), CEM cells preserve their integrity for long

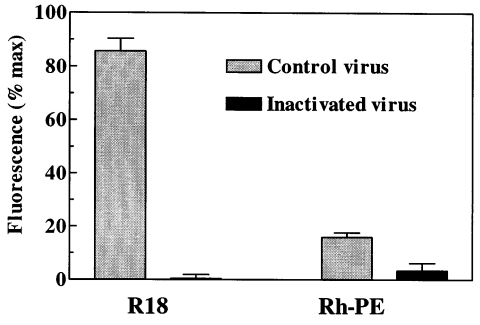

Fig. 1. Lipid mixing between intact or inactivated influenza virus labeled with either R18 or Rh-PE and CEM cells. Four μg of viral protein was added to 4×10^7 CEM cells in 2 ml of Medium A, at 37°C. After 5 min incubation at pH 7.4, the virus–cell suspension was acidified to pH 5 and further incubated for 5 min. Values represent means of at least three independent experiments performed in duplicate ± S.D.

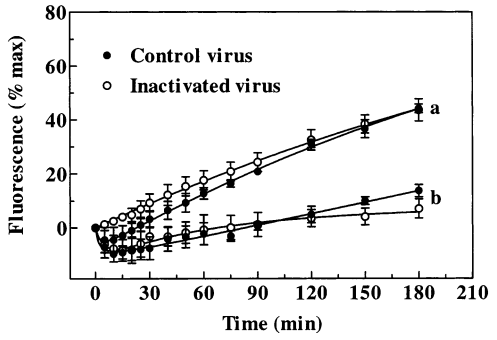

Fig. 2. Lipid mixing kinetics of influenza virus with CEM cells at neutral pH. Control or inactivated influenza virus was labeled with either R18 (a) or Rh-PE (b). Four μg of viral protein was added to 4×10^7 CEM cells in 2 ml of Medium A, at 37°C and pH 7.4. Values represent means of at least three independent experiments performed in duplicate ± S.D.

periods of time at neutral pH. Using the Trypan blue exclusion test we found that after a 90-min incubation in Medium A at 37°C and neutral pH, more than 90% of the cells were still viable, and the number of viable cells is still over 75% after 180 min incubation (data not shown). We therefore investigated the time course of virus–cell lipid mixing at neutral pH and 37°C for 180 min (Fig. 2). Under these conditions a gradual increase in fluorescence was observed with influenza virus labeled with R18, which started after 30 min of virus–cell incubation. For influenza virus labeled with Rh-PE a small increase in fluorescence was observed only after 2 h. Since it has been shown that fusion of influenza virus does not take place at neutral pH [22–24], these results suggest that the increase in fluorescence observed at neutral pH most likely results from molecular exchange of the probes between influenza virus and CEM cells, and not from membrane fusion. In fact, the results obtained from parallel experiments carried out with inactivated influenza virus (Fig. 2) showed that the kinetics of fluorescence increase in this case are very similar to those obtained with non-inactivated influenza virus. Hence, the dequenching observed with the control virus at neutral pH represents molecular exchange of the probes between viral and cellular membranes, and not membrane fusion. Non-specific probe transfer assumes significant values for R18 after a 3 h incubation at pH 7.4, in contrast to what happens with Rh-PE (Fig. 2). However, the ratios between the fluorescence increase in Fig. 2 after 180 min and in Fig. 1 are similar.

3.2. Fusion of labeled influenza virus with erythrocyte ghosts

Our results with CEM cells are in disagreement with those obtained by Arbuzova et al. [21]. These authors reported that influenza virus labeled with R18 or Rh-PE exhibits the same dequenching kinetics, as well as the same final extent of fluorescence dequenching, upon fusion with erythrocyte ghosts. To elucidate the reason for the different results obtained using CEM cells, we also examined the fusion activity of influenza virus with erythrocyte ghosts. For the sake of comparison we used the same methodology as the one employed with CEM cells (Fig. 1), as well as the experimental conditions used by Arbuzova et al. [21]. Following the same experimental procedure used with CEM cells, influenza virus fusion activity was induced by lowering the pH to 5, after virus binding to the erythrocyte ghosts at pH 7.4 and 37°C for 5 min. Following the approach described by Arbuzova et al. [21], the labeled virus was preincubated with the erythrocyte ghosts for 45 min at 0°C and pH 7.4, and the virus–cell suspension was then washed in order to remove unbound virions. Finally, fusion was induced by increasing the temperature to 37°C and lowering the pH of the medium.

Fig. 3 illustrates the extents of lipid mixing after 5 min incubation at pH 5, and clearly shows that R18 and Rh-PE exhibit different behaviors, depending on the experimental procedure used. The extents of fusion with both probes were similar only under conditions where the virus was first prebound to the erythrocyte ghosts in the cold, and unbound virus removed (Fig. 3A). Using our experimental approach, we observed a significant difference in the fusion extents obtained with the two probes after 5 min incubation at acidic pH (Fig. 3B), a result that mirrors that obtained with CEM cells (Fig. 1). It should also be noted that the higher extents of fusion observed when the virus is prebound at 0°C are in agreement with previous results [3]. Similar observations were also described for Sendai virus [4].

3.3. Fluorescence characteristics of the probes R18, Rh-PE and DORh-B when incorporated into the influenza virus membrane

As our results with CEM cells and erythrocyte

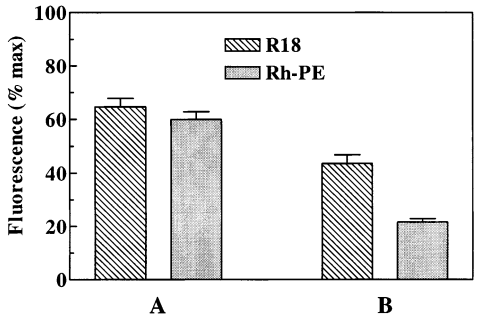

Fig. 3. Low pH-induced lipid mixing between influenza virus and erythrocyte ghosts under different experimental conditions. Influenza virus (2 μg viral protein) was labeled with either R18 or Rh-PE and added to erythrocyte ghosts (26 μg protein) in 2 ml of 10 mM HEPES/145 mM NaCl. (A) Influenza virus was incubated with the erythrocyte ghosts for 45 min at 0°C. Unbound virus was removed by centrifugation in the cold, and the virus–erythrocyte ghost suspension was further incubated for 5 min at 37°C and pH 5. (B) Influenza virus was incubated with erythrocyte ghosts for 5 min at pH 7.4 and 37°C, and the medium was then acidified to pH 5, and the virus–cell suspension further incubated for 5 min. Values represent means of at least five experiments ± S.D.

ghosts showed that R18 and Rh-PE exhibit different kinetics of fluorescence dequenching, we examined the fluorescence characteristics of the two probes. In a further attempt to determine an appropriate probe to follow membrane fusion, we also analyzed the rac-2,3-dioleoylglycerol ester of rhodamine B (DORh-B).

The excitation and the emission spectra of R18, Rh-PE and DORh-B incorporated into the influenza virus envelope at 5 mol% of viral lipid showed a significant overlap (data not shown). These results are in agreement with previous reports [1,2,14] and suggest that resonance energy transfer between DORh-B molecules may occur, and that, as R18 and Rh-PE, this probe may also be useful for monitoring membrane fusion. For a probe to be useful in monitoring membrane fusion it should exhibit a high efficiency of energy transfer, defined by the following equation [25]:

$$E = 1 - F/F_0 \quad (2)$$

where F and F_0 are the values of fluorescence at the wavelength of maximum emission in the absence and presence of detergent, respectively.We compared the efficiency of energy transfer obtained with R18, Rh-PE and DORh-B incorporated into the virus membrane at 5 mol% of total viral lipid. The value of energy transfer obtained with R18 was higher than that obtained with Rh-PE and significantly higher than that observed using DORh-B (Table 1). Since the values of fluorescence intensity obtained in the presence of detergent were similar for the three probes, these results suggest that DORh-B and Rh-PE molecules incorporated in the viral membrane may acquire an orientation that would yield less energy transfer. Alternatively, the relative orientation of the probe molecules after fusion may become more favorable for energy transfer than in the viral membrane (due to some constraints), resulting in reduced quenching. It should be noted that similar values of maximal fluorescence were obtained using either $C_{12}E_8$ (2 mM) or Triton X-100 (0.5% v/v).

3.4. Fusion of labeled influenza virus with unlabeled liposomes

To investigate a possible effect of the target membrane proteins on the differential fluorescence dequenching of R18 and Rh-PE incorporated in the viral membrane observed with CEM cells (Fig. 1) and erythrocyte ghosts (Fig. 3), we examined the fusion activity of influenza virus using a simpler system, namely liposomes composed of PC/PE/Ganglioside GD1a (6:3.5:0.5). Experiments were performed at 20°C and 37°C, since temperature is one of the factors that can affect the behavior of fluorescent probes, and thus influence non-specific probe exchange.

Following influenza virus binding to the liposomes at pH 7.4, the virus fused rapidly upon lowering the pH to 5.0, as revealed by the increase in fluorescence observed for the three probes tested,

Table 1
Efficiency of energy transfer[a]

Probe	E
R18	0.767
Rh-PE	0.521
DORh-B	0.295

[a]The efficiency of energy transfer was determined according to Eq. 2, using the fluorescence values obtained from the emission spectra of the three probes at 590 nm, when incorporated in the viral membrane, in the absence and in the presence of $C_{12}E_8$ (2 mM).

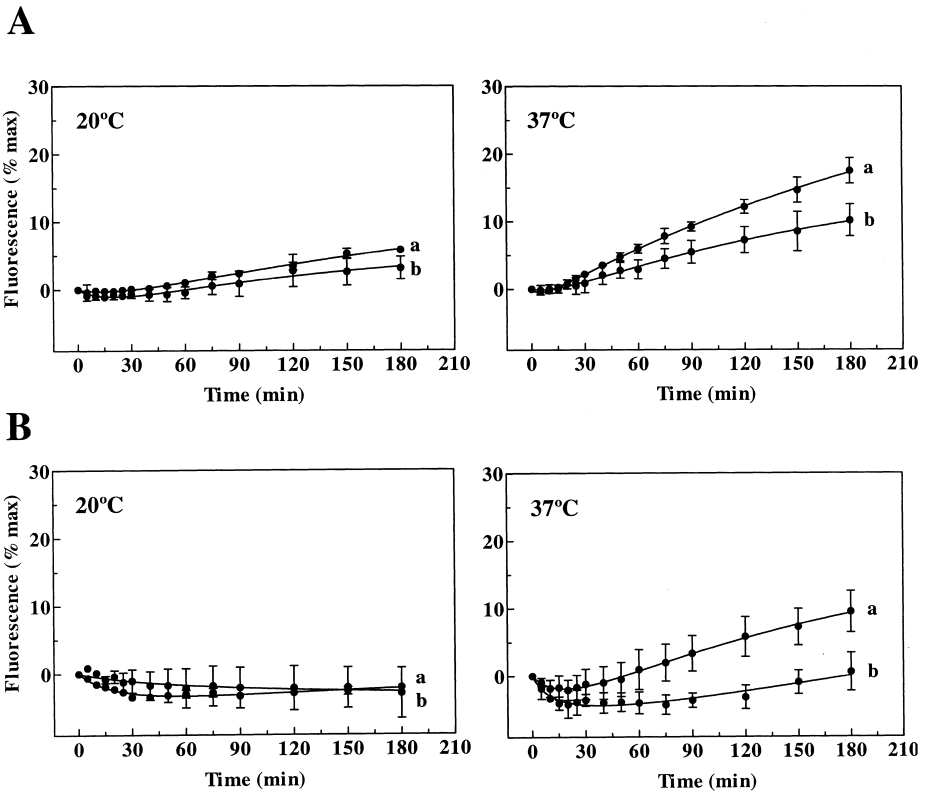

Fig. 4. Lipid mixing kinetics of labeled influenza virus with liposomes at 20°C and 37°C. (A) Interaction of labeled influenza virus with liposomes at neutral pH. (B) Interaction of labeled inactivated influenza virus with liposomes at acidic pH. Influenza virus was labeled either with R18 (a) or Rh-PE (b). Two μg of viral protein was added to 40 nmol of liposomes PC/PE/GD1a (6:3.5:0.5) in 2 ml of 10 mM HEPES/145 mM NaCl. Fluorescence increase was monitored at pH 7.4 (A) and at pH 5.0 (B). Values represent means of at least five experiments ± S.D.

notably at 37°C (data not shown). This increase was much more pronounced for R18 than for Rh-PE or DORh-B, especially at 20°C. The slow dequenching observed with DORh-B also confirmed our previous hypothesis regarding the low efficiency of this probe as a membrane fusion indicator. In these experiments the liposomal lipid was 55-fold in excess of viral lipid. Hence Eq. 1 can be adequate in estimating the percentage of lipid mixing as with cells or with erythrocyte ghosts.

To assay for non-specific probe transfer in this system we again used both virus–liposome incubations at neutral pH, and inactivated virus. At pH 7.4, a slow but gradual increase in the R18 and Rh-PE fluorescence levels was observed, after a 30-min incubation at 37°C. However, at 20°C no significant increase in fluorescence was detected (Fig. 4A). The results obtained from parallel experiments carried out with inactivated influenza virus showed that the kinetics of fluorescence increase were very similar to those obtained with non-inactivated virus (data not shown), which indicates that, at neutral pH and at 37°C, non-specific probe transfer occurs, especially for R18, after a 30-min virus–target incubation. To evaluate whether non-specific probe transfer would also occur at pH 5.0, we performed parallel experiments with inactivated influenza virus. Fig. 4B shows that under these conditions an increase of fluorescence was observed only for R18 at 37°C. However, the putative dilution of the R18 molecules into the target membrane started after 60 min, and was less pronounced than the one obtained at neutral pH (Fig. 4A). The fact that membrane hydrophobicity increases at acidic pH, due to the neutralization of negative charges on viral membrane proteins [26], may help to explain this observation.

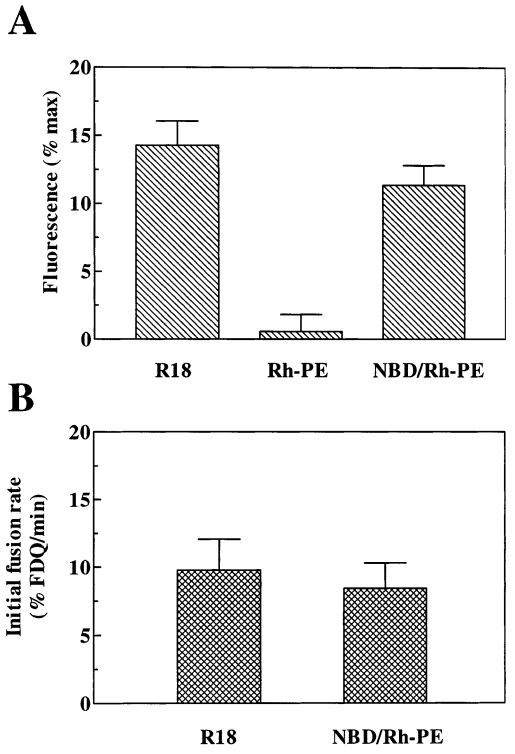

Fig. 5. Lipid mixing between unlabeled influenza virus and labeled liposomes. Liposomes were labeled either with R18, Rh-PE or NBD/Rh-PE. Influenza virus (5 nmol of viral lipid) was added to 5 nmol of labeled liposomes composed of PC/PE/GD1a (6:3.5:0.5) in 2 ml of 10 mM HEPES/145 mM NaCl. Fluorescence increase was monitored for 5 min at pH 5, following virus–liposome preincubation for 15 min at pH 7.4. (A) Extent of fusion after 5 min at pH 5. (B) Initial fusion rate. Values represent means of at least four experiments ± S.D.

3.5. *Fusion of unlabeled influenza virus with labeled liposomes*

The use of liposomes as target membranes for influenza virus allows for the direct comparison of R18 and Rh-PE with the NBD/Rh-PE assay, an assay in which non-specific probe exchange most likely does not occur [18]. To confirm the results obtained with labeled influenza virus and unlabeled liposomes, we used a reverse system, and performed fusion experiments using unlabeled virus and liposomes labeled with R18, Rh-PE or NBD/Rh-PE. In the application of the RET fusion assay [1,18] NBD-PE and Rh-PE are incorporated in the liposome bilayer, and fusion of liposomes with unlabeled virus is monitored by an increase in the fluorescence intensity of NBD, due to its dilution within the fusion products, and to a less efficient quenching of its fluorescence by Rh-PE.

The extents of fluorescence increase for R18 and Rh-PE after a 5-min incubation at pH 5.0 were very different, and almost negligible for Rh-PE (Fig. 5A). However, the extent of membrane fusion and the initial fusion rate obtained with R18 and NBD/Rh-PE were nearly identical (Fig. 5A,B). It is worth noting that the extents of fluorescence increase obtained here were much lower than those obtained with the reverse system (labeled virions fusing with unlabeled liposomes), although the materials were the same. This was expected, since using this experimental system, where the suspension consists of equal numbers of fusion-active virions and liposomes, the extent of fluorescence intensity increase due to the relief of probe quenching is limited to 40%, as shown by Nir et al. [27]. However, if labeled virus interacts with an unlabeled target membrane that is present in large excess, as is the case illustrated in Figs. 1, 3 and 4, and if all virions are capable of fusing, the increase in fluorescence can approach 100% [28,29].

4. Discussion

Experiments performed with influenza virus and CEM cells showed that the dequenching of Rh-PE is one quarter of that observed with R18 (Fig. 1). This difference is not due to non-specific probe transfer of R18, since no fluorescence increase was detected using inactivated virus (Fig. 1). These results seem to indicate that Rh-PE molecules do not distribute homogeneously in the newly formed membrane (virus+cell), or that their diffusion into this membrane is slower. Malinin et al. [30] found that the rate of NBD-PE/Rh-PE dilution is considerably slower than that of acyl chain-tagged probes during poly(ethylene glycol)-mediated fusion of small unilamellar liposomes, and suggested that the bulky headgroups of the former inhibit the movement of the probes through the fusion stalk. One would expect, however, that the headgroup volumes of Rh-PE and R18 would be similar. Nevertheless, Rh-PE possesses two fatty acyl chains, while R18 has only one, resulting in different dynamic molecular shapes. Since the molecular shapes of exogenous agents, including ly-

sophosphatidylcholine and oleic acid, may affect the structure of the fusion intermediate [31], probe molecules could potentially affect the fusion reaction. Assuming that R18 is only in the outer monolayer of the virus, it would be expected to act like lysolipids and inhibit the 'stalk intermediate', while Rh-PE would not affect this structure. Our data indicate, however, that lipid mixing monitored by R18 is more rapid than that by Rh-PE, suggesting that R18 does not inhibit the fusion intermediate. If, however, R18 is translocated into the inner monolayer, it may facilitate the fusion stalk. It is of interest to note that the presence of 2% R18 in erythrocyte membranes fusing with cells expressing lipid-anchored HA enhanced fusion pore formation [32]. The fluorescence of Rh-PE can also be affected by membrane cellular components. In fact, it has been described that cholesterol increases the fluorescence quenching of R18 and Rh-PE [10]. Although this effect cannot explain our results, other cellular components may preferentially affect Rh-PE fluorescence. A decrease in fusion activity of Rh-PE-labeled influenza virus as a consequence of a possible interaction between this probe and viral fusion proteins is not likely, since viral hemolytic activity, which is directly related to fusion activity, is not affected by either R18 or Rh-PE in the range of concentrations used in this study [21].

At neutral pH we observed a continuous increase in R18 fluorescence, which starts earlier, and reaches higher values, when compared to Rh-PE (Fig. 2). This phenomenon is due to non-specific probe transfer between the labeled virus and the plasma membrane of CEM cells, since similar results were obtained when we used inactivated virus (Fig. 2).

The results obtained with CEM cells do not agree with a previous study [21], which reported that the fluorescent probes R18 and Rh-PE exhibited similar kinetics of fluorescence dequenching, when monitoring fusion between labeled influenza virus and erythrocyte ghosts. In an attempt to resolve this issue, we also performed experiments using erythrocyte ghosts as target membranes for influenza virus. These experiments demonstrated that R18 and Rh-PE have different behaviors depending on the experimental procedure used. Both probes exhibit similar extents of fusion when the virus is prebound to the target membrane at 0°C and unbound virions are then removed [21] (Fig. 3A). However, with our experimental approach the probes showed differential behaviors, nearly identical to what was observed using CEM cells as viral targets (Fig. 3B). A possible explanation for these results may arise from the fact that, after a 45-min incubation at 0°C, the percentage of bound virus is much higher than after a 5-min incubation at 37°C [5]. Thus, when the pH is lowered, the majority of the virus already bound to the target membrane can undergo membrane fusion, which may facilitate a more extensive transfer of Rh-PE to the plasma membrane.

Using liposomes as target membranes, we found that R18 and Rh-PE exhibited different kinetics of fluorescence dequenching (data not shown), although the difference was less pronounced than that observed with CEM cells. These results suggest that the higher complexity of the CEM plasma membrane, namely the presence of membrane proteins, may hinder the homogeneous distribution of Rh-PE molecules after fusion. The dequenching detected using DORh-B was always very reduced (data not shown), thus confirming initial suspicions (Table 1), that this probe is not appropriate to study the kinetics of membrane fusion.

Our experiments with liposomes at neutral pH (Fig. 4A) demonstrate that some non-specific probe transfer takes place at 37°C, especially with R18. Experiments performed with inactivated virus at acidic pH (Fig. 4B) yield a similar result. Once more, this effect is less pronounced than the one obtained with CEM cells (Fig. 2), which again may be explained by the presence of membrane proteins, namely proteins involved in lipid transfer or others that may, by similar mechanisms, favor the non-specific transfer of probe molecules. It is relevant to note that a lysosomal protein was already described as being responsible for non-specific probe transfer of R18 in a reconstituted fusion assay [33]. The fact that we found less non-specific probe transfer for Rh-PE than for R18, both with CEM cells and liposomes, is most likely explained by the fact that R18 is a smaller molecule. It is quite possible that this small size is responsible, both for the quick detection of membrane merging achieved with this probe, and for the non-specific transfer that takes place at later stages.

The use of liposomes as target membranes for in-

fluenza virus allowed us to study virus-mediated membrane fusion in a reversed system (i.e., fusion of labeled liposomes with unlabeled virions), and thus to compare the results obtained with R18 and Rh-PE with those obtained with the NBD/Rh-PE assay. These experiments confirmed that the kinetics of fluorescence dequenching observed with Rh-PE are much slower than those monitored with R18. On the other hand, and more importantly, a comparison of the results obtained with R18 and NBD/Rh-PE shows that the extents of fusion and the initial fusion rates obtained with both assays are very similar, and that R18 dequenching thus accurately follows membrane fusion. In an earlier work [28] these two types of labeling yielded almost the same percent of influenza virus fusion activity at 20°C and 37°C.

In summary, membrane fusion can be detected with both R18 and Rh-PE, but non-specific probe transfer can also take place with either probe. However, R18 seems to be the probe of choice to monitor membrane fusion when it is necessary to label preexisting bilayers. R18 is the fluorescent probe that offers a larger working scale, due to the high efficiency of fluorescence energy transfer that occurs between probe molecules. Therefore, this probe efficiently detects rapid membrane mixing, which is very important for accurately following the kinetics of the fusion process. Furthermore, unlike Rh-PE, the R18 assay compares favourably with the NBD/Rh-PE assay in the same system. Whatever the cause for fast or slow probe transfer, it is of course essential to perform control experiments with the goal of establishing a reliable time frame for each experiment in any given system. Furthermore, one should always be aware that this time frame is temperature-dependent. In this context, it is important to note that non-specific R18 transfer does not take place appreciably until about 30 min of virus incubation with unlabeled target membranes at 37°C. Although non-specific probe transfer is less of an issue when Rh-PE is used, the detection of membrane merging is also grossly impaired in this case, and quantification of the initial kinetics of membrane merging will likely result in severe inaccuracies. Indeed, when fusion was monitored with Rh-PE and R18, identical results were obtained only with virus–target membrane prebinding at 0°C, followed by the removal of unbound virions. This protocol, besides being physiologically questionable, cannot be carried out in many other systems that involve fusion of vesicles with similar sizes. However, since non-specific transfer of Rh-PE takes place after longer incubation times, this probe may have some advantage over R18 if the fusion activity to be monitored occurs over a lengthy period.

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