Inactivation of PR8 Influenza Virus through the Octadecylrhodamine B Chloride Membrane Marker

Heidi Wunderli-Allenspach,* Maja Günthert, and Susanne Ott

Department of Pharmacy, Biopharmacy, Swiss Federal Institute of Technology, CH-8092 Zürich, Switzerland

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ABSTRACT: The octadecylrhodamine B chloride (R18) membrane marker was incorporated into PR8 influenza viruses and virus receptor (G_{D1a}-) containing small unilamellar vesicles (SUV). Both were tested in a fusion/lipid transfer assay [Wunderli-Allenspach, H., & Ott, S. (1990) *Biochemistry 29*, 1990–1997] to find out whether incorporation into artificial and biological membranes yields equivalent results. The R18 assay is based on incorporation of quenched concentrations of the label into donor membranes and monitoring of the dequenching upon its dilution into unlabeled acceptors. With PR8 viruses and R18-labeled SUV, a fast, hemagglutinin-specific fusion takes place at pH 5.3, independently of the initial quenching. At neutral pH, a slow, nonspecific R18 transfer occurs. Both processes follow second-order kinetics. Upon incubation of R18-labeled PR8 viruses with unlabeled SUV or LUV in neutral buffer, transfer is also found. At pH 5.3, a complex dequenching curve best described with superposition of two second-order functions was encountered: a fast, hemagglutinin-specific component and a slow, nonspecific component. A decreasing proportion of the fast fusion was found with increasing initial quenching of labeled virus. Extrapolation showed that full fusion activity is obtained only with low initial quenching (20–30%). The gradual inactivation of the virus by increasing amounts of R18 was confirmed with biological assays (e.g., infectivity). The R18 surface density is much higher in viruses than in liposomes to obtain the same initial quenching. Analysis with the Stern–Volmer plot revealed that R18 monomers and dimers contribute to the quenching in labeled PR8 viruses, whereas only dimers determine the quench curve in liposomes.

For kinetic studies on membrane interactions in vitro, octadecylrhodamine B chloride (R18)[1] has been introduced as a marker by Hoekstra et al. (1984). The aspect that this label was suitable not only for artificial but also for preexisting biological membranes was emphasized. The R18 assay is based on the incorporation of R18 into lipid bilayers at quenched concentrations. Dilution upon interaction with an unlabeled target membrane leads to dequenching, which can be measured quantitatively in a fluorescence spectrophotometer. In the meantime, this approach has been used for numerous interaction studies with cells and viruses (e.g., Nussbaum & Loyter, 1987; Stegmann et al., 1987; Clague et al., 1990). In most cases, R18 was used as a fusion marker since no evidence was found for spontaneous exchange of the molecule between erythrocyte ghosts or LUV (Hoekstra et al., 1984).

We have previously incorporated R18 into virus receptor (G_{D1a}-) containing SUV to study membrane interactions between these liposomes and PR8 influenza viruses. Two types of dequenching reactions could be distinguished kinetically; a fast, hemagglutinin- (HA-) specific fusion process at low pH and a slow, nonspecific lipid transfer at neutral pH (Wunderli & Ott, 1990; Ott and Wunderli, unpublished results). Now we have investigated the equivalence of R18 incorporation into either viruses (biological membranes) or SUV (artificial membranes). Our results indicate that the marker exchange observed with labeled SUV occurs with R18-labeled PR8 viruses as well. However, as we can show, incorporation of the R18 marker into viral membranes affects the fusion activity. The HA-specific dequenching reaction is significantly reduced and also other viral activities like infectivity, hemolysis, and hemagglutination are lowered by the R18 labeling. Comparison of the quench curves of PR8 viruses and liposomes shows that much more R18 is needed in viruses than in SUV or LUV to reach the same initial quenching. Analysis with the Stern–Volmer plot reveals that dimers determine the amount of quenching in labeled liposomal preparations, whereas monomers and dimers contribute in the case of R18-labeled PR8 viruses.

MATERIALS AND METHODS

Chemicals. Egg yolk phosphatidylcholine (PC) and phosphatidic acid (PA), both grade 1, were purchased from Lipid Products (Nutfield, Great Britain). Ganglioside G_{D1a} (disialoganglioside) was from Bachem (Bubendorf, Switzerland), octadecylrhodamine B chloride (R18) was from Molecular Probes (Junction City, OR), [*methyl*-^{14}C]choline chloride was from NEN (NEC-141; specific activity 2 GBq/mmol), and 1,2-dipalmitoyl-L-3-phosphatidyl[*N-methyl*-^{3}H]choline was from Amersham (TRK.673; 3 TBq/mmol). All materials for tissue culture were from Gibco, cholesterol and EDTA (ethylenediaminetetraacetic acid) were from Sigma, and the other chemicals were from Merck (all analytical grade). Buffers used were as follows: for dequenching assays, PBS (phosphate-buffered saline) (10 mM Na_2HPO_4/KH_2PO_4) containing 130 mM NaCl, at pH 5.3 or 7.4 as indicated; for virus stocks, NTE buffer (10 mM Tris-HCl, 50 mM NaCl, and 1 mM EDTA), pH 7.4; for hemolysis assays, citrate–NaCl buffer (50 mM trisodium citrate hydrochloride and 100 mM NaCl), pH 5.0.

* To whom correspondence should be addressed.

[1] Abbreviations: ^{14}C-choline, [*methyl*-^{14}C]choline chloride; ^{3}H-DPPC, 1,2-dipalmitoyl-L-3-phosphatidyl[*N-methyl*-^{3}H]choline; EDTA, ethylenediaminetetraacetic acid; G_{D1a}, disialoganglioside; HA, hemagglutinin; MDCK, Madin Darby canine kidney; NA, neuraminidase; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; R18, octadecylrhodamine B chloride; RSV, respiratory syncytial virus; SUV and LUV, small and large unilamellar vesicles; TCID, tissue culture infective dose.

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Virus. PR8 influenza virus [A/PR/8/34 (H1N1)] was grown on MDCK (Madin Darby canine kidney) cells. After partial purification, virus suspensions in NTE buffer were frozen in liquid nitrogen (Wunderli & Ott, 1990; Wunderli et al., 1990). For all assays, aliquots of PR8 viruses were freshly thawed, kept on ice, and used within the next 6–8 h. Viral proteins were measured according to Bradford (1976) using the Bio-Rad microassay (Bio-Rad Laboratories, Munich, FRG). For certain experiments (R18 quench curves, see below), ^{14}C-choline-labeled PR8 viruses were produced as follows: MDCK cells were diluted 1:4 from confluent cultures and labeled with 10^5 Bq of ^{14}C-choline/75-cm^2 flask (i.e., per $\sim 5 \times 10^6$ cells in 15 mL of Eagle's minimal essential medium with 10% fetal calf serum). After incubation for 5 days at 37 °C (5% CO_2), the prelabeled cells were infected with PR8 viruses as usual (Wunderli & Ott, 1990). Labeling was continued by addition of fresh ^{14}C-choline (amount as above) to the infected cultures. Viruses were harvested 72 h after infection and partially purified, and the specific activity was determined. Labeled viruses reproducibly contained about 80–100 Bq/μg of viral protein. Bromelain (EC 3.4.22.4) digestion was used to remove the two surface proteins, hemagglutinin (HA) and neuraminidase (NA), from the viruses (Brand & Skehel, 1972). Residual protein after this treatment amounted to ~40% of the original content. According to Klenk (1991) the molar ratio of HA to NA is between 4:1 and 5:1. This means that HA accounts for more than 80% of the bromelain-removed, soluble protein fraction. Lipid was estimated assuming a protein content of 70% (w/w) and a lipid content of 20% (w/w) with respect to total virus (Barrett & Inglis, 1985). Calculations for particle numbers were based on a molecular weight of 2.5×10^8 for influenza virus (Klenk, 1991). The mean diameter of viral envelopes (devoid of HA) had previously been determined as ~55 nm (Wunderli & Ott, 1990). Viral activities were studied by standard procedures [summarized in Wunderli et al. (1990)]. In brief, hemagglutinating units were determined with chicken erythrocytes (Fazekas de St. Groth & Webster, 1966). The hemolytic activity was tested according to Yewdell et al. (1983) with human erythrocytes (O/Rh$^+$) within 2 days after sampling of the blood. The infectivity of influenza viruses was measured by infection of MDCK cells with serial dilutions. The procedure described previously (Wunderli et al., 1990) was followed with the exception that the neuraminidase treatment was omitted. Fifty percent end points ($TCID_{50}$, tissue culture infective dose) were calculated according to Reed and Muench (1938) taking positive HA titers in the supernatants as criterion.

Liposomes. Small unilamellar vesicles (SUV) were prepared in PBS, pH 7.4, with the detergent dialysis method (Zumbühl & Weder, 1981) with a Liposomat (Dianorm, Munich, FRG). Sodium cholate was used at a lipid to detergent ratio of 0.6 (mol/mol). Details on the production have previously been described (Wunderli & Ott, 1990). Residual cholate in the liposomes amounted to less than 1 molecule/280 lipid molecules (total lipid). The diameter of the SUV was ~27 nm (≥99% of the vesicles) as determined by dynamic light scattering (Nicomp 370 submicron particle sizer; Skan, Basel, Switzerland). The following standard lipid composition was used: PC/PA/cholesterol/G_{D1a} = 0.625/0.075/0.231/0.069 (mol/mol/mol/mol) at a final total lipid concentration in the liposome stocks of about 0.7 mg/mL (R18 labeling, see below). Recoveries were calculated for each batch by means of ^{3}H-DPPC. Large unilamellar vesicles (LUV) were produced by a repetitive freezing, thawing, and filtration method similar to the extrusion method described by Mayer et al. (1986). Briefly, a dry lipid film was produced without detergent and manually dispersed in PBS, pH 7.4. Liposomes were then frozen eight times in a solid CO_2/ethanol mixture and thawed at 40 °C, followed by eight filtration cycles through a 0.2-μm pore size membrane (Minisart, Sartorius, FRG). For LUV the lipid composition as well as the final total lipid concentration in the stocks were the same as for the SUV (see above). Two size populations could be determined by dynamic light scattering: ~110 nm and ~300 nm (77.5% and 22.5% of the vesicles, respectively). Calculations for LUV were based on a mean diameter of 153 nm. Liposomes were stored at 4 °C in the dark and used within 2 weeks. Particle numbers for the stocks were calculated according to Huang and Mason (1978).

R18 Labeling. The specific fluorescence of the commercially available R18 varies. It was therefore determined for each batch, and the quench curves for liposomes and viruses were established. Liposomes were labeled by either of the two following procedures. With the so-called endogenous labeling method, R18 was incorporated into the lipid films at various concentrations. The marker was thus integrated into the liposomal membranes upon their formation. Alternatively, preexisting liposomes were labeled exogenously with the same procedure as applied for PR8 viruses, i.e., ethanolic R18 stock solutions (4 μL) with various R18 concentrations were added to 100-μL aliquots of viruses (~8 μg of viral lipid/mL) or liposomes (~70 μg of liposomal lipid/mL). Samples were incubated in the dark for 1 h at room temperature and the quenching was determined (see below). This value is defined as initial quenching ($Q_{initial}$) of a labeled species. To calculate correct R18 ratios for each sample, ^{3}H-DPPC-labeled liposomes and ^{14}C-choline-labeled PR8 viruses were used. For direct comparison between the quench curves of labeled liposomes and PR8 viruses, the R18 concentration was expressed as molecules of R18 per lipid bilayer surface area (sum of inner and outer surface). The particle diameters used for calculations are indicated above.

Fluorescence Measurements. For fluorescence measurements a LS-5B spectrophotofluorometer (Perkin-Elmer) equipped with a thermostated circulating water bath was used. To avoid bleaching of the samples, the shutter was kept closed between measurements. The excitation and emission wavelengths were 545 and 585 nm, respectively; the slit width was 10 nm. Quenching of R18-labeled preparations was measured at room temperature (22 °C) for the quench curves or at 37 °C for all preparations used in the various experiments. Calculations were performed as described by Hoekstra et al. (1984). In brief, measurements in PBS, pH 7.4, were related to a calibration standard which was obtained as follows: Vesicles (liposomes, viruses) were diluted into a 1% Triton X-100 solution in PBS, pH 7.4, which causes complete solubilization of membranes and therefore maximal increase in fluorescence. Interaction of G_{D1a}-containing liposomes with PR8 influenza viruses was monitored by measuring the dequenching kinetics of R18 at pH 7.4 and/or pH 5.3 (see Results). The R18 marker was incorporated into viruses or SUV as indicated. Standard dequenching assays were run as follows: liposomes (volumes as indicated) were added to PBS, pH 7.4 or 5.3, prewarmed to 37 °C, directly in the cuvette. HCl was added as needed to keep the pH at 5.3. The dequenching reaction was started by addition of PR8 virus. Virus/liposome particle ratios and the corresponding lipid ratios for the various incubations as well as the total particle concentrations are given in Results. The final volume of the

incubation mixture was 1 mL. R18 dequenching, i.e., the increase in fluorescence, was monitored as a function of time up to 400 min. The fluorescence intensity at 0 min was subtracted from all subsequent measurements as background. Measured intensities were expressed as DQ(t), i.e., fraction of the fluorescence at infinite dilution, the latter being obtained by dilution of R18 liposomes or viruses (same concentrations as used in the assay) in 1% Triton X-100 and subtraction of Triton X-100 background fluorescence. This calibration standard was incubated in a parallel cuvette (37 °C) and checked throughout the incubation time.

Data Analyses. Kinetic data were analyzed with the NLFit 3.0 program (QuantumSoft, Zürich, Switzerland). All dequenching curves [DQ = $f(t)$] could be fitted with either of the two following formulas: one second-order function

$$DQ = \frac{DQ_{max} \times t}{(1/^2kDQ_{max}) + t} = \frac{DQ_{max} \times t}{t^* + t}$$

or superposition of two second-order functions

$$DQ = \frac{DQ_{maxI} \times t}{t^*_I + t} + \frac{DQ_{maxII} \times t}{t^*_{II} + t}$$

where DQ_{max} is the maximal dequenching reached, t^* is the time to reach half-maximal dequenching, and ^2k is the second-order rate constant [(concentration·time)$^{-1}$] (Wunderli & Ott, 1990). Decisions about optimal fits for a particular curve were based on χ^2 values.

With the Stern–Volmer plot, quench curves can be analyzed for the respective contributions of fluorescent probe monomers, dimers, trimers, or aggregates to the fluorescence quenching (Arbeloa, 1981; McDonald, 1990). The general equation is

$$(DQ_{initial})^{-1} = (DQ_{max,initial})^{-1} + a_1 r + a_2 r^2 + a_3 r^3$$

where $DQ_{initial} = (1\text{-}Q_{initial})(\text{fraction})$, $DQ_{max,initial}$ is the maximal possible dequenching (equals 1), a_1, a_2, and a_3 are constants (see text), and r is the R18 surface density (R18 molecules per square nanometer). Data were fitted with the NLFit 3.0 program (see above). All curves analyzed showed best fits with either

$$(DQ_{initial})^{-1} = 1 + a_1 r + a_2 r^2 \quad (1)$$

called "dimers plus monomers" fit, or

$$(DQ_{initial})^{-1} = 1 + a_2 r^2 \quad (2)$$

called "dimers only" fit. Quench curves of various labeled species (liposomes, viruses) were compared by plotting the respective residuals from eq 1 and eq 2 fits together. Residuals are plotted as the differences between measured values and those calculated from the respective fits.

RESULTS

Dequenching Assays with R18-Labeled PR8 Viruses or R18-Labeled SUV. R18-labeled PR8 viruses and R18-labeled SUV were tested in the dequenching assay previously established to study membrane interactions between PR8 influenza viruses and virus receptor (G_{D1a}-) containing liposomes (Wunderli & Ott, 1990). The respective dequenching kinetics are compared in Figure 1. In both cases, the initial quenching of the labeled species was about 94%. Incubations were performed at pH 5.3 (37 °C) in the presence of an excess of unlabeled acceptor membrane (see legend to Figure 1). Particle ratios were chosen on the basis that one virus can fuse

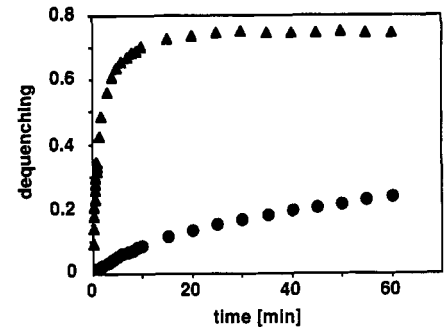

FIGURE 1: Dequenching assay with R18-labeled PR8 viruses and R18-labeled, G_{D1a}-containing SUV, respectively. G_{D1a}-containing SUV were incubated in PBS, pH 5.3, at 37 °C with PR8 viruses. (●) R18-labeled PR8 viruses, particle concentration 5×10^{12}/mL and virus to liposome particle ratio 1/13 000; (▲) R18-labeled SUV (endogenous labeling), particle concentration 6×10^{10}/mL and virus to liposome particle ratio 2/1. The initial quenching for both labeled species was 94%. Data points are means from four independent experiments.

with several liposomes, but each liposome can fuse with only one virus (Nir et al., 1986). It is striking to note that the dequenching measured with R18-labeled viruses is much slower than with R18-SUV and does not reach the same maximal value. This result was also obtained with lower virus to liposome particle ratios (tested down to ~1/100). In this case, however, due to low fluorescence readings, curves were not as well defined (not shown). For quantitative comparison, kinetic analysis of the data was performed with a nonlinear fit program (see Materials and Methods). Both dequenching curves can best be fitted with a second-order function; however, curve parameters (DQ_{max}, i.e., maximal dequenching, and t^*, i.e., time to reach half-maximal dequenching) differ significantly (Table I). Data are also included for incubations of R18-labeled viruses with LUV instead of SUV as acceptor membranes. From all combinations tested (pH 5.3, 37 °C), a fast dequenching process is only found with R18-labeled liposomes. For R18-labeled viruses the reaction is 30–40 times slower, independent of the type of unlabeled acceptor membrane (SUV or LUV) used in the assay.

In Table I, data are also listed for assays performed at neutral pH (37 °C). It is obvious that R18 dequenching at pH 7.4 occurs not only with R18-labeled SUV but also with R18-labeled viruses. The process is about 3 times slower (t^* = 80–120 min for R18-labeled PR8 as compared to t^* = 30–40 min for R18-labeled SUV) and only reaches ~15% (LUV) to ~25% (SUV) of the maximal possible value.

To exclude the possibility that the labeling conditions (exogenous versus endogenous) were influencing the dequenching behavior, SUV were also exogenously labeled and tested in the assay at both pH 5.3 and 7.4 (data not shown). No significant difference was found between fitted curve parameters of exogenously and endogenously labeled liposomes (same initial quenching, same assay conditions).

Influence of Initial Quenching on the Dequenching Kinetics. SUV were labeled (endogenously) with various amounts of R18 to study the influence of the extent of quenching. The initial quenching was determined for each preparation and the dequenching kinetics were measured in PBS, pH 5.3, under standard assay conditions (see Materials and Methods). Quench values between 50% and 95% were tested and the corresponding curves were analyzed (Table II). Independently of the initial quenching, a fast reaction occurs with $t^* < 2$ min. DQ_{max} values reach between 80% and 100% of the maximal possible values. R18-labeled PR8 viruses with initial quench values between 50% and 95% were then tested in

Table I: R18 Dequenching Kinetics upon Interaction of PR8 Viruses and G_{D1a}-Containing Liposomes[a]

		incubation at pH 5.3				incubation at pH 7.4			
R18-labeled species	acceptor membrane	n	$Q_{initial}$ (%)	DQ_{max}	t^* (min)	n	$Q_{initial}$ (%)	DQ_{max}	t^* (min)
SUV (A)	PR8 virus	4	94	0.769 ± 0.005	1.18 ± 0.04	4	92	0.750 ± 0.005	33.45 ± 1.02
PR8 virus (B)	SUV	4	94	0.448 ± 0.006	50.57 ± 2.25	2	92	0.252 ± 0.014	125.90 ± 2.90
PR8 virus (C)	LUV	3	95	0.365 ± 0.006	36.82 ± 2.06	2	95	0.154 ± 0.004	83.60 ± 5.60

[a] PR8 influenza viruses were incubated with G_{D1a}-containing SUV or LUV in PBS, pH 5.3 or 7.4, at 37 °C as indicated. Experimental details for the incubations with SUV (A and B) are given in the legend for Figure 1. For incubations with LUV (C), the total particle concentration was 1.2 × 10^{11}/mL and the virus to liposome particle ratio was about 1/400. Curves were fitted with the NLFit 3.0 program (see Materials and Methods). Fitted parameters are listed with their 90% confidence limits. n represents the number of independent experiments.

analogous experiments (pH 5.3, 37 °C) with LUV as acceptor membranes. Analysis of the dequenching curves revealed that for this series best fits were obtained with superposition of two second-order functions instead of a single one (Table III). In the case of 95% initial quenching, the difference between a one- or two-component fit was small as can be judged from the curve parameters (see Table III). For lower initial quench values, two-component fits showed significantly lower χ^2 values than the one-component fits. In all tested cases, a fast kinetic component can be defined with $t^* < 2$ min which accounts for up to about a third of the total dequenching reaction. The second component is much slower (t^* ~40–50 min). Total DQ_{max} ($DQ_{maxI+II}$) only reaches about 40% of the possible value with high initial quenchings (>75%). With lower initial quenching it increases to about 70% of the maximal possible value. To sum up, two dequenching reactions occur simultaneously upon incubation of R18-labeled PR8 viruses and G_{D1a}-containing liposomes. The fast reaction is increasingly favored with a decreasing amount of R18 in the respective virus preparation. With 50% initial quenching the fast component accounts for about a third of the total. Incubations of SUV instead of LUV with R18-labeled PR8 viruses showed the same result; i.e., best fits were always obtained with two components, a fast one ($t^* < 2$ min) and a slow one (t^* = 40–50 min), the proportion of the fast process being decreased with increasing initial quenching (data not shown). Figure 2 illustrates the correlation between initial quench values for R18-labeled PR8 virus preparations and the contribution of the fast reaction to the total dequenching. The latter was calculated from fitted parameters of assays run with LUV as acceptor membranes and is expressed as percent fusion in relation to the totally reached dequenching [(DQ_{maxI} × 100)/$DQ_{maxI+II}$]. This curve can be fitted with an exponential function. With increasing amounts of R18 incorporated into PR8 viruses, ie., with increasing initial quenching, the fast component of the dequenching reaction, i.e., the fusion process, is reduced (to below 10% for initial quench values >90%). From the fitted curve an initial quenching of about 20% can be extrapolated which would allow for full fusion activity, i.e., DQ_{maxI} = 100%.

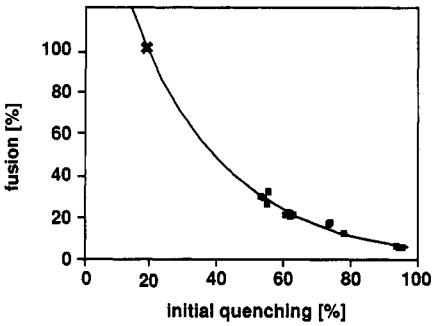

FIGURE 2: Inactivation of the fusion activity of labeled PR8 virus as a function of initial quenching of R18. Experiments were performed with R18-labeled PR8 viruses and G_{D1a}-containing LUV as described in Table III. The extent of fusion, i.e., of the fast dequenching reaction (DQ_{maxI}), was calculated from the parameters of the fitted dequenching curves. It is expressed as percentage of the total dequenching ($DQ_{maxI+II}$) measured in the respective assays and plotted versus the initial quenching of the labeled viruses. Results from single experiments are plotted. × represents the extrapolated initial quenching for a fully active R18-labeled virus preparation.

Table II: Dequenching Kinetics as a Function of Initial Quenching: PR8 Influenza Viruses Incubated with R18-Labeled, G_{D1a}-Containing SUV at pH 5.3[a]

n	$Q_{initial}$ (%)	DQ_{max}	t^* (min)
2	95	0.790 ± 0.005	1.17 ± 0.03
2	91	0.793 ± 0.004	1.31 ± 0.03
2	82	0.817 ± 0.007	1.63 ± 0.05
1	50	0.589 ± 0.010	1.62 ± 0.13

[a] R18-labeled, G_{D1a}-containing SUV were prewarmed in PBS, pH 5.3, at 37 °C. The initial quenching of the preparations was as indicated. The reaction was started by addition of the unlabeled PR8 viruses. Viruses were always in excess, i.e., the ratio of viruses to liposomes was about 2/1. The total particle concentration in all assays was 6 × 10^{10}/mL, corresponding to about 200 ng of liposomal lipid/mL and about 3 μg of viral lipid/mL. Dequenching was measured in a spectrofluorometer and the kinetics were analyzed with the NLFit 3.0 program (see Materials and Methods). Fitted parameters are listed with their 90% confidence limits. n represents the number of independent experiments.

Table III: Dequenching Kinetics as a Function of Initial Quenching: R18-Labeled PR8 Influenza Viruses Incubated with G_{D1a}-Containing LUV at pH 5.3[a]

n	$Q_{initial}$ (%)	$DQ_{max\ I}$	t^*_I (min)	$DQ_{max\ II}$	t^*_{II} (min)
3	95			0.365 ± 0.006	36.82 ± 2.06
3	95	0.025 ± 0.004	0.87 ± 0.35	0.353 ± 0.004	47.65 ± 2.00
1	79	0.034 ± 0.010	1.10 ± 0.67	0.252 ± 0.008	41.04 ± 6.40
3	62	0.088 ± 0.006	0.58 ± 0.11	0.324 ± 0.006	52.91 ± 4.31
2	50	0.110 ± 0.013	1.05 ± 0.26	0.214 ± 0.011	44.64 ± 9.64

[a] PR8 viruses were labeled with various amounts of the R18 marker (initial quenching as indicated) and incubated with G_{D1a}-containing LUV (pH 5.3, 37 °C). Liposomes were always in excess, i.e., the particle ratio of viruses to liposomes was 1/400. The total particle concentration was 1.2 × 10^{11}/mL in all experiments. Dequenching was measured in a spectrofluorometer and the kinetics were analyzed with the NLFit 3.0 program (see Materials and Methods). Fitted parameters are listed with their 90% confidence limits. n represents the number of independent experiments.

Viral Activities of R18-Labeled PR8 Viruses. To directly study the effect of R18 incorporation into the PR8 membrane, viral activities like infectivity, hemolysis, and hemagglutination were tested. PR8 influenza viruses were labeled with various amounts of R18 yielding initial quench values between 37% and 94%. Infectivity was tested on MDCK cells by means of 50% end point titrations based on positive HA titers in the supernatants. The virus production was followed during 70 h. As listed in Table IV, R18 incorporation into viruses reduced their infectivity significantly. Already with an initial quenching of 37%, infectivity was about 2-fold reduced as compared to the ethanol control. Initial quenching of about

Table IV: Infectivity of R18-Labeled PR8 Influenza Viruses on MDCK Cells[a]

		infectivity $TCID_{50}$			
n	$Q_{initial}$ (%)	22 h[b]	30 h	46 h	70 h
6	94	$10^{-2.18}$	$10^{-3.54}$	$10^{-4.56}$	$10^{-4.71}$
10	79	$10^{-3.43}$	$10^{-5.08}$	$10^{-5.68}$	$10^{-5.71}$
8	60	$10^{-4.26}$	$10^{-5.52}$	$10^{-6.23}$	$10^{-6.27}$
2	37	$10^{-4.65}$	$10^{-5.79}$	$10^{-6.58}$	$10^{-6.58}$
10	PR8 control	$10^{-4.76}$	$10^{-6.06}$	$10^{-6.95}$	$10^{-6.95}$

[a] The infectivity of R18-labeled and unlabeled PR8 influenza viruses was measured by $TCID_{50}$ titration on MDCK cells (see Materials and Methods). Aliquots of PR8 viruses were labeled with various amounts of R18 and the initial quenching was determined (see Materials and Methods). Control viruses were treated identically except that the R18 was omitted. End points were determined, taking positive HA titers in the supernatant as criterion. n represents the number of independent experiments. [b] Time after infection.

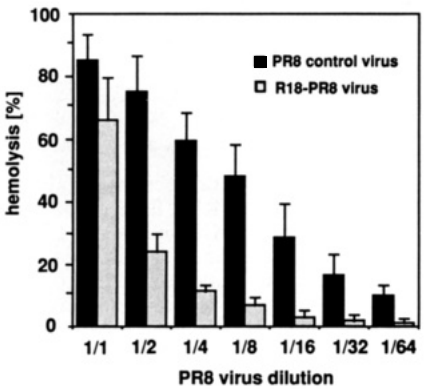

FIGURE 3: Hemolytic activity of R18-labeled PR8 influenza viruses. Aliquots of PR8 influenza viruses were labeled by addition of an ethanolic solution of the R18 marker (see Materials and Methods). The initial quenching of the preparations was between 92 and 98%. R18-labeled PR8 viruses (dilutions between 1/1 and 1/64 as indicated, starting from the labeled stock) were adsorbed to human erythrocytes (30 min, 0 °C) and duplicate samples were incubated with citrate–NaCl buffer, pH 5.0 (30 min, 37 °C). Absorbance was measured at 405 nm in the supernatants and the hemolytic activity was calculated (see Materials and Methods). Control viruses were incubated under labeling conditions without R18 before being used in the assay. Data are from six independent experiments.

60% led to a 4-fold reduction, quenching of about 79% to a 17-fold reduction, and finally, quenching of about 94% to a 170-fold reduction. Unlabeled viruses which had been incubated in 3.8% ethanol in PBS, pH 7.4, analogous to the labeling procedure, served as a control. The infectivity of these control viruses was within a factor of 2 of the infectivity of PR8 virus kept in PBS, pH 7.4 (data not shown). Highly quenched R18-virus preparations ($Q = 94\%$) were also tested for other viral activities. The hemolytic activity was significantly reduced (Figure 3). Even hemagglutination was slightly reduced by a factor of about 2 as compared to the control viruses (data not shown).

Quench Curves. Increasing amounts of R18 were incorporated into PR8 viruses and G_{D1a}-containing liposomes (SUV and LUV). The initial quenching of all preparations was determined (see Materials and Methods) and plotted against the respective R18 surface densities (Figure 4A). The latter were calculated as number of R18 molecules (using the specific fluorescence) per square nanometer surface area (inner plus outer; see Materials and Methods). To reach about 96% initial quenching, ~0.15 R18 molecules/nm^2 are needed in the case of labeled liposomes (SUV or LUV), whereas in the case of PR8 viruses the number is about 10 times higher.

To distinguish between the contributions of R18 monomers, dimers, and trimers to the fluorescence quenching in various

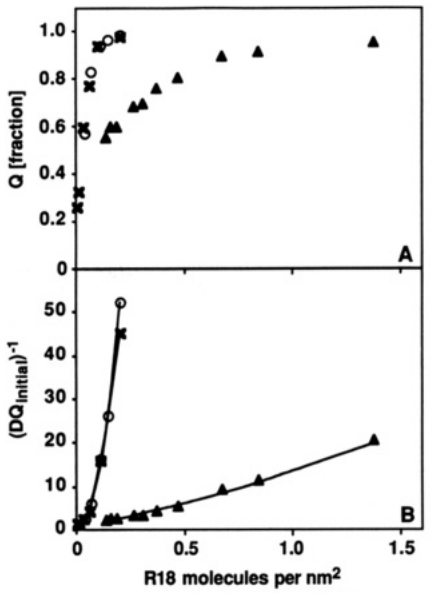

FIGURE 4: R18 quench curves of PR8 viruses and G_{D1a}-containing liposomes. R18 was incorporated into PR8 influenza viruses and G_{D1a}-containing SUV at various concentrations. Quenching was determined for all preparations (see Materials and Methods) and plotted against the corresponding relative R18 contents (A). The latter is expressed as number of R18 molecules/square nanometer of membrane surface (inner and outer) (see Materials and Methods) to permit comparison between liposomes and viruses. (▲) Exogenously labeled PR8 viruses: (×) exogenously labeled SUV; (○) endogenously labeled SUV. The same data were analyzed with the Stern–Volmer plot (B) to study the influence of monomers and dimers on fluorescence quenching (see Materials and Methods).

vesicles, quench curves were analyzed with the Stern–Volmer plot described in Materials and Methods (Arbeloa, 1981; McDonald, 1990). This plot is illustrated in Figure 4B for the same samples as shown in Figure 4A. To get a clue as to the mechanism of R18 quenching, a comparative study was performed using exogenously and endogenously labeled SUV and LUV, respectively, and exogenously labeled PR8 viruses. In addition, labeled intact viruses were also compared with PR8 viruses which had been bromelain-treated after R18 labeling to investigate the role of the HA spikes on quenching. In all tested cases, best fits for the Stern–Volmer plots were obtained by either the "dimers plus monomers" formula or the "dimers only" formula (see Materials and Methods). This indicates that neither trimers nor aggregates of R18 are involved in the fluorescence quenching. Regarding the contribution of R18 monomers and dimers, comparison between labeled liposomes and PR8 viruses can best be achieved by plotting for each quench curve the respective residuals for eq 1 fit, i.e., $DQ^{-1} = 1 + a_1 r + a_2 r^2$, and eq 2 fit, i.e., $DQ^{-1} = 1 + a_2 r^2$ (Figure 5). With this approach no indication for a contribution of monomers is obtained for either exogenously labeled SUV (Figure 5A) or endogenously labeled SUV (Figure 5B). In both cases the residuals with both fits are small and scatter randomly around zero within the experimental limits. The same picture was also found with LUV independent of exogenous or endogenous labeling (not shown). In all liposomes, a constant value of 1154 ± 116 (nm^2/R18 molecules)^2 ($n = 7$) was found for a_2. The situation was quite different for R18-labeled PR8 viruses. In this case, the "dimers only" fit leads to systematic deviations of calculated from experimental values, and a significantly improved fit was only obtained with the "dimers plus monomers" formula as illustrated with the respective residuals (Figure 5C). To study the influence of the HA spikes on R18 quenching in labeled influenza viruses, the following experiment was performed:

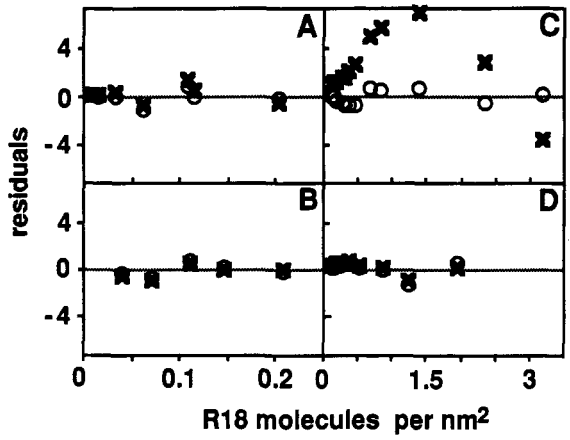

FIGURE 5: Contribution of R18 monomers and dimers to fluorescence quenching. R18 was incorporated at various concentrations into PR8 influenza viruses and G_{D1a}-containing liposomes (SUV or LUV as indicated) and the quenching was determined. For experimental details see legend to Figure 4 and Materials and Methods. Stern–Volmer plots of all preparations were fitted (see Materials and Methods) and the residuals plotted for a "dimers only" fit (×) as compared to a "dimers plus monomers" fit (○). (A) Exogenously labeled SUV; (B) endogenously labeled SUV; (C) exogenously labeled PR8 viruses; (D) exogenously labeled, bromelain-treated PR8 viruses.

PR8 viruses were labeled as usual with increasing amounts of R18. Before quenching was determined, the R18-labeled viruses were treated with bromelain to remove HA spikes (see Materials and Methods). As illustrated in Figure 5D, the contribution of monomers to the R18 quenching was significantly reduced. An adequate fit was obtained with the "dimers only" equation with an a_2 constant of ~10. This change in the quench curve is strictly related to the removal of the HA spikes, as no such effect was found with the control viruses incubated in parallel without bromelain (not shown). We also tried to cleave HA molecules from PR8 viruses with bromelain prior to R18 labeling. In this case no significant difference was found in the quench curves of bromelain-treated and untreated viruses (data not shown). The implications of this will be discussed.

DISCUSSION

As shown in this paper, R18 by no means acts as an inert marker for biological membranes. The most striking evidence for this stems from biological assays performed with R18-labeled PR8 viruses. The viral infectivity on MDCK cell cultures is significantly reduced as compared to unlabeled control virus. Less than 1% of the infectivity is left with 94% quenched virus preparations, and even with as little as 37% initial quenching about a 2-fold reduction as compared to the infectivity of control viruses is found. Hemolysis, known to be less susceptible to inactivation, is significantly reduced by R18 labeling and so is even hemagglutination, which is least affected by inactivation procedures, e.g., low pH treatment (Yewdell et al., 1983). The interference of R18 with viral membrane functions is also reflected in the dequenching kinetics under fusion conditions (pH 5.3, 37 °C). Dequenching curves differ significantly between R18-labeled PR8 viruses incubated with SUV and R18-labeled SUV incubated with PR8 viruses. In the latter case, best curve fits were obtained with a single second-order function independent of the initial quenching of the labeled SUV (see Table II). The time to reach half-maximal dequenching was determined as $t^* < 2$ min, in agreement with the previously described fast, HA-specific fusion reaction (Wunderli & Ott, 1990). With R18-labeled PR8 viruses, however, kinetic analysis of the dequenching curves revealed two striking features: dequenching remained incomplete in most cases, and best curve fits were obtained with superposition of two second-order functions, a fast one with $t^* < 2$ min and a slow one with $t^* \sim 40$–50 min (see Table III). The fast component is interpreted as specific virus fusion for two reasons: (1) the t^* value is in the same range as found for fusion of labeled SUV with unlabeled viruses and (2) it can be completely abolished when bromelain-treated viruses are labeled with R18 and used in the assay (not shown). The slow dequenching process persists with HA-depleted viruses, which is indicative for R18 marker transfer in agreement with previous results (Wunderli & Ott, 1990). Superposition of two dequenching components could theoretically result from limited marker dilution as encountered if R18-labeled LUV ($\phi \sim 150$ nm) fuse with PR8 viruses (ϕ lipid bilayer ~ 55 nm) (Ott and Wunderli, unpublished results). For incubations with R18-labeled viruses this explanation does not hold for the following reasons: on one hand, the two kinetic dequenching components appear independent of the size of the acceptor membrane, i.e., with SUV or LUV, and on the other hand, as several liposomes can fuse with one virus (Nir et al., 1986), the large excess of unlabeled membranes in our assays precludes limitation of R18 dilution. The most likely explanation for the mixed dequenching reaction is the inactivation of PR8 viruses through R18 marker incorporation. This confirms the results obtained with biological assays (see above). By plotting the amount of fusion $[(DQ_{maxI} \times 100)/DQ_{maxI+II}]$ versus the initial quenching of the respective virus preparations, an inactivation curve for the R18-labeled PR8 viruses is obtained. With increasing concentration of the R18 marker in the viral membrane, the contribution of the fast dequenching reaction decreases exponentially. The extrapolated initial quenching which would still permit full fusion activity in the dequenching assay is ~20% (see Figure 2), in good agreement with the extrapolated value from infectivity assays ($Q_{initial}$ = 20–30%). On a molecular basis this means that inactivation of PR8 viruses starts above ~2.8 R18 molecules/HA trimer. This calculation is based on an average number of 500 HA trimers/virus (Klenk, 1991) and on the assumption that R18 is incorporated into the inner and outer leaflet of the viral lipid bilayer. For initial quench values between 60% and 80% used in most assays with R18-labeled viruses, the molar ratio of R18 to HA trimers is between 8:1 and 12:1. As HA molecules account for >80% of the soluble protein fraction removed from the virus surface by bromelain treatment, and seeing the one-to-one interaction of R18 with HA, we postulate that the inactivation of PR8 influenza viruses results from direct interaction of R18 molecules with HA molecules, which leads to blocking of the pH-induced conformational change. A partial reversibility of this inactivation could be demonstrated with a modified infectivity assay in which unadsorbed viruses were not removed before incubation at 37 °C (not shown). Under these conditions, virus titers after infection with 96% quenched R18-labeled PR8 viruses were increased by a factor of about 8 but still remained ~20-fold below the control titers obtained with unlabeled viruses. We interpret this as R18 transfer from receptor-bound viruses to the MDCK cell membrane before the virus particles reach a low pH compartment. In those viruses which can get rid of most of the R18 molecules in time, the HA trimers can later undergo a conformational change in the prelysosome, leading to fusion.

Evidence has recently come from various groups that inactivation through R18 is not only found with PR8 influenza viruses. In studies with RSV (respiratory syncytial virus) a

residual infectivity of 10% is mentioned after R18 labeling (Srinivasakumar et al., 1991). Very recently, Wilschut et al. (1991) reported on a slower and less extensive fusion reaction of R18-labeled X-99 influenza viruses with erythrocytes as compared to pyrene-PC-labeled influenza virosomes. As possible reason they suspected that exogenous R18 labeling did not result in uniform marker distribution on the viruses. Screening other publications on fusion assays with R18-labeled viruses, it is striking to note that fast fusion reactions occur, but DQ_{max} values for specific fusion do not exceed 0.4 (e.g., Nussbaum & Loyter, 1987; Pal et al., 1988; Sinangil et al., 1988; Clague et al., 1990), whereas in our standard assay with unlabeled viruses and R18-labeled liposomes (Wunderli & Ott, 1990) they reproducibly reach between 0.7 and 0.9. The low values measured with R18-labeled viruses are compatible with the hypothesis that although the extent of fusion is reduced through partial inactivation of the virus, estimates for fusion rates are not necessarily affected unless, as in our case, a slow dequenching reaction, namely, R18 transfer, takes place concomitantly. Our results provide strong evidence that, depending on the type of interacting partners, R18 labeling of biological membranes may yield qualitative rather than quantitative information. If very low initial quench values are chosen to avoid inactivation, very low DQ_{max} values result and the dequenching curves are not well enough defined to permit kinetic data analysis. In agreement, Morris et al. (1989) noted that dequenching was quite variable among experiments, and they suspected that no absolute measure for either rate or extent of fusion could be obtained with R18-labeled biological membranes.

Another aspect to be considered when using R18 as a membrane marker is the influence of the membrane composition on self-quenching of the fluorescent label (Scheule, 1987; Loyter et al., 1988; MacDonald, 1990). Quench curves of bilayers may differ significantly depending on the respective contents of cholesterol and other membrane-condensing lipids. No significant difference can be found between the quench curves of exogenously and endogenously labeled SUV and LUV, respectively. This is evidence against the hypothesis that with exogenous labeling R18 would be inserted into the outer layer only, whereas with endogenous labeling the marker would be evenly distributed in the two layers (Aroeti & Henis, 1986). In the case of PR8 influenza viruses, R18 surface densities are about 10 times higher than those of exogenously and endogenously labeled liposomes (SUV and LUV) to reach the same initial quench values. Detailed analysis of the various quench curves with the Stern–Volmer plot reveals a significant difference in the contribution of R18 monomers and dimers to the dequenching. In the case of liposomes, dequenching can be fully attributed to monomer–monomer interactions [$DQ^{-1} = f(r^2)$]. The a_2 constant is the same for all R18-labeled liposomes tested (standard lipid composition, see Materials and Methods) independently of vesicle size and the type of labeling (exogenous vs endogenous). This constant contains three parameters, i.e., the unquenched fluorescence lifetime, τ^0, the dimerization constant, K_d, and the rate constant for dequenching by nonfluorescent dimers, k_{qd} (Arbeloa, 1981). The reproducibility in the production of R18-labeled vesicles as reflected by the constant value for a_2 permits comparative, quantitative membrane interaction studies with these liposomes. The situation with R18-labeled PR8 viruses is quite different. Fluorescence dequenching results not only from monomer–monomer interactions (r^2) but also from monomers only (r), i.e., [$DQ^{-1} = f(r, r^2)$]. The contribution of monomers to R18 quenching can, however, be suppressed by removal of the HA spikes with bromelain. This indicates that HA interferes with R18 fluorescence and leads to a quenching which is independent of R18 intermolecular interactions. Clear evidence for the particular role of HA in the quenching reaction was only obtained if PR8 viruses were labeled with R18 prior to bromelain digestion. The same result would be expected independent of whether removal of HA was performed before or after labeling with R18. However, in the case of R18 labeling after HA removal, monomer contribution to quenching did not disappear, indicating that the newly exposed epitopes of the membrane-bound residual HA molecules, i.e., after bromelain digestion, did also bind R18 and lead to R18 monomer quenching. The a_2 constant in the case of viruses was around 10, i.e., 100-fold lower than for R18-labeled liposomes. We also did experiments to exclude the possibility that the particular labeling behavior of PR8 could be due to the fact that we did not pass the labeled viruses over a Sephadex column to separate aggregates and micelles from the incorporated marker as suggested by Hoekstra et al. (1984). Before and after passage over a column, preparations in our hands did not differ regarding the initial quenching, and likewise no quantitative difference in the fusion/transfer kinetics was found. We attribute this to the fact that we use a 25 times lower protein concentration ($\sim$30 μg/mL) in the labeling mixture as compared to other groups. With higher concentrations we got macroscopically visible aggregates.

It has long been claimed that R18 does not undergo transfer to unlabeled membranes (Hoekstra et al., 1984; Morris et al., 1989). Evidence mainly came from studies with erythrocyte ghosts or LUV as donor and/or acceptor membranes. As we have previously shown, R18 is transferred efficiently from R18-labeled SUV to PR8 viruses (Wunderli & Ott, 1990). The results presented here indicate that transfer also takes place from R18-labeled PR8 viruses to SUV and LUV, although to a limited extent and by a factor of about 3 more slowly than from R18-labeled SUV to viruses. A systematic study revealed that the time to reach half-maximal dequenching for transfer increases with increasing size of the donor vesicle (Ott and Wuderli, unpublished experiments). R18 transfer can possibly interfere with the kinetic analysis of dequenching curves. In case of partial inactivation of the viral fusion activity, for instance, superposition of dequenching originating from R18 transfer can occur, which may go unnoticed. This occurs more as t^* for transfer varies with pH, as demonstrated for R18 transfer from SUV as well as from PR8 viruses. In both cases, t^* at pH 7.4 is about twice the t^* at pH 5.3 (data not shown). Therefore, the decision whether dequenching in a particular case is due to fusion or marker transfer cannot be made on the basis of pH dependence only. Control experiments with acid-inactivated or bromelain-treated viruses at low pH are crucial for the identification of a specific fusion reaction.

To sum up, our data suggest that caution is needed when using direct labeling of biological membranes. With the PR8 influenza virus, inactivation through incorporation of the R18 marker has been demonstrated in biological as well as fluorescence assays. It can be traced to an association of R18 molecules with HA molecules. Inactivation of the virus starts if >90% of the HA molecules have an R18 molecule bound. Unless the opposite can quantitatively be proven, it must be assumed that the R18 concentrations needed for self-quenching will possibly interfere with any membrane function. Low R18 concentrations, which do not interfere with viral activities, are not recommended because they lead to scattering of data points. Furthermore, quantitative kinetic dequenching studies

on membrane interactions are based on R18 dimer quenching. Contribution of the monomer quenching as demonstrated in labeled PR8 viruses may lead to superposition of at least two dequenching reactions and hamper quantitative analysis. Another aspect which cannot be neglected is lipid transfer. Indications are that it is influenced not only by the type of molecule but also by the size of the donor vesicle and the pH of the milieu (see above). It should be noted here that the t^* values for marker transfer also vary as a function of the initial quenching of the labeled species (Ott and Wunderli, unpublished results). Appropriate controls have to be performed for each experimental system. As it stands, the ideal method to study interactions with and in particular between biological membranes still has to be found. The R18 marker remains a very valuable tool to study membrane interactions, if used under carefully controlled conditions. For instance, integration into defined, artificial bilayers allows quantitative kinetic studies with unlabeled, intact PR8 influenza viruses (Wunderli & Ott, 1990; Ott and Wunderli, unpublished results). With the latter approach, extent and rates of fusion and lipid transfer under various conditions can be determined reproducibly.

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