Volume 243, number 2, 251–258 FEB 06668 January 1989

pH-dependent fusion of reconstituted vesicular stomatitis virus envelopes with vero cells

Measurement by dequenching of fluorescence

Marie-Therese Paternostre, R. Joel Lowy[+] and Robert Blumenthal

Section on Membrane Structure and Function, LTB, NCI, and [+]*Laboratory for Kidney and Electrolyte Metabolism, NHLBI, NIH, Bethesda, MD 20892, USA*

Received 10 November 1988

Reconstituted vesicular stomatitis virus (VSV) envelopes were formed by solubilization of the viral envelope with Triton X-100 followed by removal of detergent by direct addition of SM2 biobeads. We provide direct demonstration of fusion of reconstituted VSV with cells using fluorescent lipid and aqueous probes incorporated into the VSV virosomes during reconstitution. We show a direct comparison of the kinetics and pH profile of fusion with cells between reconstituted VSV and fluorescently labeled intact virus. With this preparation it is now possible to gain additional information about the role of cooperativity in viral protein-mediated fusion, and to permit construction of efficient vehicles for delivery of drugs and other materials into cells.

VSV; Membrane fusion; Reconstitution; Fluorescence probe

1. INTRODUCTION

To study molecular mechanisms by which the viral spike glycoprotein induce membrane fusion [1,2], it is necessary to manipulate the components involved. Reconstitution of the viral fusion proteins into lipid vesicles (virosomes) offers several advantages for these sorts of studies. Although a large number of viral spike glycoproteins have been incorporated into lipid vesicles, only in a few cases it has been shown that the reconstituted product was functionally active in the same way as intact virus in inducing membrane fusion (see [3] for a review). Procedures have been developed for formation of reconstituted viral envelopes, whose fusogenic activity was very similar to that of the intact virus [4–6]. A successful method, first applied to Sendai virus, involves extraction of the viral envelope by Triton X-100 followed by detergent removal by direct addition of SM2 biobeads [6]. This method was then successfully applied to influenza virus [7–9]. Metsikko et al. [10] recently reported formation of vesicular stomatitis virus (VSV) envelopes by solubilization in the detergent *n*-dodecyl octaethylene monoether, followed by removal of the detergent in two steps. These virosomes induced polykaryons in BHK-21 cell monolayers at a pH threshold of 5.8.

In the present study we show that functionally active VSV envelopes were formed by solubilization in Triton X-100, followed by removal of the detergent in one step. As monitored directly by fluorescence techniques, these virosomes fused with Vero cells at a pH threshold of 6.3, with a kinetics similar to that of the intact virus.

Correspondence address: R. Blumenthal, Section on Membrane Structure and Function, LTB, NCI, Bethesda, MD 20892, USA

[+] *Present address:* Physiology Department, Armed Forces Radiobiology Research Institute, Bethesda, MD, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2. EXPERIMENTAL

2.1. *Materials*

N-4-Nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) was obtained from Avanti (Birmingham, AL), Triton X-100 (TX100) from Aldrich (Milwaukee, WI), *n*-dodecyl octaethylene monoether from Calbiochem (La Jolla, CA), cell culture media and Trypsin-EDTA were obtained from Gibco (Grand Island, NY), Biobeads (SM2) were purchased from Bio-Rad (Richmond, CA) and ^{3}H-TX100 from Dupont NEN (Wilmington, DE).

2.2. *Cell cultures*

Vero cells were grown to confluency in Dulbecco's minimal essential medium supplemented with 10% calf serum in 75 cm^2 plastic dishes. The fusion experiments were carried out with the cells in suspension. Incubation for 10 min at 37°C in 1 ml phosphate-buffered saline containing 0.5 mg trypsin and 0.2 mg EDTA yielded 2×10^7 cells/ml. Before the experiments the cells were washed three times by centrifugation in a solution containing 145 mM NaCl and 10 mM Hepes, pH 7.4 (NaCl-H).

2.3. *Virus*

VSV (Indiana) was grown on monolayer cultures of baby hamster kidney (BHK-21) cells and purified by sucrose velocity and density gradients, to approx. 1 mg of VSV protein per ml [11]. The sucrose solutions were made in a buffer containing 10 mM Tris, 1 M NaCl, and 1 mM EDTA, pH 7.5 (TNE). ^{3}H-labeled VSV was prepared by infecting BHK cells in the presence of [^{3}H]leucine or [^{3}H]glucosamine (5 μCi/ml growth medium). The specific activity ranged from 200–2000 cpm/μg VSV protein. The purified virus was stored at −70°C in a TNE buffer containing about 30% (w/v) sucrose.

2.4. *Solubilization of VSV and reconstitution of viral envelopes*

TX100 was added from a 10× concentrated solution to a suspension of VSV (about 1 mg/ml protein ≅ 0.2 mg/ml lipid). The final concentration of TX100 used for solubilization was calculated on the basis of:

$$([TX100] - CMMC)/[Lip] = 5.5 \quad (1)$$

where [TX100] is the total concentration of TX100; CMMC, the critical mixed micelle concentration of this detergent (0.18 mM) [12]; and [Lip], the total concentration of lipids. The formula was used to ensure an excess of TX100 for solubilization without denaturing the protein. The virus in the presence of the TX100 was maintained under gentle and continuous stirring for 1 h at 4°C for the solubilization to be complete. After solubilization the sample was centrifuged in a 70 TI rotor (Beckman Instruments) at 30000 rpm for 1 h at 4°C in order to remove the nucleocapsid. The supernatant containing the solubilized protein was collected, it contained about 75% of the total viral radioactivity. The virus preparation was labeled with [^{3}H]leucine, which labels all the proteins. Since VSV G-protein only comprises about 20% of total viral protein, the high recovery of radioactivity indicates that other proteins in addition to the G-proteins are 'solubilized' by the treatment with TX100. SDS-polyacrylamide gel electrophoresis performed on the TX100-solubilized preparation (not shown) indicated that VSV M and N proteins were extracted by this procedure in about similar amounts to the G-protein.

Incorporation of the fluorescent lipid probe NBD-PE into the virosome was performed by drying NBD-PE from a chloroform solution in a glass vial under N_2 to form a thin film. The amount of NBD-PE used was calculated such that the final concentration of the probe in the viral envelope would be 20% of total viral lipid. Then, the supernatant containing viral protein and lipid, as well as detergent was added to the NBD-PE film, and the sample was vortexed.

After solubilization of the fluorescent lipid, SM2 biobeads were added to the sample at a biobead (wet wt):detergent ratio of 20:1 (w/w). The biobeads were prewashed with methanol, distilled water and buffer, and always kept in buffer. The elimination of detergent was performed by direct contact with the biobeads under gentle stirring for 2 h on ice followed by 2 h at room temperature. Before any experiment, the samples were filtered through a 0.22 μm Millipore type Millex GV filter to remove aggregated virosomes.

Preparation of calcein-encapsulated virosomes involved addition of a calcein solution (200 mM) to the detergent-solubilized viral envelope before adding the biobeads. The final concentration of calcein in the detergent solution was 40 mM. Before use, the calcein-encapsulated virosomes were passed through a PD-10 column to remove any unencapsulated calcein.

2.5. *Fluorescence microscopy*

Virosomes were bound to subconfluent Vero cells which had been previously cultured on coverslips. Binding to cells was done at 4°C in pH 7.4 buffer (see below) for 45 min by flooding coverslips with virosome suspensions at 1/10 the concentration used in the spectrophotometric studies (see below). Coverslips were then mounted in a perfusion chamber on the stage of a microscope. The preparation was examined both with Schlieren

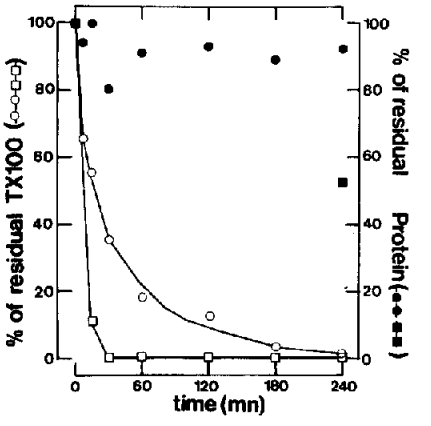


Fig.1. Elimination of TX100 by absorption to biobeads. The biobeads (50 mg/ml) were added to a suspension of VSV envelope (1.2 mg/ml protein, 0.24 mg/ml lipid) in TX100 (5 mg/ml). The elimination of TX100 (open symbols) and absorption of viral protein (closed symbols) were followed by radioactivity measurements. Experiments were performed at 4°C (circles) and 23°C (squares).

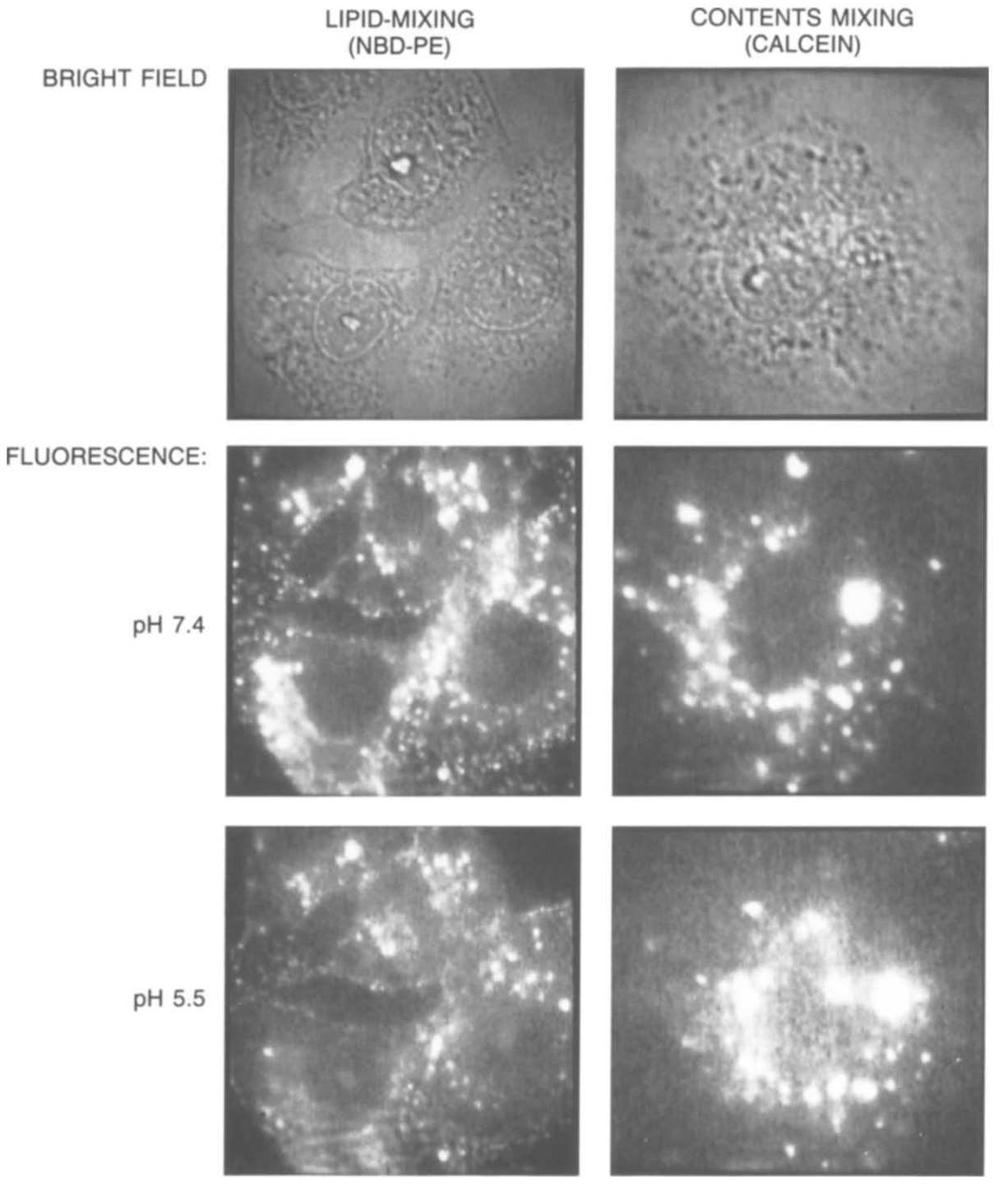


Fig.2. Fusion of VSV envelopes with Vero cells as detected by video fluorescence microscopy. Virosomes bound to Vero cells, which had been previously cultured on coverslips, were observed (see section 2) at 4°C and pH 7.4 (second row), and after 10 min incubation at 37°C and pH 5.5 (third row). The fields were the same as shown in the top row (bright field). The panels on the left represent virosomes containing the lipid probe NBD-PE, and those on the right the aqueous probe, calcein.

bright field optics [13] and fluorescence to select a group of cells for the experiment. The temperature was maintained at less than 10°C and pH 7.4 during chamber assembly and cell selection. The fusion experiments were begun by simultaneously switching the perfusate to the pH 5.5 buffer and increasing the chamber temperature to 37°C. The $t_{1/2}$ is 60 s and 13 s for the temperature change and chamber mixing, respectively. Alternating bright field and fluorescent images were recorded for 10 min. Fluorophores were excited with an argon laser light source using the 488 nm and 496 nm lines. The emission was filtered using a 510 nm dichroic and 514 nm long pass filter. The detector system was an intensified video camera system [14]. The image processor (Recognition Concepts Inc., Incline Village, Nevada) was used during the experiment to generate a 256 frame average of the images before recording. Images were subsequently digitally enhanced by black level thresholding and grey scale compression, to increase contrast, and then photographed from a television monitor. Care was exercised to use the same digital processing on all sets of images and that no loss of visible objects occurred due to these processing steps.

2.6. *Spectrofluorometry*

Fusion of R18-labeled VSV with Vero cells was carried out as described previously [15]. The assay with the NBD-PE-labeled virosomes was carried out in a similar fashion. The virosomes (100–200 μg protein) were added to 2×10^7 Vero cells in 1 ml buffer, pH 7.4, containing 145 mM NaCl and 10 mM Hepes, and incubated at 4°C for 45 min to form virosome-Vero complexes. The cells were washed in the same buffer and centrifuged at 300 × *g*. For kinetic experiments 50 μl of the virosome-Vero suspension was pipetted into a cuvette containing pH 7.4 buffer prewarmed to 37°C. After about one minute, sufficient to warm up the cells, the pH was changed by injecting 2–60 μl of 1 M Mes. The pH was always measured at the end of the experiment. Fluorescence was measured using an SLM 8000 spectrofluorometer with 1 s time resolution at 473/520 nm (excitation/emission) wavelengths for the virosomes containing NBD-PE, and at 565/590 nm (excitation/emission) wavelengths for intact VSV labeled with R18. Cut-off filters (510 for NBD and 570 for R18) were used on the emission to reduce scatter contributions.

We express our data as the % fluorescence dequenching (FDQ) calculated from the fluorescence changes according to:

$$\% \text{ FDQ} = 100 \times (F - F_0)/(F_d - F_0) \quad (2)$$

where F is the fluorescence of virosome-Vero complex at a given

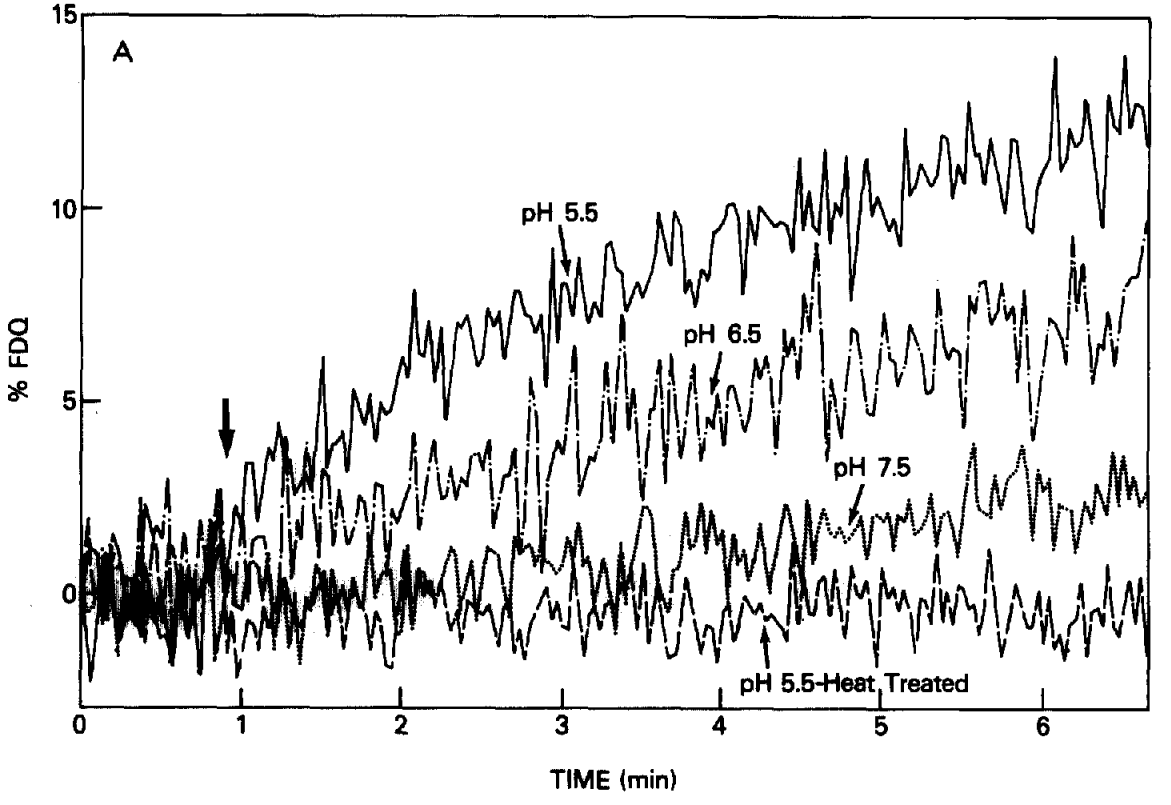


Fig.3. Kinetics of fluorescence dequenching (FDQ) of virosomes or intact virus with Vero cells. NBD-PE-labeled virosomes (A) or R18-labeled VSV (B) were bound to Vero cells, washed, and suspended with trypsin-EDTA as described in section 2. 50 μl of the complex was injected into 2 ml PBS, pH 7.4, prewarmed at 37°C. About 1 min later, the pH in the medium was changed by adding 6–20 μl of 0.5 M Mes (indicated by the arrow). Percent FDQ was calculated according to eqn 2. The traces at different pH are marked on the figure. A control is shown with virosomes treated for 15 min at 56°C.

time point, F_0 is the fluorescence of the complex at time zero, and F_d the total fluorescence measured by disrupting the cells in the detergent *n*-dodecyl octaethylene monoether.

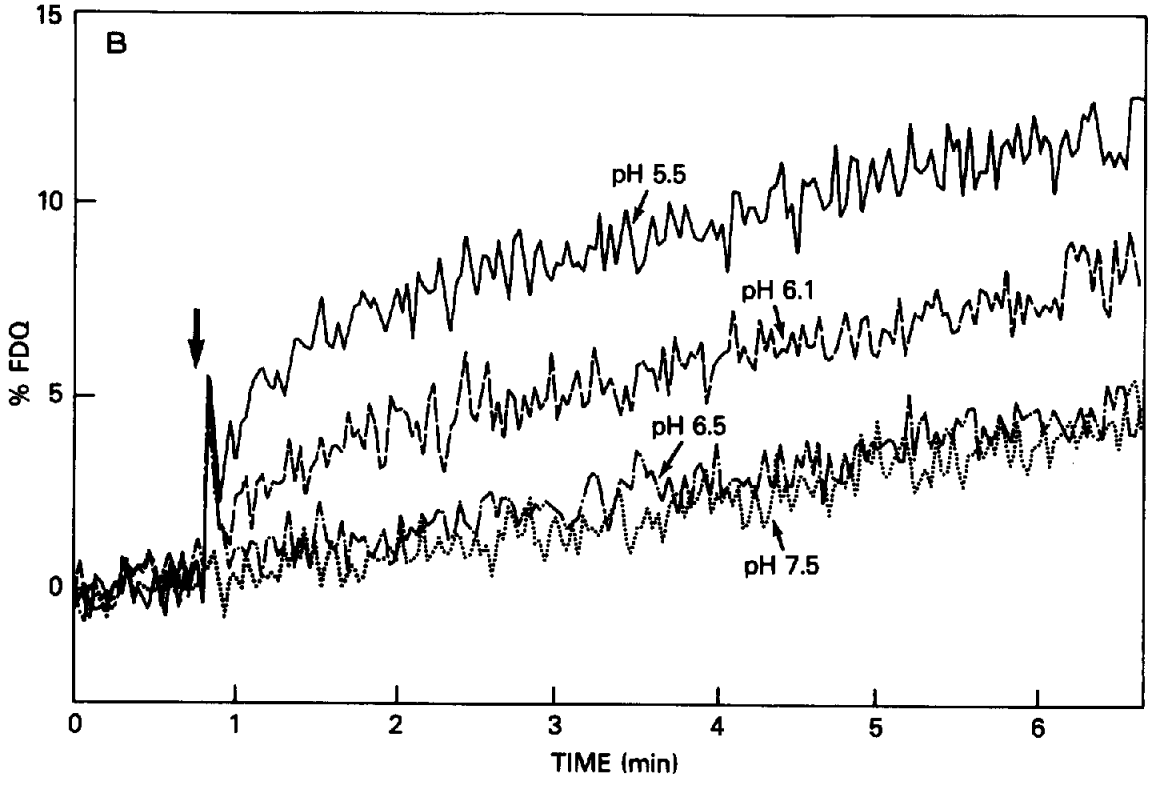
3. RESULTS

3.1. *Elimination of detergent and formation of virosomes*

The amount of detergent and protein remaining in the suspension as a function of time after addition of biobeads is shown in fig.1. The figure shows a rapid removal of detergent at 23°C, with a loss of about 45% protein after 4 h. On the other hand, at 4°C the removal of detergent was slower with little loss of protein. Removal of detergent at 23°C for 4 h led to the formation of particles which were not fusogenic (not shown). On the other hand, removal of TX100 at 4°C was incomplete even after 4 h. In order to balance these two factors, we incubated the TX100-solubilized VSV mixture with biobeads for 2 h at 4°C, followed by 2 h at 23°C, leading to the formation of fusogenic virosomes (see below) without residual TX100. Centrifugation of the preparation on a discontinuous sucrose gradient for 20 h at 30000 rpm in a SW55 rotor (Beckmann Instruments, Palo Alto, CA) resulted in banding of 80% of the lipid associated with 80% of protein in the fraction containing 20–25% sucrose (not shown).

3.2. *Fusion of virosomes with Vero cells detected by fluorescence microscopy*

Fig.2 shows the light micrographs for the fusion experiments with both NBD-PE and calcein labeled virosomes. The field is 40 and 60 μm for the NBD-PE and calcein experiments, respectively. The pH 5.5 image in both experiments is from 10 min following the change from pH 7.4. In both cases the fluorescent particles are very heterogeneous in size, although in general more very small particles were observed in the NBD labeled preparation. A similar widely varying size distribution of particles was observed for both labels when virosomes attached to poly-lysine coated coverslips, in the absence of cells, were examined. It is assumed that this distribution of fluorescence probe is due to aggregates of different



numbers of virosomes, although heterogeneity of individual virosome size and/or labeling intensity cannot be excluded. After 10 min there is a considerable reduction in the number of either NBD or calcein labeled particles visible on the cell surface.

Quantitation of particle loss was determined for both dyes by counting all objects visible in the field before and after the pH change. For both calcein and NBD-PE virosomes about 25% of the fluorescent particles disappeared from the cell surface after exposure to pH 5.5 for 10 min at 37°C. For both virosome preparations the decrease appeared to be predominantly of small particles; this is particularly evident in the NBD-PE-labeled preparation (see fig.2).

Concomitant with this reduction in punctate fluorescence there was an increase in the diffuse fluorescent labeling of the cells. This change can be most readily seen with the volume marker, calcein, in regions of the cell corresponding to the nucleus and cell periphery which became considerably brighter. These changes in the pattern of fluorescence are consistent with a fusion mediated redistribution of the probes, providing visual evidence of virosome-vero cell fusion. With both labels numerous punctate objects, generally being the largest particles, remain associated with the cells even after 10 min at low pH. This is consistent with the spectrofluorometric data (see below) and confirms that not all of the fluorescent probe is associated with fusion competent particles. The data also suggest that association into large aggregates can inhibit the fusion process.

3.3. *Kinetics of fusion as measured by fluorescence dequenching*

Fig.3 shows the kinetics of fusion of reconstituted VSV as well as of intact VSV with Vero cells. At pH 5.5 fluorescence of virosome-vero cell complexes increased with an approximately exponential time course reaching, after 6 min, a value which corresponding to 13% fluorescence dequenching, calculated according to eqn 2 (fig.3A). As a control, we treated virosomes for 15 min at 56°C, since this treatment inactivates fusogenic activity of intact VSV, although binding to cells is not affected [16]. There was no fluorescence change at pH 5.5 with virosomes treated for 15 min at 56°C (fig.3A), indicating that the fluorescence dequenching observed with untreated virosomes was due to the fusogenic activity of the VSV envelope proteins. The patterns of increase in fluorescence were remarkably similar for virosome (fig.3A) and intact virus (fig.3B) fusion, given the fact that different probes were used.

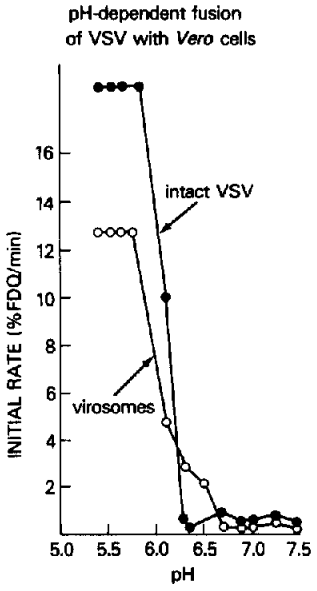


Fig.4. pH dependence of fusion. The rate of FDQ as a function of pH calculated from maximal slopes of kinetic curves such as those shown in fig.3. Open and closed circles represent virosomes and virus, respectively.

In fig.4 the initial rate of fluorescence dequenching is plotted as a function of pH both for virosomes and intact VSV.

The threshold for fusion of virosome with cells is at pH 6.3, similar to that of intact virus, and to VSV-induced cell fusion [17].

4. DISCUSSION

We show that functionally active VSV envelopes were formed by solubilization in Triton X-100, followed by removal of the detergent in one step by direct addition of biobeads. We provide a direct demonstration of fusion of the virosomes with cells using a lipid mixing probe as well as an aqueous compartment mixing probe. The virosomes showed the same pH thresholds and extents of fusion as intact virus. Moreover, control virosomes treated at 56°C showed no fluorescence changes, indicating that the effect we observed was

specific for viral fusion activity. The fluorescent lipid probe we incorporated during reconstitution is more similar to phospholipids, than the fatty acid probe, R18, used for studies with intact virus. Nevertheless the kinetics of fluorescence increases were very similar.

The VSV envelopes formed by solubilization in the detergent *n*-dodecyl octaethylene monoether, followed by removal of the detergent in two steps induced polykaryons in BHK-21 cell monolayers at a pH threshold of 5.8 [10]. In that study it was noted that direct addition of biobeads to solubilized VSV envelopes at 23°C resulted in the formation of virosomes with inconsistent fusogenic properties [10]. That agrees with our observation concerning removal of TX100 at 23°C. As shown in fig.1, TX100 removal at 23°C occurs quite fast and with protein loss. Presumably too rapid a removal of detergent results in aggregation of protein and lipid into separate structures, and formation of multilamellar vesicles [3,18]. However, when we initially incubated the TX100-solubilized envelopes with biobeads at 4°C, and subsequently incubated them at 23°C to remove residual detergent, we obtained virosomes, whose fusogenic properties were very reproducible. Five different virosome preparations yielded the same fluorescence dequenching kinetics with Vero cells as seen in figs 3 and 4.

We have not yet optimized conditions for solubilization of VSV by detergent and removal of detergent. For instance, the preparation contains about the same amount of VSV M and N protein as the intact virus. Presumably extraction in high salt releases those proteins from the nucleocapsid [11]. However, Walter (unpublished) has observed that solubilization of VSV by TX100 in normal saline also results in extraction of VSV M and N protein. Another problem with the reconstitution procedure is formation of virosome aggregates. We have partially overcome that problem by filtration of the product through a 0.22 μM Millipore filter.

The patterns of increase in fluorescence were remarkably similar for virosome (fig.3A) and intact virus (fig.3B) fusion. However, some differences were noted. Although the extents of fluorescence dequenching were the same for virosome and intact virus, the initial rates were faster with intact virus. Moreover, the steepness of the pH dependence was higher for intact virus (fig.4). We have previously related rates of viral spike glycoprotein-mediated fusion to cooperative interactions between such proteins [2,19]. Perhaps the highly organized disposition of the G-protein in the intact virus gives rise to a higher degree of protein cooperativity, and consequently to a faster fusion rate and steeper pH dependence. We also note that the rate of fluorescence dequenching at pH 7.5, which we have interpreted as entry via the endocytic pathway [15], was higher with intact virus. This difference might be attributed either to cooperativity of fusion, or to the fact that virosomes form larger aggregates, and consequently do not readily enter the endocytic compartment via coated pits.

Improving procedures for solubilization of VSV by detergent and removal of detergent might result in better organization of the G-protein in the membrane, and consequently a higher degree of cooperativity in mediating membrane fusion. Such products would enable us to gain additional information about the role of cooperativity in viral protein-mediated fusion, and to permit construction of efficient vehicles for delivery of drugs and other materials into cells.

Acknowledgements: We thank Drs Anne Walter, Anu Puri, Debi Prasad Sarkar and Michel Ollivon for their valuable help throughout the course of this work. We also thank Dr Kenneth R. Spring for generously making available to us the low light level fluorescence microscopy and image processing system developed in his laboratory. M.-T.P. was partially supported by the NSF-CNRS exchange of scientists fund (USA87003) and by funds partly provided by the International Cancer Research Data Bank Programme of the National Cancer Institute, NIH (US), under contract no. No1-CO-65341 (International Cancer Research Technology Transfer – ICRETT) of the International Union Against Cancer.

REFERENCES

[1] White, J., Kielian, M. and Helenius, A. (1983) Q. Rev. Biophys. 16, 151–195.
[2] Blumenthal, R., Puri, A., Sarkar, D.P., Chen, Y., Eidelman, O. and Morris, S.J. (1988) in: Cell Biology of Virus Entry, Replication and Pathogenesis (Compans, R. et al. eds) UCLA Symposia on Molecular and Cellular Biology 90, Alan Liss, New York, in press.
[3] Walter, A., Eidelman, O., Ollivon, M. and Blumenthal, R. (1988) in: Cellular Membrane Fusion: Fundamental Mechanisms and Applications of Membrane Fusion Techniques (Wilschut, J. and Hoekstra, D. eds) Marcel Dekker, New York, in press.

[4] Uchida, T., Kim, J., Yamaizumi, M., Miyake, Y. and Okada, Y. (1979) J. Cell Biol. 80, 10–20.
[5] Loyter, A. and Volsky, A. (1982) Cell Surf. Rev. 8, 215–266.
[6] Vainstein, A., Hershokovitz, M., Israel, S., Rabin, S. and Loyter, A. (1984) Biochim. Biophys. Acta 773, 181–188.
[7] Nussbaum, O., Lapidot, M. and Loyter, A. (1987) J. Virol. 61, 2245–2252.
[8] Lapidot, M., Nussbaum, O. and Loyter, A. (1987) J. Biol. Chem. 262, 13736–13741.
[9] Stegmann, T., Morselt, H.W., Booy, F.P., Van Breemen, J.F., Scherphof, G. and Wilschut, J. (1987) EMBO J. 6, 2651–2659.
[10] Metsikko, K., Van Meer, G. and Simons, K. (1986) EMBO J. 5, 3429–3435.
[11] Newcomb, W.W., Tobin, G.J., McGowan, J.J. and Brown, J.C. (1982) J. Virol. 41, 1055–1062.
[12] Paternostre, M.T., Roux, M. and Rigaud, J.L. (1988) Biochemistry 27, 2668–2677.
[13] Axelrod, D. (1981) Cell Biophys. 3, 167–173.
[14] Spring, K.R. and Smith, P.D. (1986) J. Microsc. 147 (pt 3), 265–278.
[15] Blumenthal, R., Bali-Puri, A., Walter, A., Covell, D. and Eidelman, O. (1987) J. Biol. Chem. 262, 13614–13619.
[16] Puri, A., Grimaldi, S. and Blumenthal, R. (1989) in preparation.
[17] White, J., Matlin, K. and Helenius, A. (1981) J. Cell Biol. 89, 674–679.
[18] Rigaud, J.L., Paternostre, M.T. and Bluzat, A. (1988) Biochemistry 27, 2677–2688.
[19] Blumenthal, R. (1988) Cell Biophys. 12, 1–12.