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Biochimica et Biophysica Acta 1278 (1996) 98–104

BBA Biochimica et Biophysica Acta

Effect of X31 influenza virus fusion on phosphatidylserine asymmetry in erythrocytes

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Received 13 March 1995; accepted 17 July 1995

Abstract

Influenza virus fusion is mediated by its fusion protein, hemagglutinin (HA). HA undergoes a low pH dependent conformational change that results in insertion into the cell membrane bilayer, formation of a fusion pore, and merging of membrane lipids and establishment of cytoplasmic continuity. Erythrocytes, which can serve as targets for influenza virus fusion, display an asymmetric transbilayer arrangement of their phospholipids. The effect of influenza virus fusion on erythrocyte phosphatidylserine asymmetry was determined. Influenza virus were bound to erythrocytes containing the fluorescent membrane probe NBD-PS in the inner leaflet. Induction of fusion by exposure to a low pH environment resulted in movement of PS to the outer leaflet of the cell as well as hemolysis. Insertion of the fusion protein into erythrocytes and subsequent fusion can be distinguished from hemolysis by examining the interaction of a soluble form of HA (BHA) with cells and by monitoring viral fusion at low temperatures. No hemolysis was observed under either condition. BHA binding and insertion into cells did not affect the asymmetry of PS. Incubation of influenza virus fusion at pH 5, 0°C resulted in complete fusion but no outward movement of PS was observed. These findings suggest the viral fusion pore does not involve a rearrangement of the transbilayer phospholipid organization of the target membrane.

Keywords: Influenza; Fusion; Phosphatidylserine; Asymmetry; Hemagglutinin; Hemolysis; Erythrocyte

1. Introduction

Following endocytosis influenza virus fuses with cells in a pH dependent manner (for review see [1–3]). The fusion protein of influenza virus, hemagglutinin (HA), undergoes a conformational change upon exposure to an acidic environment that results in insertion into cellular membranes. Complete fusion results in the merging of viral and cellular membranes and the establishment of cytoplasmic continuity. HA appears to mediate fusion through the formation of fusion pores. Several differing models for influenza fusion have been postulated [2,4–7]. However, almost all share the characteristic of proceeding through lipid intermediates. Although these intermediate structures have not been defined, intermediates that have been suggested are an inverted micellar structure and formation of a 'stalk' monolayer [7,8]. Influenza fusion does not appear to require an inverted micelle intermediate as liposomes incapable of forming such structures are nevertheless suitable targets for influenza virus fusion [9].

Erythrocytes and platelet cells exhibit an asymmetric localization of their phospholipids, with phosphatidylcholine (PC) and sphingomyelin primarily in the outer leaflet and the majority of phosphatidylethanolamine (PE) and virtually all of the phosphatidylserine (PS) in the inner leaflet [10]. The asymmetry of the aminophospholipids appears to be maintained by a Mg-ATP dependent translocase that selectively transports PE and PS from the outer to the inner leaflet [11]. Certain pathological and physiological situations leads to exposure of PS on the outer leaflet. Erythrocytes from patients with sickle cell anemia have elevated levels of PS on their outer leaflet [12] which contributes to enhanced clotting [13]. It has also been

Abbreviations: BSA, bovine serum albumin; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; PDA, pyridyldithioethylamine; PBS, phosphate-buffered saline; RBC, red blood cells; HA, hemagglutinin; BHA, bromelain digested hemagglutinin; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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Elsevier Science B.V.
SSDI 0925-4439(95)00193-X

shown that undifferentiated erythroid cells [14,15] and aged erythrocytes [16] present PS on their outer leaflet. Activation of platelets resulting in an increase in cytoplasmic calcium levels induces the rapid exposure of PS on the outer leaflet of these cells (reviewed in [17]). This physiological response is crucial for formation of a blood clot at the site of injury.

PS movement to the outer leaflet can also be induced experimentally. Hypotonic lysis results in the formation of hemolytic pores and the loss of PS asymmetry [18,19]. Electrically-induced fusion of erythrocytes has been shown to result in the exposure of PS at the cell surface [20]. We wished to determine if influenza virus fusion, because of its interaction with the membrane bilayer and formation of a fusion pore, induces a reorganization of the transmembrane phospholipid arrangement of the erythrocyte plasma membrane bilayer. Influenza virus can be induced to fuse with the plasma membrane bilayer of cells by subjecting the bound virus-cell to an acidic environment [21]. In this study we monitored levels of a fluorescent PS analog (NBD-PS) on the outer leaflet of RBC after interaction with influenza virus. We have determined that significant amounts of this probe move to the outer leaflet following viral fusion and hemolysis. Under conditions where hemolysis is not observed NBD-PS remains in the inner leaflet in spite of the establishment of complete fusion between virus and cells. Therefore, influenza virus fusion appears to induce little or no rearrangement of PS asymmetry in target erythrocytes other than through hemolysis.

2. Materials and methods

2.1. Reagents

The following reagents were purchased: purified influenza virus (X31 strain) from SPAFAS (Storrs, CT); C6-NBD-PS from Avanti Polar Lipids (Alabaster, AL); bromelain, ricin-Sepharose, neuraminidase, and bovine serum albumin (BSA) from Sigma (St. Louis, MO); NBD-taurine and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) from Molecular Probes (Eugene, OR). Pyridyldithioethylamine (PDA) was graciously provided to us by Alan Schroit. GP4F cells (bovine papilloma virus-transformed NIH 3T3 cells constitutively expressing HA) were the kind gift of Judy White. Fresh human blood was obtained from the NIH blood bank. Phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) was prepared by the NIH media section.

2.2. Effect of influenza virus fusion on phosphatidylserine (PS) asymmetry

RBC were washed three times with PBS containing 10 mM glucose. Cells were resuspended at 50% hematocrit. To load NBD-PS on the inner leaflet RBC were labeled with NBD-PS (2 μg NBD-PS/10 μl EtOH/$2 \cdot 10^8$ RBC/ml PBS) for 1 h at 37°C. Cells were centrifuged to a pellet, washed once with 1 ml of 1% BSA in PBS to remove NBD-PS from the outer leaflet, then washed twice with PBS to remove residual BSA. The location of the NBD-PS with respect to the inner and outer leaflets was determined by centrifuging cells in the presence or absence of 1% BSA. % NBD-PS inside was calculated as: [with BSA/without BSA] × 100. X31 influenza virus was incubated with labeled RBC at 4°C to promote binding, then washed with PBS to remove unbound virus. Citric acid was added to achieve the fusogenic condition of pH 5. Samples were pelleted through PBS with or without 1% BSA in an Eppendorf microcentrifuge (Brinkman Instruments, Westbury, NY) to determine the amount of NBD-PS remaining in the inner leaflet. The supernatant was collected and hemoglobin content monitored by absorbance at 540 nm on a Bio-Tek Microplate reader (Winooski, VT). % Hemolysis was calculated as: [(RBC + virus) − (RBC only)/(RBC + Triton X-100) − (RBC only)] × 100. RBC pellets were solubilized with 1% Triton X-100 and the NBD fluorescence quantitated on a Jasco spectrofluorometer (Easton, MD) set at 470 nm excitation and 530 nm emission wavelengths.

2.3. Fusion of GP4F cells with NBD-taurine loaded RBC at 4°C

Loading of RBC with NBD-taurine and subsequent fusion with GP4F was performed essentially as described [22]. Briefly, GP4F cells in T-75 flasks were treated with 0.22 mg/ml neuraminidase, washed, then incubated with NBD-taurine loaded RBC ($5 \cdot 10^8$ total, 5 ml) for 30 min at room temperature to promote binding. After extensive washing cells were harvested with either 0.5% trypsin or enzyme-free buffer. Following a wash with PBS cells were resuspended and stored on ice. Continuous NBD fluorescence dequenching was monitored with a SLM 8000 spectrofluorometer (SLM Instruments, Urbana, IL) attached to a water/ethanol bath set to 0°C. The temperature in the cuvette was 2–4°C. Excitation and emission wavelengths were set at 470 and 540 nm, respectively. Aliquots of GP4F-RBC complexes were added to 2 ml PBS in a cuvette, after which citric acid was added to lower the pH to 5. Fusion extents were normalized to maximal fluorescence dequenching, determined by the addition of Triton X-100 (0.05% final concentration).

2.4. Preparation of bromelain digested hemagglutinin (BHA)

BHA from X31 influenza virus was prepared as described [23,24]. Briefly, virus was incubated with bromelain overnight, 37°C, at a 1:2 virus/bromelain ratio in 0.1 M Tris, 1 mM EDTA, 50 mM 2-mercaptoethanol, pH 7.2.

Supernatant was collected following centrifugation of the mixture and BHA was isolated by ricin-sepharose chromatography. BHA was eluted with 0.2 M galactose, dialysed, and concentrated using a Centricon 30 microconcentrator. Protein concentration was determined by BCA protein detection assay (Pierce, Rockford, IL).

3. Results

3.1. Influenza virus fusion induced loss of PS asymmetry

Influenza virus fusion is assumed to proceed through the formation of fusion pores in the target membrane (reviewed in [1,25]). To determine if the fusion of influenza virus with erythrocytes induces a perturbation in the PS bilayer asymmetry the X31 strain of influenza was induced to fuse with RBC containing a fluorescent analog of PS in the inner leaflet of the plasma membrane bilayer. RBC were incubated with NBD-PS, which is rapidly translocated to the inner leaflet [26,27]. Residual NBD-PS on the outer leaflet is removed by washing with 1% BSA. Such a treatment results in 95–99% of the exogenous PS in the inner leaflet [18]. X31 influenza virus was bound to these labeled RBC and exposed to either fusogenic or non-fusogenic conditions. At pH values that are known to result in membrane fusion (pH 5, 37°C) [28] a significant loss of PS asymmetry was observed (Fig. 1). Under conditions where there is no membrane fusion (neutral pH or 5 min incubation at pH 5, 4°C) there was no change in the asymmetry of PS. The loss of PS asymmetry was independent of a direct effect by the low pH since incubation without virus at either pH values did not affect the PS asymmetry.

The kinetics of the loss of PS asymmetry as well as the concentration dependence of X31 virus was determined. PS asymmetry was lost over the course of 30 min at 37°C (Fig. 2). The movement of PS to the outer leaflet was inversely proportional to the hemolysis of the target erythrocytes. This correlation raised the possibility that the loss of PS asymmetry was due to movement of phospholipids through hemolytic pores and not the fusion pores. The concentration dependence showed maximal loss of PS asymmetry at 1–10 μg X31 virus/$2 \cdot 10^8$ RBC, again corresponding to the levels of hemolysis induced by influenza fusion (Fig. 3).

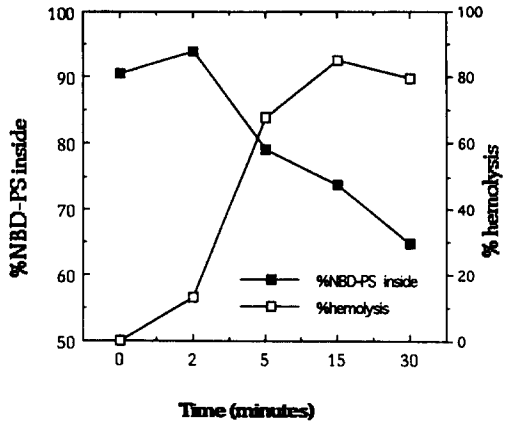

Fig. 2. Kinetics of loss of PS asymmetry and hemolysis induced by X31 influenza virus. RBC containing NBD-PS on the inner leaflet were bound to X31 influenza virus as described in Fig. 1. After addition of citric acid to lower the pH to 5 samples were incubated at 37°C for given times. Aliquots were centrifuged through PBS ± 1% BSA and %NBD-PS inside was calculated. Absorbance of supernatants were monitored at 540 nm to monitor hemoglobin release. RBC ± Triton X-100 served as negative and positive controls, respectively. % Hemolysis was calculated as: [(RBC + virus) − (RBC only)/(RBC + Triton X-100) − (RBC only)] × 100.

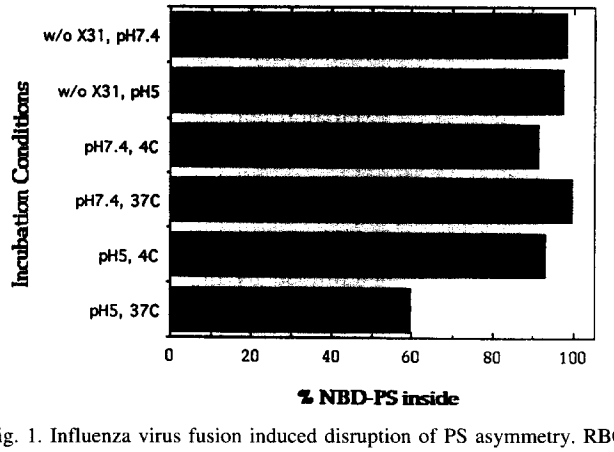

Fig. 1. Influenza virus fusion induced disruption of PS asymmetry. RBC were loaded with NBD-PS on the inner leaflet as described in Section 2. 10 μg of X31 influenza virus was bound to $4 \cdot 10^7$ RBC for 30 min on ice, washed, and incubated under the given conditions for 5 min. Aliquots were collected and centrifuged through PBS ± 1% BSA. % NBD-PS inside was calculated as: [with BSA/without BSA] × 100.

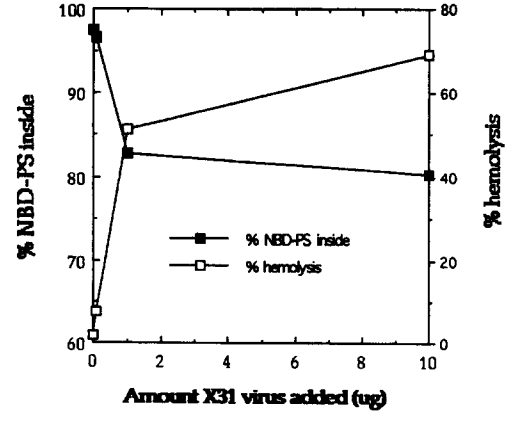

Fig. 3. Titration of X31 influenza virus and effects on PS asymmetry and hemolysis. 0, 0.1, 0, or 10 μg of X31 influenza virus was incubated with RBC/NBD-PS for 30 min on ice to promote binding. After washing samples were adjusted to pH 5 and incubated for 10 minutes at 37°C. % NBD-PS inside and % hemolysis was calculated as described in Section 2.

3.2. Separation of fusion events from hemolysis

Incubation of X31 influenza virus with erythrocytes at pH 5, 37°C results in fusion but it also induces hemolysis via hemolytic pores [29]. In order to distinguish the effects on PS asymmetry from fusion and hemolysis we attempted to define conditions under which influenza virus interacted with cells but did not induce hemolysis. It has been previously shown that bromelain digestion of influenza virus yields a soluble form of HA (BHA) that exhibits identical low pH dependent conformational changes as that observed for the intact HA [24,30]. Although BHA does not induce fusion, it does insert into liposomes [31–34] and erythrocyte ghosts [35]. However, BHA does not induce any hemolysis of erythrocytes, even at high concentrations [29]. Therefore, the interaction of BHA with erythrocytes can determine if the initial insertion of the fusion protein into target membranes could induce a loss of PS asymmetry. BHA was incubated with NBD-PS loaded erythrocytes and incubated at pH 5, 37°C. Even after the addition of 50 μg of BHA to $4 \cdot 10^7$ RBC no change in PS asymmetry was observed (Table 1). This corresponded with a lack of hemolysis. Incubation of 10 μg intact X31 virus resulted in 80% hemolysis and movement of 45% of the NBD-PS to the outer leaflet. The possibility that the apparent lack of disruption of PS asymmetry is compensated by the aminophospholipid translocase was addressed by treating cells with inhibitors of the translocase (sodium fluoride and pyridyldithioethylamine) prior to influenza virus interaction. Treatment with inhibitors did not lead to the appearance of PS in the outer leaflet (Table 1). Therefore the insertion of HA into the erythrocyte membrane is insufficient to induce perturbation of the phospholipid asymmetry.

Cold fusion of influenza virus is described as the diffusion of fluorescent probes from the virus membrane into target liposomes or cells at pH 5, 0°C [6]. Under these conditions we did not observe any loss of PS asymmetry nor any hemolysis, even at incubations of 100 μg virus with RBC for 1 h at pH 5, 4°C (Fig. 4). Significant membrane fusion is observed within 30 min under similar conditions [6,35]. The absence of any perturbation in PS asymmetry in the presence of membrane fusion suggests viral fusion does not involve widespread redistribution of the inner leaflet lipids.

Table 1
Effect of BHA on NBD-PS asymmetry and hemolysis

BHA (μg)	%NBD-PS inside [a]		%Hemolysis [a]	
	− inhibitor	+ inhibitor [b]	− inhibitor	+ inhibitor [b]
0	94	87	2	3
2	97	87	1	2
5	94	90	4	4
10	99	91	6	7
20	98	90	5	5
50	97	88	4	5
+ X31 virus	55	58	80	77
pH 7	90	94	3	1

[a] Listed amounts of BHA or 10 μg of intact X31 virus were incubated with RBC (± inhibitors), washed, and exposed to pH 5 or pH 7.4 conditions for 30 min. %NBD-PS inside and %hemolysis were calculated as described in Section 2.

[b] RBC were incubated with 10 mM NaF for 30 min at 37°C prior to addition of BHA and subsequently incubated with 1 mM PDA for 30 min at 4°C.

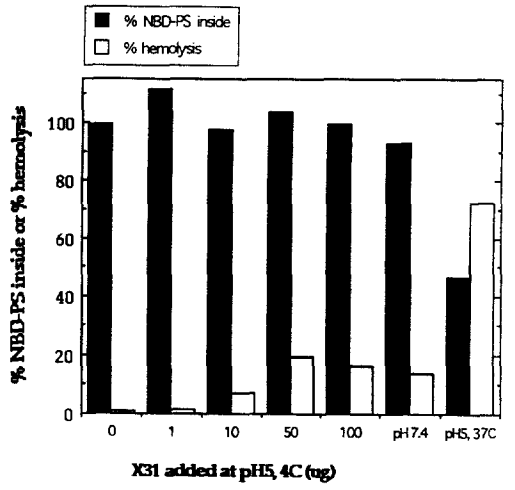

Fig. 4. Incubation of X31 influenza virus at pH 5, 4°C with NBD-PS loaded RBC. RBC loaded with NBD-PS were bound to 0, 1, 10, 50, or 100 μg X31 influenza virus and incubated at pH 5 or pH 7.4 for 60 min on ice. One sample was incubated with 10 μg virus at pH 5, 37°C as a positive control. After washing %NBD-PS remaining and %hemolysis was determined.

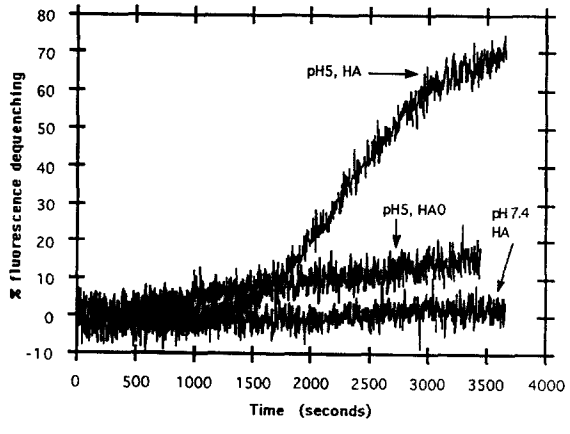

Fig. 5. Fusion of NBD-taurine loaded RBC with GP4F cells. RBC were loaded with NBD-taurine and bound to GP4F cells. After washing aliquots were added to cuvettes containing PBS preadjusted to 4°C. Continuous measurement of fluorescence was initiated and the pH adjusted to 5 after 20 s. HA samples had been treated with trypsin to render the fusion protein active whereas HA0 samples still contained non-fusogenic HA0 proteins. Fusion extents were normalized to maximal fluorescence dequenching, as determined by addition of Triton X-100.

3.3. Complete fusion occurs at pH 5, 4°C

An alternative possibility for the maintenance of PS asymmetry despite viral fusion is that the redistribution of fluorescent membrane probes is due to hemifusion, i.e., a connection between virus and cell that is restricted to outer leaflet lipid movement without continuity between the aqueous contents. In a hemifusion state the inner leaflet lipids remains intact and therefore no loss in PS asymmetry is expected. Hemifusion can be discerned from complete fusion by monitoring the transfer of fluorescent aqueous markers. Because of the technical difficulties of monitoring contents mixing from virus to cell we monitored the movement of the aqueous probe, NBD-taurine, from erythrocytes to cells constitutively expressing HA. These GP4F cells fuse with erythrocytes in a pH dependent manner identical to that of intact virus [36,37]. Fig. 5 shows the RBC loaded with NBD-taurine fuses with GP4F cells, albeit after approximately a 20 min delay. The ability of HA to mediate complete fusion at pH 5, 4°C demonstrates the maintenance of PS asymmetry under these conditions (Fig. 4) is not due to a lack of fusion pore formation.

4. Discussion

The hypotonic lysis of erythrocytes in the absence of divalent cations has been shown to result in the complete randomization of PS [18]. Although use of a long chain insoluble PS analog results in a smaller amount of PS ($\sim$ 15%) on the outer leaflet following hypotonic hemolysis [19] it is nevertheless significantly greater than the untreated erythrocyte. Disruption of the erythrocyte membrane by electrically induced fusion also results in movement of PS to the outer leaflet [20]. As influenza virus involves perturbation of the cell membranes and formation of a fusion pore we were interested to determine if viral fusion could also affect the PS asymmetry of erythrocytes. Our results show that influenza virus fusion results in movement of PS from the inner to the outer leaflet (Fig. 1). It is known that influenza virus fusion results in extensive hemolysis [21,29], which is readily observed as hemoglobin release into the supernatant (Figs. 2 and 3). Therefore, the loss of PS asymmetry may be attributed to movement of PS through fusion pores, hemolytic pores, or some combination of the two.

In order to determine the contribution of the fusion pore to perturbation of PS asymmetry we selected two conditions under which influenza HA interacts with erythrocyte membranes without inducing hemolysis. These are soluble HA insertion and 4°C fusion. BHA is similar to intact HA in that both undergo a low pH dependent conformational change that results in a greater association with lipid vesicles [24,30]. However, no hemolysis occurs when BHA is added to erythrocytes, even at high concentrations [29]. Direct photolabeling studies have demonstrated that BHA from X31 virus is capable of inserting into target membranes prior to the onset of fusion [31,35] but fusion is not induced by BHA. Therefore the interaction of BHA with erythrocytes could monitor the effect of HA insertion on PS asymmetry. In the absence of hemolysis there appears to be no randomization of PS (Table 1). It was recently shown that phospholipid flip-flop can be induced in liposomes by peptides capable of forming pores [38]. However, other peptides appeared to intercalate into membranes without perturbing the asymmetrically loaded lipids. Thus it appears that the interaction of the HA ectodomain with erythrocytes is not sufficient for fusion pore formation. This agrees with photolabeling studies that showed the HA fusion peptide appears to penetrate only into the outer leaflet of the membrane bilayer [39]. It is interesting that a lipid anchored form of HA constitutively expressed in cells mediates a state of hemifusion, i.e., membrane continuity without cytoplasmic connection [40]. In a state of hemi-fusion the asymmetric location of NBD-PS in the inner leaflet precludes it from being directly perturbed by BHA insertion.

The X31 strain of influenza virus is capable of fusing with liposomes and cells at pH 5, 0°C, after a 4–8 min delay period [6]. A 1 h incubation of virus with erythrocytes at pH 5, 4°C, does not result in any significant hemolysis (Fig. 4). This distinction allows us to distinguish the effects of the hemolytic pores from the fusion pores on the perturbation of the PS asymmetry. The absence of PS movement under conditions where membrane fusion occurs but hemolysis does not (Fig. 4) suggests that formation of viral fusion pores does not perturb the transbilayer asymmetry of the target membrane. As with the BHA experiments the aminophospholipid translocase does not appear to be counteracting outward PS movement since translocation of PS is blocked by a 4°C incubation [26].

A general estimation of the number of fusion pores involved in pH 5, 4°C fusion shows the lack of PS redistribution is unlikely to be due to insufficient numbers of virus particles associating with the erythrocyte. The highest amount of X31 virus that we have added to the erythrocytes under pH 5, 4°C, conditions was 100 μg (Fig. 4). Since each virus particle has a mass of $1.61 \cdot 10^8$ Da [41], 100 μg is equivalent to $3.7 \cdot 10^{11}$ particles. This amount of virus was incubated with $4 \cdot 10^7$ cells, resulting in 9250 virus particles per erythrocyte. Approx. 20% of the virus added is bound to the cells under our experimental conditions (unpublished data), which translates to $\sim$ 1850 virus particles/cell. Under optimum conditions 100% of bound influenza virus can fuse with erythrocyte ghosts [42]. However, incubation at pH 5, 4°C, leads to $\sim$ 40% of the fusion efficiency in comparison to pH 5, 37°C [35]. Therefore we estimate $\sim$ 740 virus particles fuse per RBC, if each virus particle forms one fusion pore. As there is no change in PS asymmetry under these conditions (Fig. 4) we conclude that either no disturbance of the transbilayer

asymmetry occurs during influenza virus fusion or ~ 740 fusion pores are not sufficient to induce the transport of a significant amount of PS.

An alternative explanation for the absence of PS movement to the outer leaflet is that pH 5, 4°C, incubation of virus with cells induces hemifusion. In this scenario the fluorescent membrane probe diffuses from the virus into the target membrane without movement of lipids in the inner leaflet. However, 4°C fusion appears to represent mixing of both membrane and aqueous components since the aqueous fluorescent probe, NBD-taurine, undergoes dequenching during cold fusion (Fig. 5). Hemifusion, by definition, would not permit the continuity of aqueous contents between erythrocytes and GP4F cells. It should be noted that we are investigating cell-cell fusion instead of virus-cell fusion. The two systems present differences in lag times prior to the onset of membrane fusion. Nevertheless, the general characteristics of influenza fusion appear to be sustained in a cell expressed system [22,43,44].

A limitation of this approach is the inability to detect small amounts of PS that may have translocated to the outer leaflet. Stable fusion pores may be localized events that do not permit widespread exchange of inner leaflet lipids. It is also possible that the entry of influenza RNA may not require numerous fusion pores. However, it should be noted that transient fusion pores that could serve as additional sites for PS transfer have been detected [45–47]. Fusion also appeared to be tight [45], which corresponds with our observation of lack of PS movement. Another difficulty in identifying PS on the outer leaflet is the rapidity of viral fusion at physiological temperatures which in turn limits the time available for bulk flop of PS. Contradicting this argument is the absence of PS on the outer leaflet (Fig. 4) following the slow kinetics of complete 0°C fusion (Fig. 5), suggesting outward movement of PS is not a significant component of this process. It appears that there is no bulk redistribution of the inner leaflet phospholipids of the erythrocyte membrane bilayer during viral fusion independent of hemolysis.

The use of C6-NBD-PS, a short acyl chain fluorescent PS analog, raises questions as to whether this probe is representative of endogenous PS, with its two long acyl chains. It is quite possible that endogenous PS may behave differently than C6-NBD-PS following influenza virus fusion. However, the distribution of C6-NBD-PS and endogenous PS were closely correlated in erythrocyte ghosts [18] and aged erythrocytes [16] suggesting that this probe may reflect, at least qualitatively, the movement of native PS.

These findings indicate that during influenza fusion the inner leaflet lipids mix via undetermined lipid intermediates without any significant transbilayer lipid exchange of the target membrane. Because the lipid anchored form of HA does not mediate complete fusion [40] it is assumed that the transmembrane domain of HA is required for either the inner leaflet mixing or the separation of the leaflets giving rise to complete fusion and cytoplasmic continuity. The fusion pores at this point appear to be tight, to the extent that capacitance patch clamp measurements monitor a continuous membrane [45,47] and the transbilayer asymmetry remains intact (Fig. 4). Only after continued incubation does the erythrocyte membrane become leaky and large molecules such as hemoglobin diffuse out of the cell.

Acknowledgements

We are grateful to Alan Schroit for his insightful suggestions and his interest that initiated this study, as well as his kind gift of PDA. We would also like to thank Stephen Morris, Joel Lowy, and members of the Blumenthal laboratory for discussions.

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