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Effect of substitution of hemagglutinin-neuraminidase with influenza hemagglutinin on Sendai virus F protein mediated membrane fusion

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Abstract Recombinant virosomes containing fusion protein (F) of Sendai virus and the envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA) of influenza virus within the same membrane were prepared. Such hybrid vesicles were found to hemolyse red blood cells both at pH 7.4 and pH 5.0. Hemolysis induced by hybrid vesicles was much higher than seen with F-virosomes in the presence of WGA, but was about two-fold less than the hemolysis caused by F,HN-virosomes. Reconstituted influenza virus envelopes and F-virosomes failed to induce hemolysis at pH 7.4. Using a fluorescence probe-based lipid mixing fusion assay, hybrid virosomes were found to fuse with cultured HeLa cells both at pH 7.4 as well as pH 5.0. The data indicate that the presence of Sendai virus HN protein in the virosomal membrane is not absolutely essential for the virosome–cell fusion process.

Key words: Sendai; Influenza; Reconstitution; Virosome; Fusion

1. Introduction

Sendai, a member of the paramyxoviridae family, is well characterised as an enveloped animal virus with two integral membrane proteins known as the hemagglutinin-neuraminidase (HN) and the fusion factor (F) [1]. Penetration of the virus into the host cell is mediated by a membrane fusion process. The F protein is absolutely essential in mediating the fusion of virion envelope with the cellular plasma membrane after initial attachment has been provided by the HN protein [2]. However, there is a considerable degree of uncertainity about the detailed role played by the HN protein of paramyxoviruses in the virus-membrane fusion step, and also about the molecular mechanism of F protein-induced fusion [3]. Whereas reports with some paramyxoviruses document that F protein alone is sufficient to cause virus–cell and cell–cell fusion [4], several other reports involving expression of envelope proteins on the cell surface indicate that both HN and F are required for these processes [5,6]. Most of these studies are based on syncytium assays.

Reconstituted membrane vesicles containing viral envelope glycoproteins are a convenient tool to investigate structure–function relationships of transmembrane proteins as well as molecular mechanism of virus–membrane fusion [7,8]. Using reconstitution techniques and a direct online lipid mixing fusion assay, we have recently shown that F protein in the presence of an attachment moiety is sufficient for virosome–cell fusion; HN if present in the virosomal membrane enhances only the initial rate but does not affect the extent of fusion [8]. It has been suggested that a homologous pair of F and HN proteins may be required for the recruitment of the F protein to the appropriate contact region for cell–cell fusion [8]. On the other hand, in the virosome the F protein is recruited in a limited area of the vesicle by the reconstitution technique itself, and therefore HN is not strictly required. Nonetheless, the transmembrane disposition of HN in the virosome enhances the rate of fusion [9], presumably by placing F in its proper microdomain structure [10]. In order to gain a better understanding of the role of molecular interactions between F and HN glycoprotein in Sendai virus-mediated fusion process, we have prepared hybrid virosomes containing Sendai viral F protein and influenza hemagglutinin. Evaluation of hemolytic and fusogenic activities of these hybrid vesicles at neutral pH indicates a nonstringent requirement for HN protein in virosome–cell fusion.

2. Materials and methods

2.1. Reagents

NBD-PE was purchased from Avanti (Birmingham, AL), SM2-Biobeads were obtained from Bio-Rad (Richmond, CA); Triton X-100 was from Aldrich (Milwaukee, WI); WGA, DTT, trypsin (type III), sodium azide, and EDTA were purchased from Sigma. D-MEM, D-PBS, fetal bovine serum and penicillin/streptomycin were obtained from Life Technologies Inc. Other reagents used were of highest grade commercially available.

2.2. Virus

Sendai virus (Z strain) and influenza virus X:31 (A/Aichi/68;H3N2) were grown in the allantoic sac of 10–11-day-old embryonated chicken eggs. The virus was harvested and purified as described earlier [8,11]. Virus yield was estimated in terms of protein according to Markwell et al. [12] and its activity was checked by agglutination and lysis of mouse red blood cells [8].

2.3. Cells

HeLa cells were grown at 37°C, 5% CO_2 in D-MEM supplemented with 10% fetal bovine serum as described earlier [10]. Fresh red blood cells were obtained from healthy Swiss albino mice.

2.4. Preparation of reconstituted hybrid vesicles

Hybrid virosomes were prepared by first reducing 20 mg Sendai virus with 3 mM DTT as previously described [8], followed by dialysis at 4°C for 16 h against three changes of 2 litres of buffer A (150 mM NaCl, 10 mM Tris, pH 7.4, 2 mM Mg^{2+}, 2 mM Ca^{2+}). To the dialysed sample

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Abbreviations: F, Fusion factor; Tx100, Triton X-100; DTT, dithiothreitol; HA, hemagglutinin; NA, neuraminidase; HN, hemagglutinin-neuraminidase; WGA, wheat germ agglutinin; NBD-PE, *N*-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine; D-PBS, Dulbecco's phosphate-buffered saline; D-MEM, Dulbecco's modified Eagle's medium.

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2 mg of influenza virus was added followed by centrifugation (100,000 × g for 1 h at 4°C) and the pellet obtained was resuspended in 2 ml buffer A containing 44 mg of Tx-100. After incubation for 1 h at 20°C, the Tx100-solubilized fraction was reconstituted by stepwise addition of SM2 Biobeads and centrifuged [8]. The pellet containing hybrid virosomes (1.0–1.2 mg protein) was resuspended in 1 ml buffer A and stored at 4°C. Influenza-virosomes were prepared by reconstituting Tx100-solubilized fraction of influenza virus in the absence of Sendai virus. F- and F,HN-virosomes were prepared as described earlier [8]. Protein analysis showed that hybrid virosomes prepared from a mixture containing Sendai and influenza virus particles at a ratio 10:1 (w/w), respectively, exhibited the maximum activity. NBD-PE was incorporated into the hybrid virosomes following our published procedure [8].

2.5. Electrophoretic analysis of virosomes

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used under reducing conditions to check the purity of hybrid virosomes. The method of sample preparation and the discontinuous buffer system used was as described by Laemmli [13]. The analysis was done in a separating gel containing 10% acrylamide and 0.27% bisacrylamide in the presence of 1.0% SDS.

2.6. Hemolysis assay

Hybrid virosomes (varying amount of protein) were incubated with 0.5 ml of 0.5% mouse RBC's in ice for 40 min at pH 7.4 in a final volume of 1 ml in PBS. The pH of the medium was then adjusted to the desired value (either pH 7.4 or pH 5.0) and further incubation was done at 37°C for 2 h with periodic shaking. At the end of the incubation, the extent of hemolysis was determined at 540 nm. Hemolysis assay of F-virosomes was done in the presence of wheat germ agglutinin following our published method [8].

2.7. Spectrofluorometric measurements

The fusion of NBD-PE labelled virosomes with HeLa cells was carried out as reported earlier [8]. In brief, the virosomes (10 μg protein) were mixed with 1×10^7 HeLa cells in 1 ml of D-PBS and incubated at 4°C for 40 min. The virosome–cell complexes were washed three times at 300 × g in the same buffer to remove unbound virosomes. Heat-treated and trypsin-treated virosomes were employed as controls to determine the specificity of interaction of hybrid virosomes with target cells [14]. Changes in fluorescence resulting from the fusion of hybrid virosomes with HeLa cells were measured continuously with a spectrofluorometer (model 8000; SLM-Aminco, Urbana, IL) either at pH 7.4 or pH 5.0 as described elsewhere [15]. The fluorescence dequenching (%FDQ) was calculated according to the following equation.

$$\%FDQ = 100 \times \frac{F - F_o}{F_t - F_o}$$

where F and F_t are fluorescence units at a given time point and in the presence of 0.1% Tx-100 respectively and F_o is fluorescence at time zero.

3. Results

3.1. Characterization of hybrid virosomes

The protein composition of the virosomes was examined by SDS-PAGE under reducing conditions. Coomassie blue staining of the gel revealed prominent bands corresponding to influenza NA and HA2. Sendai viral F_1 protein and influenza viral HA_1 protein appeared at the same position in the gel as a very intense band (lane 2, Fig. 1). The HN protein has earlier been shown to be completely retained in the Triton-insoluble fraction after DTT treatment of the virus particles [8]. From negatively stained preparations and electron microscopic studies, it appeared that the vesicles obtained were relatively homogeneous and their size was similar to that of intact virus particles ranging in diameter from 100–300 nm (data not shown). The structural integrity and non-leaky nature of the vesicles were determined as described earlier by encapsulating aqueous fluorescent probes like NBD-taurine, calcein, and lysozyme; these were found to be retained in the virosomes for a significant period of time [8,14].

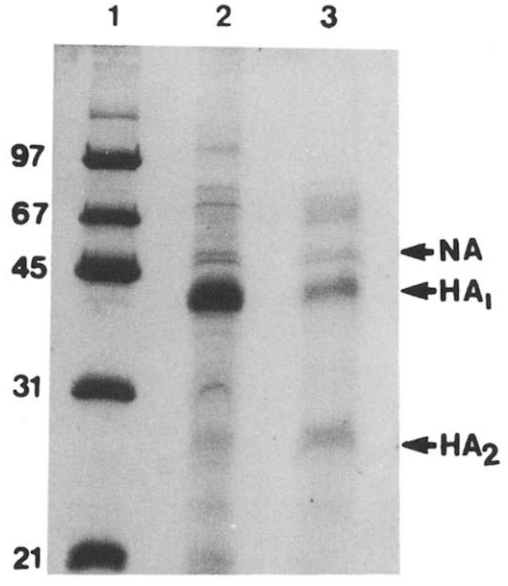

Fig. 1. Electrophoretic analysis of hybrid virosomes. Coomassie stained gel following SDS-PAGE reveals molecular weight markers (lane 1), hybrid virosomes (lane 2) and influenza-virosomes (lane 3). 25 μg protein of each virosomes was loaded. In lane 3 the position of NA, HA_1 and HA_2 are shown. In lane 2, F_1 and HA_1 migrate to the same position under reducing conditions.

3.2. Functional interaction of hybrid virosomes with mouse erythrocytes

It is well established that the pH dependence of hemolytic activity induced by Sendai and influenza viruses is closely related to their membrane fusion potential [7]. Hence, the fusogenic activity of hybrid vesicles was measured by their ability to cause hemolysis. Fig. 2A and B show the concentration dependence of the extent of hemolysis induced by hybrid virosomes at pH 5.0 and pH 7.4, respectively. (Note the different scales on the abcissa.) As can be seen in Fig. 2A, a low degree of hemolysis is observed at pH 5.0, even at a very high dose of protein in hybrid virosomes. Fig. 2B shows that the hybrid virosomes could efficiently cause hemolysis at pH 7.4. Fig. 3 shows that the rate of hemolysis levels off within 20 min after incubation of virosomes with mouse erythrocytes at pH 7.4. Virosomes prepared by reconstitution of only influenza virus (influenza-virosomes) did not have any hemolytic activity at pH 7.4; however, they were found to be active in agglutinating mouse erythrocytes. On the other hand, F-virosomes alone failed to induce either agglutination or lysis of mouse erythrocytes at pH 7.4 (data not shown). Since binding is a prerequisite for the fusion process to occur, it was strongly indicated by these experiments that in the case of hybrid virosomes, the binding function is provided by HA protein of influenza virus followed by fusion induced by Sendai virus F protein at pH 7.4. Heat treatment and proteolysis by trypsin treatment are known to cause complete inactivation of Sendai viral fusion protein thereby abolishing the fusogenic and hemolytic activities [14].

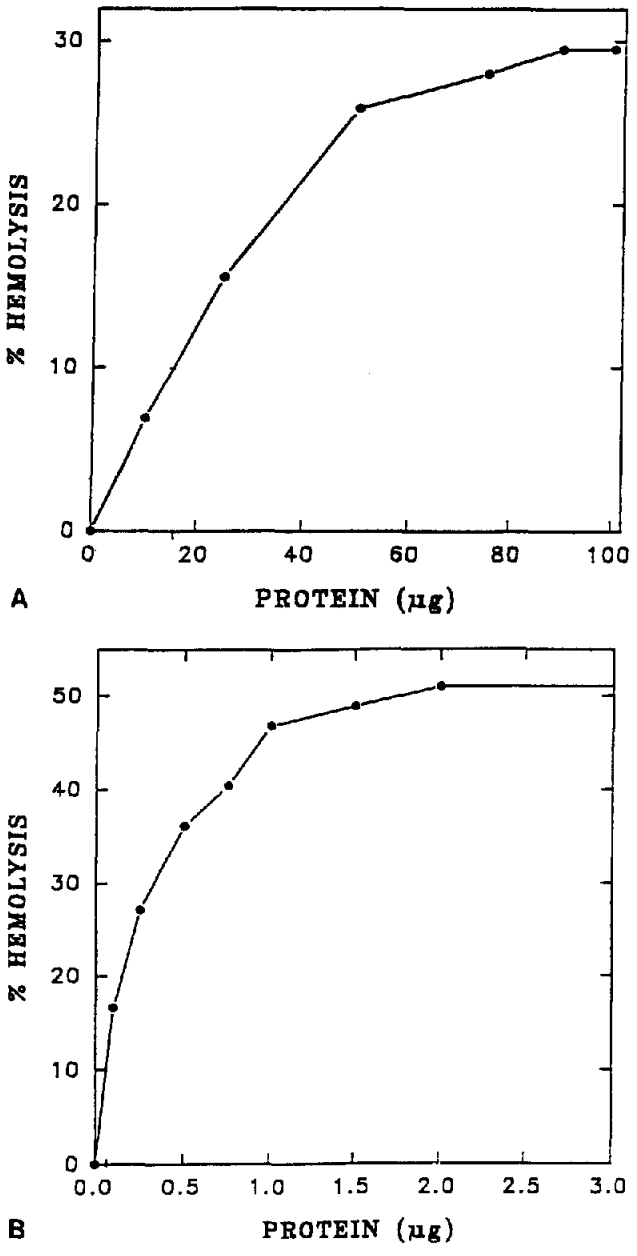

Fig. 2. Hemolysis induced by hybrid virosomes at pH 5.0 (A) and pH 7.4 (B). Increasing amounts of virosomes were added to a mixture of mouse erythrocytes (0.5% v/v) in a total volume of 1 ml of PBS, pH 7.4. The amounts of virosomes are shown as μg protein. Hemolysis was measured as described in section 2. The points are an average of three independent experiments.

However, trypsin treatment is known to have no inhibitory effect on the biological activity of influenza virus [16]. Indeed heat-treated and trypsin-treated virosomes were found to be completely inactive in hemolysing mouse erythrocytes at pH 7.4. No such inhibition of hemolysis was observed at pH 5.0 after trypsin treatment of virosomes (data not shown). It may therefore be inferred that hemolysis observed at pH 5.0 with hybrid virosomes is due to the presence of an active HA protein of influenza. Moreover, polyclonal antiserum (rabbit) against intact influenza virus blocked lysis at both neutral as well as acidic pH (data not shown), strongly suggesting that only HA mediates the initial attachment at both pH values. A comparison of the extents of hemolysis brought about by hybrid virosomes, F-virosomes and F,HN-virosomes is shown in Fig. 4. At a fixed concentration of virosomal protein, the hemolysis caused by hybrid virosomes was found to be significantly higher than F-virosomes in the presence of WGA. The F,HN-virosomes were however found to be about two-fold more active in hemolysis than hybrid virosomes.

3.3. *Interaction of hybrid virosomes with cultured cells*

The kinetics of fusion of hybrid virosomes with HeLa cells was studied both at pH 5.0 and pH 7.4. Fig. 5 shows that a relatively high degree of fluorescence dequenching was observed following incubation of virosomes with target cells. The rate of fusion at low pH was found to be higher than that at neutral pH, although the extent was more at pH 7.4. Treatment of hybrid virosomes either by trypsin or heat inhibited the fusogenic activity almost completely at pH 7.4, indicating the major contribution of F protein to the fusion process. F-Virosome alone could not bind and fuse with HeLa cells. Fusion kinetics at both neutral and acidic pH was not altered in the presence of 10 mM sodium azide (an inhibitor of endocytosis), demonstrating that the fusion occurred only at the plasma membrane level.

4. Discussion

In the present study, we have clearly shown that HN protein of Sendai virus is not an absolute requirement for the viral fusion process. The fusion can be efficiently brought about by providing initial attachment with the target cells through a heterologous binding molecule such as HA of influenza virus. It is well known that although influenza virus HA binds strongly to the target cells (e.g. mammalian red blood cells, HeLa cells, etc.) in a broad pH spectrum, it fuses only at

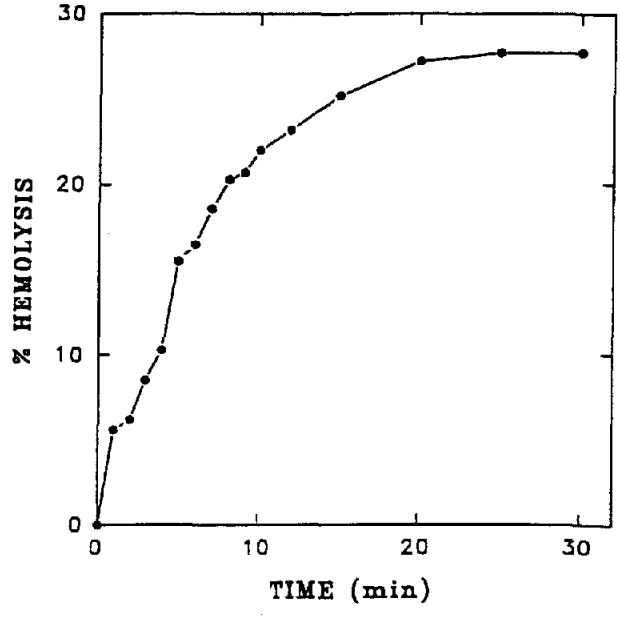

Fig. 3. Kinetics of hemolysis induced by hybrid virosomes at pH 7.4. Virosomes (250 ng) were added to mouse RBCs (0.5% v/v) in a total volume of 1 ml in PBS (pH 7.4) and hemolysis was measured as a function of time at 37°C as described in section 2. The points are an average of three independent experiments.

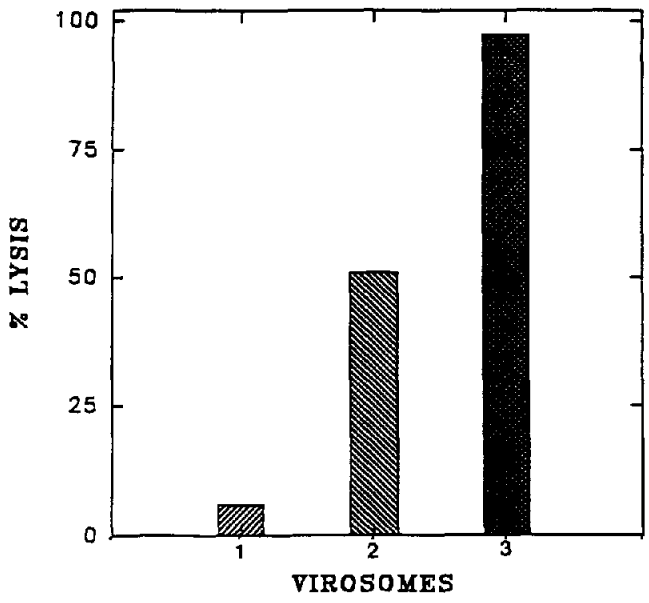

Fig. 4. Extent of hemolysis induced by various virosomes. Hemolysis was measured as described in Fig. 2 following incubation at 37°C for 2 h at pH 7.4. In all virosome preparations 2 μg protein was used. The points are average of three independent experiments. F-virosomes + WGA (1); hybrid virosomes (2); F,HN-virosomes (3).

pH 5.0. Moreover, its fusogenicity is completely abolished at neutral pH [11]. Hence, hybrid virosomes were used as a very suitable system to examine the Sendai virus–cell fusion process in the absence of its native binding protein. The fusogenicity of hybrid virosomes with target cells has been further compared with that of F- (in the presence of a soluble attachment factor, WGA) and F,HN-virosomes (containing the native binding protein, HN). Although the hybrid virosomes were enriched in F protein, the presence of influenza HA_2 was evident from the SDS-PAGE analysis under reducing conditions (Fig. 1). The F_1 and HA_1 (known to be of similar molecular weights) appear as single intense band as a result of comigration (lane 2). The staining of F_2 was very weak in agreement with previous reports [8]. In lane 2, out of a total of protein (25 μg) loaded, a majority is contributed by F protein, whereas in lane 3, 25 μg protein was from influenza envelope glycoprotein only. It is well known that the Coomassie blue staining property varies with different proteins [17], and this presumably is the reason for the intensity variation of bands between lane 2 and 3. The presence of HA in the hybrid virosomes was also confirmed by the inhibition of binding and fusion of hybrid vesicles with target cells by anti-influenza virus antiserum (data not shown). The presence of F and HA within the same membrane in hybrid virosome was further checked by coprecipitation of both the proteins by anti-influenza antiserum (data not shown) as described by Lapidot and Loyter [18]. As evident in Fig. 2, 200–300-fold more virosomes (in terms of protein) was required for 30% lysis at pH 5.0 as compared to neutral pH, which can be explained by the presence of only small amounts of influenza glycoprotein (one-tenth the amount of F protein) in the hybrid virosomes. The low fusion activity at pH 5 also reflects that hemolysis induced by F protein is quite reduced at low pH [19]. The initial rate of lysis induced by hybrid virosomes was about two-fold less than that observed earlier in case of F,HN-virosomes [8] (Fig. 3). Fig. 4 also reflects similar behaviour in terms of extent of lysis. It is important to notice that lysis induced by hybrid virosomes was about eight-fold more than that of F-virosomes when a water-soluble factor, WGA, was used to provide attachment to the target cells. This may be due to the very tight binding conferred by HA being present in its native juxtaposition in the virosomal membrane, in contrast to WGA being present as a soluble binding protein. This idea is further supported by the consideration that very tight binding of two membranes is a prerequisite for efficient fusion per se [19].

The online membrane mixing assay gives a direct idea about the initial event and the molecular mechanism of fusion [15]. The initial rate of fusion (%DQ) of hybrid virosomes was found to be about three-fold higher at pH 5.0 than at pH 7.4 (Fig. 5), whereas the final extent of fusion is about two-fold more at pH 7.4. This behaviour has a slight disparity from the observed lysis. However, it is known that subtle differences exist in the mechanism of hemolysis and lipid mixing process during membrane fusion induced by viral envelope proteins [8]. We may explain the initial higher fusion rate at low pH as an involvement of both F and HA protein in fusion in terms of membrane mixing. The higher extent of fusion at pH 7.4 after 20 min may be ascribed to the ten-fold more F protein present in hybrid virosomes. The low pH-induced inactivation of HA protein (X:31 strain) is well known from the work of Puri et al. [11]. Hence, the lower extent of fusion at pH 5.0 may be due to a time-dependent low pH-catalyzed inactivation of HA protein. However, the real mechanism of this kind of behaviour is not fully understood from this work. HeLa cells are known to be devoid of any surface membrane bound galactose receptor. The inability of F-virosomes alone to interact with HeLa cells is in agreement with the absence of any galactose receptors that may act as attachment sites for F protein's terminal galactose moieties as was earlier observed in case of a liver cell type [20].

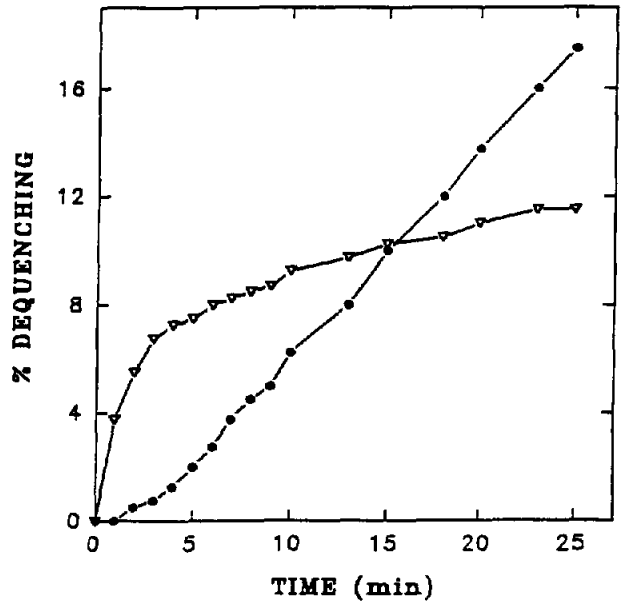

Fig. 5. Kinetics of fluorescence dequenching of NBD-PE labelled hybrid virosomes with HeLa cells. An aliquot (50 μl) of virosome–cell suspension was placed into a cuvette containing 2 ml D-PBS, pH 5.0 (open triangles) or pH 7.4 (solid circles) prewarmed to 37°C, and fluorescence measurements were made within 2 s as described in section 2. The values are average of three independent experiments.

Our main conclusion remains that the presence of HN in the reconstituted viral membrane is not obligatory in virosome–cell fusion. We however do confirm a type of homologous interaction between F and HN of paramyxoviruses in terms of virosome–cell fusion as was also observed by Hu et al. in case of cell–cell fusion [6]. We have established earlier that a high affinity binding of F protein to target cells through a sugar receptor is sufficient for an active membrane fusion in the absence of HN [20]. Our present work establishes for the first time that a heterologous membrane ligand like HA with strong binding affinity with target cells is sufficient to replace the role of HN to a high extent if not fully.

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References

[1] Okada, Y. (1988) Curr. Top. Membr. Transp. 32, 297–336.
[2] Scheid, A. and Choppin, P.W. (1977) Virology 80, 54–66.
[3] Lamb, R.A. (1993) Virology 197, 1–11.
[4] Horvath, C.M., Paterson, R.G., Shaughnessy, M.A., Wood, R. and Lamb, R.A. (1992) J. Virol. 66, 4564–4569.
[5] Wild, T.F., Malvoisin, E. and Buckland, R. (1991) J. Gen. Virol. 72, 439–442.
[6] Hu, X.L., Ray, R. and Compans, R.W. (1992) J. Virol. 66, 1528–1534.
[7] Hsu, M.C., Scheid, A. and Choppin, P.W. (1979) Virology 95, 476–491.
[8] Bagai, S., Puri, A., Blumenthal, R. and Sarkar, D.P. (1993) J. Virol. 67, 3312–3318.
[9] Bagai, S. and Sarkar, D.P. (1993) FEBS Lett. 326, 183–188.
[10] Bagai, S. and Sarkar, D.P. (1993) Ind. J. Biochem. Biophys. 30, 395–399.
[11] Puri, A. Booy, F.P., Doms, R.W., White, J.M. and Blumenthal, R. (1990) J. Virol. 64, 3824–3832.
[12] Markwell, M.A., Haas, S.M., Tolbert, N.E. and Bieber, L.L. (1981) Methods Enzymol. 72, 296–303.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Bagai, S. and Sarkar, D.P. (1993) Biochem. Biophys. Acta 1152, 15–25.
[15] Morris, S.J., Zimmerberg, J., Sarkar, D.P. and Blumenthal, R. (1993) Methods Enzymol. 221, 42–58.
[16] Klenk, H.D., Rott, R., Orlich, M. and Blodorn, J. (1975) Virology 68, 426–439.
[17] Congdon, R.W., Muth, G.W. and Splittgerber, A.G. (1993) Anal. Biochem. 213, 407–413.
[18] Lapidot, M. and Loyter, A. (1989) Biochim. Biophys. Acta 980, 281–290.
[19] Sarkar, D.P. and Blumenthal, R. (1988) Membr. Biochem. 7, 231–247.
[20] Bagai, S. and Sarkar, D.P. (1994) J. Biol. Chem. 269, 1966–1972.