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Studies of Influenza Haemagglutinin-Mediated Membrane Fusion

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A resonance energy transfer assay of membrane fusion developed by P. S. Uster and D. W. Deamer (*Biochemistry* 24, 1-8 (1985)) was used in a study of influenza haemagglutinin-mediated fusion. The characteristics of fusion and haemolysis by X-31 (H_3N_2) virus, a number of mutants of X-31 which fuse membranes at higher pH, and purified haemagglutinins released from virus particles either by detergent dissociation or by bromelain digestion were compared with particular regard to pH and temperature dependence. The finding that membrane fusion activity, haemolysis, and changes in haemagglutinin conformation covary with pH and temperature provide support for the role of haemagglutinin in fusion and are discussed in relation to the stability of its structure. © 1986 Academic Press, Inc.

INTRODUCTION

The initial stages of influenza virus infections appear to involve binding of virus particles to sialic acid-containing receptors at the cell surface, endocytosis, and fusion of virus membranes with the membranes of the endosomes. The haemagglutinin of the virus membrane participates in both receptor binding and membrane fusion processes (reviewed in Lamb and Choppin, 1983; Ward, 1981). Evidence for its involvement in the latter comes from a variety of experiments (reviewed in White *et al.*, 1983) amongst which are the observations that at the pH in endosomes (about pH 5) haemagglutinin acquires the capacity to interact with membranes as a consequence of a pH-dependent change in its structure (Skehel *et al.*, 1982). We are investigating the mechanism of membrane fusion particularly with regard to the role of haemagglutinin in the process using X-31 (H_3N_2) influenza virus since the three-dimensional structure of X-31 haemagglutinin at pH 7 is known (Wilson *et al.*, 1981). In this paper we describe the application of a resonance energy transfer procedure to a study of *in vitro* virus-mediated membrane fusion and use it to confirm the coincidence of changes in haemagglutinin conformation and membrane fusion and to compare the temperature and pH dependence of fusion, haemolysis, and haemagglutinin structure.

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METHODS

Materials. *N*-4-Nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulphonyl)-dioleyl phosphatidylethanolamine (RHO-PE) were purchased from Avanti Polar Lipids Inc., Birmingham, Alabama; cholesteryl anthracene-9-carboxylate (CAC) from Molecular Probes Inc., Junction City, Oregon; and Amberlite XAD-2 beads from BDH Ltd., Poole, Dorset, U. K. All lipids were obtained from Sigma Chemical Company Ltd., Poole, Dorset, and were checked for purity by thin layer chromatography. Gangliosides, Type III from bovine brain, were found to contain G_{M1}, G_{M2}, G_{D1a}, G_{D1b}, G_{D2}, and G_{T1b}.

Preparation of virus. X-31 virus and the amantadine resistant mutants were isolated, grown in eggs, and purified as previously described (Daniels *et al.*, 1985).

Preparation of liposomes. Liposomes were prepared by the method of McCabe

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and Green (1977): 5 mg of the lipid mixture: phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, gangliosides, and cholesterol, at molar ratios of 10, 3, 3, 1, 0.5, 1.75, and 14, and 2 mol% of the fluorescent probes CAC and NBD-PE were dried *in vacuo* and, after addition of 5 ml 0.15 *M* NaCl, 0.01% (w/v) sodium azide, 10 m*M* Tris/HCl, pH 8, the mixture was sonicated for six 1-min periods at 4° using a probe sonicator (MSE) set at 12 μm peak to peak. The liposomes were centrifuged at 100,000 *g* for 45 min and the supernatant, which electron microscopy showed to contain predominantly small unilamellar liposomes, was used in the subsequent assays.

Preparation of haemagglutinin rosettes and bromelain-released haemagglutinin. Virus (1 vol, approximately 10 mg/ml viral protein) in 0.15 *M* NaCl, 0.01% sodium azide, 10 m*M* Tris/HCl, pH 8, was added to 10 vol 15 m*M* Tris/HCl, pH 8, 0.5% (w/v) Brij 36T, and incubated for 30 min at 4°. After centrifugation at 100,000 *g* for 60 min, the supernatant, which contained only virus glycoproteins, was passed down a protein A-Sepharose 4B column to which antineuraminidase monoclonal antibodies had been bound, in order to remove the neuraminidase. Brij 36T was exchanged for octyl glucoside (Helenius *et al.*, 1977) by centrifugation through a 10 to 30% sucrose gradient containing 1% (w/v) octyl glucoside for 18 hr at 100,000 *g* at 20°. Haemagglutinin-containing fractions were dialysed against three changes of 5 litres 0.15 *M* NaCl, 0.01% sodium azide, 10 m*M*, Tris/HCl, pH 8, containing approximately 25 g of Amberlite XAD-2 beads to adsorb the detergent. The resulting haemagglutinin rosettes were concentrated to 1 mg/ml prior to use. Bromelain released haemagglutinin was prepared as previously described (Brand and Skehel, 1972).

Haemolysis and trypsin susceptibility. Haemolysis was estimated as before (Daniels *et al.*, 1985). X-31 virus and haemagglutinin rosettes were used at equal haemagglutinating activities. Bromelain-released haemagglutinin was used at the same concentration as haemagglutinin rosettes. Haemolysis was measured after 1, 5, and 24 hr incubations at 37° by determining the optical density at 520 nm of the cell supernatant. Values for autohaemolysis were subtracted from the observed results. Twenty percent autohaemolysis was observed after 5 hr incubation at pH 4.9 and after 24 hr at pH 5.4. The trypsin susceptibility of haemagglutinin was determined as previously described (Skehel *et al.*, 1982).

Assay for fusion. The resonance energy transfer method of Uster and Deamer (1985) was used which involves the nonexchangeable probes CAC as fluorescent donor and NBD-PE as fluorescence acceptor. Each probe was used at a concentration of 2 mol%. Fusion of fluorescent liposomes with unlabelled bilayers results in dilution of the fluorescent probes and consequent decrease in resonance energy transfer detected by increase in CAC fluorescence and decrease in NBD-PE fluorescence. Fluorescence was measured with a Perkin Elmer MPF 4 spectrofluorimeter with excitation and emission slit widths set at 10 nm. Excitation of the donor probe, CAC, was at 368 nm and filters 9863 and UV39 were used in the excitation and emission paths, respectively, so as to minimise the effect of light scattering. Viruses in 950 μl phosphate-buffered saline (haemagglutination titre approximately 2560) were incubated with 50 μl of fluorescent liposomes for 5 min at 4°. The pH of the solution was adjusted using 0.15 *M* sodium citrate pH 3.5 and mixtures were incubated at the appropriate temperature for 5 min. The fluorescence spectra of the two probes were measured and expressed as the ratio of CAC (460 nm) to NBD-PE (532 nm) fluorescence. The pH at which a 50% change in this ratio occurred was taken as the pH of fusion for each virus. This value was not dependent upon the amount of virus used. Energy transfer in the absence of virus was not affected by pH but NBD-PE fluorescence decreased more than CAC fluorescence with increasing temperature. Although this changed the ratio of CAC to NBD-PE fluorescence at different temperatures the pH at which a 50% change in ratio occurs is not changed.

We employed the probes used by Uster and Deamer (1985) rather than those of Struck *et al.* (1981) which have also been used in studies of influenza virus-mediated fusion (Wilschut and Hoekstra, 1984) because, although in the latter system using NBD-PE as donor and RHO-PE as acceptor energy transfer was greater, we found that virus, haemagglutinin rosettes, and bromelain-released haemagglutinin decreased the fluorescence of RHO-PE when this was singly included in the liposomes. This decrease only occurred at a pH below that expected for haemagglutinin–lipid association to occur. In addition, haemagglutinin rosettes and bromelain-released haemagglutinin, which contain no exogenous lipid to dilute out the fluorescent probes, increased the fluorescence of NBD-PE at low pH with liposomes containing both NBD-PE and RHO-PE. This was a secondary effect of the quenching of RHO-PE fluorescence as NBD-PE fluorescence in liposomes containing only this probe was unaffected by virus, haemagglutinin rosettes or bromelain-released haemagglutinin at any pH. In this connection, fluorescence polarisation studies showed that when virus was added to liposomes containing one fluorescent probe and the pH decreased to 5.0 anisotropy of CAC decreased by 7%, NBD-PE increased by 14%, but that of RHO-PE increased by 47%. Haemagglutinin rosettes and bromelain-released haemagglutinin also increased anisotropy of RHO-PE. These results suggest that haemagglutinin in the lower pH conformation associates with the rhodamine probe decreasing its mobility and its fluorescence.

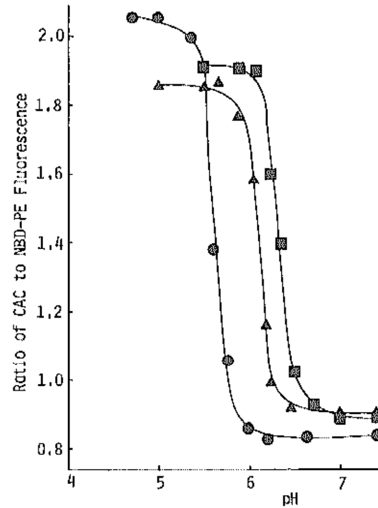

FIG. 1. pH dependence of membrane fusion by X-31 virus and amantadine resistant mutants 1 and ab4. Membrane fusion was measured by the resonance energy transfer procedure at 37° described under Methods. The ratio of CAC/NBD-PE fluorescence was used as the measure of fusion and the pH at which a 50% change in this ratio occurred was 5.60 for X-31 virus. The average of 11 determinations of this value for X-31 virus was 5.60 with a standard deviation of 0.02. Details of the mutants are given in Table 1. All three viruses were present at equal haemagglutination titres. ●, X-31 virus; ▲, mutant 1; ■, mutant ab4.

RESULTS

Assays of Membrane Fusion Using Resonance Energy Transfer

The resonance energy transfer procedure selected and described under Methods involves CAC and NBD-PE which are excited and emit fluorescence at 368 and 460 nm, and at 468 and 532 nm, respectively (Uster and Deamer, 1985). Using this procedure fusion of labelled liposomes by X-31 virus was observed to occur at pH 5.6 (Fig. 1), and results obtained in a similar way for a number of amantadine hydrochloride-resistant mutants of X-31 previously described (Daniels *et al.*, 1985) are shown in Table 1. Fusion by X-31 virus was also observed to occur at pH 5.6 using liposomes of different lipid compositions, e.g., phosphatidylcholine/cholesterol alone, and in experiments in which all of the lipids were singly removed from the liposome formation mixture none was observed to be essential for fusion (cf. Doms *et al.*, 1985). This also applies to gangliosides which were incorporated to allow binding of virus to liposome via sialic acid receptors. Omission of ganglioside had no effect on the rate of fusion or the pH at which it occurred and a mutant of X-31 virus which recognizes sialic acids in α-2:3 linkage to galactose rather that α-2:6 linkages which are recognized by wild-type X-31, behaved indistinguishably from X-31. Observations of X-31 fusion to liposomes containing sialic

TABLE 1

INCREASES IN THE pH OF MEMBRANE FUSION AND HAEMOLYSIS BY AMANTADINE RESISTANT MUTANTS OF X-31 VIRUS

Virus mutant	Residue	Amino acid substitution	Membrane fusion ΔpH	Haemolysis ΔpH
1	HA_2 6	I → M	0.5	0.3
1a	HA_2 112	D → G	0.5	0.5
2a	HA_2 54	R → K	0.3	0.3
5a	HA_2 114	E → K	0.5	0.6
4x	HA_2 81	E → G	0.4	0.3
6x	HA_2 9	F → L	0.4	0.5
12x	HA_2 112	D → N	0.8	0.6
aa1	HA_2 47	Q → R	0.5	0.4
aa4	HA_2 105	Q → K	0.3	0.3
aa9	HA_2 112	D → E	0.4	0.3
aa2	HA_1 17	H → Q	0.9	0.9
ab4	HA_1 17	H → R	0.8	0.8

Note. Membrane fusion was assayed by the resonance energy transfer procedure described under Methods and haemolysis as described previously (Daniels *et al.*, 1985). Isolation of the mutants and sequence analyses of their HAs were described in Daniels *et al.* (1985). Wild-type X-31 virus fused membranes at pH 5.6 and caused haemolysis at pH 5.5. All values shown are simple differences from these figures.

acid residues in α-2:3 linkage have also been made by Van Meer *et al.* (1985).

The kinetics of the fusion process are shown in Fig. 2a which shows the increase with time of incubation of fluorescence at 460 nm of CAC brought about by the dilution of the probes. The process was essentially complete 3 min after adjusting the pH to 5.1. Virus–liposome mixtures incubated at pH 8 showed no change in fluorescence, neither was a change seen when liposomes were incubated at pH 5.1 with X-31 virus which had previously been incubated at pH 5.1 for 15 min at 37°, a procedure which destroys virus infectivity (Daniels *et al.*, 1985). Figure 2b shows the initial and final spectra of the probes in X-31 mediated fusion and in the presence of acid-treated X-31 virus. Acid-treated virus suspensions were more opaque than untreated virus; however, the initial spectra of the probes were very similar in experiments using either virus preparation. Therefore increases in light scattering brought about by acidification of virus suspensions appear not to influence measurements of membrane fusion when the light filtering system described above is used.

The kinetics of fusion are very similar to those observed for the conformational change as determined by the acquisition of the characteristic trypsin susceptibility of soluble haemagglutinin after incubation at low pH (Fig. 3). In these experiments it was necessary to maintain bromelain-released haemagglutinin at low pH for at least 30 sec to allow changes in the conformations of a significant proportion of molecules.

Haemolysis as a Measure of Membrane Fusion

The results presented in Table 1 for the pH at which different mutants fused to labelled liposomes are similar to those obtained for the pH at which the mutants lysed erythrocytes. In all cases haemolysis occurred at slightly lower pH than fusion. Overall, however, the resonance energy transfer procedure has proved to be more reproducible. This is primarily because of difficulties in determining the pH of maximum haemagglutinin-dependent haemolysis since spontaneous haemolysis also occurs at low pH. The largest discrepancies

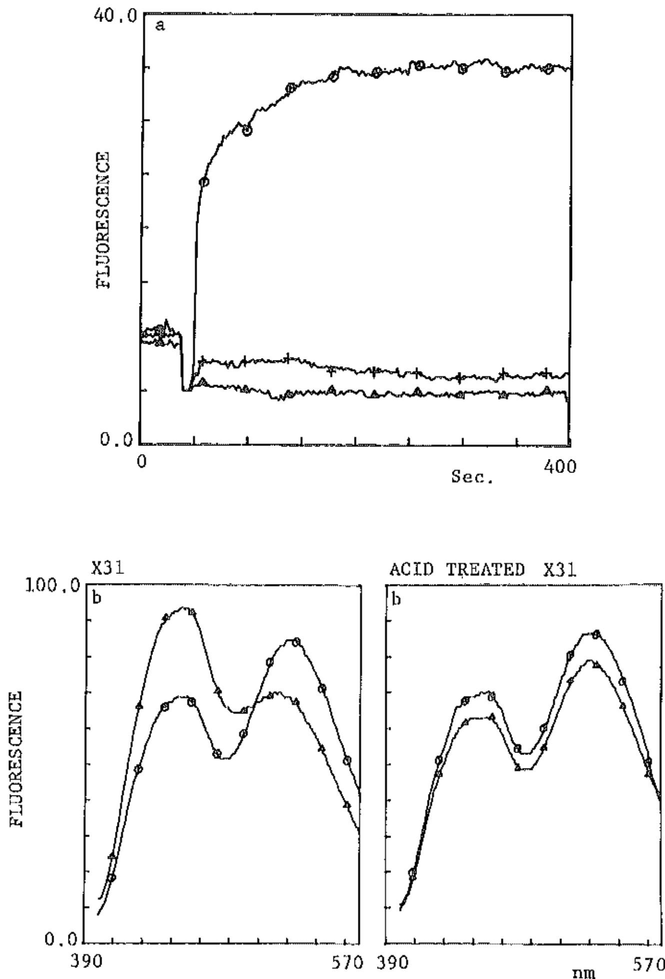

FIG. 2. Membrane fusion by X-31 virus. (a) The kinetics of fusion by X-31 at pH 5.1 and pH 7.8 and by acid treated X-31 virus. Fusion is recorded by increases in CAC fluorescence at 460 nm. Acid treatment of X-31 was by incubation at pH 5.1 for 15 min at 37°. ○, Fusion by X-31 virus at pH 5.1; +, fusion by X-31 virus at pH 7.8; △, fusion by acid treated X-31 virus at pH 5.1. (b) The initial and final fluorescence spectra of the fusion assay suspensions from (a). ○, Initial spectra; ▲, spectra after a 5-min incubation at 37°, pH 5.1.

from previously reported haemolysis results are for mutants aa2 and 6x (Daniels *et al.*, 1985) but these were not confirmed by the haemolysis tests performed in this study. The reason for this particular variability may be due to the blood sample which was used to test both of these viruses or may be a consequence of the different relative stabilities of these viruses on storage.

Variations in Membrane Fusion and Changes in Haemagglutinin Structure with pH and Temperature of Incubation

It was observed previously that the pH at which influenza viruses lysed erythrocytes was systematically higher than that at which their haemagglutinins were changed in structure as judged by their

of lipid charge neutralization, and although dependent on haemagglutinin–lipid interaction appears to be secondary to it.

The ability of the haemagglutinin preparations to fuse mixtures of fluorescently labelled and unlabelled liposomes was also determined. Detergent isolated haemagglutinins mediated fusion at similar pH values to the viruses from which they were prepared (Fig. 5). Bromelain-released haemagglutinins, however, did not cause vesicle fusion even at the longer incubation times employed in the haemolysis studies possibly because of their inability to interact with more than one liposome.

In these experiments with purified haemagglutinins fluorescence energy transfer was also observed using two populations of liposomes, one of which contained CAC and the other NBD-PE providing additional evidence of haemagglutinin-mediated liposome–liposome fusion.

Antibody Inhibition of Liposome Fusion

Evidence for the involvement of haemagglutinin in liposome fusion was also obtained from experiments using monoclonal antibodies. The results presented in Fig. 6 show that anti-haemagglutinin antibodies which bind to different regions of the molecule inhibit liposome–liposome fusion mediated by haemagglutinin rosettes whereas anti-neuraminidase antibodies included as controls were without effect. Similar results were obtained using intact virus. Furthermore, using either virus or haemagglutinin rosettes, inhibition was observed whether or not the liposomes contained gangliosides.

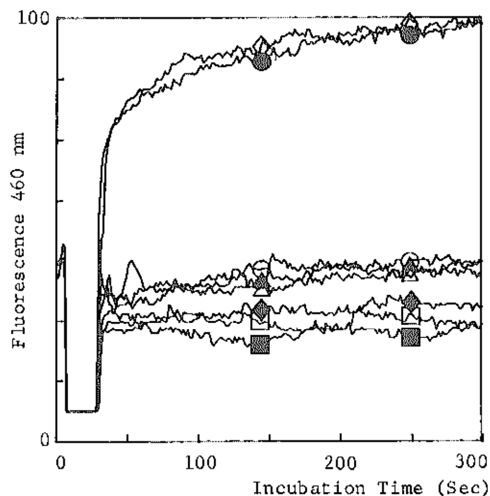

FIG. 6. Inhibition of membrane fusion by anti-haemagglutinin monoclonal antibodies. Aliquots (100 μg) of X-31 haemagglutinin rosettes in PBS were incubated with 100 μg of different monoclonal antibodies purified by affinity chromatography (Ey *et al.*, 1978) for 5 min at 37°. A liposome mixture containing 50 μg fluorescent liposomes and 200 μg unlabelled liposomes in PBS was then added and 5 min later the pH was adjusted to 5.1 by adding citrate buffer, pH 3.5. Increases in fluorescence of CAC at 460 nm were recorded as indices of membrane fusion. The anti-X-31 haemagglutinin monoclonal antibodies used HC19, HC21, HC24, and HC45 recognize HA_1 residues 147, 198, 144, and 63, respectively. Antibody NC 56 reacts with X-31 neuraminidase. Control assays without haemagglutinin addition at pH 5.1 and containing haemagglutinin but incubated at pH 7.8 were included. ▲, HC 19; △, HC 24; ◇, NC 56; □, X-31 haemagglutinin pH 7.8; ○, HC 21; ◆, HC 45; ●, X-31 haemagglutinin without antibodies; ■, no haemagglutinin pH 5.1.

DISCUSSION

This is a report of the application of a resonance energy transfer assay developed by Uster and Deamer (1985) to a study of influenza haemagglutinin-mediated fusion of model membranes. The results presented in general confirm conclusions drawn previously concerning the role of haemagglutinin in fusion from studies in which haemolysis was used as an assay of fusion. In particular, the observed similarities between the kinetics of fusion and the rate of change in haemagglutinin conformation at pH 5.6 and the similarities between the temperature and pH dependence of fusion and the changes in conformation provide further support for the role of haemagglutinin in fusion and for the requirement for a low pH-triggered change in its structure for activity.

These conclusions are also supported by the observations that purified detergent isolated haemagglutinin rosettes mediate liposome fusion particularly since fusion

occurs at appropriately different pH using the haemagglutinins of different virus mutants. The possibility that bound detergent is responsible for this activity is made unlikely by these results, by the finding that haemolysis is also mediated by bromelain-released soluble haemagglutinins, and by the observations that anti-haemagglutinin antibodies specifically prevent fusion.

Analysis of the location of amino acid substitutions in the haemagglutinins of the X-31 mutants listed in Table 1 led to the basic conclusion that haemagglutinins containing mutations which decrease the stability of the trimer by modifications in either side-chain size or charge undergo the required conformational change and fuse membranes at higher pH than wild-type virus (Daniels *et al.*, 1985). The observations of the increase in pH of fusion with increasing temperature described here are in accord with this conclusion and substantiate our previous interpretation of the differences observed between the pH of haemolysis at 37° and the pH at which haemagglutinin becomes susceptible to proteolysis at 20°. The biological significance of the membrane fusion activity of mutant viruses at elevated temperatures and of the stability of the functional haemagglutinin at this temperature is presently being studied.

After submission of this manuscript Stegmann *et al.* (1985) reported similar resonance energy transfer studies of the fusion of X-47 influenza virus with cardiolipan liposomes using the probes of Struck *et al.* (1981) which are discussed under the Methods section.

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