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Fusion of Newcastle disease virus with liposomes: role of the lipid composition of liposomes

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We demonstrate here that fusion occurs between the membrane of the Newcastle disease virus (NDV) and liposomes. Fluorescence dequenching studies (using Rhodamine-bearing viral envelopes) revealed the mixing of the lipids constituting the viral and liposomal membrane. The digestion of internal viral proteins by trypsin-containing liposomes indicated the mixing of the internal aqueous compartments. This last assay is independent of exchange of lipids between liposomal and viral membrane in the absence of fusion. Investigation of the effects of liposomal composition indicated that the presence of phosphatidylethanolamine and gangliosides are essential to optimize fusion. The fact that the Newcastle disease virus membrane can fuse with liposome also confirms that fusion must be determined by the viral proteins and could be mostly independent of the nature or presence of the host proteins.

Penetration of paramyxovirus into the host cell occurs by fusion of the viral membrane with the plasma membrane at the cell surface [1]. The envelope of paramyxoviruses such as Newcastle disease virus (NDV) consists of a lipidic bilayer and two glycoproteins (HN and F) which project from the viral surface. The HN protein is responsible for attachment of the virus to neuraminic acid-containing receptors at the cell surface and also has neuraminidase activity [2–4]. The F protein is responsible for several biological activities of the virus, all which result from fusion of the viral membrane with cell membranes [5–7]. Cleavage of the F protein is required for membrane fusion activity [5,7]. The F_1 protein N-terminus generated by proteolytic cleavage of the F_0 protein is characterized by a sequence of 15 hydrophobic amino acids [8]. Because oligopeptides with sequences resembling the N-terminus of the F_1 protein inhibit the action of the F protein [9] and because cleavage of the F_0 protein to form the F_1 protein is accompanied by an increase in the exposed hydrophobic surface, it has been hypothesized that the F protein acts by inserting its hydrophobic moiety into the target membrane [10].

However, the molecular mechanism of this process remains largely unknown. It is the purpose of this paper to investigate the role of the host membrane lipids in the fusion process between NDV and the host cell. Liposomes were used as host models, since lipid composition of such simplified systems can be easily modified. Moreover, studies employing liposomes benefit from the availability of sensitive and quantitative assays to monitor the fusion reaction [11].

In this paper, the ability of NDV to fuse with liposomes is demonstrated. Fusion implies mixing of the internal aqueous compartments and mixing of the lipid phases [12,13].

Mixing of the lipid phases of virus and lipo-

somes has been demonstrated using the self-quenching properties of a fluorescent probe (Rhodamine) covalently linked to a lipid (phosphatidylethanolamine (PE)) and incorporated in the viral membrane. This procedure has been applied successfully to follow the kinetics of fusion between influenza virus and phospholipid vesicles [11], or between reconstituted Sendai virions (virosomes) and liposomes [14] and is now currently used for continuous and sensitive monitoring of membrane fusion in both artificial and biological systems.

Liposomes made of phosphatidylcholine (PC), phosphatidylethanolamine (PE), total gangliosides, cholesterol and sphingomyelin (1 : 1 : 0.7 : 0.8 : 1, w/w) [15] were incubated 30 min at 37° with Rhodamine-PE labelled viruses. Fluorescence of the suspension was followed at 586 nm. To determine the amount of Rhodamine-PE incorporated into the viral membrane, viral lipids were extracted [11] with a chloroform/methanol-0.1 M HCl (1 : 1, v/v) mixture. The fluorescence was measured on an aliquot of the lipid extract and calibrated with chloroform solutions of known Rhodamine-PE concentrations. The surface density of Rhodamine-PE in the viral membrane was 2.6% (w/w) of total viral phospholipids. Hoeckstra et al. [11] have demonstrated (1) that under these experimental conditions, self-quenching efficiency is proportional to the probe's surface density and (2) that upon fusion between labelled and non-labelled membranes, decrease in surface density of Rhodamine-PE results in a proportional increase in fluorescence intensity (dequenching).

Table I shows a strong increase of the Rhodamine-PE fluorescence when viruses were incubated 30 min at 37°C in the presence of liposomes as compared to the fluorescence level ob-

TABLE I

EFFECT OF INCUBATION WITH LIPOSOMES ON THE FLUORESCENCE OF RHODAMINE-PE CONTAINING NDV

Fluorescence of Rhodamine-PE containing NDV after an incubation of 30 min at 37°C of the virus (0.001 mg protein/ml) with liposomes (0.1 mg lipid/ml) of different compositions in a final volume of 600 μl of buffer (20 mM Hepes 0.15 M/NaCl (pH 7.8)). Fluorescence values are given in %. O% is the fluorescence of Rhodamine-PE containing virus before incubation, 100% is the fluorescence of Rhodamine-PE after addition of 1% (v/v) Triton X-100. Fluorescence was measured at 586 nm (λ_{ex} 564 nm) with a ISA Jobin Yvon J3D spectrofluorimeter. NDV (Italian type) was provided by the National Institute for Veterinary Research (Dr. Meulemans). The virus was isolated from the allantoic fluid of infected embryonated chickens eggs and stored in phosphate-buffered saline (pH 7.4) at −20°C. Liposomes of various lipid compositions were freshly prepared for each experiment. Lipids were dissolved in $CHCl_3$. The solution was dried under a nitrogen atmosphere at 20°C and further dried under reduced pressure for at least 2 h. Multilamellar liposomes were formed by mechanical stirring (vortex mixer) of the lipid film in buffer. The liposomal suspension was sonicated (Branson Sonic Power Sonifer B12) for 10 min at 0°C and the preparation was centrifuged 10 min at 600 × *g*. Insertion of the probe into the viral membrane was accomplished as described [11]. 10 μl ethanolic solution of Rhodamine-PE (2 mg/ml) were injected under vortexing into 1 ml of a solution containing NDV (0.7 mg viral protein). This solution was incubated 1 h at room temperature in the dark. To remove non incorporated Rhodamine-PE, the solution was applied to a column of Sephadex G150 (1.8 × 20 cm). PC (egg), PE (ovine brain), sphingomyelin (Sm) (bovine brain), cholesterol (Chol) (porcine liver), total gangliosides (Gt) (bovine brain) and trypsin were purchased from Sigma (U.S.A.). *N*-(lissamine Rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine was furnished by Avanti Polar Lipid (U.S.A.).

No.	Liposome composition	Weight ratio	Fluorescence (%) after 30 min of incubation at 37°C
1	PC	1	4.4
2	PC; Chol	1 : 0.8	2.0
3	PC; Gt	1 : 0.07	6.4
4	PC; Gt; Chol	1 : 0.07 : 0.8	6.8
5	PC; PE	1 : 1	11.6
6	PC; PE; Chol	1 : 1 : 0.8	10.3
7	PC; PE; Chol; Sm	1 : 1 : 0.8 : 1	8.4
8	PC; PE; Gt	1 : 1 : 0.07	14.4
9	PC; PE; Gt; Chol	1 : 1 : 0.07 : 0.8	16.7
10	PC; PE; Gt; Chol; Sm	1 : 1 : 0.07 : 0.8 : 1	17.0

tained with viruses alone. This increase in fluorescence, related to the dilution of the probe in the lipid bilayer, demonstrated that the membrane of the virus had fused with the liposomal membrane. To determine whether a specific lipid could be made responsible for the observed effects, identical experiments were performed using vesicles with different lipid compositions (Table I).

Table I shows that NDV did not fuse significantly with liposome only made of PC. Addition of cholesterol to PC did not increase the level of fusion. Also, presence of sphingomyelin in different liposomal compositions as PC, PE, cholesterol or PC, PE, cholesterol, total gangliosides did not modify significantly fusion properties. The observation that cholesterol is not involved in the fusion process between NDV and liposomes may be in contradiction with results obtained by others [16] for Sendai virus-liposomes fusion, but suggests that their mechanism of interaction with the host cell could be different. We have however, no evidence that binding of the virus to the liposomes, rather than fusion itself, may not be inhibited by certain liposomal compositions showing low extents of fusion.

Table I indicates that total gangliosides and PE could play a major role in the interaction between virus and liposomes. Indeed, comparison of fusion obtained with PC, cholesterol liposomes and PC, cholesterol, PE liposomes demonstrates the major role of PE. The same observation was made for other liposomal compositions with an without PE (Table I). The role of PE in the fusion process has been related to the ability of this lipid to assemble in inverted micelles structures. Since it is difficult to imagine that the bilayer structure of lipids is continuously preserved during the fusion process, it has been assumed that at some stage, the lipids will have to locally adopt a nonbilayer configuration. This is the basis for the hypothesis that H_{II} preferring lipids such as PE, by virtue of their ability to adopt nonlamellar lipid structures, actively participate in the fusion process [17]. This hypothesis is supported by the experiments of Cullis et al. [18,19] indicating that addition of fusogenic lipids (glycerolmonooleate) to erythrocytes induced membrane fusion, which process is accompanied by hexagonal H_{II} phase.

Gangliosides also enhance the fusion capacity. Presence of total gangliosides in PC, cholesterol liposomes induced 6.8% of dequenching against 2% in absence of total gangliosides. This observation was verified for all liposomal systems (Table I). The HN protein of paramyxovirus recognizes *N*-neuraminic acid (NANA) as receptor [20]. Haywood et al. [21] demonstrated that Sendai virus used gangliosides (containing NANA) as receptors when these gangliosides were incorporated into liposomes. Our results strongly suggest that HN protein of NDV can use a ganglioside as receptor too. Total gangliosides could increase first binding and then fusion between NDV and liposomes.

However, it is surprising to observe fusion in absence of receptor. In the present work, because of the high concentration of liposomes around the virus, the high number of collisions between them makes the fusion possible without the need of a specific receptor. Hsu et al. [22] have indeed shown that F protein inserted into liposomes induces fusion even in the absence of HN glycoprotein, if a means of attachment of the liposome (virosome) to the cell is provided (lectins for instance).

To show that fusion was induced by viral glycoproteins and not by viral lipids, we monitored fusion between liposomes of PC, PE, sphingomyelin, cholesterol, total gangliosides and vesicles made with viral lipids extracted from the viral membrane. We controlled by electrophoresis that no detectable proteins were associated to this extract. No increase of fusion was observed after 30 min of incubation at 37°C (data not shown), demonstrating that fusion is not induced by viral lipids. Moreover, this observation indicates that the increase of fluorescence obtained with the virus is due to a virus-liposome fusion and not to a lipid-lipid exchange.

Mixing of the aqueous phase of virus and the aqueous phase of liposome was demonstrated biochemically by trapping trypsin within the liposomes. If fusion between the two internal phases occurs, trapped trypsin will digest the internal proteins of the virus. This method has been used to demonstrate the pH dependence of the fusion between Semliki forest virus (SFV) and phospholipid vesicles [15].

To demonstrate that the mixing of aqueous phases of NDV and liposome accompanied the

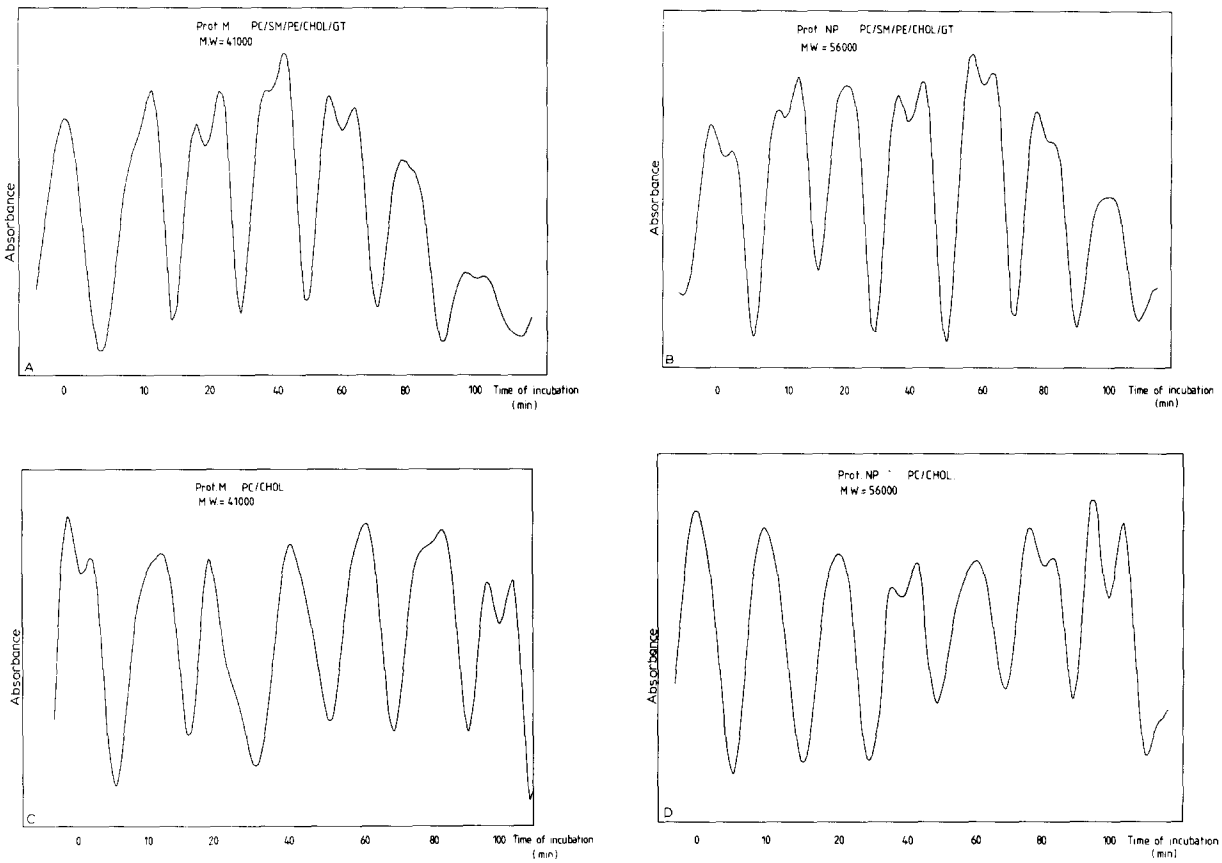

Fig. 1. SDS-polyacrylamide gel electrophoresis of viral proteins. The gel was scanned horizontally, each positive peak corresponding to the same protein on a different lane. NDV (0.0025 mg protein/ml) and liposomes (1 mg lipid/ml) in a final volume of 400 μl of buffer containing 125 μg/ml of soybean trypsin inhibitor were incubated at 4°C for 20 min and then 100 min at 37°C. Aliquots were taken at different intervals of time and mixed to a sample buffer containing 0.05 M Tris, 1 mM EDTA, 1% SDS, 1% β-mercaptoethanol, 0.8 mg/ml PMSF freshly added. SDS-polyacrylamide gel electrophoresis were performed as described by Laemmli [23]. Gels were stained with $AgNO_3$ as described by Morrissey [24]. To obtain trypsin containing liposomes, 1 ml of buffer (10 mg trypsin) was added to the lipid film and the suspension was mechanically stirred vigorously at room temperature. The preparation was sonicated (Branson Sonic Power Sonifer B12) six times during 30 s at 0°C to avoid degradation of the enzyme. The liposomes were centrifuged for 10 min at $600 \times g$. To remove untrapped enzymes, the supernatant was applied to a column of Sephadex G150 (1.8×20 cm).

fusion between the two lipid bilayers, we prepared trypsin-containing liposomes. NDV were mixed with these vesicles in presence of an excess of soybean trypsin inhibitor. After different times of incubation at 37°C, samples were analysed by SDS-polyacrylamide gel electrophoresis.

Figs. 1A and 1B show that after incubation of liposomes made of PC, PE, sphingomyelin, total gangliosides, cholesterol and NDV, the internal matrix protein M (Fig. 1A) and the internal nucleocapsid protein NP (Fig. 1B) disappeared. On the contrary, Figs. 1C and 1D show that no detectable fusion occurs between viruses and liposomes of PC, cholesterol. Indeed, neither M (Fig. 1C) nor NP (Fig. 1D) were degraded which indicates that the aqueous compartments of viruses and liposomes have not been in contact. In both systems, the external proteins remained unaffected (data not shown), indicating that external proteins of the viruses were not accessible to the entrapped trypsin.

The structure of cell membrane is much more complex than that of the liposomes used in this study. However, the mixing of the aqueous and

lipid phases suggests similarities between liposome-NDV and host cell-NDV fusion. Further studies with artificial membranes of defined composition and structure should shed light on the contribution of individual components of the host cell (gangliosides, phosphatidylethanolamine) in the membrane fusion process.

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