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INITIATION OF FUSION AND DISASSEMBLY OF SENDAI VIRUS MEMBRANES INTO LIPOSOMES

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Sendai virus penetration into liposomes consists of two steps which are fusion of the viral and liposomal membranes and viral disassembly. Penetration can occur in less than one minute. The virus first causes a liposome to envelop it and then fuses with the leading edge of the developing vacuole. Viral disassembly does not follow immediately but requires release of virus-receptor binding and probably also requires changes in the association between viral proteins.

Introduction

When gangliosides are incorporated into liposomes they serve as Sendai virus receptors [1]. Sendai virus binds to gangliosides in liposomes and to the receptors of host cells in the cold, but will not penetrate cells or liposomes until the temperature is raised [2, 3]. Paramyxoviruses enter cells either by endocytosis or by fusion of their membranes with the host cell membranes [3]. Previous work has shown that when Sendai viruses were adsorbed to liposomes in the cold and then incubated at 37°C for 2 h, the viruses either became enveloped by liposomes as in the ingestion step of endocytosis [4] or became fused with liposomes [5]. Membrane fusion was determined by electron microscopy which showed that the viral ribonucleoprotein was inside the outer lamella of the liposome and the viral glycoprotein spikes were distributed throughout the surface of the liposome. This assay measures both fusion and disassembly of the virus with the liposome. In this work the initial steps of viral penetration (fusion and disassembly) are studied.

Materials and Methods

Egg phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were obtained from Avanti Biochemicals, Inc., and cholesterol, chromatography standard grade, was obtained from Sigma Chemical Co. These lipids each gave only one spot when 0.8 μmol were chromatographed with chloroform/methanol/concentrated NH_4OH (60 : 25 : 4, v/v) and with chloroform/methanol/glacial acetic acid/water (65 : 25 : 2 : 4, v/v) on Silica gel G TLC plates. Bovine brain gangliosides were obtained from Koch-Light Laboratories, Ltd., and were shown by chromatography with chloroform/methanol/2.5 M NH_4OH (60 : 35 : 8, v/v) plus 20 mg KCl/100 ml to contain five ganglioside species.

To make liposomes 0.7 μmol PC, 0.3 μmol PE, 0.66 μmol cholesterol, and gangliosides containing 0.11 μmol of *N*-acetylneuraminic acid (NAcNeu) were mixed, dried, and resuspended in 0.15 or 0.3 ml of phosphate-buffered saline at room temperature (22–24°C).

Sendai viruses were grown in eggs as previously described [1]. To infect the liposomes, 0.2 ml of Sendai virus with a hemagglutination titer of 8192 were added at 4°C. After 60 to 80 min, the liposomes and viruses were transferred to a 37°C bath for the indicated times, and then returned to the ice bath. The samples were diluted 1 :5 into 0.145 M ammonium acetate at 0°C. Fifty μl of this dilution was added to 50 μl of 2% ammonium molybdate (pH 7.1) and 10 μl of 0.005% bovine serum albumin. Ten μl

were put on a carbon grid for 1–10 min and the excess fluid removed. The samples were examined in a Phillips EM 200 electron microscope.

Results

The liposomes used for all of these experiments contained PC, gangliosides, cholesterol, and PE. When Sendai viruses are adsorbed to liposomes in an ice bucket and then transferred to a 37°C bath, 'endocytosis' and fusion occur in less than 1 min. Fig. 1 shows a liposome which has been incubated with virus at 37°C for 1 min. The virus has been partially enveloped by the liposome and has fused with the leading edge of the developing vacuole. Over 40 examples of beginning fusion have been photographed and more viewed on the microscope screen, and all initiated fusion at the leading edge of a developing vacuole with the exception of two filamentous viruses which did not induce vacuole formation and fused directly at their curved tip. Fusion was never seen to occur in the concave base of the vacuole.

To be sure that envelopment and fusion of viruses occurred during the 1 min at 37°C and not during the adsorption in the cold, samples were examined just before the temperature was raised. Viruses bound to the liposomes at 0°C were not enveloped or fused.

An occasional virus also undergoes disassembly by 1 min. Fig. 2 shows a liposome with viruses at different stages of fusion and disassembly after incubation at 37°C for 1 min. In this and the succeeding micrographs it is clear that despite the preadsorption, the course of infection is asynchronous. Several viruses have adsorbed to the liposome but have not been enveloped or fused. Two viruses (double arrows) have been partially enveloped by the liposome, and have just fused with the liposome. One of these is also beginning to disassemble. Lastly several viruses have already fused and totally disassembled as evidenced by the fact that viral glycoprotein spikes are seen distributed over the surface of the liposome (single arrows). The ratio of fused but not disassembled viruses to liposomes containing patches of glycoprotein spikes on their surface at 1 min was 6 : 1. This may underestimate the number of viruses which have disassembled since it is impossible to tell how many viruses made the patches present on one liposome, but does show that there is a lag between the fusion event and disasembly.

Disassembly is evidenced by movement of the viral glycoprotein spikes over the surface of the liposome and loss of viral contours. Since the lipids of these liposomes should be in the liquid phase at 37°C and since the liposomes have no protein or cytoskeleton, liposomes should present no barrier to free diffusion of the viral proteins. The electron micrographs, however, suggest viral disassembly is more complex than simple diffusion of viral proteins away from the site of membrane fusion. The viral membrane contains three proteins which are the M protein and two glycoproteins [6]. The glycoproteins are the F protein and the HN protein, which binds to the neuraminic acid containing viral receptors and which has neuraminidase activity. Since the initiation of fusion is at the leading edge of an 'endocytic' vacuole, and since the vacuole is formed because of circumferential binding of the ganglioside receptor to the HN proteins [4], it is evident this binding has to be released before the

Fig. 1. Initiation of membrane fusion after 1 min at 37°C. Magnification: X 76 000.

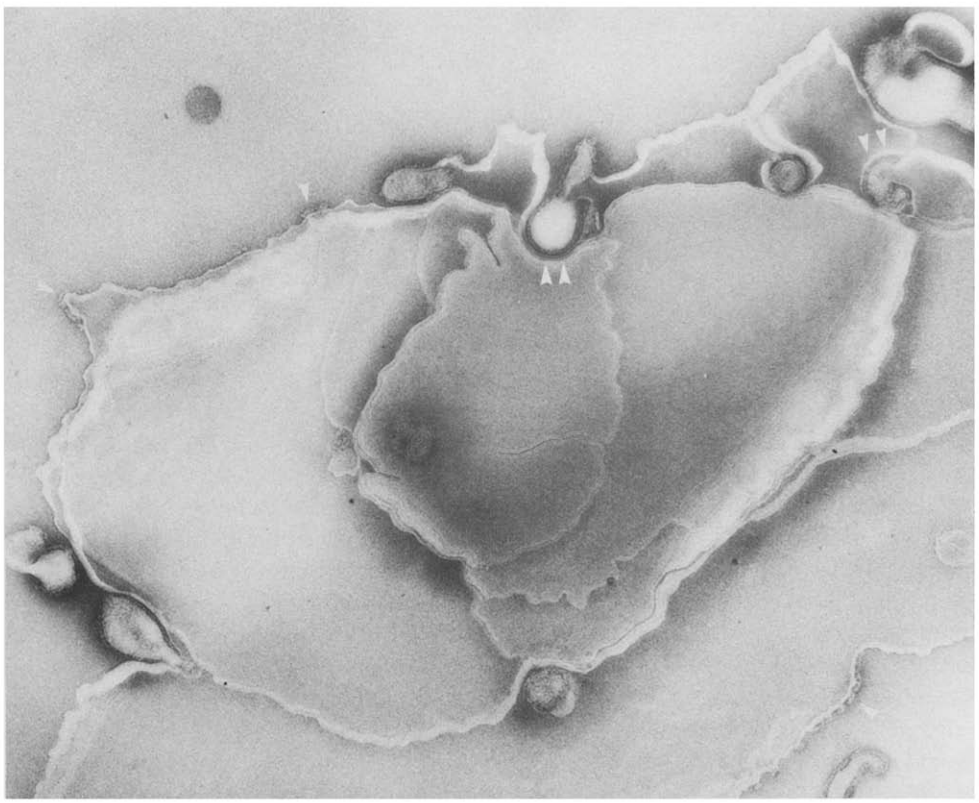

Fig. 2. Membrane fusion and viral disassembly after 1 min at 37°C. Magnification: X 60 000.

viral HN protein is free to migrate and the virus and liposome can assume the contour of the general liposomal surface. This is well illustrated in Fig. 3 where the viruses and liposomes have been incubated at 37°C for 3 min. The virus on the right (double arrow) is partially enveloped by the liposome and is just beginning to fuse with the liposome. The virus on the left (single arrow) not only has fused with the liposome, but has also partly disassembled so that most of its original contours are no longer visible. The portion of

Fig. 3. Membrane fusion and viral disassembly after 3 min at 37°C. Magnification: X 76 000.

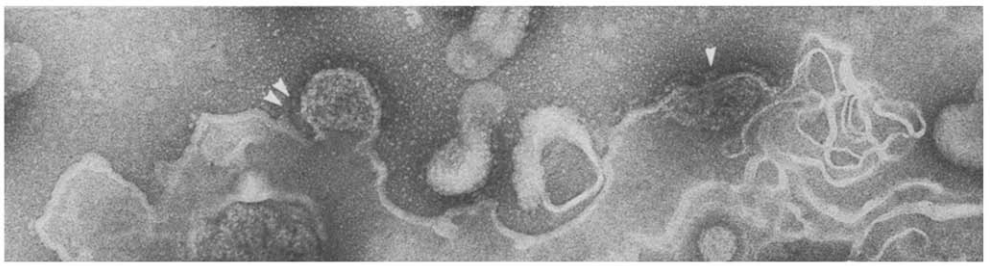

Fig. 4. Membrane fusion and viral disassembly after 12 min at 37°C. Magnification: X 95 000.

the virus which has not conformed to the general shape of the liposomal surface is the portion that still retains the binding between the viral HN protein and the liposomal receptors. Release of the binding from the ganglioside during disassembly could be due to the viral neuraminidase. This possible role for the neuraminidase has not been previously considered.

If viral proteins interact with each other in the virus, then these interactions may have to be released before the viral morphology is lost and the proteins are free to migrate. Fig. 4 shows viruses and liposomes after incubation at 37°C for 12 min. On the left (double arrow) there is a fused virus which has had all but a small area of binding released but which nevertheless retains much of its morphology. On the right is a virus (single arrow) which has progressed further in disassembly so its contours nearly conform to that of the liposome. The viral ribonucleoprotein remains attached to a region of membrane which also contains viral glycoprotein spikes. The proportion of fused viruses which maintain their morphology after 12 min at 37°C varies when different virus preparations are used, and probably relates to the integrity of the virus membrane. By two hours all the viruses have assumed the contours of the liposome and the proteins are distributed around the surface of the liposome, often in patches. The ribonucleoprotein stays attached to the membrane. This is consistent with previous work on liposomes which had been incubated 2 h at 37°C [2,5].

Discussion

The course of penetration of Sendai virus into liposomes resembles the course of penetration of Sendai virus into the cells of the chorioallantoic membrane as described by Morgan and Howe [7]. Viral penetration is composed of two steps which are membrane fusion followed by viral disassembly.

Membrane fusion occurs at the leading edge of a developing 'endocytic' vacuole. This is diagrammed in Fig. 5. The diagram pictures the attachment of viral glycoprotein spikes to the ganglioside receptors represented as dots. The sequence up to frame B occurs in the cold. When the viruses and liposomes are warmed there is circumferential progression of the ligand-receptor binding which leads to frame C. As the binding continues, the curvature of the leading

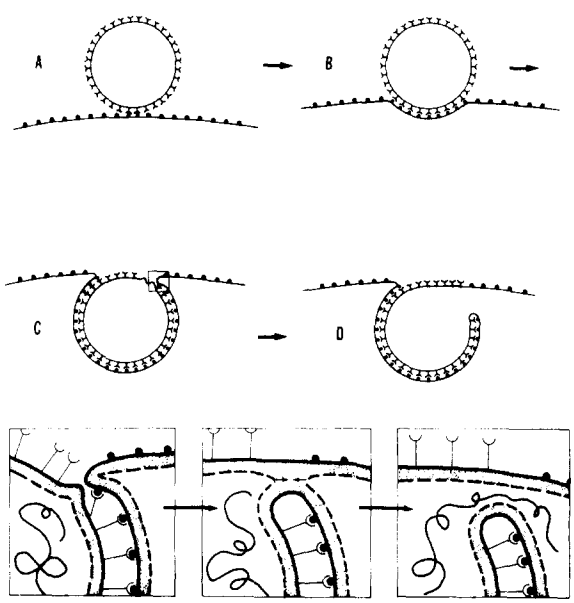

Fig. 5. Diagram of fusion of Sendai virus membrane with a liposome. The dots represent ganglioside receptors. On the insert the solid line represents the outer monolayer, the dashed line the inner monolayer and the stippled area the hydrocarbon core. The coiled line represents viral ribonucleoprotein.

edge increases. Membrane fusion occurs at this leading edge where there is a region of decreased radius of curvature. Since the liposome has a zeta potential of −18 mV and the virus has a zeta potential of −17 mV [1], the charge repulsion between the two membrane surfaces should present a barrier to the close approach necessary for membrane fusion. If a surface is highly curved, as is the case for the leading edge of the vacuole, the charge repulsion between two negatively charged surfaces is reduced [8,9]. Increasing the curvature of a membrane changes the lipid packing and increases the surface free energy [10–12], which should help drive the fusion process. Fusion of the leading edge of the vacuole with the virus leads to the structures seen in Figs. 1–3 and diagrammed in Fig. 5D. The insert on Fig. 5 presents a possible model of the events occurring at the fusion point (boxed area) between C and D. This model includes the data of Knutton [13], which indicate there is a redistribution of proteins and the formation of ridges and grooves on a fusing virus.

Once fusion has established the continuity between the viral and liposomal membrane, disassembly can take place. This disassembly requires release of the ligand binding from the receptors, which contain neuraminic acid. The release may involve the viral neuraminidase. Further restraints on migration of viral proteins appear to exist on those parts of the virus which are not bound or which have been released from binding after continued incubation. These restraints may be due to viral protein-protein interactions. The viral membrane is originally assembled in the host membrane and then buds off from the host cell. Compans and Klenk [6] in their review of viral assembly and budding have suggested that either the M protein, which is associated with the inner half of the bilayer, or the nucleocapsid, which is thought to be bound to the M protein, play a role in budding. Conversely, release of the nucleocapsid from the M protein or disruption of a matrix of M protein may be necessary for viral disassembly. Knutton and Bächi [14] have shown that when Sendai viruses fuse to red cells, the virus proteins as measured by immunofluorescence do not migrate into the red cell unless the cell is osmotically swollen. Swelling of the membrane would tend to expand the curved area around the virus and assist in releasing receptor-ligand binding. The authors observe that osmotic swelling might have an effect on viral antigen diffusion by perturbing the structural organization of either the erythrocyte membrane and/or of the incorporated viral membrane. In the red cell system, however, it was not possible to separate the two effects. Lastly, the fact that viral ribonucleoprotein remains associated with the liposomal membrane even after 2 h suggests either that viral RNA replication occurs in association with the membrane as has been previously demonstrated for RNA bacteriophage RNA [15] or that cellular components are required for the release of the ribonucleoprotein. Further work is being directed at the role of virus protein interactions and membrane expansion in virus membrane fusion and disassembly.

Acknowledgements

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