NOTES

Factors Affecting Cell Fusion Induced by Sendai Virus

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Cell fusion mediated by exogenous Sendai virus appears to occur in four temperature-dependent stages. The first two, which include viral adsorption, are pH dependent and can be inhibited by viral antibody. Viral envelope constituents remain detectable on the cell surface during the third stage and disappear only when cell-to-cell fusion supervenes. The relationship of these interactions to possible mechanisms of cell fusion are discussed.

Okada and his associates (5) demonstrated that Ehrlich ascites tumor cells could be fused with one another by exogenous Sendai virus, and that this reaction occurred most rapidly and extensively at pH 7.6 and 37 C in the presence of calcium ions. Viral antiserum could inhibit the process of fusion under these optimal conditions only during the first 5 min after virus-cell admixture (6). The present study was undertaken to examine factors governing fusion in this early, antibody-sensitive stage, as well as at later stages in which fusion is unaffected by antibody.

Concentrated Sendai virus, inactivated with 0.01% beta-propiolactone, was prepared as previously described (7). FL amnion cells were maintained in Eagle basal medium in Hanks balanced salt solution (BME), supplemented with 10% newborn calf serum. Fusion assays were carried out with cells in confluent monolayers on cover slips in Leighton tubes. Monolayers were fixed in methanol at appropriate times and stained with Lillie azure-eosin. Cover slips were placed in 5×10^{-4} M EDTA in phosphate-buffered saline (PBS) for 2 min prior to fixation and staining to cause the retraction of mononucleated cells from syncytia and thereby to facilitate cell and nuclear counts (4). Fusion indexes were expressed as the ratio of the number of nuclei counted to the number of cells in which they were found. A minimum of 500

nuclei in four different fields were examined on each stained cover slip. Antiserum (As) to egg-grown Sendai virus was prepared in rabbits, as previously described (3), and inactivated at 56 C for 30 min. Hemagglutination (HA) assays were performed with chicken erythrocytes by the pattern method (2). Neuraminidase activity was estimated on the basis of assays for free sialic acid by the thiobarbituric acid method of Warren (8).

The following experimental results are summarized in Fig. 1. Sendai virus (0.2 ml) in Earle's balanced salt solution (BSS), pH 7.6, at a concentration sufficient to yield massive cell fusion, was added to washed FL cell monolayers at 37 C. Replicate monolayers in one series were fixed in methanol and stained after various periods at 37 C. From these, it was determined that fusion first became discernible after 20 to 40 min and was maximal by the end of 2 h after addition of virus. With another series, it was found that the process could be arrested at any time after inoculation by chilling the cells and maintaining them in BSS at 4 C. Under these conditions, fusion was not seen to develop in monolayers chilled during the first 20 min of contact with virus, but was increasingly apparent as the preceding period of incubation at 37 C lengthened. The amount of virus adsorbed during the first 5 to 10 min of incubation was sufficient to cause 100% fusion, since after 10 min the viral inocula could be replaced with warm BSS without diminishing the extent of fusion observed at the end of 2 h. The addition

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FIG. 1. 100% fusing dose of Sendai virus in BSS, pH 7.6, at 37 C added to replicate FL cell monolayers. Inocula removed after increasing periods of incubation at 37 C, and (▲) monolayers fixed in methanol; (●) BSS, pH 7.6 and 4 C, added to the end of a 2-h period; (Δ) BSS, pH 7.6 and 37 C, added to the end of a 2-h period; (◊) antiviral serum in BSS, pH 7.6, added to the end of a 2-h period; (□) BSS, pH 6.0, added to the end of a 2-h period; (○) *Clostridium perfringens* neuraminidase, 100 μ g/ml in BSS, pH 7.0, added to the end of a 2-h period; (■) *Clostridium perfringens* neuraminidase, 100 μ g/ml in BSS, pH 6.0, added to the end of a 2-h period.

of viral As in BSS to monolayers after the removal of unadsorbed virus was completely inhibitory to cell fusion only during an initial 10-min period. When As was added after 20 or 30 min and cultures were incubated to the end of 2 h at 37 C, 22 and 74%, respectively, of total cell fusion occurred. Scrutiny of companion virus-challenged monolayers, to which no antiserum had been added but which were fixed in methanol at 20 and 30 min, revealed that no detectable fusion had occurred at these times. Fusion could likewise be arrested by lowering the pH to 6.0, but not beyond the first 30 min of contact between virus and cells.

To follow the fate of Sendai virus envelope constituents at the cell surface, unadsorbed virus was removed from challenged monolayers after 1.5 h, and the cell sheets were refed with BME supplemented with 0.1% lactalbumin hydrolyzate (LH medium). At various times, the LH medium was removed, the cells were rinsed with BSS, and a 0.5% suspension of guinea pig erythrocytes in PBS was added for 30 min at 4 C (Table 1). Viral HA could be detected at the syncytial surface by hemadsorption up to 4 h after the start of the experiment. By removing Sendai viral inocula from challenged FL mono-

layers and adding an excess of the substrate, Collochia glycoprotein (2) (0.4 ml of 10.9 mg/ml in BSS, pH 7.6, for 60 min), viral neuraminidase activity could also be detected until at least 2.5 h after the initial virus-cell interaction. However, inclusion in the inoculum of Collochia glycoprotein (5 mg/ml), fetuin (1 mg/ml), or normal rabbit serum (27.5%, vol/vol) did not result in decreased fusion indices.

The detection of viral neuraminidase and HA at the syncytial surface suggested that viral "fusion factor" might likewise be retained. FL monolayers at various stages of fusion were washed free of unadsorbed virus and scraped from the vessel surface into 0.5 ml of BSS. A 0.2-ml amount of the resulting suspension was then used to challenge fresh monolayers which were examined for fusion after 2 h at 37 C. Table 1 indicated that "fusion factor" was retained for up to 4 h at the surface of cells challenged with Sendai virus. This finding corresponded well with the persistence of HA and neuraminidase on the syncytial surface. All three activities disappeared *pari passu*, but not before fusion became insensitive to antibody.

From the foregoing, it appears that the fusion process may be divisible into four sequential

TABLE 1. *Presence of viral hemagglutinin, neuraminidase, and fusion factor at surface of FL cells challenged with Sendai virus*

Time after challenge (h)	Degree of fusion observed	Hemadsorption	Free sialic acid ^a	Secondary fusion index ^b
0	—	—	0.000	1.03
0.2	ND ^c	ND	ND	1.46
0.5	+	ND	0.035	2.27
1.0	2+	ND	0.030	2.47
1.5	4+	4+	0.060	ND
2.0	4+	3+	0.040	1.76
2.5	4+	3+	0.025	ND
3.0	4+	2+	0.015	1.36
3.5	4+	2+	0.010	ND
4.0	4+	+	0.010	1.26
4.5	4+	±	0.010	ND
5.0	4+	±	0.000	ND

^a Thiobarbituric acid reaction (optical density 549 nm) on supernatant fluid after addition of substrate and incubation at 37 C.

^b Fusion of secondary monolayers induced by dispersed cells previously challenged with virus and incubated at 37 C for increasing periods.

^c ND, Not done.

stages. During the first stage, lasting no longer than 10 min, viral adsorption takes place, and adsorbed virus can be inhibited by viral antibody. Following this, a second 20-min stage ensues which is pH dependent and during which addition of viral antiserum can still inhibit ultimate fusion. In the third, antibody-refractory stage, viral envelope constituents remain detectable on the surface of the cells. HA, neuraminidase, and fusion factor begin to disappear with the inception of the fourth stage, during which fusion becomes evident. Apparently only the first two stages are pH dependent, but the process can be interrupted at any point by lowering the temperature to 4 C. These sequential steps in the fusion of nucleated cells may have their counterparts in the changes observed to accompany the fusion of human erythrocytes by inactive Sendai virus (1). By electron microscopy and immunofreeze etching, virus particles attached to cells were seen to be discretely reactive with ferritin viral antibody.

On raising the temperature of the reaction to 37 C, fusion of viral envelope with cell membrane, loss of morphological identity of the virus, and rapid dissemination of envelope antigens over the cell surface occurred. This may correspond to stage three above, in which HA, neuraminidase, and fusion factor remained demonstrable, accounting for the clumping of the cells by viral antibody which, however, could no longer retard or inhibit ultimate fusion. Like fusion of cells in monolayer, fusion of erythrocytes could be arrested at any stage by lowering the temperature to 4 C. Similar mechanisms may underlie the fusion of both nucleated cells and erythrocytes by exogenous virus. One mechanism proposed is based on the concept of the membrane as a fluid mosaic, in which the free movement of lipid or glycoprotein, or both, may be influenced by exogenous factors, including viruses. The free movement and rearrangement of lipids are thought to promote fusion through temperature-dependent molecular interactions between adjacent membranes initially brought into apposition by viral HA.

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