Polykaryocyte Formation Induced by VSV in Mouse L Cells

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SUMMARY
Infection of mouse L cells with VSV leads to the formation of polykaryocytes about 4 to 12 h p.i. When anti-VSV immune serum was added during the course of infection, progression of cell fusion was soon suppressed. Cycloheximide completely suppressed the cell fusion when the drug was added within 1 h p.i., while the cell fusion was not suppressed at all when the drug was added at and after 3 h. Early polykaryocyte formation, ‘fusion from without’, was observed only at a low level in cells infected at very high multiplicities.

The development of cell fusion induced by VSV was found to be different in several cell types, although all these cells produced a rather high yield of virus: L and C-243-3 mouse cell lines showed a high level of polykaryocytosis (80 to 100 %), BHK and RK-13 cells responded at low level, and PS and Vero cells showed no cell fusion in response to VSV infection. In PS cells, however, cell fusion occurred when VSV-infected L cells were co-cultivated. From these observations, the mechanism of cell fusion induced by VSV was discussed.

INTRODUCTION
It has been reported that some lipid-containing viruses possess the ability to cause fusion of infected host cells; mumps (Henle, Deinhardt & Girardi, 1954), HVJ (Okada, 1958), measles (Warthin, 1931; Enders & Peebles, 1954), NDV (Johnson & Scott, 1964), herpes (Hoggan & Roizman, 1959), and visna virus (Harter & Choppin, 1967). However, there has been no precise study of cell fusion induced by VSV which is also a lipid-containing, enveloped virus of the rhabdovirus group that matures at the cell membrane by budding. Most studies on morphological changes in VSV-infected cells are concerned with cell rounding at both early and late stage of virus infection (David-West & Osunkoya, 1971; reviewed by Bablanian, 1975).

In the present communication, we describe the cell fusion phenomenon observed in L cells infected with VSV, and the effect of antiviral antibody and cycloheximide on VSV-induced cell fusion. Comparative cytopathic changes in several cell lines infected with VSV are also described.

METHODS
Vesicular stomatitis virus. New Jersey strain of VSV was kindly supplied by Dr Kono, the National Institute of Animal Health and was maintained in our laboratory by passaging in BHK cells. The virus, cloned through plaque purification in BHK cells, was propagated in BHK cells by inoculating at a multiplicity of 0.01 to 0.005 p.f.u./cell. After
incubation at 35 °C for 24 h, the medium was collected, centrifuged for 15 min at 3000 rev/min, and stored at -80 °C. The infectivity titre of the stock virus was about 5 to 9 × 10⁹ p.f.u./ml in BHK cells.

Cell cultures and media. Cells used in the present study were mouse L cells, BHK cells, PS cells (Porcine kidney stable cell line), Vero cells, RK-13 cells, and mouse C-243-3 cells. The cloned line, C-243-3, originally established by Bassin, Tuttle & Fischinger (1970), was kindly supplied by Dr Kawade, Institute for Virus Research, Kyoto University. Cells were grown in MEM supplemented with 10 % calf serum, 5 % tryptose phosphate broth and antibiotics. The same medium, from which calf serum was omitted, was satisfactory for maintenance of the cells and was used for virus growth.

Plaque assay. Infectivity titrations of VSV were performed by plaque assay in monolayer cultures of BHK cells. 0.2 ml of the test material were each inoculated on BHK cultures in Petri dishes, incubated at 35 °C for 1 h, and the cultures were overlaid with 1 % agar in YLE. After 2 days incubation at 35 °C, cells were stained with neutral red and the plaques were counted. Infectivity titres are expressed as plaque-forming units (p.f.u.)/ml.

Estimation of fusion as % polykaryocytosis. Cell coverslip cultures were infected with 65 to 90 p.f.u./cell of VSV. At various intervals after infection cell cultures were stained with May-Grfinwald-Giemsa. The extent of cell fusion, expressed as percentage polykaryocytosis, was estimated by counting the number of nuclei present in polykaryocytes and expressing this as percentage of total number of nuclei present in the same microscope field (Reeve & Poste, 1971).

VSV antiserum. Anti-VSV serum was obtained from rabbits by repeated intravenous injection of VSV grown in BHK cells. Sera were heated at 56 °C for 30 min prior to use. The neutralizing capacity of antiserum was determined by mixing about 100 p.f.u. of VSV with serial dilution of antiserum and inoculating L cells. The serum titre was expressed as the dilution causing 80 % plaque reduction: the neutralization titre of this serum was 80,000.

RESULTS

Growth and cytopathic effects of VSV in L cells

In a single-step growth experiment of VSV in L cells at a multiplicity of approx. 65 p.f.u./cell, virus growth and morphological alterations of the cells were studied. As shown in Fig. 1, the virus multiplied exponentially after a latent period of 2 h, and reached a maximum titre at about 6 h p.i. The first cytological change, cell fusion, occurred at about 4 h p.i., and almost all cell nuclei were found in multinucleated giant cells by about 12 h p.i. Infected cells stained by May-Grfinwald-Giemsa 12 h p.i. are shown in Fig. 2.

Similar cell fusion was observed in L cells infected with uncloned VSV of New Jersey and Indiana strain.

Effect of anti-VSV serum on fusion of L cells infected with VSV

It is known that VSV matures on the cell membrane through a budding process and the area of plasma membrane where budding occurs, as well as the virus envelope, contain virus-specific proteins in conjunction with lipids and glycolipids of cellular origin (Howatson & Whitmore, 1962; Wagner, Snyder & Yamazaki, 1970). The glycoprotein which constitutes the spike-like projections of the virus envelope binds anti-VSV neutralizing antibody (Kelley, Emerson & Wagner, 1972). With these studies in mind, attempts were made to examine the effect of anti-VSV serum on the process of cell fusion induced by VSV infection.
Fig. 1. Virus growth (○—○) and polykaryocyte formation (●—●) in L cells infected with 65 p.f.u./cell of VSV.

Fig. 2. Polykaryocyte formation induced by VSV in L cells. (a) Uninfected L cells. (b) L cells infected with VSV at 65 p.f.u./cell and fixed 12 h p.i. Magnification: ×270.
L cells in Petri dishes were inoculated with VSV at a multiplicity of 65 p.f.u./cell and incubated for 1 h at 35 °C. After washing twice with PBS, maintenance medium containing anti-VSV rabbit serum of graded concentrations was added. A parallel set of control plates was added with maintenance medium containing normal rabbit serum at the same dilutions. As shown in Fig. 3, polykaryocytosis was reduced about 50% in the presence of anti-VSV serum at a dilution of 1:80, and antiserum diluted 1:10 inhibited polykaryocyte formation almost completely.

In an attempt to find out the effect of anti-VSV serum added during the period where cell fusion was developing, antiserum was added to the culture medium at 1, 4, and 6 h i.p. to give a final dilution of 1:10. As shown in Fig. 4, the development of cell fusion was inhibited immediately after the addition of antiserum. These observations suggest that the virus glycoprotein produced on the cell surface may be involved in cell fusion of L cells infected with VSV.

**Effect of cycloheximide on cell fusion of L cells infected with VSV**

In an attempt to determine whether cell fusion could be prevented by inhibitors of protein synthesis, the following experiments were performed. L cells in Petri dishes were infected with VSV and incubated at 35 °C for 1 h. After washing twice with PBS, maintenance medium containing various concentrations of cycloheximide were added. After further incubation at 35 °C for 11 h, the culture fluid was assayed for infectivity, and the cell fusion in cells on coverslips was observed.

The results shown in Fig. 5, indicate that cycloheximide inhibited virus growth concomitant with the reduction of polykaryocyte formation. Polykaryocytosis, however, was retained rather well in comparison with the reduction of virus yield. For example, in the presence of 1 μg/ml of cycloheximide about 25% of nuclei were involved in polykaryocytes,
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while virus yield decreased to 1% of the control culture. It was also shown that the production of virus and the polykaryocyte formation were almost completely suppressed when 16 μg/ml of cycloheximide were added 1 h i.p.

In the following experiments the development of fusion was examined when 20 μg/ml of cycloheximide was added at various times post-infection. As shown in Fig. 6, the development of cell fusion was not suppressed at all when the drug was added at and after 3 h, and more than 95% of nuclei were involved in polykaryocytes within 12 h. Similar results were obtained even when higher concentrations of the drug, 200 μg/ml, were added to the medium. These results suggest that L cells are prepared for cell fusion by 3 h after VSV infection, during which protein synthesis is required. However, further cell fusion develops without concomitant protein synthesis.

Cell fusion in other cells infected with VSV

Fig. 7 shows the development of polykaryocytosis in six cell types following infection with VSV at an input multiplicity of 50 to 90 p.f.u./cell. Large polykaryocyte formation occurred in C-243-3 cells, although the development of cell fusion was slower in C-243-3 cells in comparison with L cells. In BHK cells small polykaryocytes containing 2 to 5 nuclei were formed and up to 25% of the total nuclei were involved in polykaryocytes by 10 h. RK-13 cells also showed a low level of polykaryocytosis and binucleate cells could be seen frequently. In monolayers of Vero cells few polykaryocytes could be seen. It was of interest that PS cells did not show any polykaryocyte formation throughout infection of VSV and cell rounding appeared at 3 h, involving the complete monolayer by 12 h. Further studies on cell fusion in PS cells are described below.
Holmes & Choppin (1966) using simian virus 5 (SV5) observed the inverse relationship between virus yield and the extent of cell fusion, and Alexander et al. (1973) using Newcastle disease virus indicated that the extent of cell fusion was related to the level of virus haemagglutinin at the cell surface of infected cells. In our experiments, however, no relationship could be found between the extent of cell fusion and the level of virus accumulation or virus release in six cell types inoculated at a multiplicity of 50 to 90 p.f.u./cell, as shown in Table 1.

**Cell fusion in PS cells**

As observed in preceding experiments, VSV caused cell rounding in PS cells without causing cell fusion throughout infection. However, it was found by chance that cell
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Fig. 6. Effect of cycloheximide added at various times after infection on polykaryocyte formation induced by VSV. Cycloheximide was added to the culture at 1 (○), 2 (△), 3 (□), 4 (■), and 6 (▲) h p.i. to give a final concentration of 20 μg/ml, and subsequent polykaryocytosis was measured. Polykaryocyte formation in control cultures (●) is also shown. Arrow (↓) shows the addition of cycloheximide.

Fig. 7. Polykaryocyte formation in L (○), C-243-3 (●), RK-13 (△), BHK (▲), PS (□), and Vero (■) cells infected with 50 to 90 p.f.u./cell of VSV. Polykaryocytosis in less than 10% was taken as (0), 10 to 30% as (1), 30 to 50% as (2), 50 to 70% as (3), 70 to 90% as (4), and 90 to 100% as (5).
Table 1. Comparison of virus yield and relative extent of polykaryocyte formation induced by VSV in six different cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Released virus* p.f.u./cell</th>
<th>Cell associated virus† p.f.u./cell</th>
<th>Nuclei in polykaryocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>165</td>
<td>8.8</td>
<td>90–95</td>
</tr>
<tr>
<td>C-243-3</td>
<td>25</td>
<td>0.93</td>
<td>90–95</td>
</tr>
<tr>
<td>BHK 21</td>
<td>1740</td>
<td>128</td>
<td>20–30</td>
</tr>
<tr>
<td>RK-13</td>
<td>62</td>
<td>0.38</td>
<td>20–30</td>
</tr>
<tr>
<td>Vero</td>
<td>414</td>
<td>124</td>
<td>5</td>
</tr>
<tr>
<td>PS</td>
<td>578</td>
<td>41</td>
<td>3</td>
</tr>
</tbody>
</table>

* Released virus in the culture medium at 12 h p.i.
† Cell associated virus at 9 h p.i. For measurement of cell associated virus titre, the cells, detached by EDTA, were suspended in 2 ml of buffered saline, and sonicated for 20 s.

Fig. 8. Polykaryocyte formation in PS cells co-cultivated with VSV-infected L cells. Polykaryocytosis in PS cells co-cultivated with VSV-infected L cells (●), in L cells co-cultivated with VSV-infected L cells (▲), in PS cells infected with VSV (○), in PS cells co-cultivated with normal L cells (□), and in control PS cells (△). All cultures contain 20 μg/ml of cycloheximide.

fusion occurred in PS cells when they were co-cultivated with VSV-infected L cells. These findings were further examined in the following experiments.

Monolayers of L cells in 150 ml bottles were inoculated with VSV at a multiplicity of 50 p.f.u./cell. After incubation at 35 °C for 1 h, cells were washed twice with PBS and incubated with maintenance medium for further 2-5 h at 35 °C. Cells were then detached by EDTA treatment and suspended in maintenance medium containing 20 μg/ml of cycloheximide to inhibit further virus growth. Samples of VSV-infected L cell suspension were added on PS cells grown to monolayers in Petri dishes containing coverslips. The
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Fig. 9. Polykaryocyte formation in PS cells co-cultivated with VSV-infected L cells. (a) Uninfected PS cells. (b) PS cells infected with VSV at 90 p.f.u./cell and fixed at 9 h p.i. (c) Polykaryocytes formed between PS cells and VSV-infected L cells at 6 h after co-cultivation. Magnification: ×270.

The relationship of VSV-infected L cells/total cells was about 20 to 25%. At the specified times after addition of VSV-infected L cells, coverslips were removed, stained with May-Grünwald-Giemsa, and the percentage polykaryocytosis was estimated. The results are shown in Fig. 8 and 9. It was observed that cell fusion forming binucleate cell occurred as early as 1 h after co-cultivation, and by 6 h about 45% of the total nuclei was involved in polykaryocytes, when a number of large polykaryocytes containing up to 20 nuclei were observed (Fig. 9c).

These observations infer that L cells infected with VSV may fuse with PS cells causing binucleate hybrid cells, which then fuse further with surrounding PS cells producing multinucleated giant cells.
Fusion from without by VSV

Monolayers of L cells in Petri dishes were washed with PBS and inoculated with 0.5 ml of virus suspension in maintenance medium containing 50 μg/ml of cycloheximide. The m.o.i. was 650 to 14000 p.f.u./cell. After incubation for 1 h at 35 °C, 1.5 ml of maintenance medium containing 50 μg/ml of cycloheximide was added. At 3 h p.i., percentage polykaryocytosis was estimated. L cells infected with 14000 p.f.u./cell of VSV showed low levels of polykaryocytosis in the presence of cycloheximide (about 15% of nuclei were involved in polykaryocytes within 3 h), while infection of L cells with 650 p.f.u./cell failed to induce cell fusion.

These results suggest that VSV is capable of fusing L cells by fusion from without. However, the level of polykaryocytosis by fusion from without was much lower than that by fusion from within.

DISCUSSION

Vesicular stomatitis virus (VSV) has not been shown to cause cell-cell fusion, i.e. the formation of polykaryocytes although VSV has been reported to penetrate the cell by virus-cell fusion (Heine & Schnaitman, 1969). However, the results described in this study demonstrated that infection of L cells with VSV lead to the formation of polykaryocytes about 4 to 12 h p.i., and the addition of anti-VSV serum to the cultures suppressed further development of cell fusion. Earlier papers have reported that VSV is one of the enveloped viruses that mature at host cell membrane by a budding process (Howatson & Whitmore, 1962; Zee, Hackett & Talens, 1970), and the spike-like projections containing glycoprotein are present on the surface of the virus envelope. In addition, it has been shown that the glycoprotein of VSV is the component which induces and binds anti-VSV neutralizing antibodies (Brown, Cartwright & Smale, 1967; Kelley et al. 1972). In considering these observations, it was inferred that virus envelope component, probably glycoprotein on the cell surface, may play an important role in the mechanism of cell fusion of L cells infected with VSV.

The addition of 20 μg/ml of cycloheximide to the culture at 1 h p.i. suppressed the cell fusion almost completely as well as the multiplication of viruses. However, when the drug was added at and after 3 h, even if a final concentration of cycloheximide was as much as 200 μg/ml, cell fusion was not suppressed at all. These observations may indicate that L cells infected with VSV prepared for cell fusion within 3 h p.i., during which time synthesis of protein, probably virus envelope protein, was required, and further development of cell fusion could proceed without protein synthesis.

A striking difference was found in the ability of various cells to undergo fusion after infection with VSV. From the difference in the ability of several cells to fuse following VSV infection, the cells tested here could be classified into three groups; (1) highly reactive (L and C-243-3 cells), (2) moderately reactive (RK-13 and BHK cells) and (3) non- or less reactive (PS and Vero cells).

Holmes & Choppin (1966), and Alexander et al. (1973) have discussed the relationships of virus accumulation and virus release to the process of cell fusion in different cell types. In the present studies, however, no relationship could be found between the extent of cell fusion and the level of virus accumulation or virus release in six cell types infected with VSV.

It was of interest that infection of VSV caused only cell rounding in PS cells without forming polykaryocytes, while PS cells undergo rapid fusion following co-cultivation
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with VSV-infected L cells. These results may suggest that the surface membrane of PS cells has fusion capacity relating to VSV infection. If so, why do PS cells infected with VSV not show any cell fusion? Although the reasons are still unclear, it seems to be likely that the budding process in PS cells might be involved in these phenomena. Many investigators observed that both plasma and intracytoplasmic vacuolar membrane serve as maturation sites for VSV, and Zee et al. (1970) have shown that the site of maturation of VSV is a host-dependent phenomenon. Therefore, it was thought that formation of polykaryocytes in VSV-infected cells might be influenced by the maturation site of the virus in a particular cell.

When considering the fact that the envelope of VSV fuses with the cellular plasma membrane (Heine & Schnaitman, 1969; Dahlberg, 1974), it was thought to be probable that very high m.o.i. may bring about modifications of the cell membrane causing cell fusion. However, only few early polykaryocytes were induced in L cells even when the cells were infected at a multiplicity of 14,000 p.f.u./cell. Although it is still unknown if fusion from without and fusion from within are caused by the same mechanism, fusion from without by VSV seems to be far less efficient than fusion from within in L cells.

Further morphological studies using electron microscope and experiments about VSV mutants in relation to virus growth and cytopathogenicity may shed some light on these problems. These studies are now in progress and will be published elsewhere.

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REFERENCES


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