Natural Selection of Mutants of Vesicular Stomatitis Virus by Cultured Cells of *Drosophila melanogaster*

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SUMMARY

The infection of cultured cells of the fruit fly *Drosophila melanogaster* (Schneider) with vesicular stomatitis virus (VSV) led to the establishment of persistent non-cytocidal infection. After weeks or months of persistent infection many VSV variants or mutants were detected all of which showed increased capacity for growth in these insect cells. We present a preliminary characterization of these viruses arising in the *Drosophila* cells.

INTRODUCTION

Vesicular stomatitis virus (VSV) was originally classified as an arbovirus (Taylor, 1967) on the basis of its transmission by, and isolation from, insects; it has since been reclassified as a rhabdovirus, along with rabies virus and a number of other viruses of similar bullet-shaped morphology (see review by Howatson, 1968). VSV is the prototype virus of the group and has a wide host range *in vivo* and *in vitro*, growing in cultured cells of birds, mammals and insects. The hereditary virus of *Drosophila*, Sigma virus, is a rhabdovirus which is morphologically similar to VSV (see review by Seecof, 1968). Some members of the rhabdovirus group are known to grow in plant as well as insect hosts; well-documented examples are sowthistle yellow vein virus (Richardson & Sylvester, 1968) and lettuce necrotic yellows virus (Harrison & Crowley, 1965).

Printz (1970) grew VSV in whole *Drosophila melanogaster* animals for many virus passages and observed changed plaque characteristics on primary chick embryo fibroblast cultures. Printz also used the production of CO₂ sensitivity in *Drosophila* to demonstrate the presence of virus in the flies (L'Héritier & Teissier, 1945). Yang, Stoltz & Prevec (1969) reported multiplication of VSV in cell cultures of the moth *Antheraea eucalypti*, but apparently did not continue the experiment long enough to detect mutant progeny.

The present work confirms the results of Printz (1970) and extends them to include preliminary characterization of VSV variants or mutants selected by growth in fly cells.

METHODS

*Cultivation of Drosophila melanogaster cells*. The cell line used in this study was line no. 2 derived from fly embryos by Dr Imogene Schneider (Schneider, 1972), Department of Entomology, Walter Reed Institute of Research, Washington, D.C., U.S.A., and was obtained from Dr Ruth Kavenoff. The cells were maintained on the standard media of Schneider (1964,
1967). Cells were transferred at 1- or 2-week intervals by scraping or washing as many as possible off the surface of plastic flasks (Falcon), pipetting briefly to remove clumps, diluting twofold with complete Schneider’s medium (CSM) (Gibco) plus 15 % foetal calf serum and 0.5 % proteose peptone (Difco), and plating an appropriate number of cells in plastic flasks or 4 oz glass prescription bottles. About $5 \times 10^6$ cells in 2-5 ml of medium attached completely to form loose ragged monolayers on the glass surface. The cells never became confluent with growth, but excess unattached cells grew in suspension in the medium. These suspended cells attached quickly to glass when transferred to new bottles. All cells were cultivated in an atmosphere of 5 % CO$_2$ in air.

Once cells attached to glass, they were very difficult to remove completely for counting. Therefore, experiments requiring accurate cell counts were begun within 1 day of plating a known number of cells, and the number of unattached cells was subtracted from the total. The doubling time for these cells has been estimated to be about 3 days at 25 °C (R. Kavenoff, personal communication). Cells were grown routinely at room temperature (20 to 23 °C), with the exception of experiments in which 28 °C was used.

**Infection of Drosophila melanogaster cells.** Except where indicated, cells were infected with VSV (Indiana) at an input multiplicity of 5 p.f.u./cell. All but 0.1 to 0.2 ml of medium was removed, the virus suspension was added in 0.02 to 0.1 ml vol. and allowed to adsorb for 1 to 2 h at room temperature. Except where indicated, excess virus was removed by washing five times with CSM, and 2-5 ml of fresh CSM was added. For growth curves 0.1 ml of supernatant medium was removed periodically, added to 0.9 ml complete MEM (minimal Eagle’s medium, containing 5 % calf serum), containing 10 % DMSO for preservation of virus (Wallis & Melnick, 1968), and frozen at −70 °C until assayed by plaque counting on BKH-21 cells.

**Infection of BHK-21 cells and virus purification.** Baby hamster kidney cells (BHK-21) were grown in monolayer cultures in 32 oz or 4 oz prescription bottles. When confluent, there were about $2 \times 10^7$ cells per 32 oz, or $2 \times 10^6$ cells per 4 oz bottle. For yield experiments, cells were infected with 5 p.f.u./cell of the Indiana serotype of VSV and harvested and assayed as described previously (Mudd & Summers, 1970).

Plaques were stained and counted after 1 day at 37 °C (Holland & McLaren, 1959). VSV was purified by sucrose gradient velocity and isopycnic centrifuging as described previously (Mudd & Summers, 1970), except that 1 mM-EDTA, 1 mM-tris, pH 7.0, was substituted for the 10 mM-buffer.

**Acrylamide gel electrophoresis.** Monolayers of infected BHK-21 cells labelled with radioactive amino acids were washed 3 times with cold 5 % TCA, 3 times with acetone, air-dried, and dissolved in 1 ml of electrophoresis sample buffer. Gels of 7:5 % acrylamide and 27 cm long were run as described previously for polypeptide gels (Mudd & Summers, 1970) in the SDS-phosphate system of Maizel (1969).

**RNA synthesis in HeLa suspension cells.** To facilitate sampling, monolayer cultures of HeLa $S_3$ cells were trypsinized and placed in suspension cultures at $2 \times 10^8$ cells/ml. Concentration, infection, labelling and sampling were as described previously (Mudd & Summers, 1970).

**Ouchterlony tests.** Antigenic analysis of pure virus was as described for potato yellow dwarf virus (Knudson & Macleod, 1972) except that 0.5 % or 1 % agarose was used; mouse immune serum was a gift from Dr Jacques Perrault.
Results

Long-term production of virus after primary infection of Drosophila melanogaster cells

Fruit-fly cells in culture were infected at an input multiplicity between 10 and 100 p.f.u./cell (infectivity determined in BHK-21 cells) and the inoculum was removed by repeated washing of the cells after adsorption for 1 h. We observed an initial virus yield of about $10^4$ p.f.u./ml after a week or two. No c.p.e. was observed in any inoculated cultures, and attempts to develop plaque assays or cell-killing assays with fly cells were unsuccessful.

We were able to establish persistent, non-cytocidal infections of these cultured insect cells in cases in which a high input multiplicity was employed (50 to 100 p.f.u./cell) and the inoculum was not removed after adsorption. The initial high infectivities from the inocula always disappeared after a week or 10 days. This may have been due to some active process of the cells, since the half-life of VSV in CSM alone at room temperature was about 1-2 days. Thus, at an input multiplicity of 100 p.f.u./cell, about a month would be required for thermal inactivation of input virus to a level below the limit of detectability.

Beginning about 1 month after infection we observed (Fig. 1) proportionality between
Table 1. Characteristics of VSV (Indiana) before and after passage in Drosophila cells

<table>
<thead>
<tr>
<th>Source of virus</th>
<th>Ratio of infectivities at 28 °C at 37 °C</th>
<th>Maximum infectivity ( \text{in p.f.u./ml from about } 2 \times 10^6 \text{ cells} ) in 5 ml medium: BHK-21 cells at 28 °C</th>
<th>Relative plaque size†</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSVb (original type)</td>
<td>1.0</td>
<td>( 3 \times 10^9 )</td>
<td>1.0 to 0.75</td>
</tr>
<tr>
<td>4th passage in fly cells</td>
<td>30</td>
<td>( (2 \times 10^5) )*</td>
<td>1.0 to 0.2</td>
</tr>
<tr>
<td>9th passage in fly cells</td>
<td>15</td>
<td>( (4 \times 10^8) )*</td>
<td>0.2</td>
</tr>
<tr>
<td>Mutant G plaque purified from fly cells on BHK-21 at 28 °C</td>
<td>10⁴</td>
<td>( 3 \times 10^8 )</td>
<td>0.2</td>
</tr>
<tr>
<td>Mutant T plaque purified from fly cells on BHK-21 at 22 °C</td>
<td>10³</td>
<td>( 5 \times 10^3 )</td>
<td>0.2</td>
</tr>
<tr>
<td>Mutant J plaque purified from fly cells on BHK-21 at 34 °C</td>
<td>0.5</td>
<td>( 5 \times 10^9 )</td>
<td>1.0 to 0.75</td>
</tr>
</tbody>
</table>

* Numbers in parentheses denote that the inoculum came directly from fly cells. For all others the inoculum came from BHK-21 cells.
† Maximum size of a VSVb plaque at any temperature tested is taken as 1.0; the size ratios were similar at all temperatures of plaque growth.

Fig. 3. Single-step growth curves of VSVb compared with ninth passage VSVo in fly cells at 22 °C. About \( 1 \times 10^7 \) fly cells in each glass plaque bottle were infected with 5 p.f.u./cell of VSVb or ninth passage VSVo. After adsorption of virus for 1 h at room temperature cell were washed 3 times with 2 ml CSM and then incubated at 22 °C with 2 ml of CSM. Samples were removed and stored as described in Methods and titrated at 28 °C on BHK-21 cells. The curve for VSVb is a composite of two separate experiments at 22 and 28 °C. O—O, VSVo; ▼—▼, VSVb 22 °C; ×—×, VSVb 28 °C.

input multiplicity and rate of production of infective virus at 37 °C. After about 50 days, a logarithmic increase in infectivity was evident in cultures infected at high multiplicity, and at about the same time increasing proportions of small plaques were observed in the plaque assays on BHK-21 cells at 37 °C.
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Fig. 4. Effect of temperature on the replication of VSV\textsubscript{B} and mutant G in BHK-21 and fly cells. BHK-21 cells were infected with 5 p.f.u./cell of VSV\textsubscript{B} or mutant G (as titrated at 28 \degree C on BHK-21 cells). After 15 min adsorption at 37 \degree C, excess virus was removed by washing the monolayers 5 times with Hank's balanced salt solution (BSS), and the cells were incubated at various temperatures with 5 ml MEM. Cultures were sampled at various times to establish the maximum yield and samples were stored frozen as described in Methods until titrated on BHK-21 cells at 28 \degree C. Drosophila melanogaster cells were infected and treated similarly except that adsorption was for 1 h at 22 \degree C, and excess virus was removed by washing 3 times with CSM. The supernatant media were sampled at 2 and 4 days after infection to establish the maximum yields. The ordinate, which is discontinuous for BHK-21 and fly cell yields, expresses the approximate yield of virus per cell.

\[\bullet\bullet\bullet, \text{VSV}\textsubscript{B} \text{ in BHK cells}; \circ\circ\circ, \text{VSV}\textsubscript{B} \text{ in fly cells}; \blacksquare\blacksquare\blacksquare, \text{mutant G in BHK cells}; \square\square\square, \text{mutant G in fly cells.}\]

Fig. 5. Single-step growth curve for VSV\textsubscript{B} and mutant G in BHK-21 cells at 22 \degree C. The cells were infected, treated and sampled as described in Fig. 4. \(\times\times\times, \text{VSV}\textsubscript{B}; \bullet\bullet, \text{mutant G.}\)

Temperature-sensitive mutants of VSV obtained from long-term culture in Drosophila melanogaster cells

Virus derived from the experiments shown in Fig. 1 was assayed at different temperatures, and a considerable fraction of this virus was found to have a temperature-sensitive phenotype (Fig. 2). Well-separated plaques were isolated at various temperatures and grown at low temperature on BHK-21 cells. This led to the isolation of the three types of virus clone which are shown in Fig. 2. Table 1 lists these clones and gives some phenotypic characteristics. During repeated subculturing on BHK-21 cells at an input multiplicity of 1 p.f.u./cell, these characteristics were retained. Thus the clones appear not to be phenotypic variants modified by the Drosophila melanogaster host. Ouchterlony double-diffusion tests showed three lines of antigenic identity with the original virus, so major antigenic determinants were retained. Hereafter, VSV\textsubscript{B} will be used to denote VSV of the Indiana serotype which has been grown for many generations in BHK-21 cells. Following the usage of Printz (1970), VSV\textsubscript{B} will be used to designate Indiana VSV grown for many generations in D. melanogaster.
Fig. 6. Electron microscopic comparison of mutant G virus particles with standard VSV\textsubscript{\textnormal{a}} virus particles. (A) Standard VSV\textsubscript{\textnormal{a}} grown in BHK-21 cells. (B) Mutant G virus particles grown in BHK-21 cells at permissive temperature (28 °C). Note abnormal morphology with distorted bullet shapes. (C) A mutant G virus particle from same preparation as (B). Note loss of bullet shape to give morphology resembling that of paramyxoviruses. All preparations were from purified virus particles negatively stained with phosphotungstic acid.
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Fig. 7. SDS-polyacrylamide gel electrophoresis of polypeptides of purified virus from mutant G and of BHK-21 cells infected by mutant G. Infected BHK-21 monolayers in 4 oz glass bottles were labelled between 6 and 24 h post-infection with 1 μCi each of [14C]-tyrosine, phenylalanine and valine in 1 ml MEM lacking these amino acids, or with 10 μCi [3H]-glucosamine, or with 5 μCi each of [3H]-tyrosine, phenylalanine, and valine as above. Labelled mutant G was purified as described in Methods. (a) Illustrates the electrophoretic profile of [3H]-amino acid-labelled G virus; (b) the electrophoresis of [3H]-glucosamine-labelled mutant G infected cells with [14C]-amino acid-labelled VSVG-infected cells. The anode is to the right and the letter symbols represent the location of defined virus polypeptides (Wagner et al. 1972). ○—○, [3H]-ct/min; ×—×, [14C]-ct/min.
cells. Individual clonal isolates have been given initials as in Table 1 for purposes of identification. As shown below, at least one of these isolates may be a multiple mutant.

**Single-step growth curves of VSV$_b$ and VSV$_p$ in Drosophila melanogaster cells**

A ninth passage stock of VSV$_b$, which had been continuously growing in *Drosophila melanogaster* cells through four subculturings of the cells and had been passed an additional 5 times through *Drosophila* cells, was used as inoculum in a single-step growth experiment (Fig. 3). The maximum yield of about 5 p.f.u./cell was achieved about 2 days after infection. VSV$_b$ exhibited the same growth rate but yields were 100 times lower (about 0.05 p.f.u./cell).

**Characterization of a mutant of VSV$_p$**

Of the clones listed in Table 1 only G was further characterized. It is representative of the majority of virus clones recovered from the persistently infected insect cells. It shows similar plaque size, thermal lability, and morphology in electron micrographs to uncloned stocks of VSV$_p$.

Yield of mutant G in BHK-21 and Drosophila melanogaster cells as a function of temperature. Since a plaque assay on fly cells was not possible, comparison of plating efficiencies at different temperatures could not be done. Therefore the yields of virus grown on the two cell types at different temperatures was measured. Fig. 4 compares the effects of temperature on yields of mutant G and VSV$_b$ in BHK-21 or fly cells. At 28 °C the yields per cell are the same for mutant G and VSV$_b$ grown in BHK-21 cells, but the mutant grows about 50 times better in *Drosophila melanogaster* cells than does VSV$_b$. The mutant gave poor yields on both cell types at higher temperatures.

Growth rate and yield in BHK-21 cells. Fig. 5 shows that, under single-step growth conditions, mutant G and VSV$_b$ have the same rate of increase of infectivity and the same yields when grown at 22 °C on BHK-21 cells; therefore the small plaque character is not related to the single-step yield but to some aspect of adsorption or penetration.
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Electron microscopy of mutant G. Although most mutant G particles exhibit the general bullet-shaped morphology of VSV, they are more loosely constructed and irregularly shaped than VSV\textsubscript{B} (Fig. 6). Some particles are so poorly assembled that they resemble paramyxoviruses more than rhabdoviruses. These G mutants may carry at least one structural gene lesion.

Acrylamide gel electrophoresis of polypeptides. Fig. 7(a) illustrates the electrophoretogram obtained for G virus labelled with radioactive amino acids in BHK-21 cells. The glycoprotein of the virus spikes (component G) is somewhat broader and reduced in amount as compared to ratios usually found for HeLa-grown VSV (Mudd, 1971). Comparison of \[^{3}H\]-glucosamine-labelled infected cells with VSV\textsubscript{B} infected cells, labelled with \[^{14}C\]-amino acids (Fig. 7b) also indicated a slight mobility difference between the glycoprotein of mutant G and of VSV\textsubscript{B}. There was also a large amount of apparently high mol. wt. glucosamine-labelled material present in mutant-infected cells. Its nature is not clear at present, but it is not normally observed in such quantity in cells infected with VSV\textsubscript{B}.

RNA synthesis in HeLa cells. Mutant G and VSV\textsubscript{B} incorporated radioactive uridine at the same rate at 28 °C (Fig. 8a), but 10% or less of the VSV\textsubscript{B} amount of uridine was incorporated at the non-permissive temperature (Fig. 8b). Whether this was due to a primary defect in polymerase or to secondary effects of other genetic defects is under investigation.

Reversion of phenotypes of mutant G. Three characteristics of mutant G appeared to undergo independent reversion to original phenotype during the course of these studies: (1) revertants from the temperature-sensitivity character were derived within the first low input multiplicity passage at 37 °C on BHK-21 cells; (2) revertant plaques picked from the fifth dilute passage at 37 °C appeared to be mixtures of original large plaques and 5 to 15% small plaques, neither of which was temperature-sensitive; (3) reversion of the phenotype which allowed mutant G to grow better than VSV\textsubscript{B} on fly cells was observed after a large stock was grown on BHK cells at a multiplicity of 0.1 p.f.u./cell. In three separate experiments, the excess growth character was lacking and yields as for VSV\textsubscript{B} were obtained on fly cells, but the small plaque and temperature-sensitivity characters were still present.

DISCUSSION

It must be concluded that cultivation of VSV (Indiana) in these insect cells provides selective conditions leading to the persistent growth of virus of variant or multiple mutant phenotype. In the case of mutant G these phenotypes include (a) a temperature-sensitive function, probably related to RNA synthesis; (b) a small plaque character which may be related to the structural defect observed by electron microscopy and to the changed electrophoretic mobility of the virus glycoprotein; and (c) a potential for increased yields on fly cells as compared to BHK cells. The temperature-sensitive defect could result from the low temperature of virus propagation in fly cells; experiments with BHK-21 cells at 28 °C have not yet been attempted. In order to study such mutations thoroughly, it will be necessary to isolate single point revertants for independent study.

The results described above are remarkably similar to those of Printz (1970): thirty serial passages of VSV (Indiana) through whole \emph{Drosophila melanogaster} flies led to a 200-fold reduction in the amount of virus required to infect one fly by the tenth passage; yields/fly increased tenfold by the sixth passage and about 50-fold in later passages. Small plaques on chick embryo fibroblasts appeared by the third-to-fifth passage, and temperature-sensitivity between the fifth and tenth passages. Clonal analysis of tenth passage virus revealed two types of clones in relation to the time required for the development of CO\textsubscript{2}-sensitivity in
flies. The precise relationship of Printz's clones to those described here is not clear, but it is evident that similar selections arose in both cases. It is possible that such selection may have some significance in nature for viruses of broad host range. An important factor in this selection process may be the lack of cell killing in flies or their cultured cells by a virus that is lethal to any vertebrate host-cell that it infects.

Cases of selection of virus variants or mutants in 'foreign' host cells have been described by others: poliovirus variants or mutants which were temperature-sensitive and suitable for use as live vaccines were selected by growth on various host cells at low temperatures (Dubes & Wenner, 1957; Lwoff, Tournier & Cartreaud, 1959). Temperature-sensitive variants of Newcastle disease virus were selected by persistent infection of L cells, an inefficient host (Preble & Youngner, 1972). Variants of Semliki forest virus (Peleg, 1971), chickungunya virus (Banerjee & Singh, 1969) and rabies virus (Plus & Atanasiu, 1966) were selected in cultured mosquito cells or by Drosophila melanogaster flies grown at temperatures below 37 °C. These cellular selective pressures on virus populations like VSV may be more than coincidental; they may indicate that such viruses evolved in intimate relationship with more than one type of host, so that a number of specific mutations allow adaptation to growth in the different host types.

Since RNA genomes are probably subject to much higher mutation rates than DNA genomes (Cooper, 1968) it may not take long for a large VSV population derived from animal cells to undergo that series of mutations necessary to stabilize a small subpopulation for efficient persistent infection of insect cells. For example, Flamand (1970) found that 2·3 % of VSV stocks was spontaneously temperature-sensitive, and over 80 % of the mutants characterized were in one complementation group. Much more data are required for genetic and biochemical understanding of these processes. Complementation tests with mutants from different complementation groups (Pringle, Duncan & Stevenson, 1971) should be made in double infection experiments with the insect derived viruses.

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REFERENCES


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