Isolation and Characterization of Virus-resistant Mouse Embryo Fibroblasts

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SUMMARY

A method is described for the isolation of virus-resistant mutants of mammalian cells in culture. Cells of a mouse embryo fibroblast line (3T6) were treated with a mutagen and subjected to selection for virus resistance. The cells were infected with a temperature-sensitive mutant of Sindbis virus which was allowed to proceed through one cycle of replication at the permissive temperature. On transfer of the cells to the non-permissive temperature, further cycles of virus replication were prevented and non-infected cells allowed to grow out. Repetition of this procedure over 2 months resulted in the isolation of a population of 3T6 cells which resisted infection by Sindbis virus.

Single cell clones were isolated that were resistant to the growth of Sindbis virus and also to the growth of Mengo virus, Semliki Forest virus, vesicular stomatitis virus, Newcastle Disease virus, vaccinia virus and herpes simplex virus.

INTRODUCTION

Our understanding of the regulation of protein synthesis in bacteria has been greatly aided by the isolation and study of regulatory mutants (Richmond, 1968). Even though their potential usefulness has been recognized for some time (Tomkins et al. 1969), few regulatory mutants of mammalian cells have been described (Orkin & Littlefield, 1971; Levisohn & Thompson, 1972).

The interferon system (Colby & Morgan, 1971) is suitable for biochemical and genetic study and, since it is inducible, affords a useful model for studying the regulation of protein synthesis in mammalian cells. Interferon activity can be assayed sensitively and double-stranded RNA, an inducer for interferon synthesis, is readily available in a variety of natural and synthetic forms.

We attempted to isolate cells which, as constitutive mutants, synthesized interferon or antiviral protein (Taylor, 1964) in the absence of inducer. Such cells may express a phenotype of resistance to the growth of many viruses. This paper describes the selection and isolation of virus-resistant variants of 3T6 mouse embryo fibroblasts.
METHODS

Cell culture. Mouse embryo fibroblasts of the 3T6 line (Todaro & Green, 1963) were obtained from R. Pollack (Cold Spring Harbor, N.Y.). Unless otherwise indicated, cells were subcultured in Dulbecco's modification of Eagle's medium supplemented with 5% heat-inactivated calf serum and 5% foetal bovine serum (GIBCO, Grand Island, New York). Penicillin (100 units/ml), streptomycin sulphate (200 μg/ml) mycostatin (50 units/ml) and fungizone (1 μg/ml) were routinely added. Cells were passaged serially on cell culture Petri dishes incubated at 37 °C in humidified air with 5% CO₂. Primary chick embryo (CE) cells were grown at 37 °C (Carver & Marcus, 1967).

Viruses. Newcastle disease virus, NDV (California strain), was grown at 39.5 °C in the allantoic cavity of 12-day-old embryonated eggs.

Sindbis virus (Enders strain, Carver & Marcus, 1967) was grown at 37 °C in monolayers of CE cells. Sindbis virus temperature-sensitive mutants ts6, ts11, ts15, (Burge & Pfefferkorn, 1966) were grown at 29 °C in CE cell monolayers. Semliki Forest virus, SFV (strain 25639: Rockefeller Institute, New York), was grown at 37 °C in monolayers of CE cells. Vesicular stomatitis virus, VSV (Indiana strain), was grown at 37 °C in monolayers of a continuous line of African green monkey kidney cells (BSC/B). Mengo virus (large plaque strain; Rockefeller Institute, New York) was grown at 37 °C in mouse L-929 cell monolayers. Vaccinia virus, (strain WR) was grown at 37 °C in mouse L-929 cell monolayers.

Assay of virus infectivity on mouse embryo fibroblasts (3T6). Confluent monolayers of cells were prepared in 60 mm tissue culture dishes by plating 2 × 10⁶ cells into each dish. After overnight incubation the monolayers were washed carefully with warm phosphate buffered saline (PBS) and then 0.3 ml of the virus suspension, suitably diluted in growth medium was added to duplicate plates. The virus suspension was allowed to adsorb to the cell monolayer for 30 min at 37 °C with occasional rocking. Unadsorbed virus was removed and the monolayers overlaid with 3 ml of growth medium containing 0.7% agarose. Plates were incubated for times appropriate to the particular virus. After incubation the plaques were visualized by staining the cell sheets with 2 ml of an 0.01% saline solution of neutral red for 1 to 2 h at 37 °C.

Mutation. Cells were treated with N-methyl-N'-nitro-nitroso-guanidine (MNNG: Aldridge Chemical Company, U.S.A.) in a procedure based on that of Kao & Puck (1968).
Active growing monolayers of 3T6 cells at a density of 3.5 to 4.5 × 10⁴ cells/cm² (50% confluent) were incubated with MNNG (8 μg/ml of growth medium) for 24 h at 37 °C. The surviving cells (40 to 50%) were allowed to grow out in fresh medium for several days before exposure to the selection procedure below.

Selection of cells resistant to Sindbis virus. Confluent monolayers of mutagen-treated 3T6 cells (3T6M) were prepared as described for the plaque assay of virus infectivity. These monolayers were infected with a temperature-sensitive mutant (ts15) of Sindbis virus at an input multiplicity of 5 p.f.u./cell. The virus was allowed to adsorb for 30 min at 37 °C before the cultures were incubated at 29 °C (permissive temperature) for 18 h. The cultures were then transferred to 40 °C (non-permissive temperature) and incubation continued. Microscopic examination of the cell sheets after incubation at 29 °C showed no evidence of cell death. After 18 h at 29 °C, ts15 virus completed at least one cycle of replication (Burge & Pfefferkorn, 1966). Transfer to 40 °C after 18 h does not lead to cessation of virus growth since the temperature-sensitive function in ts15 virus involves RNA synthesis rather than protein synthesis; only RNA synthesis ceases on transfer to 40 °C. Within 12 to 18 h at 40 °C extensive cell death occurred but some cells survived. These surviving cells (3T6MS) were maintained at 40 °C for 3 weeks until enough cells had accumulated to continue the selection procedure. In the next stage the above was repeated twice. The 3T6MS cells were inoculated with ts15 virus and incubated at 29 °C for 10 h before transfer to 40 °C; now less than 10% of cells died. After growth for 3 weeks, the cells (3T6MS2) were again infected with ts15 virus but now were incubated at 29 °C for 72 h when less than 1% of cells died. The cultures were incubated further at 40 °C in growth medium supplemented with rabbit anti-Sindbis virus serum for 48 h prior to transfer to growth medium at 37 °C. These cells (3T6MS3) were then allowed to multiply at 37 °C. The cells were screened for resistance to other viruses before cloning. Reserve stocks of cells resuspended in growth medium supplemented with 10% glycerol were stored at −70 °C.

Cloning of single cells. Usual methods for cloning of single cells (Puck, Marcus & Cieciura, 1956) could not be used since 3T6 cells do not form discrete colonies. Single cells were isolated by pipetting 0.1 ml amounts of a cell suspension (10 cells/ml of growth medium) into each well of 96-well micro-test tissue culture plates (Linbro, U.S.A.). The plates were incubated for 24 h at 37 °C and each well was examined microscopically for the presence of a single cell; the medium was removed from wells containing more than one cell. Incubation continued until each single cell had produced sufficient cells for transfer to larger dishes.

Haemadsorption assay. This method is an adaptation of that previously described by Marcus (1962). Partially (30 to 50%) confluent monolayers of 3T6 cells or their derivatives in 35 mm dishes were cooled to 4 °C and inoculated with 10 p.f.u./cell of NDV. Virus was allowed to adsorb for 30 min at 4 °C when the monolayers were rinsed with growth medium at 37 °C. Warm growth medium (1 ml) was added and the dishes were incubated at 37 °C for 30 min. Rabbit anti-NDV serum (a gift from P. I. Marcus) was added (0.02 ml) to each of the monolayers before incubation for a further 20 min at 37 °C. The cell sheets were then rinsed twice with warm growth medium and incubated at 37 °C for 18 h. This procedure ensured that all NDV not adsorbed by the cells was removed before final incubation. After incubation the dishes were cooled to 4 °C and 1 to 2 × 10⁶ bovine red blood cells were added to form a contiguous monolayer of settled blood cells. Incubation was continued for 20 min at 4 °C before non-adsorbed red blood cells were removed gently by washing with cold PBS. The plates were then examined microscopically and individual haemadsorption positive cells counted.

Preparation of radioactively labelled Sindbis virus. Sindbis virus RNA was labelled by
growing the virus in the presence of [5-3H]-uridine (New England Nuclear, 28.5 Ci/m-mol). Confluent monolayers containing 6 to 8 × 10^6 CE cells were treated with growth medium containing actinomycin D (1 μg/ml) for 45 min. The medium was removed and 10^7 p.f.u. of Sindbis virus in 0.5 ml of growth medium with actinomycin D was added. After 30 min at 37 °C medium containing actinomycin D (1 μg/ml) and [3H]-uridine (200 μCi) was added. After incubation for 12 h the medium was centrifuged at 1000 g for 5 min to remove cell debris. The supernatant fluid was centrifuged in the Spinco No. 40 rotor at 27000 rev/min for 30 min at 4 °C. The supernatant fluid was removed and replaced by growth medium. After 4 h at 0 °C the virus pellet was resuspended gently and layered on to 15% (w/v) sucrose (2 ml) dissolved in 0.01 M-tris, pH 7.4. This was centrifuged in the Spinco 40 rotor at 29000 rev/min for 1 h at 4 °C. The supernatant fluid was removed and the virus pellet resuspended as above. The purified virus had an infectivity of 1.3 × 10^8 p.f.u./ml on CE cell monolayers.

The radioactivity was determined on a sample of the purified virus diluted in standard buffer (0.1 M-NaCl; 0.01 M-tris, pH 7.4; 0.001 M-EDTA) to which was added SDS (0.5%) and pronase (0.5 mg/ml). After incubation at 37 °C for 30 min the RNA was extracted three times with a phenol chloroform (1:1) mixture saturated with a solution containing 0.1 M-NaCl, 0.01 M-sodium acetate, 0.001 M-EDTA. The [3H]-labelled RNA was then precipitated with ice-cold TCA (5%), collected on GF/A (Whatman) filters and the radioactivity measured in a Packard Tri Carb Spectrometer. The purified virus had a radioactivity of 2.4 × 10^5 ct/min/ml.

**Measurement of the adsorption and penetration of Sindbis virus in mouse embryo fibroblasts.** Monolayers of cells (3T6, 3T6S3A5, 3T6S3E11, 3T6S3G1) were infected at a multiplicity of 2 p.f.u./cell with Sindbis virus and incubated at 37 °C for 18 h. The virus infectivity was then assayed on CE cell monolayers. Similar monolayers of the cells were infected with purified [3H]-labelled Sindbis virus at an input multiplicity of 2 p.f.u./cell (7250 ct/min/dish). After 0.5 h at 37 °C the unadsorbed virus was removed and a sample (0.2 ml) was added to 0.5% SDS in standard buffer (3 ml). The monolayers were washed three times and incubated with growth medium at 37 °C for 1 h. The medium was removed, the monolayers washed three times with PBS and then extracted twice with 1% SDS in standard buffer and the extracts combined. Pronase was added (0.5 μg/ml) and the samples were incubated at 37 °C for 30 min. The [3H]-labelled RNA from infected cells and unadsorbed virus was prepared and assayed as above.

**RESULTS**

**Resistance to virus infection**

Uncloned cultures of 3T6MS3 cells (virus-resistant) were tested for their sensitivity to infection by several viruses. Confluent monolayers of 3T6MS3 or 3T6 cells in 60 mm dishes were infected with SFV, Mengo virus, Sindbis virus or VSV at an input multiplicity of 5 p.f.u./cell and incubated at 37 °C. At 18 h after infection the cells were examined microscopically, the media harvested and their virus infectivities determined.

The viruses grew well in 3T6 cells causing extensive damage: no surviving cells were seen in the SFV or VSV infected cultures, 50% survived in the cultures infected with Sindbis virus or Mengo virus. The viruses grew poorly in 3T6MS3 cells: all the cells survived infection with Sindbis virus, 75% survived infection with VSV, 50% survived infection with Mengo virus and 25% of the cells survived infection with SFV.

In addition to the reduced c.p.e. of these viruses in 3T6MS3 cells, the yield of infective virus was reduced (Table 1). This reduction in virus yield could be due to the selection of...
Isolation of virus-resistant fibroblasts

Table 1. Virus yield after infection of normal (3T6) and selected (3T6MS3) mouse embryo fibroblasts

<table>
<thead>
<tr>
<th>Cells</th>
<th>Virus and yield in p.f.u./cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sindbis virus</td>
</tr>
<tr>
<td>3T6</td>
<td>5.0 x 10⁶</td>
</tr>
<tr>
<td>3T6MS3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Cells were infected at a multiplicity of 5 p.f.u./cell; 18 h after infection the yield of infective virus was determined by plaque assay on CE cells.

Persistent infections

As mentioned above, the possibility that the observed virus resistance was due to a low-level persistent infection (Ginsberg, 1958) was recognized. Besides incubating clones at 29 °C for up to 2 weeks as above, other tests were employed to investigate this possibility. Cells from individual clones were plaqued as potential infective centres (Carver & Marcus, 1967) on CE cell monolayers. Suitable numbers of cells were suspended in 1 ml of growth medium containing 0.7% agarose and immediately spread over washed, confluent CE cell monolayers. After the overlay had hardened, a further 2 ml of agar overlay was added. The plates were incubated as usual for 7 days at 29 °C before staining. No plaques developed in any case.

Cells of several clones (S3A2, S3B12, S3G7, S3G3 and S3H2) were mixed with an equal number of CE cells and plated as confluent monolayers. After 11 days at 29 °C there were no cell deaths due to the outgrowth of virus. Similarly, no virus was isolated from growth medium removed from cultures of clones at 29 °C, when samples were plated on CE monolayers and incubated at 29 °C. These tests thus showed that none of the clones examined was infected persistently with Sindbis virus, ts15.

However, it is possible that non-cytopathic virus is replicating in these cells or that some part of the virus is present in the cells in an integrated form. To test these possibilities various temperature sensitive (RNA-) mutants of Sindbis virus (ts6, ts11, and ts15) were grown in S3A2 cells at the non-permissive temperature (40 °C). If S3A2 cells contained a complementation factor derived from ts15 virus then viruses of different complementation groups (ts6 and ts11) should grow to a higher infectivity than a virus of the same complementation group (ts15). No such complementation occurred and we have thus been unable to detect the presence of Sindbis virus in these cells.
Table 2. Yield of viruses in monolayer cultures of normal (3T6) and selected (3T6S3A5) mouse embryo fibroblasts

<table>
<thead>
<tr>
<th>Virus</th>
<th>Input multiplicity (p.f.u./cell)</th>
<th>Virus yield in p.f.u. per 10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal 3T6 cells</td>
<td>Selected 3T6S3A5 cells</td>
</tr>
<tr>
<td>Sindbis</td>
<td>3</td>
<td>6 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>1.4 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>1.7 × 10⁶</td>
</tr>
<tr>
<td>Semliki Forest</td>
<td>0.3</td>
<td>4 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>9 × 10⁶</td>
</tr>
<tr>
<td>Vesicular stomatitis</td>
<td>2.0</td>
<td>2 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1.3 × 10⁴</td>
</tr>
<tr>
<td>Newcastle disease</td>
<td>2.0</td>
<td>5 × 10³</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.2 × 10⁴</td>
</tr>
<tr>
<td>Mengo</td>
<td>0.02</td>
<td>1.5 × 10⁷</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>1.5</td>
<td>3 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.2 × 10⁶</td>
</tr>
<tr>
<td>Herpes simplex</td>
<td>2.5</td>
<td>6 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>6.5 × 10⁵</td>
</tr>
</tbody>
</table>

At 18 to 22 h after infection culture fluids were harvested and the virus infectivities determined on CE cells (Sindbis virus, SFV, NDV, and vaccinia virus), L cells (Mengo virus) or HeLa cells (herpes simplex virus).

**Characteristic of virus-resistant clones**

**Plaque assay of virus infectivity in 3T6 cells and derivatives**

The ability of Sindbis virus, SFV, VSV and vaccinia virus to form plaques in various virus-resistant clones of 3T6 cells was determined. After incubation for 30 h at 37 °C, Sindbis virus plaques (183 and 230) were seen on the 3T6 cell monolayers, but were not seen on the S3A2, S3G7 or S3H2 cell monolayers. The SFV plaques (81 and 115) that developed on the 3T6 cell monolayers were large (2 to 3 mm) and distinct whereas those that developed on S3G7 and S3H2 cell monolayers (21 and 31; 32 and 37, respectively) were small (1 to 2 mm) and indistinct. Vaccinia virus produced more plaques in 3T6 cell monolayers (305 and 285 plaques) than in S3A2 cell monolayers (12 and 11 plaques) or in S3G7 cell monolayers (83 and 102 plaques).

**Haemadsorption assay of NDV in 3T6 cells and derivatives**

NDV did not grow well in 3T6 cell monolayers and assay by plaque reduction was not suitable to test the growth of this virus. Instead, growth was measured by examining the adsorption of bovine red blood cells to cells that had been infected with NDV. At 18 h after infection 10% of the control 3T6 cells adsorbed red blood cells indicating the presence of replicating NDV. A similar proportion of S3H2 cells was infected but none of the S3A2 or S3G7 cells adsorbed red blood cells. These S3A2 and S3G7 cells were thus resistant to infection with NDV.

**Growth of viruses in 3T6 and S3A5 cells**

The growth of various viruses was examined in greater detail in cells of clone S3A5. Sindbis virus, vaccinia virus, Mengo virus, SFV, VSV, NDV and HSV were grown in monolayers of S3A5 and 3T6 cells and the yield of infective virus assayed.
Isolation of virus-resistant fibroblasts

Table 3. Adsorption, penetration and yield of sindbis virus in 3T6 original and selected mouse embryo fibroblast cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Extracellular [3H] (ct/min/10^6 cells)</th>
<th>Intracellular [3H] (ct/min/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T6</td>
<td>7.5 x 10^7</td>
<td>1940</td>
</tr>
<tr>
<td>3T6S3A5</td>
<td>4.1 x 10^5</td>
<td>3550</td>
</tr>
<tr>
<td>3T6S3E11</td>
<td>6.5 x 10^6</td>
<td>1810</td>
</tr>
<tr>
<td>3T6S3G1</td>
<td>3.2 x 10^6</td>
<td>2800</td>
</tr>
</tbody>
</table>

* Cells were infected at a multiplicity of 2 p.f.u./cell; 18 h after infection the yield of infective virus was determined by plaque assay on CE cells.
† Cells were infected at a multiplicity of 2 p.f.u./cell with purified [3H]-labelled Sindbis virus (7250 ct/min/dish).

All the viruses tested grew better in 3T6 cells than they did in S3A5 cells (Table 2). S3A5 cells were particularly resistant to the growth of Sindbis virus, SFV, Mengo virus and VSV (all RNA viruses). Even NDV, which grew poorly in 3T6 cells, grew less well in S3A5 cells. S3A5 cells were also resistant to the growth of the two DNA viruses vaccinia virus and HSV.

In these experiments the adsorption of the viruses by 3T6 and S3A5 cells was measured by assaying the infectivities of the input virus suspensions before and after the period of adsorption. The amount of virus adsorbed was never less than 35% and never greater than 85% of the total virus presented to the cells. Adsorption to normal or resistant cells, for any given multiplicity of infection, agreed to within 15%. Thus we detected no significant difference in the adsorption of the viruses to 3T6 or S3A5 cells.

Adsorption, penetration and yield of Sindbis virus

The possibility that the viruses could not adsorb to or penetrate resistant cells was investigated further by measuring the uptake of radioactively labelled Sindbis virus. Monolayers of 3T6, S3A5, S3E11 and S3G1 cells were infected with [3H]-uridine labelled Sindbis virus (2 p.f.u./cell; 7250 ct/min/dish) for 0.5 h and the amount of virus taken up was measured. The growth of Sindbis virus was also determined. The results (Table 3) show that Sindbis virus was taken up by all four cell monolayers. The virus grew best in 3T6 cells and most poorly in S3A5 cells. Thus, there was a 2.5 log unit reduction in the yield of infective Sindbis virus grown in S3A5 cells, but only a 38% reduction in the uptake of Sindbis virus by these cells.

DISCUSSION

The results show that by selecting for cells resistant to the growth of one virus, cells can be isolated that are resistant to viruses of several groups. What is the nature of this resistance?

Virus-resistant mutants of bacteria which affect bacteriophage receptor sites have been isolated and shown to be of two sorts: those resistant to one bacteriophage type (Luria & Delbruck, 1943) and those resistant to more than one type (Demerec & Fanu, 1945). There are specific sites for each type of bacteriophage but, since the receptors lie in a complex lipo-polysaccharide layer, alterations in the layer structure could lead to inactivation of several receptors (Wiedel, 1955). Recently, mutants of Escherichia coli unable to support the growth of lambda phages (1, 21, 82, 381, 434) have been isolated and characterized Georgopoulos, 1971; Georgopoulos & Herskowitz, 1971). Adsorption and penetration of lambda phages is unaffected in these bacterial mutants. The mutants fall into two cate-
gories: gro $N^-$ mutants fail to produce a component required for normal activity of the phage $N$ gene while gro $P^-$ mutants are deficient in a host cell function required for phage DNA synthesis.

It is unlikely that our cells are resistant to virus infection, as the result of an alteration in their cell membrane, such that virus adsorption is blocked, since we find no significant differences between original 3T6 and resistant S3A5 cells in the adsorption of any of the viruses tested. Presumably different classes of viruses adsorb at different sites on the cell surface. Furthermore, the adsorption and penetration of $[^3H]$-uridine-labelled Sindbis virus by resistant S3A5 cells differs from the normal 3T6 cells by less than a factor of two, yet there is almost a 200-fold reduction in virus yield in this experiment.

Another important clue concerning the nature of the virus resistance of these mutants may be derived from this experiment. The results before normalization on a per cell basis show that the number of TCA-insoluble $[^3H]$-ct/min not adsorbed in 30 min plus those found inside the infected cells after 1 h give the total number of $[^3H]$ct/min (± 5%) presented to the cells at zero time. This suggests that the mechanism of virus-resistance is not the immediate degradation of input virus genomes.

It also seems unlikely that our cells are persistently infected with $ts$ 15 Sindbis virus. Prolonged incubation of the mutant cells at the permissive temperature resulted in neither cell death nor production of infective material. These experiments do not rule out the possibility that virus particles defective in cytopathogenicity are replicating in the mutant cells. However, if this is true, then such particles should at least be directing the replication of their RNA and should therefore support the growth of temperature sensitive-RNA$^-$ Sindbis mutants of the other two RNA$^-$ complementation groups. We find no such complementation.

Finally, there is the question as to whether the mutants are constitutive for the interferon system. None of the mutant clones thus far examined secreted measurable quantities of mouse interferon and as yet there is no assay available for the postulated antivirus protein. The physiological nature of the alteration of these cells thus remains to be determined.

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Isolation of virus-resistant fibroblasts


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