Properties of Mouse Embryo Cells Infected with Murine Sarcoma Virus and Simian Virus 40 simultaneously \textit{in vitro}

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Serially subcultivated Balb/c mouse embryo cells pass through a non-dividing or amitotic phase prior to becoming an established, growing cell line (Aaronson & Todaro, 1968; Baker & Simons, 1971). Cells in the amitotic phase are not visibly transformed by murine sarcoma virus-Harvey (MSV-H), although there is a low level of replication of the virus (Baker & Simons, 1971). DNA synthesis and cell division are initiated in the amitotic cells by infection with SV 40 (Baker, Simons & Rankin, 1972). The amitotic cells are particularly susceptible to SV 40, 60% of the cells synthesizing DNA by 48 h after infection (Baker et al. 1972). Moreover, simultaneous infection of amitotic cells with SV 40 and MSV-H results in extensive cellular transformation and MSV replication (Baker et al. 1972). This communication describes some of the properties of cells derived from amitotic cell cultures infected simultaneously with SV 40 and MSV-H.

The origin and production of SV 40 and MSV-H, the preparation of amitotic cell cultures and the infection of these cells with SV 40 + MSV-H or with SV 40 alone, have been described in detail (Baker & Simons, 1971; Baker et al. 1972).

Unless otherwise stated, cell lines were grown and maintained in Dulbecco’s Modified Eagle’s Medium (Gibco Powder Medium H-16) supplemented with 10% inactivated calf serum. Cells were subcultured with 0.25% trypsin solution and split as required. Plastic tissue culture Petri dishes (Falcon Plastics Inc.) were used throughout.

The following cell lines were developed and used in this study:
- MT. Balb/c secondary mouse embryo cells infected with MSV-H alone under conditions identical to those used to infect amitotic cell cultures, and serially subcultured;
- SV. Balb/c embryo amitotic cell cultures infected with SV 40 alone (Baker et al. 1972);
- SMT. Balb/c embryo amitotic cell cultures infected with SV 40 + MSV-H (Baker et al. 1972).

In SV 40 rescue experiments, BSC-1 cells were used as the permissive cell line, and these cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% foetal calf serum.

To determine cell growth rates, 2 ml of the cell suspensions (10^6 cells/ml) were inoculated into 35 mm dishes. At appropriate intervals dishes in duplicate were washed with phosphate buffered saline (PBS) and the cells suspended with 0.5 ml of 0.25% trypsin solution. The cell numbers were determined by counting in a haemocytometer.

To study cellular morphology, cells were grown on cover-slips, fixed and stained with Giemsa.

The technique of Macpherson & Montagnier (1964) was used to test the ability of cells to grow when suspended in soft agar. After 14 days incubation, colonies were counted by low-power microscopy (×40).

MSV-H was assayed using a continuous line of Prince Henry (P.H.) mouse (Stanley, Dorman & Ponsford, 1953) embryo cells developed in this laboratory. Dishes (60 mm) which had been seeded with 5 × 10^6 cells 24 h previously, were treated with 2 ml of DEAE-dextran solution (25 g per ml in growth medium) for 1 h at 37 °C, washed three times with growth medium,
Figs. 1 to 3. For legend see facing page.
then infected with 0.2 ml of virus dilution. After 2 h incubation, 5 ml of growth medium was added and the medium changed every three days. The foci were counted 7 days after infection.

To rescue infectious SV40, 4 × 10^4 of the cells to be tested were mixed with 4 × 10^5 BSC-1 cells in 35 mm dishes. The medium was changed every 3 days and on the 7th day the cells were scraped into the medium, frozen and thawed three times, centrifuged at 3000 rev/min for 10 min in a bench centrifuge and the supernatant fluid filtered through a 450 nm Millipore filter. Dishes (35 mm) which had been plated with 4 × 10^4 BSC-1 cells 24 h previously were infected with 0.2 ml of filtered extract for 2 h at 37 °C and 2 ml of medium added. The medium was changed every 3 or 4 days and the cultures examined daily for signs of cytopathic effect.

The indirect fluorescent antibody technique was used to detect SV40 ' T' antigen synthesis. Cells were grown on cover-slips and fixed in cold acetone before processing.

To determine the proportion of cells synthesizing ' T' antigen, the number of fluorescent nuclei and the total number of nuclei were counted in ten randomly selected fields on each of 2 separate preparations.

To test for tumorigenicity, various numbers of freshly trypsinized cells were inoculated subcutaneously between the scapulae of 3-week-old Balb/c mice.

**Morphology of cells.** Both the SV and SMT cells grew rapidly in monolayer culture.

The amitotic cells prior to infection were very large with a sheet-like cytoplasm containing numerous longitudinal striations. The single large nucleus contained two or three prominent nucleoli (Fig. 1). Passaged SV cells were smaller with an epithelioid appearance, the nuclei possessing an increased number of nucleoli. There was not a significant degree of cell overlapping (Fig. 2). Cultures of passaged SMT cells consisted of small densely staining, spindle cells, with occasional larger epithelioid cells (Fig. 3). The cell growth was disorganized and cells showed extensive overlapping. Some indication of the difference in size can be obtained by comparing the longest diameters of the nuclei in the photographs used for Figs. 1 to 3. The real diameters have been calculated, and for amitotic cells, the average of eight such determinations is 25.0 μm; for SV cells 17.3 μm (average of 10); for SMT cells, 10.8 μm (average of 12).

**Production of virus.** SMT cells liberated large amounts of MSV-H into the culture medium. Twenty-four hour filtered fluids from passaged SMT cells (3.15 × 10^8 cells in 90 ml when culture fluid collected) contained 7.4 × 10^6 focus forming units (f.f.u.) of MSV-H per ml. For comparison, 240 ml of culture fluid from 4.3 × 10^8 passaged MT cells contained 2.5 × 10^6 f.f.u. per ml. Neither the SV nor SMT cells released infectious SV40 into the culture fluids. Virtually all nuclei (98%) of the 8th passage SMT contained SV40 ' T' antigen, whereas only 30% of 8th passage SV cells nuclei contained ' T' antigen.

Infectious SV40 could readily be obtained from both SMT and SV cells by co-cultivation with BSC-1 cells.

Infectious DNA has been isolated from Rous sarcoma virus transformed cells (Hill & Hillova, 1971) and therefore MSV specific DNA could possibly have been incorporated into SV40 virus particles. Alternatively, MSV virus particles from SMT cells could have contained SV40-specific RNA. To test the possibility of recombination of SV40 and MSV.

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**Fig. 1.** Amitotic phase mouse embryo cells with extensive sheets of cytoplasm. Giemsa stain. Note lower magnification than Figs. 2 and 3.

**Fig. 2.** SV40 infected amitotic cells (SV), subcultured eight times since infection. Giemsa stain.

**Fig. 3.** Amitotic cells infected simultaneously with SV40 + MSV (SMT) and subsequently subcultured eight times. Giemsa stain.
specific DNAs the activity of SV 40 rescued from SMT cells was tested *in vivo* and *in vitro*. Newborn Balb/c mice were inoculated intramuscularly with 0.05 ml of filtered fluids from BSC-1 cells showing c.p.e. 10 days after being inoculated with fluids from SMT and BSC-1 co-cultures. The same fluids were inoculated on to continuous lines of P.H. and Balb/c mouse embryo cells. There was no evidence of tumour formation in the mice during three months of observation and no evidence of transformation of the cells *in vitro*. P.H. and Balb/c mouse cell lines were infected and transformed by culture fluids containing MSV from SMT cells. When tested with SV 40-T antigen sera on the third and fourth days after infection, that is as soon as transformation was discernible, no cell showed positive fluorescence.

### Table 1. Growth of cells to form colonies in soft agar

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cells/dish</th>
<th>Colonies/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT (MSV)</td>
<td>$10^3$</td>
<td>0, 0</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>5, 2</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>27, 11</td>
</tr>
<tr>
<td>SV (SV 40)</td>
<td>$10^3$</td>
<td>0, 0</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>15, 14</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>1906, 1562</td>
</tr>
<tr>
<td>SMT (SV 40 + MSV)</td>
<td>$10^3$</td>
<td>46, 107</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>750, 760</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>TNDC*</td>
</tr>
</tbody>
</table>

* Too numerous to count.

Comparison of cell growth. The comparative growth rates of the cell lines in monolayer culture are shown in Fig. 4.

The results of agar suspension culture are shown in Table 1. Not only was there a difference in the number of colonies but there were considerable differences in the colony sizes, the colonies developing with MT cells were small and difficult to count or photograph.

The results of transplantation of the various cell lines into weanling mice are shown in Table 2. To circumvent virus effects in the transplantation experiments, the cells were
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Table 2. Results of transplantation of cells into weanling mice

<table>
<thead>
<tr>
<th>Cells inoculated</th>
<th>No. cells per mouse</th>
<th>No. tumours inoculated</th>
<th>No. animals dying with tumours</th>
<th>Longest time to death in days</th>
<th>Deaths after regression of primary tumour</th>
<th>Time to death (range in days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV (SV40)</td>
<td>$10^6$</td>
<td>0/7</td>
<td>0</td>
<td>---</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>$10^6$</td>
<td>0/7</td>
<td>0</td>
<td>---</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>0/6</td>
<td>0</td>
<td>---</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td>MT (MSV)</td>
<td>$10^6$</td>
<td>7/7</td>
<td>1</td>
<td>13</td>
<td>6</td>
<td>55 to 107</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>1/10</td>
<td>0</td>
<td>---</td>
<td>7</td>
<td>55 to 120</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>0/9</td>
<td>0</td>
<td>---</td>
<td>9</td>
<td>55 to 104</td>
</tr>
<tr>
<td>SMT (SV40 + MSV)</td>
<td>$10^6$</td>
<td>7/7</td>
<td>7</td>
<td>30</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>4/6</td>
<td>0</td>
<td>6</td>
<td>50 to 64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>3/6</td>
<td>2</td>
<td>28</td>
<td>57 to 165</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^3$</td>
<td>5/6</td>
<td>3</td>
<td>40</td>
<td>50 to 83</td>
<td></td>
</tr>
</tbody>
</table>

Inoculated subcutaneously into weanling mice. When administered subcutaneously to mice MSV-H has an extended latent period to tumour production (Simons & McCully, 1970) and weanling mice respond with a reduced incidence of sarcomas (Harvey, 1970). The SMT and MT cells release infectious MSV and consequently, there was a two-phase response. In some animals the injected cells grew rapidly and a proportion of these animals died bearing large tumours. The tumours regressed in the survivors, but these animals died of splenic rupture after a period of more than 50 days. Virtually all of the animals inoculated with MSV-producing cells, but not developing tumours at the site of inoculation, eventually died of splenic rupture and haemorrhage.

None of the animals receiving the SV40 cells developed tumours or died over a 6-month period.

These results show that two tumour viruses of different biological type and each of which can transform fibroblastic cells in culture, act synergistically in the same cell. Although having the morphology of MSV transformed cells (Simons, 1970), the SMT cells have an increased ability to grow in monolayer culture, agar suspension and in immunologically intact animals. As previously reported for SV40 transformed cells (Eagle et al. 1970), the SV cells did not grow on transplantation, although countable colonies appeared in soft agar. Conversely, the MT cells which did grow at high concentrations in vivo produced a few small colonies in agar. The discrepancies between growth in agar and in vivo have been discussed by Eagle et al. (1970). With SMT cells, growth in vivo did correlate with the production of large colonies in agar.

The evidence suggests that the SMT may have been synthesizing MSV at a faster rate than cells infected with MSV alone. However, the different growth rates of the cells makes interpretation difficult. Moreover, since the amitotic cells were not susceptible to MSV infection alone, the passaged MSV-infected cells are not strictly comparable, however, they do serve as an indicator of the general properties of MSV-infected cells. Clearly in the SMT cells, the presence of the SV40 genome does not result in metabolic or surface structural changes which interfere with the replication of MSV. The continuous synthesis of MSV did not prevent the production of infectious SV40 in the presence of a permissive cell line.
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REFERENCES


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