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# Contact-Inhibited Revertant Cell Lines Isolated from Simian Virus 40-Transformed Cells

## VII. Serum Detachment-Resistant Revertant Cells

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Flat revertant cells of simian virus 40-transformed mouse fibroblasts have been isolated on the basis of resistance to a selective detachment procedure. The revertants are generally similar to those isolated by other procedures.

Transformation of the normal mouse fibroblast line BALB/c 3T3 with simian virus 40 (SV40) results in many changes in cell social behavior and growth properties. From populations of these transformed SV3T3 cells, several laboratories have isolated variants that have reverted in some respects to the normal phenotype, without apparent loss of, or change in, the viral genome (2, 3, 5-7, 9, 11, 12). However, isolation of these revertants has required the use of toxic agents to select negatively against cells not conforming to normal behavior, and it is difficult to be sure that the revertants subsequently isolated have completely escaped the deleterious effects of the agent. The demonstrations of aberrant chromosomes and giant cells in some revertant lines (2) tends to reinforce such doubts.

During a study of the effect of serum on sugar transport in normal and SV40-transformed 3T3 cells (1), we discovered conditions under which SV3T3, but not confluent 3T3, cells could be induced to aggregate and detach from the dish. We have used this observation to develop a nontoxic procedure for isolation of revertant cells, which we call serum detachment-resistant (SDR) revertants. In this communication we present a preliminary characterization of the detachment effect and a description of the revertants obtained.

BALB/c 3T3 cells (clone A51), SV40-transformed BALB/c 3T3 cells (SVT2 clone), and the concanavalin A-selected revertant of SV40-transformed cells (clone 84), described previously (2), were grown in plastic dishes (Lux), in Eagle minimum essential medium (MEM) supplemented with a fourfold concentration of vitamins and amino acids and with 10% fetal calf serum. Growth conditions for these cells have been described (3). Cells were routinely shown to be free of *Mycoplasma* contamination by autoradiography of cells after incorporation of [$^3$H]thymidine (2).

When a confluent culture of SVT2 cells was incubated at 37°C in medium containing 80% serum and 20% MEM for 20 h, the morphology of the cells was not affected, but after a wash with phosphate-buffered saline (PBS; Dulbecco's A solution), subsequent incubation in PBS caused the cells to round up, aggregate, and come free from the surface of the dish within 10 min. Cells not pretreated with serum remained attached to the dish for much longer periods of time when incubated with PBS. This detachment phenomenon was not seen with confluent normal BALB/c 3T3 cells, nor was it observed with confluent concanavalin A revertant cells (1).

A preliminary characterization of this phenomenon was undertaken, and the results can be summarized as follows. (i) The detachment effect was maximum when the serum concentration was 100% and the incubation time was 20 to 24 h. (ii) The detachment is not a result of toxicity, since the cells removed by the serum-PBS treatment were 75 to 80% viable, as measured by trypan blue exclusion, and could spread out and grow when subsequently replated. (iii) BALB/c 3T3 cells were also susceptible to the serum-PBS treatment when in the subconfluent, growing state (Table 1). It is possible, therefore, that the serum-induced detachment effect does not specifically differentiate between normal and transformed cells, but rather may be a general distinction between growing and growth-inhibited cells. (iv) When $MgSO_4$ and $CaCl_2$ were added to the PBS (100 mg/liter, to make Dulbecco's A+B+C), a considerable increase in the time required for cell removal was observed (20 versus 10 min), and when five times that concentration of divalent

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TABLE 1. *Detachment of SVT2 and 3T3 cells by serum-PBS incubation*[a]

| Cell line | Preincubation (20 h) | Saline incubation | Detachment of cells[b] after (min of saline incubation): 0 | 10 | 60 |
|---|---|---|---|---|---|
| SVT2, confluent | MEM, 10% serum | PBS | − | ± | + |
| SVT2, confluent | 80% serum, 20% MEM | PBS | − | + | +++ |
| 3T3, confluent | 80% serum, 20% MEM | PBS | − | − | ± |
| 3T3, subconfluent | 80% serum, 20% MEM | PBS | − | +* | +++ |
| SVT2, confluent | 80% serum, 20% MEM | PBS + $Ca^{2+}$ + $Mg^{2+}$ [c] | − | − | − |

[a] SVT2 or BALB/c 3T3 cells were grown in 35-mm dishes in MEM containing 10% fetal calf serum. This was replaced with the medium indicated in column 2 20 h before the experiment. Cells were washed once with PBS and then incubated at 37°C in the saline solution indicated. Subconfluent cells covered only 50% of the surface of the dish and were growing exponentially.

[b] The state of detachment of the cells was assessed at 0, 10, and 60 min after the start of the saline incubation and was scored as follows: −, no effect; ±, slight effect, 10 to 50% of the cells rounded; +, 30 to 50% of cells rounded and some aggregated, with 1 to 5% of the cells detached; ++, 70 to 100% of the cells rounded and aggregated, about 10 to 30% of cells sloughed off in sheets; +++, 100% of the cells detached, in sheets. We consider ++ or +++ by 60 min a positive detachment effect. The asterisk indicates that these cells were not aggregating.

[c] PBS containing 500 mg each of $CaCl_2$ and $MgSO_4 \cdot 7H_2O$ per liter.

cations was used the cells remained attached indefinitely (Table 1). Therefore, the detachment phenomenon may involve loss of divalent cations.

To isolate revertants, SVT2 cells in their 15th passage since cloning were plated at $5 \times 10^5$ cells per 60-mm plastic dish and grown until the cells were 80 to 100% confluent. The medium was then replaced with a medium containing 80% serum and 20% MEM. After 20 h at 37°C, the serum was removed, and the cells were washed once with PBS and then incubated in PBS at 37°C for 20 min. The aggregated free-floating cells were then removed, and 2.5 ml of Eagle MEM with 10% serum was added to the dish. After 2 weeks of incubation at 37°C, the few cells that had resisted the detachment procedure had grown into colonies, several of which (5 to 20 per dish) consisted of flat, polygonal cells. In two separate experiments, the frequency of the flat colonies was between 3 and 10 per $10^6$ SVT2 cells. This is similar to the frequency reported previously (2, 3), but since a fluctuation test was not done this observed frequency is of limited significance.

Ten of these flat colonies were isolated and cloned, as has been described (3), and were characterized as follows (Table 2). All clones were morphologically indistinguishable from the flat 3T3 fibroblasts. All were T antigen positive, indicating retention and expression of at least part of the SV40 genome. Karyotypic analysis revealed a considerably elevated ploidy (4 to 6 N compared to 2 N for SVT2), a characteristic shared with revertants isolated by other means (Table 2; 2, 5, 8, 11). The saturation densities were considerably lower than that of SVT2, and the cells exhibited a complete turnoff of growth about 1 day after confluence was reached and remained viable for several days thereafter (data not shown). These latter two properties are also seen in 3T3 and in other revertants (2, 3, 11, 12) and are not characteristic of transformed cells. Another distinctive property of normal 3T3 cells, density-dependent inhibition of 2-deoxyglucose uptake (10), was also exhibited by the revertants. Whereas the reduction in uptake when cells reached confluence was less than the 10-fold drop seen in 3T3 cells (10), it was quantitatively similar to that of the concanavalin A revertant line previously studied (1; see Table 2).

Culp and Black (2) have shown that the sialic acid levels in the revertant lines they isolated were considerably higher than in the parental transformed cells. Such a change was also apparent in our revertant cells (Table 2). Indeed, the inverse relationship between saturation density and sialic acid level (2) appeared to apply within the group of SDR revertant clones isolated (some results presented in Table 2), but all such revertant clones had a considerably higher sialic acid content than either transformed or normal cells and in this respect were different from revertant lines previously studied (2; see Table 2). Another unique aspect of the sialic acid data was the decrease observed when the SDR revertant cells reached confluence. We do not at this time understand the significance of this (but see below).

Generally speaking, the data presented in this communication show that the selection

TABLE 2. *Properties of serum-resistant cells compared with those of other revertant lines*

| Cell line | T antigen[a] | Chromosome no.[b] | | Saturation density[c] (cells/60-mm dish × $10^{-6}$) | Density-dependent inhibition of 2-deoxyglucose uptake[d] (%) | Sialic acid composition[e] (μg/mg of cell protein) | |
|---|---|---|---|---|---|---|---|
| | | Range | Mean | | | Subconfluent | Confluent |
| 3T3 | – | 46–67 | 57 | 1.5 | 90 | 4.0 | 4.5 |
| SVT2 | + | 38–46 | 40 | >17 | 0 | ND[f] | 1.8 |
| SDR revertant 15 | + | 48–106 | 81 | 8.0 | 76 | 8.8 | 5.2 |
| SDR revertant 18, early passage[g] | | 72–128 | 102 | 6.3 | 66 | 9.5 | 7.2 |
| SDR revertant, late passage[g] | | ND | ND | 7.2 | 48 | 8.5 | 5.4 |
| SDR revertant 22 | + | 73–120 | 90 | 6.0 | 63 | 10.2 | 5.4 |
| ConA-resistant clone 84[h] | + | 73–108 | 90 | 2.5 | 54 | Like parental untransformed 3T3 | |
| FUdR-selected F1E[i] | + | 80–109 | 96 | 1.6 | ND | Like parental untransformed 3T3 | |

[a] Cells were grown on cover slips, fixed in cold acetone, and tested for T antigen as described previously (3), using anti-T serum, kindly provided by P. Tegtmeyer, and fluorescein isothiocyanate-conjugated anti-hamster serum, obtained from GIBCO.

[b] Subconfluent cultures were treated with colchicine overnight, and mitotic cells were removed, fixed, and spread on cold, wet slides according to the method of Hsu and Kellogg (4). Numbers shown are the range and mean of 30 metaphases.

[c] Saturation density was measured by plating $10^5$ cells in each of several 60-mm dishes and counting cells in duplicate dishes daily. At saturation, the percentage of SDR 18 nuclei incorporating [$^3$H]thymidine was 1 to 5%.

[d] Cells were seeded at $3 \times 10^4$ cells/35-mm dish. Uptake of [$^3$H]2-deoxy-D-glucose was measured, as described previously (10), every day until 2 days after the cells reached confluence. The results were plotted as uptake rate (counts per minute per milligram of cell protein) versus time (days), and the percent drop from the maximum (occurring 1 day after plating) to the minimum (at about the time the cells reached confluence) is shown.

[e] Cells were grown in 10-cm dishes, and their sialic acid was determined by a micromodification (3) of the method of Warren (13). Duplicate determinations were made on each dish, and numbers presented are averages of two to four dishes. Variation among different dishes of the same cell line was less than 10%. Subconfluent cells were at 50% of confluence and growing exponentially.

[f] ND, Not done.

[g] Early passage was passage 6; late passage was passage 15.

[h] Data taken from Culp and Black (2). ConA, Concanavalin A.

[i] Data take from Culp and Black (2) and Culp et al. (3). FUdR, 5-Fluorodeoxyuridine.

procedure we used has yielded revertant cells similar to those isolated by using other means. This similarity supports the concept that the reversion event, once established, induces several simultaneous changes in behavior of the transformed cells, and the revertant cell line selected on the basis of any one of these changes will also exhibit at least some of the others. Furthermore, since our selective agents were nontoxic, we may surmise that the properties of SDR revertants are not artifacts of cell damage.

The one property of SDR revertant cells that is apparently unique is the sialic acid content, with respect to both quantity and variation according to growth state. This effect may be associated with reacquisition of growth control, as appears to be the case with concanavalin A revertant cells (2) or, alternatively, it may be a reflection of the selection procedure. Although we have not fully characterized it, we suppose that the serum-PBS treatment selectively removes cells that are relatively poorly attached. Cells that are altered such that cell-dish adhesion is increased would therefore be resistant, and a high level of the negatively charged sialic acid moiety may well facilitate such increased adhesion. Conceivably, as the cells begin to pack after confluence is reached, the decrease in the proportion of the cell surface in contact with the dish is related to the observed drop in sialic acid content.

Our finding that the revertants have a karyotype similar to that of most other revertants, even though the selective pressure is quite different from those used elsewhere, supports other data that suggest that polyploidization is

a necessary feature of reversion (5, 8). It is clearly not a sufficient condition, however, since fusion of 3T3 and SV3T3 cells gives hyperploid hybrids with a transformed phenotype (Ozer, personal communication). Furthermore, since it is possible to isolate temperature-sensitive revertants with unchanged ploidy (9), polyploidy is not a requirement in all cases. The role that ploidy plays in determining cell phenotype is therefore unclear, and experiments are currently in progress to clarify the problem.

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