GROWTH IN VITRO OF TUMOUR CELL × FIBROBLAST HYBRIDS IN WHICH MALIGNANCY IS SUPPRESSED

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SUMMARY

We have studied the growth in vitro of a lymphoma × fibroblast hybrid and several melanoma × fibroblast hybrids in which malignancy is suppressed. The parental cells, the hybrids, and malignant segregants derived from the hybrids were analysed for serum requirement, cloning efficiency in soft agarose, density-dependent inhibition of growth, and secretion of plasminogen-activating enzyme. One malignant segregant from the lymphoma × fibroblast cross was found by a number of criteria to have a more highly 'transformed' phenotype than the hybrid from which it was derived. However, in the case of the melanoma × fibroblast crosses, none of the parameters examined could be correlated in a direct way with malignancy.

INTRODUCTION

Hybrid cells formed by fusing tumour cells with normal diploid fibroblasts have greatly reduced tumorigenicity compared with that of the parental tumour cells (Harris et al. 1969; Harris, 1971; Jonasson, Povey & Harris, 1977; Jonasson & Harris, 1977; Stanbridge, 1976). The tumours that arise from such hybrid cells originate from initially rare segregants in the hybrid cell populations (Harris, 1971; Jonasson, et al. 1977; Jonasson & Harris, 1977).

In the present study the growth characteristics in vitro of clones of such hybrid cells are compared with those of the two parental cell types and those of malignant segregants derived from the hybrid cells. The growth parameters examined included serum requirement, saturation density and ability to form clones in soft agarose. We have been unable to find a clear-cut correlation between any single aspect of the transformed phenotype in vitro and the ability of the cells to grow progressively in vivo.

MATERIALS AND METHODS

Cell lines and conditions of culture

Hybrid clones were derived from the fusion of mouse tumour cell lines with mouse embryo fibroblasts. Two tumour cell lines were used: YACIR, which is an immunoresistant variant of a Moloney leukemia virus-induced lymphoma (YAC), and a spontaneous melanoma. Two thioguanine-resistant clonal derivatives of the melanoma (Clones 2C and PG19) were studied. Details of the tumours and of the isolation and karyology of the hybrids have already been
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given (Jonasson et al. 1977). Fibroblast cultures were prepared from half-term CBAT6T6 mouse embryos and were used in passages 3–7.

Cells were routinely grown in Eagle's Minimum Essential Medium (MEM) containing 10% foetal calf serum. Growth rates were measured by plating cells in 5 ml MEM containing 1% or 10% foetal calf serum at a density of 5 x 10^4 per 6-cm diam. plastic tissue culture dish. The medium was changed every third day. At intervals the cells were removed from the plastic with trypsin, and cell numbers determined in a Coulter Counter.

**Measurement of DNA synthesis by autoradiography**

Cells were grown on 1-cm coverslips in 5-cm dishes containing 3 ml of medium. DNA synthesis was measured by adding [Me-3H]thymidine (Radiochemical Centre, Amersham; 44 Ci/mmol) to the medium at a final concentration of 1 μCi/ml (2.3 x 10^-8 M). Coverslips were removed at intervals, washed with phosphate-buffered saline and fixed in methanol. The coverslips were mounted on glass slides, treated for 1 h in cold 5% trichloroacetic acid, rinsed with distilled water, dried, and coated with autoradiographic emulsion (Ilford K5). The slides were stored in the dark for 3 days and developed with Kodak D-19 developer. The preparations were stained with a mixture of 10% Giemsa and 1% May Grünwald stains. The percentage of cells synthesizing DNA in each preparation was determined by examining at least 300 cells.

**Cloning in soft agarose**

Serial dilutions of cells were plated in 5 ml of MEM plus 10% foetal calf serum plus 0.24% agarose (BDH) in 5-cm dishes containing an underlayer of 2 ml of MEM plus 10% foetal calf serum plus 1% purified agar (Difco). One millilitre of liquid MEM plus 10% foetal calf serum was added to the dishes and changed once weekly. Colonies visible to the eye were counted after 3 weeks at 37°C. In each experiment, cells were plated at densities ranging from 10^2 to 10^5 per 5-cm dish. Each result represents the average of several experiments, each done in quadruplicate. Chinese hamster ovary cells (clone A2/H) served as a positive control in the cloning experiments in soft agarose. Under our experimental conditions these cells clone in soft agarose with an efficiency of 60%.

**Plasminogen-activating enzyme**

The activity of plasminogen-activating enzyme was determined by three methods, all of which rely on the fact that human serum has a higher content of available plasminogen than foetal calf serum (Laug, Jones & Benedict, 1975).

**Method 1.** Cells were plated in 6-cm dishes in MEM plus 10% foetal calf serum or MEM plus 10% human serum. After 48 h, 5-μl samples of medium were introduced into wells in agar diffusion plates containing fibrin (Laug et al. 1975). The diffusion plates were incubated for 24 h at 37°C, and clear zones surrounding the wells were measured.

**Method 2.** Cells were grown to confluence in 20-oz. (560-ml) glass bottles in MEM plus 10% foetal calf serum. The cells were then washed with phosphate-buffered saline, the medium replaced with 40 ml of MEM without serum, and the bottles incubated for an additional 19 h at 37°C. The medium was then collected, and 50-μl samples were assayed for plasminogen-activating enzyme by a modification of the method of Unkeless et al. (1973). 125I-labelled fibrinogen (20 μg) in a total volume of 0.1 ml of phosphate-buffered saline was added to individual wells in 24-well Linbro trays (Linbro Scientific, Inc., 1.5-cm diam. wells). The trays were dried for 24 h at 45°C, and the fibrinogen was then clotted by the addition of 1 ml of MEM plus 10% foetal calf serum to each well and incubation for 2 h at 37°C. (There is enough thrombin in the foetal calf serum to clot the fibrinogen.) The wells were washed twice with phosphate-buffered saline, and the assay mixture was added to each well in a volume of 460 μl containing 420 μl of 0.1 M Tris-HCl, pH 8.1, 50 μl of supernatant medium, and 10 μl of human serum. The trays were incubated for 24 h at 37°C, a 100-μl sample was withdrawn from each well, and the amount of 125I-radioactivity released from the fibrin clot was measured. All assays were done in duplicate, and, in each case, controls were done with human serum alone and with samples of medium alone. In addition, the total amount of releasable radioactivity
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was measured by the addition of 12 μg trypsin to each of several wells and incubation for 24 h at 37 °C. The trypsin treatment released about 95% of the radioactivity from the fibrin clots.

Method 3. Cells were plated in small Falcon flasks in 5 ml of MEM plus 10% human serum at a density of 5 x 10⁶ cells (YAC) or 10⁶ cells (all other cell types) per flask. The flasks were incubated for 24 h at 37 °C, after which the medium was removed and filtered through a Millipore filter. Samples of medium (50 μl) were assayed as in Method 2, except that the complete reaction mixture contained 350 μl of 0.1 M Tris-HCl, pH 8.1, plus 50 μl of medium. Hundred-microlitre samples were withdrawn at 4, 8 and 24 h, and the amount of 125I per sample was determined. The control in each assay consisted of 50 μl medium that had previously been incubated at 37 °C for 24 h in the absence of cells.

RESULTS

Since a major objective of the present work was to determine how the malignancy of a tumour cell was suppressed when it was fused with a diploid fibroblast, we began by examining the extent to which the growth properties of the fibroblast were expressed in the hybrid cell. In doing this, we were able to draw on the extensive information available on the regulation of fibroblast growth in culture (reviewed by Holley, 1975).

Table 1. Tumour take incidences and generation times of melanoma × fibroblast hybrid clones

<table>
<thead>
<tr>
<th>Cell type*</th>
<th>Take incidence†</th>
<th>Generation time, h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium + 1 % serum</td>
<td>Medium + 10 % serum</td>
</tr>
<tr>
<td>Untransformed diploid embryonic fibroblasts</td>
<td>0/8 (5 x 10⁶) &gt; 120, &gt; 120, &gt; 120, &gt; 120</td>
<td>43, 39, 42, 36</td>
</tr>
<tr>
<td>C57 Black melanoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone PG19</td>
<td>26/26 (5 x 10⁶)</td>
<td>24</td>
</tr>
<tr>
<td>Clone 2C</td>
<td>99/99 (5 x 10⁶)</td>
<td>37</td>
</tr>
<tr>
<td>Hybrid clone 100A, subclone 1</td>
<td>10/11 (3 x 10⁶)</td>
<td>24, 25</td>
</tr>
<tr>
<td>Clone 100A, subclone 1, tumour 3</td>
<td>51/51 (5 x 10⁶)</td>
<td>29</td>
</tr>
<tr>
<td>Hybrid clone 10</td>
<td>10/95 (3 x 10⁶)</td>
<td>24, 24</td>
</tr>
<tr>
<td>Hybrid clone 4B, subclone 9</td>
<td>39/54 (3 x 10⁶)</td>
<td>25</td>
</tr>
<tr>
<td>Clone 4B, subclone 9, tumour 4</td>
<td>N.D.</td>
<td>30</td>
</tr>
<tr>
<td>Hybrid clone 4A, subclone 9</td>
<td>14/28 (4 x 10⁶)</td>
<td>29</td>
</tr>
<tr>
<td>Clone 4A, tumour 3</td>
<td>N.D.</td>
<td>37</td>
</tr>
<tr>
<td>Hybrid clone 7 (PG19 × T13H)</td>
<td>2/18 (3 x 10⁶)</td>
<td>90, 68</td>
</tr>
<tr>
<td>Hybrid clone 19</td>
<td>1/62 (3 x 10⁶)</td>
<td>108</td>
</tr>
<tr>
<td>Hybrid clone 8 (PG19 × T50H)</td>
<td>0/20 (4 x 10⁶)</td>
<td>&gt; 120, &gt; 120</td>
</tr>
</tbody>
</table>

* Hybrid clones have been arranged in order of decreasing ability to grow in 1% foetal calf serum.
† Take incidences are expressed as the number of mice with progressive tumours over the total number injected. The inoculum (number of cells injected per mouse) in each experiment is shown in parentheses. A mouse was scored as negative if no tumour developed within 5 months.
N.D. = not determined.
Serum requirement and saturation density

The tumorigenicity of the hybrids and their log phase generation times in high and low serum media are summarized in Table 1. We found saturation density to be a much more difficult parameter to define than log phase generation time, since it is related in a complex way to surface area, serum concentration and frequency of medium change (Dulbecco, 1970; Dulbecco & Elkington, 1973). Moreover, as discussed below, the melanoma cells themselves show more marked density inhibition than the untransformed fibroblasts.

Among the various criteria used to define transformation, the one which best distinguished the growth of the fibroblasts from that of the melanoma cells was the ability of the fibroblasts to arrest growth reversibly in low serum concentrations. This was most clearly seen when cells were plated at low density (≤ 3 × 10^4 cells/cm^2) in medium containing 0.5–1% serum. Under these conditions, the fibroblasts rapidly became quiescent, while the melanoma cells grew well, although less rapidly than in
medium with more serum. This difference could be seen both in growth measurements (compare Fig. 1A with Fig. 2) and in measurements of DNA synthesis by autoradiography (Fig. 3). When more serum was added to cultures of fibroblasts arrested at low serum concentrations, DNA synthesis was resumed in the cells after a lag of 12–16 h. Melanoma cells growing in low serum concentrations could be further stimulated by the addition of more serum.

![Graph showing growth of mouse CBAT6T6 embryo fibroblasts in medium containing 1% (○—○—○) or 10% (●—●—●) foetal calf serum. Cells were plated at densities of 5 × 10⁴ cells/dish (lower curves) or 2 × 10⁵ cells/dish (upper curves).]

The difference in serum requirement between the melanoma cells and the fibroblasts was most clearly defined when the cells were plated sparsely. As cell density increased, the difference in serum requirement became less pronounced (Fig. 4). The two DNA synthesis curves in Fig. 4 actually cross at high cell densities: the melanoma cells clearly exhibit stronger density-dependent inhibition of growth than the fibroblasts. In fact, under the conditions shown in Fig. 4 we found no evidence of density-dependent inhibition of fibroblast growth. On the contrary, the growth of the fibroblasts in low serum concentrations increased at higher cell densities.

The serum requirements of the melanoma x fibroblast hybrid clones were examined at sparse cell densities. The growth rates of the hybrid clones in low serum concentrations varied widely (Table 1). At one extreme, hybrid clone 100A, subclone 1 was found to have the same growth rate in low serum concentrations as the melanoma
Fig. 3. DNA synthesis in cultures plated in low serum concentrations. Melanoma clone PG19 and CBAT6T6 embryonic fibroblasts were plated in MEM plus 0.5% foetal calf serum. Two days later (time 0) [3H]thymidine was added to all cultures, and foetal calf serum was added to some cultures to a final concentration of 10%. Coverslips were removed at the times indicated and the percentage of nuclei labelled was determined by autoradiography. Clone PG19, 0.5% serum, —•—•; clone PG19, 0.5% serum shifted to 10% serum, —■—■; embryonic fibroblasts, 0.5% serum, O—O—O; embryonic fibroblasts, 0.5% serum shifted to 10% serum, ●—●—●.

Fig. 4. Effect of cell density on DNA synthesis. Melanoma clone PG19 (— — — — —) and CBAT6T6 embryonic fibroblasts (O—O—O) were plated at the indicated density in MEM plus 0.5% foetal calf serum. Two days later, [3H]thymidine was added to all cultures. After 24 h, the percentage of nuclei labelled was determined by autoradiography.
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parent cells and as the cells of a tumour derived from the hybrid clone (Fig. 1). At the other extreme, hybrid clone 8 was found to have a high serum requirement (Fig. 5).

![Graph showing growth of hybrid clone 8 in medium containing 1% or 10% fetal calf serum.](image)

**Fig. 5.** Growth of hybrid clone 8 in medium containing 1% (○—○—○) or 10% (●—●—●) fetal calf serum.

The inability of hybrid clone 8 to grow at low serum concentration was not simply due to the poor condition of the cells, or to cell death, at low serum concentration. As shown in Fig. 6, cells of this clone plated at low serum concentration appeared to be genuinely quiescent in that a large fraction of them could be induced to resume growth when more serum was added. The lag period between the addition of serum and the initiation of DNA synthesis (8 h) was shorter than that for the fibroblasts; this may reflect the shorter overall generation time of the hybrid cells in high serum concentrations.

Growth curves for two hybrid clones with intermediate serum requirements are shown in Figs. 7, 8. At low density, hybrid clone 4A grew well at low serum concentration, but entered the stationary phase at a lower cell density than clone 100A. Hybrid clone 7 grew poorly at low serum concentration, but distinctly better than clone 8.

There was obviously a superficial correlation between the growth rate of the melanoma x fibroblast hybrids at low serum concentration and their take incidence. In Table 1, the hybrid clones are arranged in order of decreasing ability to grow in 1% serum. It is clear that those clones that grew least well at low serum concentrations...
also gave the lowest take incidences. For example the take incidence for hybrid clone 8 was 0/20 with inocula of $4 \times 10^6$ cells per mouse, while that for hybrid clone 100A subclone 1 was 10/11 with inocula of $3 \times 10^6$ cells.

![Graph](image_url)

**Fig. 6.** DNA synthesis in hybrid clone 8 in low serum concentrations. Cells were plated in MEM plus 0.5% foetal calf serum. Two days later (time 0) [$\text{H}^3\text{H}$]thymidine was added to all cultures, and foetal calf serum was added to some cultures to a final concentration of 10%. Coverslips were removed at the times indicated and the percentage of nuclei labelled was determined by autoradiography. Cultures in 0.5% serum, ○—○—○; cultures in 0.5% serum shifted to 10% serum, ●—●—●.

However, the correlation between growth at low serum concentration and take incidence does not survive more exacting scrutiny. For example, hybrid clone 10 grew well in 1% serum but its ability to grow *in vivo* was strongly suppressed. In addition, clone 100A subclone 1 and the cells of the tumour derived from it show virtually identical growth rates at low serum concentration, but the tumorigenicity of the two cell populations is very different. The cells of the tumour gave a take incidence of 51/51 with inocula of $5 \times 10^4$ cells per mouse, and tumours arose from these small inocula within 3 weeks. In the case of clone 100A subclone 1, 60-fold higher inocula ($3 \times 10^6$ cells per mouse) did indeed produce a take incidence of 10/11, but 5 of the 11 tumours only appeared after a latent period of more than 3 months. Hybrid clone 100A itself, with inocula of $5 \times 10^4$ cells per mouse, gave a take incidence of 18/78. The tumours produced from high inocula of hybrid clone 100A subclone 1 represent the selection of malignant variants from a cell population in which malignancy has been suppressed. The rapid production of tumours in 100%
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of recipients with small inocula of cells from the clone 100A subclone 1 tumour reflects progressive growth of the mass of the cell population injected. The detailed evidence for this conclusion has already been presented (Jonasson et al. 1977).

![Graph showing growth of hybrid clone 4A](image)

Fig. 7. Growth of hybrid clone 4A in medium containing 1 % (O---O---O) or 10 % (●---●---●) foetal calf serum.

It has been demonstrated that, in the melanoma \( \times \) fibroblast hybrids, there is selection pressure directed specifically against the two chromosomes 4 derived from the fibroblast (Jonasson et al. 1977). The presence or absence of the fibroblast chromosome 4 does not, however, appear to affect growth in low serum concentrations. For example, hybrid clone 100A subclone 1 contains one copy of the fibroblast chromosome 4, while hybrid clone 10 has lost both copies. Both clones grew well in 1 % serum.

Cloning in soft agarose

It has been reported that the ability of virus-transformed fibroblasts to form clones in soft agar or methocel is correlated with tumorigenicity in nude mice (Freedman & Shin, 1974; Shin, Freedman, Risser & Pollack, 1975). It was therefore of interest to examine this parameter in the hybrid cell system.

An interesting result was obtained with the lymphoma \( \times \) fibroblast hybrid clone 1G (Table 2). This hybrid initially produced no tumours in 12 recipients with inocula of \( 4 \times 10^6 \) cells per mouse. On continued cultivation of the hybrid cell popula-
tion in vitro, the take incidence rose progressively in the usual fashion (for details, see Jonasson et al. 1977). Clone iG itself does not form colonies at all in soft agar, whereas the cells from a tumour derived from clone iG do clone under the same conditions, albeit at low efficiency. The malignant derivative also shows two other features normally associated with transformation in vitro. First, it grows more rapidly than clone iG in both high and low serum concentrations (Fig. 9). Second, whereas clone iG grows in flat monolayers on surfaces, cultures of the malignant derivative consist of mixed populations of criss-crossed flattened cells and cells that detach from the surface and grow in suspension.

However, the correlation breaks down in the melanoma hybrid series. The melanoma tumour cells cannot grow in soft agar or methocel (cloning efficiency $< 0.5 \times 10^{-6}$, Table 2) irrespective of plating density. They also fail to form clones in soft agar when plated over a feeder layer of Balb/c 3T3 cells, according to the method of Sato, Slesinski & Littlefield (1972).

*Plasminogen-activating enzyme*

Reich and co-workers have described an extracellular enzyme which converts plasminogen to plasmin and which is produced in large quantities by virus-transformed fibroblasts (Unkeless et al. 1973; Ossowski et al. 1973; Pollack, Risser,
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Conlon & Rifkin, 1974) and some other types of transformed cells (Laug et al. 1975). We have assayed the activity of this enzyme in populations of tumour cells, fibroblasts, hybrids in which malignancy is suppressed and malignant derivatives of these hybrids (Table 2).

Table 2. Cloning efficiencies in soft agarose and plasminogen-activating enzyme activities of the parental cells and the hybrid clones

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cloning efficiency in soft agarose</th>
<th>Method 1*</th>
<th>Method 2†</th>
<th>Method 3‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse embryo fibroblasts</td>
<td>&lt; 0.5 x 10⁶</td>
<td>—</td>
<td>14</td>
<td>428</td>
</tr>
<tr>
<td>Melanoma clone PG19</td>
<td>&lt; 0.5 x 10⁻⁶</td>
<td>—</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hybrid clone 100A, subclone 1</td>
<td>&lt; 0.5 x 10⁻⁶</td>
<td>—</td>
<td>N.D.</td>
<td>90</td>
</tr>
<tr>
<td>Hybrid clone 100A, subclone 1, tumour 3</td>
<td>&lt; 0.5 x 10⁻⁶</td>
<td>—</td>
<td>N.D.</td>
<td>996</td>
</tr>
<tr>
<td>YAC lymphoma</td>
<td>7-8 x 10⁻⁴</td>
<td>—</td>
<td>N.D.</td>
<td>0</td>
</tr>
<tr>
<td>Hybrid clone 1G</td>
<td>&lt; 0.5 x 10⁻⁶</td>
<td>+(1 mm diam.)</td>
<td>1981</td>
<td>1136</td>
</tr>
<tr>
<td>Hybrid clone 1G, tumour 4</td>
<td>3-7 x 10⁻³</td>
<td>+ + + + (6 mm diam.)</td>
<td>10725</td>
<td>2435</td>
</tr>
</tbody>
</table>

* HS = human serum, FCS = foetal calf serum.
† Activities are expressed as cpm of 125I released per 100-μl sample in 24 h. The blank (human serum alone) was 1565 cpm and has been subtracted from all measurements. The radioactivity released by 12 μg of trypsin in 24 h was 11 028 cpm.
‡ Activities are expressed as cpm of 125I released per 100-μl sample in 4, 8 and 24 h. Blank values were determined from an assay of 50 μl of MEM + 10% human serum and have been subtracted from all measurements. The amount of radioactivity released by 12 μg of trypsin per 100-μl sample was 7393 (2 h), 7466 (4 h) and 8354 (24 h) cpm.

Our initial assay of the lymphoma cross indicated that the hybrid clone 1G and its malignant derivative produced detectable amounts of the enzyme, whereas neither parent cell did (Table 2, Methods 1 and 2). However, with the most sensitive assay procedure (Table 2, Method 3), we found that the fibroblasts produced some enzyme activity, while the lymphoma tumour cells appeared to produce none at all. In all assays the malignant derivative of clone 1G produced much more enzyme than clone 1G itself. In the case of the melanoma series, the outstanding finding was that the untransformed fibroblasts produced more plasminogen-activating enzyme than the highly malignant melanoma cells.

Discussion

In this study we have used hybrid cells to test the degree of correlation between transformed phenotype in vitro and tumorigenicity in vivo. We found the growth characteristics of the melanoma cell line in vitro to be at variance with some current
views about the relationship between transformed phenotype and tumorigenicity. The melanoma cells are highly malignant in vivo, producing rapidly growing and metastasizing tumours from small inocula (Jonasson et al. 1977). However, in culture, the melanoma cells do not show the features usually associated with transformation in vitro. First, the melanoma cells, although they grow very well in sparse cultures (14–16 h generation time), show very strong density-dependent inhibition, as illustrated in Fig. 4. Second, they do not clone at all in soft agarose or methocel, thus demonstrating that this property is not a general characteristic of tumour cells, as proposed by Freedman & Shin, 1974.

In the melanoma × fibroblast crosses the property most extensively studied was serum requirement. We found that the difference in serum requirement between the melanoma cells and the fibroblasts was most clearly demonstrated when the cells were plated sparsely in low serum concentrations. At higher cell densities, the difference in serum requirement disappeared. This is probably due to a combination of factors including the well known 'helper' or medium-conditioning effects observed in fibroblast cultures (Rubin, 1966; Dulbecco & Elkington, 1973) and the greater density-dependent inhibition of growth of the melanoma cells.

Among the seven melanoma × fibroblast hybrid clones examined in detail, there was a superficial correlation between serum requirement and tumorigenicity, but this
correlation did not survive more stringent examination. Hybrid clone 100A subclone 1, which has a greatly reduced tumorigenicity compared with the parental melanoma cells or with the cells of tumours derived from the hybrid clone, nonetheless grows as well as either of these in low serum concentrations. In this connexion the recent study by Stiles, Desmond, Sato & Saier (1975) is of interest. These workers found that a number of clones of human cells transformed by Simian Virus 40 and showing all the characteristics of transformation in vitro nonetheless failed to grow progressively in immunosuppressed mice. C. J. Gee, in this laboratory, has recently obtained similar results with SV40-transformed mouse cells. It is clear that transformation in vitro is not in itself sufficient to confer on a cell population the ability to grow progressively in the animal. It may well be that for some cell types, the changes associated with transformation in vitro are preliminary events leading eventually to tumorigenicity in vivo; but the growth characteristics of the melanoma cells themselves indicate that even this proposition cannot be generalized.

The results obtained with the lymphoma × fibroblast hybrid clone 1G were quite different from those obtained with the melanoma × fibroblast hybrids and conformed more closely to the common experience. In the lymphoma cross, the hybrid in which malignancy was suppressed failed to clone in soft agar, whereas the malignant derivatives of this hybrid did clone under these conditions. The malignant derivative also showed a more irregular morphology in monolayer culture and a faster generation time in high and low serum concentrations; it also produced higher levels of plasminogen-activating enzyme.

We suspect that the transformed phenotype of the lymphoma cells is initially suppressed in the fibroblast cross and that hybrid clones are obtained only after some additional transforming event has occurred. This notion is supported by two observations. The first is that hybrid clones are recovered at extremely low frequency in the lymphoma × fibroblast cross: only one hybrid clone was obtained from several independent cell fusion experiments. The second is that this single hybrid clone secretes plasminogen-activating enzyme. This enzyme is known to be inducible in untransformed fibroblasts (Wigler & Weinstein, 1976) and to be present in all transformed mouse fibroblast lines, including those of low tumorigenicity (Pollack et al. 1974); but it is totally absent from the parental lymphoma tumour cells. These findings are explained most simply by the intervention of an additional transforming event affecting the fibroblast component of the hybrid clone 1G.

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