The Characterization of SV40-Transformed Cell Lines Derived from Mouse Teratocarcinoma: Growth Properties and Differentiated Characteristics [1]

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ABSTRACT Mouse teratocarcinoma cells derived from embryoid bodies of 129SVsl mice were cultured in vitro to permit their differentiation. These cells were then infected with simiam virus 40 (SV40) and 31 cloned cell lines (SVTER) were derived from these cultures. All 31 SVTER cell lines contained the SV40 tumor (T) antigen and grew as permanent lines in culture. Mock-infected embryoid body cultures did not give rise to permanent cell lines. The morphology of each SVTER cell line was distinct and did not change during successive subclonings.

The growth properties and tumorigenic potential of all 31 SVTER cell lines were investigated. None of these lines produced tumors in 129SVsl mice. Each cell line was tested for its ability to (1) grow in medium containing 1% serum, (2) plate on a cell monolayer, and (3) form clones in methocel suspension. Only three of the SVTER cell lines were transformed with respect to all three of these criteria. Most of these cell lines were minimal transformants.

The SVTER cell lines were tested for creatine phosphokinase (CPK), an enzyme activity characteristic of mouse brain and muscle tissue, and the protease, plasminogen activator (PA) which is found in embryoid bodies and several differentiated cell types. Some of the SVTER cell lines contained high levels of CPK, while others had high levels of PA and a third group of cells contained neither enzyme activity. No SVTER cell line was found with high levels of both these enzyme activities. This result suggests that mutually exclusive sets of genes are expressed in these cells as might be expected from the distinct tissue distribution of the two enzyme activities studied. These SVTER cell lines may be useful in reconstructing developmental pathways of differentiating teratomas in vitro.

Testicular teratomas of mice are particularly useful for the study of development and tumorigenesis. These tumors are composed of malignant embryonal carcinoma cells which under the appropriate conditions give rise to a variety of benign, differentiated cells. (Stevens, '67a,b; Pierce, '67). Differentiation of embryonal carcinoma cells has also been reproduced in tissue culture, allowing the study of development in vitro under experimentally controlled conditions. Both cloned embryonal carcinoma cell lines (Jakob et al., '73; Martin and Evans, '74; Jami and Ritz, '74) and cultures of embryoid bodies (the ascites form a transplantable teratoma) (Teresky et al., '74; Levine et al., '74; Gearheart and Mintz, '74, '75) have been shown to differentiate in vitro into a variety of cell types. The criteria employed to demonstrate differentiation in vitro have included morphological alterations (Teresky et al., '74; Martin and Evans, '74, '75), the acquisition or loss of enzymatic activities characteristic of differentiated tissues (Levine et al., '74; Gearheart and Mintz, '74,

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'75; Hall et al., '75) and the presence or absence of cell surface antigens that are found on embryonal carcinoma or differentiated cells (Artz et al., '73, '74; Babinet et al., '75; Edidin and Gooding, '75; Stern et al., '75). In addition, differentiated cells derived in culture from teratocarcinoma cells are no longer able to form tumors in animals, indicating a concurrent loss of tumorigenic potential with the acquisition of differentiated functions (Pierce, '75; Hall et al., '75).

One important goal of these in vitro studies has been to analyse the events which occur as a teratocarcinoma cells becomes committed to a specific developmental pathway. This task has proven to be difficult because the differentiated cells derived from teratocarcinoma cells have low cloning efficiencies and rarely can be established as permanent cell lines in cultures (Hall et al., '75). Only a few cases of teratocarcinoma-derived permanent cell lines with differentiated functions have been reported: parietal yolk sac (Lehman et al., '74); myoblasts (Boon et al., '74), keratinocytes (Reinwald and Green, '75).

In this communication we describe the isolation and characterization of cloned cell lines derived from a population of differentiating teratocarcinoma cells. In order to establish these permanent cell lines, we have employed the tumor virus SV40, because primary cells transformed by this virus are capable of infinite growth in culture (Pollack et al., '74). Embryonal carcinoma cells appear to be refractory to SV40 infection (Swartzendruber and Lehman, '75). However, differentiated cells present in cultures of teratoma cells are susceptible to infection and allow expression of viral gene products in particular the virus tumor antigen (Swartzendruber and Lehman, '75). Based on these observations it should be possible to transform differentiated teratoma cells or even those cells at intermediate stages in development. These SV40 transformed, teratocarcinoma-derived cells might then give rise to stable cloned cell lines which are either fixed at a particular developmental stage or are capable of further differentiation under the appropriate conditons. Studies with these cloned cell lines might then allow the reconstruction of some events in the developmental pathways of different cell types.

MATERIALS AND METHODS

Culture conditions. Cells were grown on disposable petri dishes in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum. Incubators were gassed with 10% CO_2 and 90% air and maintained at 37°C. Cloned cell lines were frozen in plastic vials in DME, 10% fetal calf serum and 10% glycerol at liquid nitrogen temperatures approximately two months after cloning.

Growth properties. To determine the growth rates of cloned cell lines, 10^3 cells were plated in 35-mm dishes and replicate plates counted every two days for 12 days. To test for growth in low serum, plates were rinsed and refed on day 1 with DME supplemented with 1% fetal calf serum. Plating efficiencies were determined by plating dilutions of cells of monolayers of NIH-Swiss 3T3 cells (Todaro and Green, '63) or on plastic surfaces. These cells were then refed every three days, stained and counted after 12 to 18 days. To determine the ability of cells to grow in suspension, 10^5 and 10^4 cells were plated in growth medium containing 1.2% methyl cellulose (Methocel, 4,000 cps, Dow) on plates coated with growth medium containing 0.9% agar (Difco). Methocel plates were refed once a week with 4 ml methocel medium and the number of colonies were counted with a dissecting scope using dark-field illumination four weeks after plating.

Teratocarcinoma cells. The transplantable tumor OTT6050A, a pluripotent teratoma, was the gift of Doctor L. Stevens of the Jackson Laboratory to AJL. This ascites tumor, consisting of embryoid bodies, was harvested from the peritoneal cavity of strain 129SVsl mice, in which it was passaged at 3-week intervals (Teresky et al., '74). Embryoid bodies were washed three times in growth medium and 0.1 cc packed cells was used to seed five, 100-mm plates. PCC4 aza-1 is an embryonal carcinoma cell line isolated by Jakob et al. ('73). Under normal culture conditions, it remains an embryonal carcinoma cell with little evidence of differentiation.

SV40 Infection and cloning techniques. One-month-old cultures of embryoid bodies were rinsed once with medium and infected with 0.25 ml SV40 (10^8 PFU/ml, strain 776, a gift of Doctor Rex Risser) or mock infected for two hours at 37°C. Plates were then rinsed, fresh growth medium was added and the cultures incubated at 37°C for three weeks. Cells from isolated regions of a plate were trypsinized using steel cylinders and plated in 60-mm

dishes. From each of these petri dishes, well-isolated colonies were picked and recloned by plating 0.3 cells/well in Linbro microwells.

T-antigen staining. 10^4 cells were plated into a 35-mm culture dish containing 12-mm coverslips. After several days, coverslips were washed in serum free medium fixed in methanol (10 minutes room temperature) and rinsed in PBS. The coverslips were treated at 37°C with hamster antibody against SV40 T-antigen against SV40 T-antigen (Flow, diluted 1:4 in phosphate buffered saline [PBS]) for one hour, rinsed with PBS, and reincubated one hour with fluorescein-conjugated goat anti-hamster IgG. The coverslips were finally rinsed with PBS, mounted in elvanol, and examined with ultraviolet epi-illumination on a Zeiss Photomicroscope II.

Complement fixation test. The complement fixation tests were performed in microtiter wells essentially by the procedures described by Sever ('62). The CF titer refers to the highest dilution of antigen in which the red blood cells were not lysed. Each titer is the average of duplicate tests where serial 2-fold dilutions of 25 μl antigen were employed with two units of complement.

Tumorigenicity of cell lines. Cells were rinsed three times with PBS and scraped from the surface of a culture dish. Approximately 10^6 cells were injected either subcutaneously or intra-peritoneally into individual male or female 129SVsl or 129 mice. The mice were examined for tumors up to eight months after injection.

Creatine phosphokinase assay. Creatine phosphokinase activities were determined by methods described by Levine et al. ('74). The brain and muscle forms of these enzymes were fractionated by polyacrylamide gel electrophoresis (Levine et al., '74). Protein determinations were performed by the method of Lowry et al. ('51).

Plasminogen activator assay. Assays for soluble plasminogen activator were performed as described by Pollack et al. ('74). Cells were plated (10^6 cells per 100 mm petri dish) and allowed to grow for one day in DME 10% fetal calf serum. The medium was then removed, the monolayer washed twice with PBS, and 10 ml of DME without serum placed on the cells. The cells were then incubated for 8 and 16 hours at 37°C. The medium was removed, clarified of debris by centrifugation and frozen at −20°C until use. Fifty μl of this medium was then assayed at 37°C in Linbro plates coated with ^{125}I-fibrin. Purified fetal calf plasminogen was used as a source of plasminogen. Additional assays were performed to determine the level of plasminogen activator bound to the cell surface as well as intracellular plasminogen activator. These data are not presented but were in agreement with the activities illustrated.

RESULTS

SV40 transformation of teratocarcinoma-derived cells

Embryoid bodies from the transplantable teratocarcinoma OTT6050A were permitted to attach to the surface of a culture dish. Under these conditions, cells divide or migrate out of these bodies and after prolonged incubation produce a variety of morphologically distinct cells with differentiated characteristics (Teresky et al., '74; Levine et al., '74; Gearheart and Mintz, '74, '75). After four weeks in culture, when the majority of cells appeared differentiated, these cells were infected with SV40 (at approximately 30-100 PFU/cell). Three weeks after viral infection, the cells were cloned and cell lines (designated SVTER followed by a clone number) from 31 independent clones were obtained for study. Mock-infected, teratocarcinoma-derived cultures were also isolated and cloned, but these cells failed to sustain growth in culture, whereas greater than 95% of the clones from SV40-infected cultures were easily established as permanent cell lines. Thus, SV40 infection permitted the establishment of cloned lines derived from the embryoid body cultures.

All 31 cells lines (SVTER) were shown to contain SV40 tumor (T) antigen by fluorescent antibody binding and complement fixation tests (results not presented). With one exception (SVTER 42), greater than 95% of the cells contained T-antigen. With SVTER 42 only about 60% of the cells contained T-antigen even after a second cloning procedure.

The morphology of each of these 31 transformed, teratocarcinoma-derived cell lines was quite distinct. Examples of the morphologies of several cell lines are presented in figure 1. During recloning the characteristic morphology of each cell line was retained. Based upon morphological considerations alone, it was not possible to equate

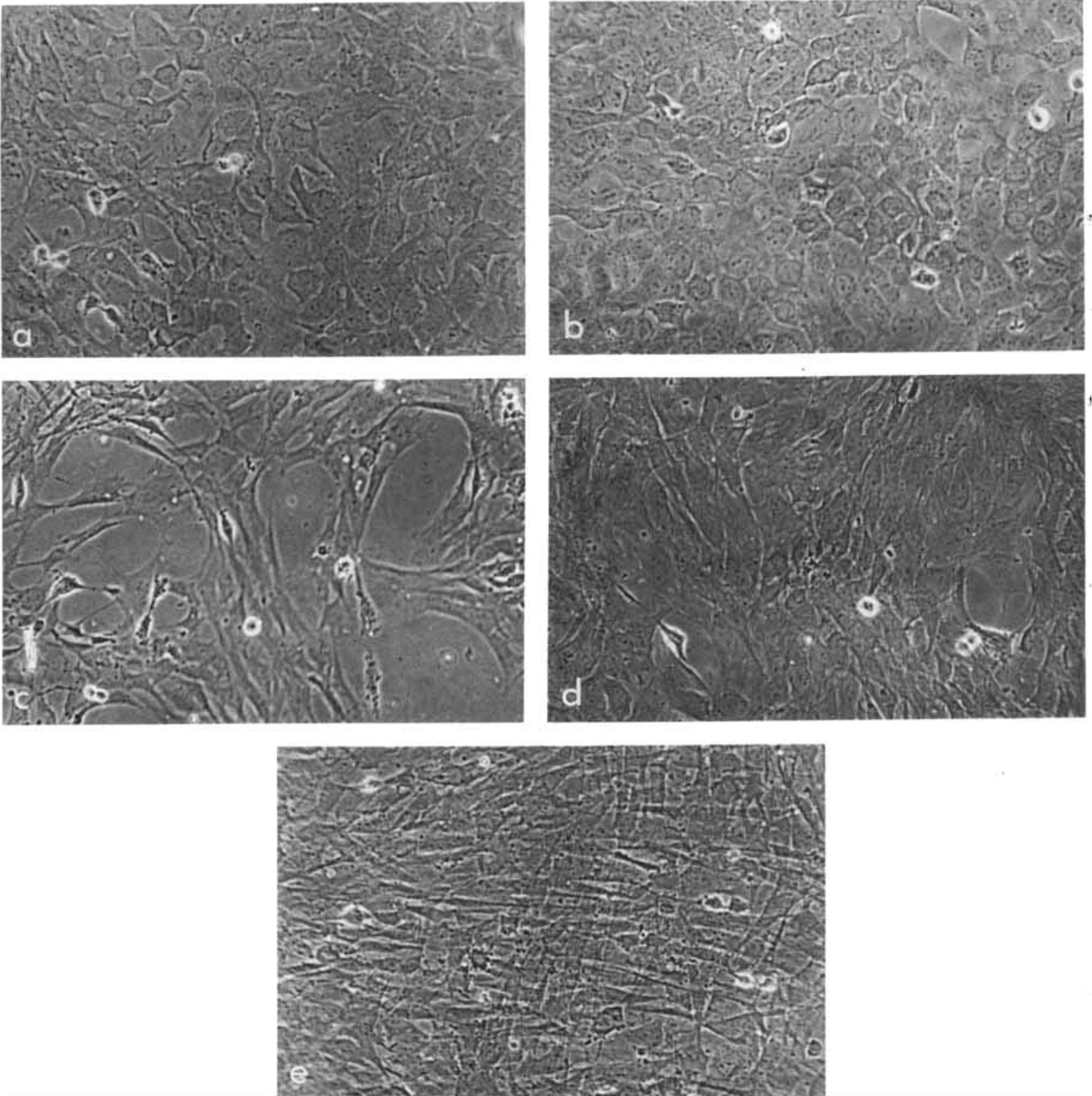

Fig. 1 The morphology of five of the SVTER cells lines. Embryoid bodies growing on the surface of a culture dish were infected with SV40. Three weeks later cells were cloned and SV40 tumor antigen containing cell lines were established; 31 cell lines (SVTER-clone number) were produced in this fashion and each line had a characteristic morphology that retained this property even after subsequent clonings. Five examples of these cell lines are presented: (a) SVTER 33; (b) SVTER 16; (c) SVTER 14; (d) SVTER 44; (e) SVTER 62.

the cells in these cultures with any given differentiated cell type.

Growth properties and tumorigenic potential of the SVTER cell lines

SV40 transformants are commonly defined by an alteration in growth potential. Cells are considered transformed if they possess (1) doubling times in medium containing 1% serum that do not exceed two times the generation time in 10% serum (2) are able to form colonies on top of a cell monolayer with at

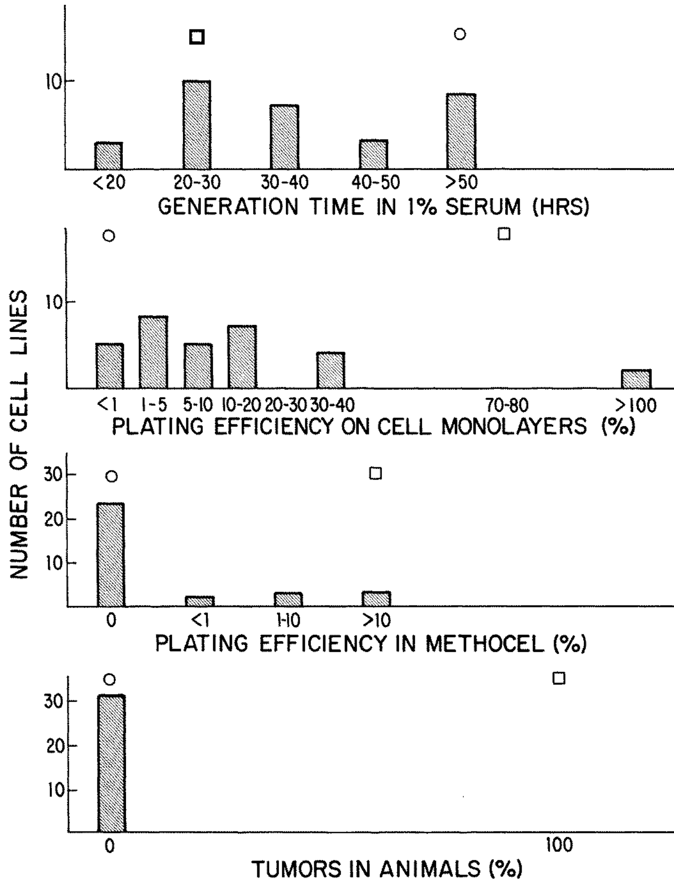

Fig. 2 The distribution of growth potentials of the SVTER cell lines. The 31 SVTER cell lines were analysed for (1) The generation time for growth in medium containing 1% serum, (2) The efficiency of plating on top of cell monolayers, (3) The efficiency of plating in methocel and (4) Tumor formation in 129 mice. The distribution of cell lines with these properties is presented. ○, Balb 3T3; □ SV-Balb 3T3-T-2-TUM.

least 5% of the efficiency of plating on plastic surfaces and (3) are able to form colonies in methocel with efficiencies at least 1% of that obtained on plastic surfaces. Each of the SVTER cell lines was tested for these three criteria of transformation. The experimentally determined values for all cell lines are compiled in figure 2. For comparison, figure 2 also shows the values obtained for an untransformed mouse cell line (Balb 3T3) and a tranformed, tumorigenic mouse line (SV-3T3-T2-TUM).

Twenty-one of the 31 SVTER cell lines grew rapidly in medium containing 1% serum. Nineteen cell lines were able to plate (with a 5% or better efficiency) on monolayer cultures, while only 6 of 31 cell lines grew well (1% or greater efficiency) in methocel. Only 3 out of 31 SVTER cells lines were transformed with respect to all three of these growth properties (SVTER 32, 104, 112). Eleven additional cell lines were transformed with respect to two of these phenotypes and 15 cell lines possessed only one of the transformed growth properties. Two cell lines were not transformed by any of these criteria despite the retention of SV40 T-antigen.

All of the 31 SVTER cell lines were injected into 129 mice (1-3 × 10^6 cells per mouse). Over a 7- to 8-month period neither subcutaneous nor intraperitoneal injections resulted in a tumor (fig. 2). By comparison, the injection of the embryonal carcinoma cell line (PCC4 aza-1), embryoid bodies, or SV40 transformed Balb/c cells (SV-3T3-T2-TUM) at the same cell concentrations into isogenic mice

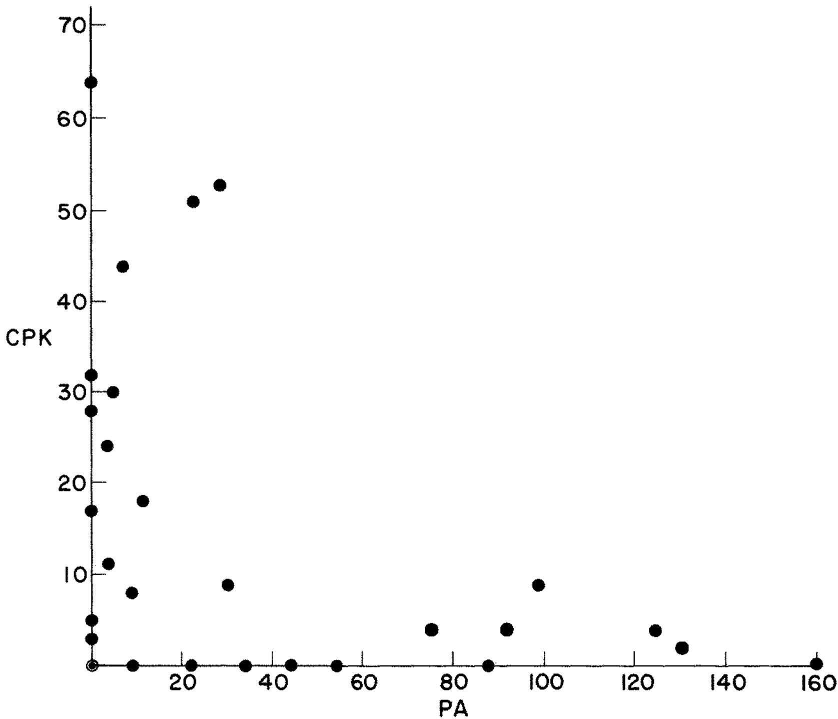

Fig. 3 The specific activity of plasminogen activator and creatine phosphokinase in 29 SVTER cell lines. The specific activity of creatine phosphokinase (OD_{250}/min/mg protein) and plasminogen activator (^{125}I-fibrin cpm hydrolysed/μgm protein) was determined for each of 29 SVTER cell lines. The specific activity of each enzyme in a cell line was then expressed as a percentage of the specific activity found in mouse brain (for CPK) or a standard cell line (4-71-1) with high plasminogen activator levels (for PA activity). This permitted the same standards to be included in every assay as a reference for a compilation of all the results. Each point represents the level of CPK and PA in one of the 29 SVTER cell lines tested.

resulted in 100% tumor formation in two to four weeks (results not presented). Based upon this criterion for transformation, none of the SVTER cell lines appear to be transformed.

Enzymatic activities characteristic of embryoid bodies and differentiated cells

Embryoid bodies obtained from the peritoneal cavity of a mouse have been shown to contain phasminogen activator (Hall et al., '75). This protease activity also appears in a number of differentiated cell types (endodermal cells of embryoid bodies [Sherman et al., '76], activated macrophages [Unkeless et al., '74], vascular endothelium [Todd et al., '59; Riseberg et al., '75], ovarian granulosa [Beers et al., '75] and viral transformed cells [Unkeless et al., '73; Pollack et al., '74]). Creatine phosphokinase is an enzyme activity characteristic of fetal and adult brain and muscle tissue. The brain form of this enzyme is expressed in differentiated cells derived from embryoid bodies in culture (Levine et al., '74). Since these enzyme activities have been observed in differentiating cultures of embryoid bodies, the SVTER cell lines were also examined for the presence of these enzyme activities. In each case, the specific activity (enzyme activity per mg protein) was determined. The results of the creatine phosphokinase assay are expressed as a percentage of the specific activity of this enzyme found in the adult mouse brain tissue (fig. 3). The levels of plasminogen activator in the SVTER cell lines are reported as a percentage of the specific activity of plasminogen activator found in a standard, high-activity cell line (4-71-1 isolated by Imada and Sueoka, unpublished results) (fig. 3). In either case only levels greater than 15% of these control values represent significant quantities of these enzymes activities. The activities of these enzymes in each of the SVTER cell line

are compared in figure 3. This graph indicates that the expression of these two enzymatic activities in any one cell line appears to be mutually exclusive.

DISCUSSION

When embryoid bodies derived from a murine transplantable testicular teratoma are permitted to settle on the surface of a culture dish, a variety of differentiated cell types are produced in culture (Teresky et al., '74). In order to isolate and analyse teratoma-derived cells which are either fully differentiated or in process of development, SV40 infection was employed. Viral transformation allowed the establishment of cloned cell lines (SVTER) derived from differentiating teratocarcinoma cell cultures. These cell lines have been analysed for their transformed phenotypes and two enzyme activities characteristic of embryoid bodies or differentiated cells.

SV40 infection is clearly required for establishment of these cell lines in culture. All of the SVTER lines express the SV40 tumor antigen. In addition, since mock-infected teratocarcinoma-derived cells failed to produce cells that grew in culture for more than a few passages, the virus must exert an effect on these cells, permitting the establishment of permanent cell lines. However, with respect to virally-induced transformation of growth potential, the great majority of these teratoma-derived SVTER cell lines were only minimally transformed.

The growth properties of the embryonal carcinoma cell in culture resemble those of a fully-transformed cell: high plating efficiency in methocel and on monolayer cell cultures, moderate growth in medium containing 1% serum and tumor formation in animals. None of the SVTER teratocarconoma-derived lines, even though SV40-transformed, have growth properties like the embryonal carcinoma cell.

The collection of SVTER cell lines may be valuable in analysing several stages of teratoma differentiation. In an attempt to characterize these cell lines for properties associated with differentiated cells, we have assayed them for two enzyme activities, creatine phosphokinase and plasminogen activator. Creatine phosphokinase is characteristic of differentiated cells (brain or muscle). Plasminogen activator is easily detected in endodermal cells of embryoid bodies, some differentiated cell types (macrophage, granulosa cells, endothelial cells) as well as in certain virally-transformed cells. An analysis of plasminogen activator activity and creatine phosphokinase levels in 29 SVTER cell lines (fig. 3) reveals three classes of cell lines: (1) those cell lines with high levels of plasminogen activator and little or no creatine phosphokinase, (2) those cell lines with high levels of creatine phosphokinase and little or no plasminogen activator and (3) those cell lines with low levels of both the enzyme activities. All of the 29 cell lines tested fit into one of these three categories, as might be expected for clones derived from teratoma cells developing in vitro. There is clearly a reciprocal relationship between cell lines containing plasminogen activator and creative phosphokinase. The third class of SVTER lines exhibiting neither enzyme activity could represent cells that were obtained from pathways of development not leading to muscle, to brain or to cell types containing plasminogen activator. The reciprocal distribution of the enzyme activities of creatine phosphokonase and plasminogen activator in each cell line suggests that defined sets of gene expression can be detected in these clones. These cell lines may therefore represent intermediates in developmental pathways during differentiation of teratocarcinoma cells.

It should be acknowledged, however, that these cells could represent aberrant examples of development. The role of the virus, SV40, in establishing cloned cell lines is not understood and could lead to unusual patterns of gene expressions. Easton and Reich ('72) and Holtzer et al ('75) have shown that infection of chick embryo myoblast cultures with Rous sarcoma virus precludes either cell fusion or differentiated enzyme expression. Only further study of these cell lines will indicate whether they can be useful in elucidating the pathways of development in the teratocarcinoma system.

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