Differential Tumorigenicity of 3T3 Cells Transformed in Vitro with Polyoma Virus and in Vivo Selection for High Tumorigenicity

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ABSTRACT

BALB/c 3T3 cells transformed in vitro with a temperature-sensitive mutant of polyoma virus were cloned. Forty-eight clones examined demonstrated heterogeneity with respect to doubling-time in vitro and tumorigenicity in syngeneic mice in vivo. Observation periods that lasted in certain cases as long as 2 years showed that some clones exhibited a relatively high tumorigenicity, i.e., they yielded a relatively high incidence of tumors following a small inoculum of cells and a relatively short latency period. Other clones were relatively low tumorigenic: even high tumor cell inocula yielded a relatively low tumor incidence following a relatively long latency period. These results indicate that at least in this system variation in tumorigenicity is generated independently of host factors.

An intraclonal heterogeneity with respect to the length of the precancer latency period was seen. Some tumors appeared relatively early following inoculation of cloned cells, whereas others appeared considerably later following an identical inoculum of the same clone.

Cloned in vitro transformed cells were passaged once in syngeneic mice and recultured. The single in vitro passage cycle augmented considerably the tumorigenicity of these cells as compared to their in vitro maintained clonal ancestors. The increased tumorigenicity of the in vivo passed cells is due, most probably, to the in vivo induction and/or selection of high tumorigenic intraclonal variants. The survival time of mice bearing high tumorigenicity variants was very similar to that of mice bearing low tumorigenicity variants.

INTRODUCTION

Heterogeneity of cells residing in tumors is well established (1–14). Heterogeneity was reported to occur with respect to many characteristics such as morphology, karyotype, enzymatic profile, ability to metastasize, in vitro growth pattern, responsiveness to drugs, antigenicity etc. The consensus of many researchers (e.g., ref. 11) is that tumor heterogeneity is a manifestation of tumor progression. The present study, as others (e.g., refs. 15–17), is based on the model proposed by Nowell (18–19) that the progression of primary tumors is driven by a dynamic combination of variant cell generation and selection of those variants best fitted to survive and proliferate in vivo.

Cellular variant generation seems to be a stochastic, highly unordered and multidirectional process which involves both genetic (4, 11–12, 14, 18–21) and epigenetic (11, 14, 22–25) changes. Several factors, such as the genetic instability of malignantly transformed cells (14, 19, 26–27), environmental conditions (14, 28) and the influence of other cells (11, 14, 29) probably play an important role in variant generation. This process would thus drive a homogenous cell population to become a highly heterogeneous one. The large variety of cellular types generated by this process would increase the probability that certain transformed populations will survive various selection pressures.

Selection is the directing factor in tumor progression. A large proportion of the variant variety is probably selected out and the remaining cell populations may be more homogenous compared to the nonselected one with respect to the potential to propagate under these particular conditions.

Previous studies from this laboratory (30–38) were concerned with interactions between the immune system and transformed cells at early stages of the progression process. In order to further understand these interactions we find it necessary to establish the relative contribution of host-derived selective pressures in shaping the phenotype of such transformed cells. Investigation of the role of host factors in directing tumor progression poses some experimental difficulties. At early stages of progression the initial transformed cell population is too small to be detected or to be analyzed by the available experimental methods. On the other hand a sufficiently large tumor cell population, as is the case with human cancer or with most of the experimental animal tumors has already undergone a selection process of unknown magnitude at the time of analysis. In order to circumvent, at least partially, this difficulty we did not use in vivo grown tumors—the phenotype of which is determined both by the intrinsic heterogeneity of the cells and by host mediated selection pressures. Instead, we utilized in vitro transformed cells which have never encountered any in vivo operating selective pressures. We studied some phenotypic characteristics of clonal variants of such in vitro transformed cell populations before and after a single in vivo passage. This approach allows an evaluation of the consequences of selective pressures operating in vivo.

The system used in this study includes cells transformed in vitro by PyV.2 This transformation system was used for two major reasons. The first is that the molecular basis for transformation and tumorigenesis is well established in this system (39–50). The second reason is that immune responses play a highly significant role in controlling the tumorigenesis of PyV-transformed cells (for review see refs. 51–53).

MATERIALS AND METHODS

Mice. BALB/c mice (H-2*) were bred and maintained in the animal quarters of Tel Aviv University.

Cell Lines. BALB/c 3T3 and the 3T3-TSC PyV transformed cell lines were kindly provided by Dr. M. Fried, The Imperial Cancer Research Fund, London. The 3T3-TSC line was cloned from a BALB/c 3T3 cell line which had been transformed by a temperature sensitive mutant of PyV-deficient in late viral genes (54). This deficiency did not affect the expression of early antigens (54).

Cell Culture Medium. Cell cultures were grown in DMEM (Maagar, Beit Haemek, Israel) supplemented with glutamin, antibiotics, and 10% fetal calf serum (Maagar, Beit Haemek, Israel).

Cloning Procedure. The 3T3-TSC cell line was subcloned by means of a limiting dilution procedure as described previously (55). The cells were dispersed at a concentration of 0.25 cells/well into 96 microtiter plates and cultured for 2 weeks.

2 The abbreviations used are: PyV, polyoma virus; dt, doubling time; C-cells, cells maintained in culture; CTC cells, cultured cells that were passaged once as tumors in syngeneic mice and recultured after explantation; DMEM, Dulbecco's modified Eagle's medium.

3 M. Fried, personal communication.
wells containing 10^6 thymocytes per well as a feeder layer. With this procedure less than 20% of the wells yielded colonies. Statistically, there is a 98% chance that each of these colonies originated from a single cell. The wells were examined 4-5 days after subcloning by a binocular microscope and the few wells which contained more than one colony were discarded.

**Measurement of **$d_t$** in Vitro.** Growth rate in the present system was density dependent. At low cell densities the rate was very slow and was accelerated as the densities grew. Close to the confluent state the growth rate slowed down again. The cells were seeded in duplicates in bottles with 25-cm² growth area with 2.5-ml growth medium. 10^5 viable cells were seeded per bottle and were harvested after about 3-5 generations at a density of about 10^6 cells per bottle. The bottles were trypsinized three times and washed twice with 3-ml medium without serum or with sterile phosphate buffered saline after every trypsinization until no cells could be detected on the bottom of the bottles after an extensive examination with a binocular microscope. 10^5 viable cells from the same pool in 2.5-ml growth medium were then seeded into each bottle for another cycle of $d_t$ measurement. The $d_t$ was calculated on the basis of viable counts using a hematocytometer and a count of minimum 200 cells by the formula (56):

$$d_t = \left( \frac{T - \text{lag}}{\ln \left( \frac{N_T}{N_s} \right)} \right) = \left( \frac{T - \text{lag}}{\ln \left( \frac{N_T}{10^5} \right)} \right)$$

where $N_T$ is the average number of cells removed from one bottle, $N_s$ is the number of cells seeded in one bottle (10^5 cells), and $T$ is the time interval (hours) between seeding and harvesting. The lag is the time (hours) from seeding until the cells are flattened and adhere to the bottom and the first round cells of the metaphase can be seen. Usually the lag interval lasted 6-8 h.

Inoculation of Cells to Mice. Logarithmically dividing cells were trypsinized and washed three times in cold DMEM. The appropriate number of cells ($5 \times 10^5 - 5 \times 10^6$ cells/ml) in a volume of 0.2 ml was injected s.c. into the hind leg of 8-week-old syngeneic female BALB/c mice. Viability of the injected cells was 95% or higher. The time of appearance of palpable tumors was recorded.

Survival time of tumor bearers was the interval (days) between tumor appearance and death of the host.

Preparation of CTC Cultures. 3T3-TSC cells or cells which were cloned from this culture were inoculated into syngeneic BALB/c mice. Tumors which appeared at the site of inoculation were dissected, minced to pieces with a diameter of about 0.5 mm, washed three times with 5 ml DMEM through an iron mesh to remove debris, small pieces, and single tumor cells. Only pieces that remained on the net were used for $in vitro$ growth. Two to three pieces were seeded per well in 96 wells tissue culture plates. Cells originating in the tumor pieces migrated out and created a ring of monolayer around each piece. At that time the tumor pieces and the cells close to them were removed mechanically with a pasteur pipet. The remaining cells (forming the rings) were allowed to spread and fill the microtiter wells. Most (if not all) of the cells which remained in the wells were generated $in vitro$ and they were free (or almost free) from cells associated with the tumor. Cells grown continuously $in vitro$ were designated C-cells. Cells after one passage $in vivo$ were designated CTC cells.

Statistical Analysis. The statistical evaluation of differences between tumor appearance curves was done by using the Logrank test (57).

**RESULTS**

Heterogeneity within a Polyoma Virus-Transformed 3T3 Cell Line

We subcloned a 3T3 cell population transformed $in vitro$ with PyV and maintained the new clones in culture. The obtained clones were tested for various phenotypic traits. Clonotypes differing in cell morphology and size, in cell density at confluency, in growth rate $in vitro$ ($d_t$) and in tumorigenicity were obtained. Below we present data on the two latter phenotypic characteristics.

Distribution of and the Change in the $in Vitro$ Doubling Time of Polyoma Virus-transformed 3T3 Cells. Nearly a 1000 clones of PyV-transformed 3T3 cells were obtained. Forty-eight clones were chosen randomly and their doubling time was measured at early stages after cloning, i.e., shortly after the cells filled the wells and reached analyzable quantities. Fig. 1 shows the $d_t$ distribution of these 48 clones. This sample of clones was found to be rather heterogeneous with respect to $d_t$ values. These ranged from 10.5 to 65 h with a standard deviation of 7.5 h. The $d_t$ value distribution showed a high peak close to the fast edge at about 14.0 h and a long tail of slow clones. The average $d_t$ was 14.7 h.

The $d_t$ of the parental PyV-transformed 3T3 line was 14.5 h. This value was very close to the average $d_t$ value and to the peak of the tested sample of clones. It did not change during a period of more than 100 passage generations.

We used the $d_t$ of these clones as an $in vitro$ parameter to examine the phenotypic stability of the clones. $d_t$ measurements of the above mentioned 48 clones were performed at about every third generation for two to three months during which the clones underwent 20-40 generations. The change in $d_t$ of representative clones grown in culture is plotted in Fig. 2. These may be summarized as follows. Slow and medium growing clones demonstrated a decrease in their $d_t$. This occurred...
Differences in the Tumorigenicity among Polyoma Virus-transformed 3T3 Cells. A sample of 18 clones was inoculated into syngeneic BALB/c mice at a cell dose of $10^3$, $10^4$, and $10^5$ cells per mouse. The clones were inoculated as early as possible after cloning and as soon as sufficient quantities of cloned cells were obtained. Tumorigenicity was measured by two criteria: incidence (number of tumor bearers per number of mice inoculated) at certain time intervals following inoculation and the kinetics of tumor appearance. The aggressiveness of tumors was assessed by measuring the length of the survival time of tumor bearers.

The results showed that uniformly the dose of $10^5$ cells was below the tumorigenicity threshold in all the clones for an observation period of at least 250 days. The tumor incidence in mice injected with $10^4$ or $10^5$ cells indicates a heterogeneity among PyV-transformed 3T3 clones with respect to this tumorigenicity parameter.

The tumorigenicity of 11 clones was followed for periods sometimes as long as 2 years after the inoculation of the cloned cells. In addition to tumor incidence the kinetics of tumor appearance was evaluated. This parameter included the average length of the precancer latency period; the length of the period from cell inoculation until the appearance of the first tumor in the inoculated mouse group; the length of the period separating the appearance of the first and the last tumor in the group and the average time interval between the appearance of subsequent tumors. The results presented in Table 1 (an inoculum of $10^5$ cells) show that the different clones were heterogeneous with respect to incidence 200 days following inoculation and with respect to kinetics of tumor appearance. For each clone the tumorigenicity parameters are correlated: high tumor incidence is correlated with relatively short latency periods. Also in the high tumorigenic clones the average interval between subsequent tumors was relatively short compared to low tumorigenic tumors.

The data in this table also indicate that although the inoculated cells were of monoclonal origin, an intraclonal heterogeneity could be observed. The tumors originating from any particular clone, but especially from the relatively low tumorigenic ones, were highly variable with respect to the kinetics of tumor appearance. Some tumors appeared relatively early following cell inoculation while others appeared considerably later. Increasing the inoculum size tended to decrease these differences. These results suggest that independent progression mechanisms may operate with different variants generated from descendants of a single clone. The time required to achieve a certain set of characteristics determining the tumorigenic phenotype is therefore different for different subclonal variants.

The observed heterogeneity in parameters of tumorigenicity of different clones is not due to chance. Fig. 4 shows curves of cumulative tumor incidence from three different inoculated clones in two separate experiments performed 8 months apart. The striking similarity between the kinetics of tumor appearance of a particular clone in both experiments shows that the heterogeneity pattern of these clones is stable for at least this time period.

In contrast to the correlation found between the kinetics of appearance of tumors from a given clone and tumor incidence (i.e., high incidence correlated well with rapid kinetics), no such correlation could be found between the two parameters of tumorigenicity (incidence and kinetics of tumor appearance) and the aggressiveness (survival time of tumor bearers) of a given clone. The survival time of tumor bearing mice, irrespective of bearing a tumor from a high tumorigenic clone or a tumor from a low tumorigenic one were in general surprisingly similar (Table 2). Moreover the length of the survival time of mice bearing tumors which appeared early following the inoculation of transformed cells from a particular clone was similar to that of mice bearing late tumors from the same clone.

Lack of Correlation between Doubling Time in Vitro and Tumorigenicity. One of the mechanisms which could account for differences between high or low tumorigenicity is an intrinsic difference in the proliferation capacity of different clones. We tested this possibility by comparing the $dt$ of various PyV-transformed clones with their tumorigenicity. Since no such correlation could be found we may conclude that at least in this tumor system these phenotypic traits are independent.

Increased Tumorigenicity of Polyoma Virus-transformed 3T3 Cells after a Single in Vivo Passage

If variants best fitted to survive in vivo are indeed selected during tumor progression, it is to be expected that cells which underwent such a postulated selection process would exhibit an increased tumorigenicity compared to cells which were not exposed to such selection pressures. This hypothesis was tested and confirmed by the set of experiments described below.
Differential Tumorigenicity of 3T3 Cells

Table 1 Tumorigenicity Parameters of PyV-Transformed Clones

<table>
<thead>
<tr>
<th>Clone/culture</th>
<th>Tumor incidence after 200 days</th>
<th>Latency perioda to first tumor (days)</th>
<th>Mean latencyb period (days)</th>
<th>Interval betweena first and last tumor (days)</th>
<th>Mean intervalb between successive tumors (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A18</td>
<td>10/10</td>
<td>71</td>
<td>86 ± 11.3</td>
<td>33</td>
<td>3.9</td>
</tr>
<tr>
<td>A10</td>
<td>9/10</td>
<td>62</td>
<td>79.6 ± 10.6</td>
<td>34</td>
<td>4.9</td>
</tr>
<tr>
<td>A8</td>
<td>10/10</td>
<td>60</td>
<td>108 ± 28.1</td>
<td>98</td>
<td>10.1</td>
</tr>
<tr>
<td>A9</td>
<td>10/10</td>
<td>94</td>
<td>120 ± 17.1</td>
<td>53</td>
<td>5.9</td>
</tr>
<tr>
<td>A7</td>
<td>9/10</td>
<td>73</td>
<td>110 ± 24.1</td>
<td>87</td>
<td>10.9</td>
</tr>
<tr>
<td>A12</td>
<td>10/10</td>
<td>89</td>
<td>119 ± 25.5</td>
<td>87</td>
<td>9.7</td>
</tr>
<tr>
<td>A13</td>
<td>9/10</td>
<td>83</td>
<td>119.8 ± 25.6</td>
<td>78</td>
<td>9.8</td>
</tr>
<tr>
<td>A3</td>
<td>4/10</td>
<td>120</td>
<td>&gt;204.0</td>
<td>&gt;350</td>
<td>35.0</td>
</tr>
<tr>
<td>C10</td>
<td>3/8</td>
<td>157</td>
<td>&gt;242.3</td>
<td>&gt;230</td>
<td>38.3</td>
</tr>
<tr>
<td>A6</td>
<td>1/10</td>
<td>154</td>
<td>&gt;274.0</td>
<td>&gt;300</td>
<td>30.0</td>
</tr>
<tr>
<td>A1</td>
<td>1/10</td>
<td>168</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-TSC</td>
<td>10/10</td>
<td>60</td>
<td>85.0 ± 12.8</td>
<td>42</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* The inoculated dose was 10⁶ cells/mouse.

The inoculated dose was 10⁶ cells/mouse.

Results obtained at the end of the observation period (about 450 days).

Since at the termination of the experiment not all mice had tumors, the sign more than was included.

BALB/c 3T3 cells transformed in vitro by PyV (designated C-cultured cells) were inoculated to syngeneic mice. Some of the resulting tumors were explanted and maintained in culture until a sufficient number of cells for reinoculation was obtained. The cells which underwent a single in vivo passage were designated CTC (culture-tumor-culture) cells. Various doses of CTC cells were reinoculated into syngeneic BALB/c mice. Identical numbers of C-cells from the same clone were inoculated in parallel. The tumorigenicity (tumor incidence and kinetics of tumor appearance, see above) and aggressiveness (length of the tumor-bearer period, see above) of C and CTC cells from the same clones were compared. Fig. 5 shows cumulative tumor incidence curves of C and CTC cells from the parental PyV-transformed population and from five separate clones. In all cases CTC cells demonstrated an increased tumorigenicity. This increase was very pronounced when low-tumorigenic clones such as A3 and A6 (Fig. 5) were employed. In all cases the difference between the tumor appearance curves of C and CTC cells were highly significant (P < 0.01).

Tumors arising from CTC cells of a particular clone showed a remarkable similarity to each other with respect to the kinetics of tumor appearance (Table 3). Thus the tumors arising from CTC cells from a particular clone appeared about the same time after inoculation, in contrast to tumors from the homologous C-cells which appeared, in general, and as indicated above at long intervals from each other (Table 3). Moreover, the difference in kinetics of tumor appearance between high and low tumorigenic clones essentially disappeared when CTC cells were inoculated. Thus comparing the growth curves of CTC cells from different clones, a remarkable resemblance in incidence of tumor appearance (invariably fast) was seen (Table 3).

Although tumorigenicity (i.e., incidence and kinetics of ap-
pearance) increased in CTC cells compared to C-cells from the same clone, the aggressiveness of the tumors arising from CTC cells did not increase and was similar to that of tumors from C-cells (Table 4).

Effect of a Second in-Vivo Passage of PyV-transformed CTC Cells on Tumorigenicity

CTC cells from the parental PyV-transformed 3T3 population were inoculated into syngeneic BALB/c mice. The resulting tumors were explanted and again propagated in culture as described above. These cells together with the corresponding C-cells and the original CTC cells were assayed for tumorigenicity. The results presented in Fig. 6 and Table 5 demonstrated that the additional "selection cycle" in vivo had only a marginal effect on tumorigenicity. There were no statistical differences between the cumulative tumor incidence curve of CTC cells and that of twice-transplanted CTC cells. This indicates that the crucial and important selective events took place during the first in vivo residence period of the transformed cells, with little room for additional positive selection traits.

DISCUSSION

The results of this study provide further support for the concept that the progression of (at least certain) tumors is directed by a process of variant generation and by a selection of those variants best fitted to propagate in vivo.

In this study we attempted to avoid a difficulty encountered in other studies dealing with the influences of host-derived pressures on tumor progression. We utilized in vitro transformed cells which, although undoubtedly subject to numerous and various selective pressures related to propagation in culture, have not encountered selective pressures specifically relevant to in vivo growth, i.e., to tumorigenicity. By subjecting such cells to host-derived pressures during a single in vivo passage we are in a position to delineate between two types of cellular characteristics: those which are influenced by the transformation process and by culture-associated selective pressures on the one hand and those which are induced or selected by the host on the other hand.

The polyoma system studied in this work confirms results that were achieved in the past concerning one of the basic rules of progression namely the "independent progression of characters" (58, 59). We could thus find no strict correlation between growth rate in vitro and tumorigenicity in vivo showing the results of certain investigators (59–62), but differing from the results of others (63). Similarly we did not find any correlation between the length of the latency period and the survival time of tumor bearers.

Malignancy-associated characteristics of cultured transformants tend to change as a function of time in culture (64). The two characteristics of cultured PyV-transformed cells examined in this study, namely doubling time in vitro and tumorigenicity in vivo showed different patterns of alterations. Whereas tumorigenicity was stable, at least for a period of several months, doubling time in vitro, especially of those clones which deviated considerably in this respect from the uncloned population, changed rather rapidly. Most clones had, at the end of this process, doubling times similar to those of the initial population. Even then the tumorigenicity pattern of the different clones did not change (data not shown). This again illustrates the independent behavior of malignant characteristics (58–62).

Heterogeneity induction with respect to tumorigenicity in in vitro transformed cells seems to be a relatively common event. Even populations of cells that were recently cloned demonstrated, upon inoculation, a varied tumorigenicity behavior. Thus an analysis of the tumorigenicity curves of various inoculated clones indicates quite clearly that certain cellular members within a (so called) clone are more efficient than others in their ability to form tumors in vivo. In extreme cases when low tumorigenic clones were inoculated into syngeneic mice, an interval of more than a year could occur between the appearance of the first tumor and the last tumor (see Table 1). These drastic differences between the latency periods of different tumors within the same clonal population may be the result of an extremely low frequency within such a population of cellular variants with the full capacity to form tumors. If such a cellular variant is present in a certain cell inoculum, the latency period will depend by and large on the in vivo doubling time of this variant. If the cell inoculum does not contain such a variant, the latency period will depend upon the time required to gen-

Table 4 Tumor latency period and survival time of mice inoculated with C and CTC cells from PyV-transformed 3T3 clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Average latency period (days)</th>
<th>Average survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>A8</td>
<td>116.0 ± 30.8</td>
<td>56.0 ± 12.0</td>
</tr>
<tr>
<td>A9</td>
<td>136.4 ± 52.2</td>
<td>63.0 ± 10.4</td>
</tr>
<tr>
<td>A3</td>
<td>&gt;186.5</td>
<td>44.5 ± 16.5</td>
</tr>
<tr>
<td>A6</td>
<td>&gt;249.0</td>
<td>37.4 ± 6.7</td>
</tr>
</tbody>
</table>

* The inoculated dose was 10⁶ cells/mouse.

Fig. 6. Kinetics of tumor appearance of 3T3-TSC cells kept in culture (C), after one passage in vivo (CTC), or after two passages in vivo (CTC x 2). For details see legend to Figs. 4 and 5.

Table 5 Changes in tumor incidence of 3T3-TSC cells after one or two in vivo passages

<table>
<thead>
<tr>
<th>Cell inoculum per mouse</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>CTC</td>
<td>2 x CTC</td>
<td>C</td>
</tr>
<tr>
<td>10⁶</td>
<td>0/10</td>
<td>0/9</td>
<td>0/10</td>
<td>5/10</td>
</tr>
<tr>
<td>10⁵</td>
<td>0/9</td>
<td>0/9</td>
<td>2/9</td>
<td>8/9</td>
</tr>
</tbody>
</table>

* The values are number of mice with tumors out of the total number of mice inoculated with the indicated number of cells at the indicated days post inoculation.
erate such a variant and on the dt of this variant after it was generated.

Progression towards malignancy is a multistep process (2, 4, 11–12, 14, 18–21, 58). If n progression steps following the initial transformation event are required for a transformed cell to become a tumorigenic variant, it is logical to assume that an inoculum of cells in which the most progressed variant is that with n-2 progression steps will form a tumor more rapidly than an inoculum in which the most progressed variant has undergone n-5 progression steps. This explanation is supported by the very well established fact that by increasing the size of the cell inoculum an increased tumorigenicity (i.e., a more rapid kinetics of tumor appearance and thus a higher incidence of tumors at a given time) is achieved. This explanation obviously does not distract from the importance of the time factor required for the progression from one progression step to another (diversification rate, 14). Obviously this factor could vary from one clone to another.

It is of obvious importance to establish the nature of the progression steps required to render a transformed cell tumorigenic. In the polyoma system some of the steps have been identified. Progression steps essential for transformation involve the expression of two early proteins of PyV, the large and middle T (43, 50). Coexpression of certain oncogenes, such as src (47) or c-yes (49), seems also to be involved in progression with n-2 progression steps will form a tumor more rapidly than of in vivo passaged tumors obtained in the PyV-transformed cells. It is important to emphasize that the relative homogeneity diversified intra and interclonal tumorigenicity patterns of C-CTC cells does not distract from the importance of the time factor required for the progression from one progression step to another (diversification rate, 14). Obviously this factor could vary from one clone to another.

The availability of relatively low tumorigenic C-cells and of highly tumorigenic CTC cells derived from a common clonal ancestor will enable a genetic and phenotypic identification of traits selected against or favored by the in vivo conditions. As an example for such a selection it had been shown by Fried et al. (41) that the in vivo passage of cells transformed in vitro by PyV selects against the expression of free viral genomes and against the expression of the large T antigen.

Whatever the in vivo environment induced or selected against, it seems that it limited diversification at least with respect to tumorigenicity and at least in the PyV-transformation system. This is evident from the comparison of tumorigenicity parameters between the various CTC clones. These cells, all of which have undergone a single in vivo passage as tumors, have acquired a similar tumorigenicity pattern, both within the clone and interclonally. This is a strikingly opposing situation to the diversified intra and interclonal tumorigenicity patterns of C-cells. It is important to emphasize that the relative homogeneity of in vivo passaged tumors obtained in the PyV-transformed cells can be related, so far, only to the tumorigenicity phenotype. Other properties connected with the structural or functional behavior of the CTC cells have not been compared as yet to those of C-cells. It is quite possible that in addition to the documented effects of the microenvironment of the tumor in the generation of diversification (14, 29), microenvironmental factors, such as other cells may limit diversification in certain cases (29, 65).

While tumorigenicity of CTC cells is drastically increased (compared to C-cells), the aggressiveness of the CTC cells (measured by the survival time of the tumor-bearing mice and based on the assumption that increased aggressiveness of tumor cells is expressed by their ability to rapidly kill their host) is not selected for in vivo. These results are comparable to those obtained from the comparison between the aggressiveness of high and low tumorigenic C-cells. All these results indicate that high or low tumorigenicity is dependent on the ability to overcome certain in vivo existing barriers. Once this is achieved, the selection for accelerated growth capabilities or malignancy is of relatively small magnitude.

The mechanism(s) responsible for the increased tumorigenicity of the in vivo passaged CTC cells is (are) unknown. However, in view of the high immunogenicity of PyV cells (52) and the evidence showing that immune surveillance mechanisms are heavily involved in controlling the development of PyV-induced tumors (51–53), one may assume that immunoselection is a dominant factor in increasing the tumorigenicity of such cells. It is not unlikely that highly tumorigenic CTC cells may lose the PyV-specific tumor specific transplantation antigen or express altered amounts of viral proteins which determine tumorigenesis. Other mechanisms may involve a decreased susceptibility to lysis mediated by killer cells of various types (66). Initial results of Northern blot analyses reveal however no differences in PyV mRNA expression between PyV-transformed cells that were or were not subjected to in vivo selection. These preliminary results are in accordance with those of Van Roy et al. (67).

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REFERENCES
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