Two Distinct Types of SV40-transformed Human Amnion Cells

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SUMMARY

Human epithelial amnion cells infected in primary culture with simian virus 40 transform to give rise to two morphologically different cells (T and R). R cell foci appear later, after infection, and the proportion of R to T cell foci increases when older amnion cultures are infected. T cells may be easily subcultured by routine trypsinization methods. The more slowly dividing R cells are very resistant to treatment with enzymes and chelating agents, and require special subcultivation procedures. Cultures of both transformed cell types produce SV40; R cell cultures show virus only in the supernatant fraction. Chromosome numbers of R cells are near tetraploid in the early stages of cultivation. However, T cells remain in the diploid range for longer periods. R cells do not enter a typical "crisis" stage. They produce more growth than T cells in the cheek pouch of weanling hamsters conditioned with cortisone. It is proposed that the amount of R cell transformation is related to the formation of a population in older uninfected primary cultures with an abnormal genetic constitution.

INTRODUCTION

Observations on the transformation of human epithelial amnion cells by simian virus 40 (SV40) have been reported frequently by this laboratory (6-8, 10-12, 14, 19, 20). A comprehensive review of this transformation and the subsequent growth of the transformed amnion cells has been published (15). Previous reports have mainly dealt with a SV40-transformed cell type, now referred to as the T cell, which divides frequently and is easily subcultured until "crisis" occurs about 200 days after infection. A 2nd type of transformed epithelial cell, designated R cell, has now been recovered from SV40-infected primary amnion cultures (16). This paper compares the 2 cell types in growth and morphology. In addition, data are presented on virus production, chromosome changes, and the tumor-producing capacity of these cells.

MATERIALS AND METHODS

SV40. The SV40 strain VA 45-54 GMK 4, obtained by courtesy of M. R. Hilleman, the Merck Institute for Therapeutic Research, Rahway, N. J., was passed in cultures of human foreskin cells and from 2 to 4 times in Hopps' BS-1 line of African green monkey cells (21) in preparation for the virus inocula used in the present experiments. For a few of the experiments the virus was passed 2 to 4 times in CV-1 cells as well (22).

Cells. Primary cultures of human amnion cells were prepared by trypsinization of amniotic membranes obtained from local hospitals. Cultures were established in Medium 512 (15), supplemented with 15% fetal bovine serum; penicillin, 100 units/ml; and streptomycin, 100 \( \mu g/ml \). After 10 to 36 days, confluent monolayers were infected with SV40 at a multiplicity of exposure of 0.3 to 3000 I.D.\(_{50} \)/cell in McCoy's Medium 5a (24) with 10% agamma newborn calf serum. This was also the medium during subsequent serial cultivation.

Viruses. Virus infectivity titers were based upon the I.D.\(_{50} \)/ml as calculated by the method of Reed and Muench (30). Tube cultures of CV-1 cells maintained on McCoy's Medium 5a with 10% agamma calf serum were each inoculated with 1.0 ml of serial 10-fold dilutions of the virus sample in the same medium with 2% agamma calf serum. Cytopathic effects were recorded for a minimum of 28 days.

Infectious Center Assay. SV40-infected cultures were trypsinized and washed 3 times with fresh medium and the cells were counted. One-ml samples of serial 10-fold dilutions were introduced by inoculation onto monolayers of CV-1 cells in 3-oz (approximately 90-ml) prescription bottles. After 6 hr the fluid was removed and the cultures were overlaid with a mixture of 4 parts 3% special Noble agar (Difco Laboratories, Inc., Detroit, Mich.), 4 parts 2X LY medium (13), and 1 part McCoy's Medium 5a supplemented with 20% agamma newborn calf serum. The highest number of plaques counted at 7, 10, or 14 days was used to express the number of virus-producing cells. Samples of the supernatant fraction from cell dilution tubes were routinely included as controls for extracellular virus.

Chromosome Analysis. The SV40-transformed cells were prepared for chromosome studies by incubation for 2 hr in 0.0005% colchicine by a method based on the suspension technique with ignition of the fixative (25).

Tumor Production. Golden Syrian hamsters were ob-
tained as weanlings and inoculated in the right cheek pouch with 0.4 ml cell suspension. These hamsters received 5 mg cortisol acetate s.c. the day before and the day after the cell inoculation. Terramycin was added to the drinking water of all hamsters.

**Immunofluorescence Technique.** The presence of T antigen in SV40-transformed cells was determined by the indirect technique (29). Acetone-fixed cultures were incubated with hamster SV40 tumor antiserum (Flow Laboratories, Rockville, Md), washed with phosphate-buffered saline, and reincubated with anti-hamster globulin conjugated with fluorescein isothiocyanate (by courtesy of Dr. R. Holdenried, National Cancer Institute, Bethesda, Md.). The conjugated anti-hamster globulin was adsorbed twice with mouse liver powder and once with human liver powder. The details of this indirect immunofluorescence technique have been reported previously (28).

**RESULTS**

**Transformation and Growth.** Primary cultures of human epithelial amnion cells infected with SV40 for a number of weeks contained 2 morphologically distinct types of foci. The 2 types, referred to as R foci and T foci, were easily observed in hematoxylin and eosin-stained cultures, as illustrated in Figs. 1 and 2. Foci of T cells could be distinguished in stained preparations 17 days after infection with SV40 at a multiplicity of exposure of 3000 I.D.50/cell; however, foci of R cells were not observed until 28 days after infection. The multiple exposure in most instances. T cell foci predominated when 10 or 34 days old with 3 multiplicities and stained was determined from primary amnion cultures infected previously (28).

**Table 1**

<table>
<thead>
<tr>
<th>Amnion No.</th>
<th>Multiplicity of</th>
<th>Number of discrete R and T cell foci from 2 cultures 5 and 6 weeks after SV40 infection: three multiplicities of exposure of 10- and 34-day-old primary amnion cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>256</td>
<td>I.D.50/cell</td>
<td>35 days</td>
</tr>
<tr>
<td>3000</td>
<td></td>
<td>post-infection</td>
</tr>
<tr>
<td>R</td>
<td>T</td>
<td>R</td>
</tr>
<tr>
<td>30</td>
<td>9</td>
<td>55</td>
</tr>
<tr>
<td>0.3</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

*Age of culture on day of infection (4.7 X 10⁶ cells/culture).*

R and T cells were recovered from SV40-transformed primary amnion cultures by trypsinization. T cells were isolated as single cells by treating primary cultures for as little as 5 min with 0.25% trypsin at pH 7.0. R cells, which could not be isolated as single cells, were removed as clumps from the same primary cultures by subsequent trypsinization at pH 8.0 for 15 to 30 min. Thus, subcultures of both cell types could be established from the same transformed primary culture and studied simultaneously.

**Subculture and Morphology.** T cells in culture passage were easily trypsinized as single cell suspensions and transferred with weekly dilution varying between 1:2 and 1:10. The cultures reached cell densities between 5 X 10⁶ and 2 x 10⁷ cells (per T-30 culture flask containing 5 ml medium). Crisis occurred about 200 days (average of 10 experiments) after SV40 infection of 10-day-old primary amnion cell cultures. T cell cultures could not be maintained without frequent passage, because dense cell sheets which formed by the 3rd or 4th week rolled away from the glass.

During the first few passages following transformation, T cells maintained a homogeneous morphology and contained large nuclei, with many dense nucleoli (15). As the age of the cell strain increased there was an increased variability in the shape and size of the nucleus. A few passages before crisis, the cell growth appeared less organized, nuclei became very large and irregular, and there were frequent tri- and tetrapolar mitoses.

R cell cultures could be transferred weekly at a 1:2 dilution by trypsinization at high pH for long periods. Weekly transfer, however, placed great stress on their growth capabilities, so that fewer cells were recovered with each subsequent passage. These frequent subcultures resulted in the formation of a large percentage of giant cells (Figs. 3 and 4), and such a culture could not be transferred beyond the 13th passage. Five strains of R cells originating from 4 different amniotic membranes have been maintained with intervals of more than 4 weeks between passages and are over 500 days old. Only very dense cultures, containing more than 1 x 10⁶ cells/ml culture fluid, were passed. The R cell strains obtained from different amnions had similar characteristics.

R cells have a distinct epithelial-like morphology and contain large round or oval nuclei with several nucleoli (Fig. 3). Cell size depended on the density of the culture. Those cells which survived enzyme treatment formed discrete colonies of epithelial cells within a few days of subculture. As the mass of the colonies became larger, the more centrally located cells became smaller. In spite of the high cell density at 3 weeks, the cells still formed only a monolayer. After further growth, this membrane-like cell layer appeared to form folds to compensate for the increased number of cells. Even at 6 weeks following subculture, no nuclear overlapping could be seen.
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The characteristic of R cells to maintain a great cell to cell adhesion has made it difficult to obtain single cell suspensions for cloning, growth studies, and chromosome analyses. Attempts to separate more single cells from the cultures with EDTA, Pronase, elastase, collagenase, and a combination of EDTA and trypsin have failed.

**Virus Production.** All strains of both T and R cells continued to produce SV40 after transformation and during subsequent culture passages. This was also true for primary cultures of R cells from which the T cells had been removed by a short period of trypsinization. The virus production picture for R and T cell cultures in serial passage is illustrated by the data in Chart 1. In this experiment T and R cells were isolated as individual strains from the same primary culture 83 days after SV40 infection with a multiplicity of 300 I.D.50/cell. R cells and 1 strain of T cells (A) were subcultured with a weekly dilution of 1:2. A 2nd T cell strain (B) from the same primary culture was transferred each week by inoculating a constant number of cells (2 x 10^5)/ml at each transfer (cell seeding constant). The supernatant virus titers were determined at each culture passage and were expressed as I.D.50/10^6 cells.

T cell cultures produced virus during many passages at titers between 10^5 and 10^6. As crisis approached, the titers increased for both strains (12). The B strain of T cells in crisis yielded more than 1 x 10^8 cells/ml showed titers between 10^1.5 and 10^4 I.D.50/ml.

**Chromosome Analysis.** A detailed account of the chromosome abnormalities observed for T cells at different cultivation stages has been reported previously (15). During many culture passages after transformation the distribution of chromosome numbers was in the near diploid range. In general, abnormalities were frequent. Chromosome abnormalities, as well as chromosome number, increased with increasing passage.

In view of the difficulties in making suitable chromosome preparations of R cells, only a few preparations have been available for analysis. In 1 experiment the chromosomes of R cells could be counted and analyzed at 2 different passage levels. T cells, originating from the same amniotic membrane, were analyzed after the same period of cultivation as the later R cell preparation (Passage 8, 147 days postinfection). The number of chromosomes for one-half the cells examined were still in the diploid range. R cells contained a great majority of cells with high chromosome numbers (90 to 110) as early as 114 days after infection in the 4th culture passage and at 147 days after infection in Passage 8 (Chart 2). The chromosome abnormalities observed for T cells were also seen in R cell preparations. There was a high number of metaphase plates containing 1 dicentric chromosome or...
more. Minute chromosomes and chromosome breaks were frequently observed. A large telocentric chromosome, observed in T cell preparations at various frequencies in different culture passages, has not been observed in the presently examined R cell preparations.

**Tumor Production.** Uninfected primary amnion cells have never produced any tumor growth with the present technique, and previous attempts to study the tumor-producing capacity of T cells gave very limited results (15). For example, 3 to 7 million T cells from various culture passages were inoculated into the cheek pouches of weanling hamsters conditioned with cortisone. Large tumors were never observed, but approximately one-half of the animals showed traces of tumor growth within the first 2 weeks after inoculation. Our attempts to produce R cell tumors with the same technique have been more encouraging. Inocula of 2 to 5 million cells/animal from either of 2 R cell strains resulted in definite cell growth. Although some giant cells and cells with bizarre nuclear morphology were seen in most tumors, the majority of cells were epithelial and contained large nuclei with little cytoplasm (Fig. 6). Mitosis was not uncommon, even 14 days postinoculation. However, there was apparently no invasion of adjacent tissues (Fig. 5). All tumors regressed by the 3rd week, leaving a necrotic area in the pouch. The lack of invasive growth in the pouch tissue with transformed amnion cells correlates with the results of other SV40-transformed human cell types (23).

**T Antigen.** In both R and T cultures 100% of the cells contained T antigen, as determined by fluorescent antibody staining.

**DISCUSSION**

Although foci of both T and R cells were observed in SV40-infected primary cultures of amnion cells in earlier investigations, it recently became apparent that R cells were not represented in the serially cultured strains previously studied. It was also a new observation that R cells were present in high quantities only when old primary cultures were exposed to SV40. Thus, T cells, which were more abundant in infected younger primary cultures and responded more favorably to subculture by routine procedures, have been the main object of research for several years.

It has been a general observation that SV40-transformed human cell strains enter a crisis period a number of months after transformation. Crisis has been demonstrated for both transformed fibroblasts (17, 18) and transformed amnion cells (12, 15). Strains of R cells, if subcultured a number of times, and especially when passage is attempted at frequent intervals, also reach a limiting stage of cell growth characterized by abnormal cell division, cell death, and lack of increase in the population. However, R cell strains which are not subcultured or are passed only a few times at less frequent intervals continue to divide and show a slow increase in cell number. Primary cultures and cultures which have been passed infrequently are still viable more than 500 days after transformation. Chang and Sinskey (1), in their recent report on the SV40 transformation of amnion cells, have made the similar observation that confluent monolayers of multiplying cells, presumably R cells, could be maintained for at least 14 months in cultures not subjected to serial subcultivation. The difference between T cells, which always enter crisis, and R cells, which can be prevented from entering a similar crisis period, appears to be correlated with the pattern of growth in the culture. R cells demonstrate a great cell to cell adhesion and move as an intact membrane-like growth, which at high cell densities folds away from the glass surface. It may also be true that the high chromosome numbers observed for R strains early after transformation and in T cell strains recovered from crisis (Gaffney et al., in press) are an expression of survival potential.

R cells do not represent spontaneously altered amnion cells subsequently infected with SV40. They have been present in every SV40-infected primary culture of human amnion cells, but no similar cell type has ever been seen in uninfected cultures. T antigen was found in all R cell nuclei, whereas, cultures of spontaneously transformed amnion cells (FL line) infected with SV40 were negative for T antigen and virus production a few passages after infection (unpublished results). In addition, many years of experiments with cells from hundreds of amniotic membranes in this laboratory have proven that spontaneous alteration is an extremely rare occurrence. The growth behavior of R cells, for example, their slow rate of growth, and their stability in culture for extended periods is distinctly different from that of spontaneously transformed amnion cell lines.

Other reports on the growth of 2 morphologically distinct transformed cells following simian virus 40 infection stem from studies in cultures originally containing mixed cell types (2–5). We have no evidence that there are 2 epithelial cell types in the amnion.

Some preliminary observations have shown that chromosome changes do occur in uninfected amnion cells after longer periods of cultivation. Uninfected cells during the first 2 weeks of culture divide frequently so that, in most cases, a confluent monolayer may be seen between the 7th and 10th days. During this period the cells contain exact diploid or tetraploid chromosome numbers (27). The mitotic index then decreases and the cells may be maintained as a monolayer for extended periods. Minor chromosome abnormalities and changes in the distribution of chromosomes within the different chromosome groups could be distinguished in cells cultured for more than 1 month (unpublished results). Studies of the chromosome distributions in T cells have shown that during many passages before crisis the distribution of numbers fell in the near diploid range (15). R cells, however, contain an abnormal number of chromosomes in the first few culture passages. Also, the amount of transformation...
which results in R and T cells, represented by the number of foci, was related to the age of the amnion at the time of infection (Table 1). One attractive hypothesis on the origin of 2 transformed cell types may be that the amount of transformation which results in R cell growth is related to the formation of a population in older primary cultures with an abnormal genetic constitution. This hypothesis may also have a bearing on the more pronounced growth of R cells as tumors in the cheek pouch of weanling hamsters treated with cortisone as compared to other SV40-transformed human cells (15, 26). The amount of growth of R cells has not been comparable to that observed for "spontaneously" transformed human amnion cells, for example those of the FL line (9).

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REFERENCES


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Fig. 1. Focus of SV40-transformed R cells in primary human amnion culture; 34 days after infection with 300 I.D.50/cell. × 113.
Fig. 2. Focus of SV40-transformed T cells in primary amnion culture; 28 days after infection with 300 I.D.50/cell. × 103.
Fig. 3. Higher magnification of R cell subculture illustrating the uniform appearance of cells maintained with intervals of more than 4 weeks between passages. × 780.
Fig. 4. High incidence of giant cells in R cell culture passed at frequent intervals. × 118.
Fig. 5. Section of cheek pouch of weanling hamster treated with cortisone, 6 days after inoculation of 5 × 10⁴ R cells. × 120.
Fig. 6. Higher magnification of hamster cheek pouch 6 days after inoculation of R cells. × 470.
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