# SV40 Transformation of Human Amnion Cells Under Different Culture Conditions[1] (34753)

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The general events following SV40 infection of human epithelial amnion cells *in vitro*, including cytopathogenicity and transformation, have been previously described (1, 2). Two distinct types of transformed cell foci appear in the infected primary amnion cultures. Most of our experiments have dealt with cells from one of the two types of foci (T cells). T cell foci are more frequent after infection of young cultures. The cells derived from T cell foci divide faster, can be cultured by routine methods, and require frequent culture transfers. However, after a number of transfers they always reach a stage at which the population does not increase and morphological changes are observed ("crisis"). In contrast, R cells require special subculture procedures (trypsinization at high pH); they are difficult to remove from the glass surface and show a strong cell to cell adhesion which affect their pattern of growth. R cell foci occur most frequently after infection of older cultures, and when not subcultured frequently, these more stable cells do not enter a "crisis" stage. The characteristics of R cells have been reported separately (3). The present paper reports on certain quantitative aspects of the transformation in this epithelial system and the subsequent growth in subculture of the SV40 transformed T cells under varied conditions of multiplicity of infection, culture age at the time of infection, culture medium, and transfer schedule.

*Materials and Methods. Virus.* The SV40 strain VA 45-54 GMK 4, used in all experiments, was passed in several lines of African green monkey kidney cells. The details of the methods for determining the infectious unit, $TCID_{50}$/ml in African green monkey kidney cells, have been described (2).

*Cells.* Cultures of human amnion cells were prepared by trypsinization of human amniotic membranes from placentas at term. The primary cultures were grown in medium 512 (2) containing 15% fetal bovine serum; penicillin, 100 units/ml; and streptomycin, 100 μg/ml. Immediately before infection the monolayer cultures were washed three times with saline A (4); subsequent to infection and during all following subcultivation the culture medium routinely was McCoy's medium 5a (5) with 10% agamma newborn calf serum. In most experiments the SV40 infected amnion cultures were transferred 6 weeks after infection; subsequent culture transfers were carried out at weekly intervals. The number of cells available for transfer was determined. A dilution factor was applied so that the number of cells seeded in the new transfer culture was constant, $2 \times 10^5$ cells corresponding to 1 ml of medium (CSK method). In the following data, the time of "crisis" is indicated to begin when no increase in cell population had been observed during 2 weeks following that culture transfer at which the cell number recovered was less than or equal to the cell number seeded, and morphological changes, including increases in nuclear and cell sizes, odd nuclear and cell shapes, and frequency of abnormal mitoses were observed.

The two media, McCoy's medium 5a and medium 512 with either fetal bovine or agamma calf serum, were compared for their ability to promote the SV40 transformation.

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Medium 512 is a qualitatively complex medium, mainly due to its content of NCTC-109 (6). Although McCoy's medium 5a is less complex, it contains a greater concentration of most of the essential amino acids and some of the vitamins.

*T antigen.* The presence of T antigen in SV40 transformed cells was determined by the indirect immunofluorescence technique (7). Acetone fixed cultures were incubated with hamster SV40 tumor antiserum (Flow Laboratories, Rockville, Md.), washed with phosphate-buffered saline, and reincubated with adsorbed antihamster globulin conjugated with fluorescein isothiocyanate (by courtesy of Dr. R. Holdenried, National Cancer Institute). The details of this indirect technique have been previously reported (8).

*Results.* With increasing multiplicity of SV40 exposure (M. of Exp.) of primary cultures of amnion cells, transformed cell foci, as expected, appeared earlier. Their number and size also increased, as did the percentage of cells containing T antigen, at similar times after infection. These parameters were affected by culture age and by the type of medium used.

*Time of focus appearance.* Table I shows the earliest times at which foci of transformed cells were observed in hematoxylin and eosin stained cultures derived from 5 different amnions. Slides were stained at various times after infection and the presence of foci scored by a + or —. The variables in these experiments included: four different multiplicities of exposure (3,000 to 0.3 $ID_{50}$/cell); two media, McCoy's and medium 512, with either fetal bovine or agamma calf serum supplement; and a difference in the age of the amnion culture at the time of infection (10, 20, and 36 days). At a multiplicity of exposure of 3000 in McCoy's medium with agamma calf serum, the earliest appearing discrete transformed foci were observed 12 days after infection of 36-day-old amnion cultures, and 15 days after infection of 10-day-old cultures. Under similar conditions but at a multiplicity of exposure of 0.3, the first observation of foci was made twenty days after infection of 36-day-old cultures and 7 weeks after infection of 10-day-old cultures. These data indicate that increasing culture age at the time of infection and increasing multiplicity of exposure reduced the time of earliest focus appearance. The time of earliest focus appearance also differed in the two culture media. For example, the ap-

TABLE I. Time Postinfection of First Observed Transformed Foci in 5 Experiments (A183, A243, A244, A255, A261) Including 4 Multiplicites of Virus Exposure (M. of Exp.).

Variation of day of infection (10, 20, 36 days); two media: McCoy's and Medium 512 with fetal bovine or agamma calf serum. (+), foci observed; (—) no foci observed.

| M. of Exp. ($ID_{50}$/cell) | Exp. no. | Day of infection | Medium | Days | | | | | | | Weeks | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 10 | 12 | 14 | 15 | 17 | 19 | 20 | 4 | 5 | 6 | 7 |
| 3000 | A261 | 10 | McCoy's aγ calf s. | — | — | — | + | + | | | | | | |
| | | 20 | McCoy's aγ calf s. | — | — | + | + | | | | | | | |
| | | 36 | McCoy's aγ calf s. | — | + | + | | | | | | | | |
| 300 | A183 | 10 | McCoy's aγ calf s. | | | — | | + | | | | | | |
| | A243 | 10 | 512 aγ calf s. | | — | | | | — | | — | — | + | |
| | | 10 | 512 fet. bov. s. | | — | | | | — | | — | — | + | |
| 10 | A255 | 10 | 512 fet. bov. s. | | | | — | | | | — | — | | + |
| | | 10 | 512 aγ calf s. | | | | — | | | | — | — | | + |
| | | 10 | McCoy's aγ calf s. | | | | — | | | | + | | | |
| | | 10 | McCoy's fet. bov. s. | | | | — | | | | + | | | |
| 0.3 | A244 | 10 | McCoy's aγ calf s. | | | | | | | — | | — | — | + |
| | | 20 | McCoy's aγ calf s. | | | | | | | — | | — | + | |
| | | 36 | McCoy's aγ calf s. | | | | | | | + | | | | |

pearance of transformed foci was delayed in medium 512 with either serum, as compared to McCoy's medium with either serum. Thus, in the latter medium there was focus formation during the third and fourth weeks at multiplicities of 300 and 10, respectively, when no foci were observed during the fifth week in medium 512. The data shown for multiplicities of 3000 and 0.3 $ID_{50}$/cell were confirmed with cells from different amnions,

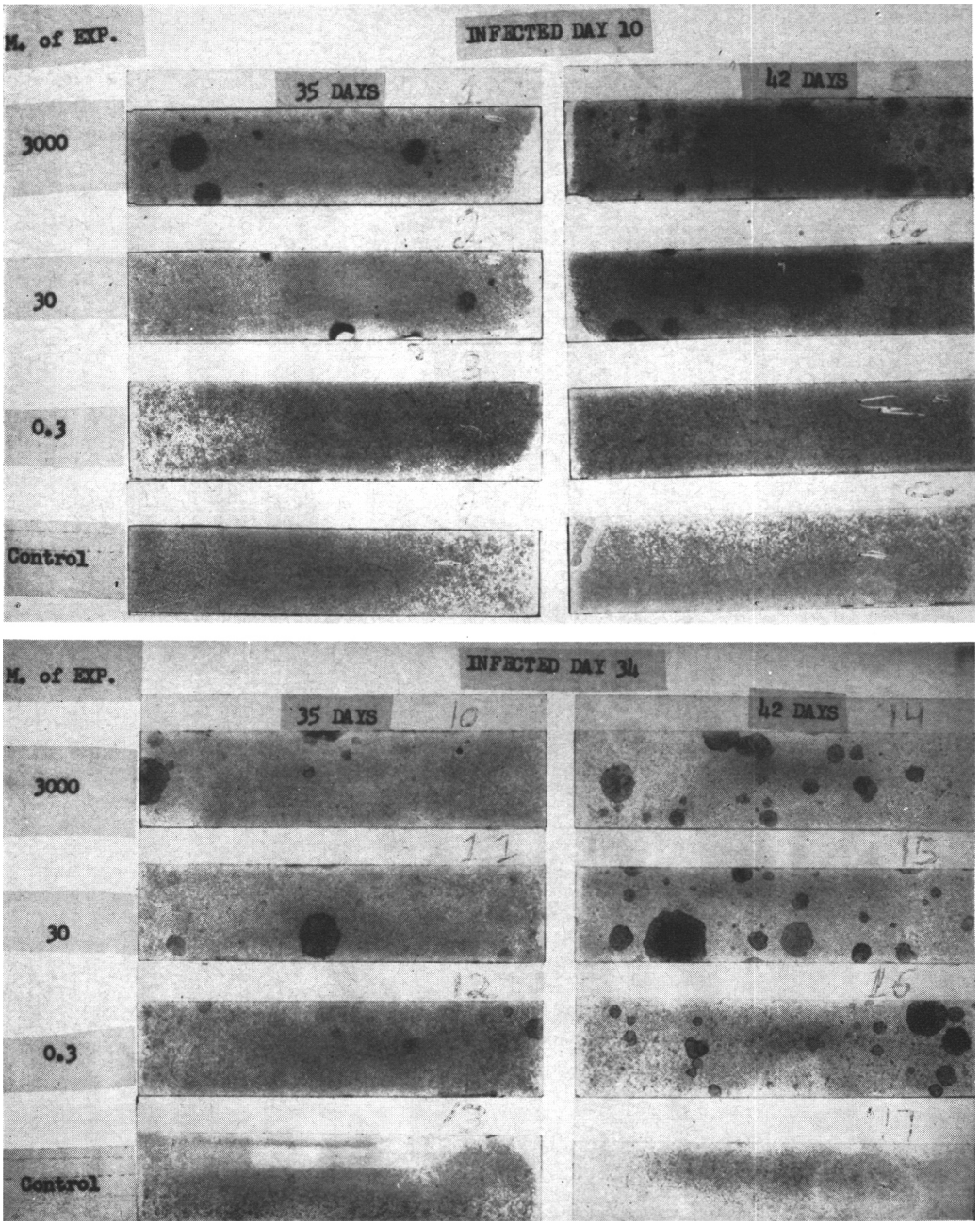


FIG. 1. Foci of SV40 transformed amnion cells in primary cultures infected at days 10 or 34 of culture with different virus multiplicities (M. of Exp.). Stained with hematoxylin and eosin 35 or 42 days after infection.

indicating that the variation in the time of earliest appearance of foci is minor.

*Focus number and size.* Primary cultures derived from the same amniotic membrane were infected when 10 or 34 days old with three multiplicities of exposure (0.3, 30, or 3000 $ID_{50}$/cell). Figure 1 is a composite photograph of one culture of each, fixed and stained 35 or 42 days after infection. The number of discrete foci of transformed cells are indicated in Table II. When observed 5 weeks after the infection of 10 or 34-day old cultures, they increased with increasing multiplicity of exposure. When the cultures infected at 10 days were observed 6 weeks after infection, the number of foci had increased from 13 at a multiplicity of 0.3 to 43 at a multiplicity of 3000 (total of two cultures). However, cultures infected when 34 days old and observed 6 weeks after infection showed a similar number of foci (44, 52, 46 foci per two cultures) at all three multiplicities, apparently because a maximum number of foci had been formed. The apparent decrease in number of foci at 42 days after infection of 10-day cultures with a multiplicity of 3000 is explained by the detachment of some T cell foci, not included in the count.

Focus size increased with the period of time after infection and with increasing multiplicity of exposure. For similar periods of infection, the foci were larger in cultures infected when 34 days old than in those infected when 10 days old.

The colonies of transformed cell growth shown in Fig. 1 represent foci of both T and R cells. It has been reported (3) that the proportion of R to T cell foci increased when older amnion cultures were infected. In all the cultures infected at day 10, more T cell than R cell foci were observed. This was also the case when they were observed as late as 76 days after infection. There were more R cell foci, both 5 and 6 weeks after infection of 34-day-old primary cultures.

TABLE II. Number of Discrete Cell Foci from Two Cultures 5 and 6 Weeks After SV40 Infection of 10- and 34-Day-Old Primary Amnion Cultures.

| A256 M. of Exp. ($ID_{50}$/cell) | Postinf. (days): | 10 Day[a] 35 | 10 Day[a] 42 | 34 Day[a] 35 | 34 Day[a] 42 |
|---|---|---|---|---|---|
| 3000 | | 64 | 43 | 33 | 44 |
| 30 | | 17 | 17 | 21 | 52 |
| 0.3 | | 6 | 13 | 9 | 46 |

[a] Age of culture on day of infection ($4.7 \times 10^5$ cells/culture).

TABLE III. Percentage Amnion Cells Containing T Antigen After SV40 Infection at Four Multiplicities of Exposure.

Medium: McCoy's with 10% agamma calf serum.

| Postinfection (day) | M. of Exp. ($ID_{50}$/cell) 3000 | 300 | 30 | 0.3 |
|---|---|---|---|---|
| 3 | 5.3 | 0.3 | 0.7 | 0 |
| 7 | 7.3 | 4.8 | 0.3 | 0 |
| 14 | 13.7 | 17.9 | 1.6 | 0 |

*Early T antigen.* The percentage of cells containing T antigen early after infection increased with the multiplicity of exposure. As shown in Table III the number of positive cells increased from 0 cells per thousand at 14 days after infection with a multiplicity of 0.3 to approximately 15% at the same time after multiplicities of 300 or 3000. Other experiments have shown that T antigen positive cells appeared earlier after infection in McCoy's medium than in medium 512 with either serum. Under comparable conditions, the proportion of cells with T antigen varied for cells from different amnions. The data in Table II are the highest obtained.

*Growth in subcultures.* The number of transformed T cells available for weekly transfer can be expressed by the transfer dilution factors. In one experiment six transformed strains, originating from one amniotic membrane, were cultured in McCoy's medium with 10% agamma calf serum. The multiplicity of exposure was 3000 for three strains; 0.3 for the other three strains. First culture passage was at day 38 after infection. The number of cells available for transfer in the low multiplicity strains permitted less weekly dilution than in the three strains exposed to the high virus multiplicity, and increasing dilution factors were delayed. The three strains exposed to the high multiplicity

TABLE IV. Variation of Multiplicity of Exposure (3000 or 0.3 $ID_{50}$/cell) and Day of Infection (10, 20, or 36 days of culture).

Comparison of passage numbers, time of crisis, and number of divisions for 6 SV40 transformed strains from one amnion (A244).

| A244 | M. of Exp. ($ID_{50}$/cell) | Day of infection | No. of passages | Crisis (day postinf.) | No. of divisions[a] |
|---|---|---|---|---|---|
| Med.: McCoy, aγ calf s. | 3000 | 10 | 21 | 190 | 43 |
| Tr. dil.: CSK[b] | | 20 | 20 | 190 | 39 |
| | | 36 | 21 | 190 | 37 |
| Av | | | 20.7 | 190 | 39.7 |
| | 0.3 | 10 | 19 | 180 | 34 |
| | | 20 | 14 | 170 | 25 |
| | | 36 | 19 | 190 | 32 |
| Av | | | 17.3 | 180 | 30.3 |

[a] Based upon transfer dilutions.

[b] Cell seed constant: $2 \times 10^5$/ml.

underwent 40 population doublings (av based upon transfer dilutions) before "crisis" occurred. The three strains exposed to the low multiplicity underwent only 30 doublings (Table IV). The range of population doublings for all our experiments in McCoy's medium with agamma calf serum has been from 25 to 49 in the range of multiplicities of exposure from 3000 to 0.3 $ID_{50}$/cell. In the present experiment, one strain for each multiplicity of virus exposure was infected at either 10, 20, or 36 days after seeding of the primary cultures. No relationship between this variation and the number of transformed cells, as expressed by the dilution factors or population doublings, could be established. The number of culture passages before "crisis" was 21, 20, and 21 (av, 20.7) for the three high multiplicity strains as compared to 19, 14, and 19 (av, 17.3) for the three strains exposed to the low virus multiplicity (range for all comparable experiments: 14 to 27 passages). "Crisis" occurred 10 days later in the three high multiplicity strains.

In another experiment, one strain of transformed cells was maintained at a constant split ratio of 1:2 at each culture passage, whereas a comparable strain was passed according to the CSK schedule. The first culture passage was not until 83 days after infection. The first strain underwent 34 population doublings. This value was determined from the weekly increase in cell number per culture, and by converting these values into the total cell yield potential. By the same calculation method, the second strain had a potential of 38 population doublings. The first strain showed "crisis" after 220 days and 17 passages; the second strain was in "crisis" after 180 days and 13 passages (Table V).

*Virus production.* Independent of a varia-

TABLE V. Variation of Transfer Dilution (CSK or 1:2).

Comparison of passage numbers, time of crisis, and number of divisions for two SV40 transformed strains from one amnion (A248).

| A248 | Transfer dilution | No. of passages | Crisis (day postinf.) | No. of divisions[a] |
|---|---|---|---|---|
| M. of Exp.: 2500 $ID_{50}$/cell | CSK[b] | 13 | 180 | 38 |
| Med.: McCoy, aγ calf s. | 1:2 | 17 | 220 | 34 |

[a] Based on cell increase per passage.

[b] Cell seed constant: $2 \times 10^5$/ml.

tion of the original multiplicity of virus exposure from 3000 to 0.3 $ID_{50}$/cell, virus production, expressed as log $ID_{50}$/ml culture supernatant, established rapidly (within 3 weeks) at a level between 6.5 and 7.5 (Fig. 2). This level was apparently also unrelated to a variation of culture age (from 10 to 36 days), at the time of infection. After the first culture passage, SV40 was also produced at all cultivation stages prior to and including the stage of "crisis" (2). In all experiments in McCoy's medium supplemented with agamma calf serum, the initial high virus production period was followed by a period of several months of low virus yields. An increase (up to 4 logs/ml) to virus titers equivalent to those observed in the initial lytic stage has been consistently observed in the passage prior to "crisis" (9). This pattern was independent of the multiplicity of exposure and of the age of the culture at the time of infection.

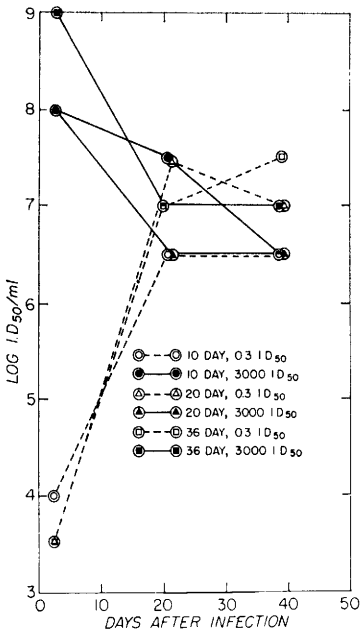


FIG. 2. SV40 production in primary cultures of amnion cells infected with SV40. Multiplicity of virus exposure: 3000 or 0.3 $ID_{50}$; culture age at time of infection: 10, 20, or 36 days.

*Discussion.* Previously described methods for SV40 transformation assays (10–12) are not directly applicable to primary amnion cultures which are difficult to transfer quantitatively. Attempts to determine the amnion transformation frequency by the agar suspension culture methods (12) have so far been unsuccessful. The present measurements of transformation were made on monolayer cultures, infected and subsequently fixed and stained. As expected, it could be observed that increasing virus inoculum increased the number of transformed cell foci. Within the range of multiplicities examined, and for the periods chosen, the number of foci did not increase proportionately with the multiplicity of virus exposure. Since the lowest multiplicity of exposure was insufficient to infect all cells and the maximum number of foci resulted from this multiplicity after 42 days, there is evidence of late infection by virus propagated in the cultures.

Early virus production was pronounced so that sufficient virus to infect all cells not initially infected was present in the culture supernatant within 3 weeks, regardless of the original virus exposure (Fig. 2). It has been previously shown that the distinction between the infected cells which transform and those that did not, occurred early after infection. This agrees with observations of SV40 infected human fibroblast cell strains, for example by Aaronson and Todaro (13). It has also been shown that transformed amnion cells apparently originate from a very minor part of the originally infected cell population (2). Since the number of foci observed was similar in cultures infected at 34 days at all multiplicities, and in cultures infected after 10 days with the highest multiplicity, it appears that this is the total number of transformed foci that can be expected. Since, on an average, 47 foci were present in cultures containing $4.7 \times 10^5$ cells at the time of infection (34 days), the transformation event occurs in each $10^{-4}$ cell if one focus originates from one cell. This value agrees with the frequency determined for SV40 transformed human diploid fibroblasts (11). The transforming unit of virus in the amnion system cannot be determined until similar assays have been performed in the presence of SV40 antiserum.

The early recognition of transformed amnion cell foci (12 days after infection at optimal conditions) differs markedly from the first reported months-long latent periods for SV40 transformation of human fibroblasts (14). However, transformed colonies have been observed 3 weeks after SV40 infection of human foreskin cells (8), and it is reported that fibroblast strains derived from human skin have shown colonies of transformed cells within 10 days following SV40 infection (11). It has been observed for other cell types that increasing virus multiplicity accelerates the morphological SV40 transformation (15, 16), and that the latent period before transformation (of hamster cells) depended on the culture medium and serum supplement (15). It has also been observed for human fibroblast strains that cells, cultured for a longer period of time before infection, responded earlier with transformation (17), and very old cultures of amnion cells (166 days) have contained transformed foci at the 12th day after infection, while the latent period for younger cultures was reported to be 24–84 days (18).

The theory that defective cells, which predominate in late culture passages, are the target cells for SV40 transformation (17), seems to be applicable to explain the results observed with very old fibroblast cultures. However, the differences observed with the amnion system concerns an age span of only a few weeks. It seems reasonable to propose that the paucity of normal untransformed cells with inhibitory properties in older cultures (2, 19) can, in part, explain the difference in response between 10-day and 5-week-old amnion cultures. However, it is conceivable that the "defective cell" explanation may also be valid for the amnion cultures. Within this period the uninfected cultures change from readily dividing to stationary cultures, and chromosome changes have been observed to occur with extended cultivation (3). The change, which may be the basis for a more effective, abortive, noninfectious type of relationship, may also have a bearing on the increasing proportion of R to T cell foci observed when older amnion cultures are infected.

Compared to data on human fibroblast strains (20, 21, 13), the percentage of amnion cells containing T antigen within the first 2 weeks after infection is high in this amnion preparation (Table III). We may assume that the test is highly sensitive, and that the cell cultures employed in the present test are "susceptible" (13). The early increasing proportion of T antigen positive cells may be a reflection of the special conditions in the primary, untransferred amnion cultures, not containing antiserum (13).

The subcultured T cells were affected by the transfer schedule (Table IV), and a low split ratio permitted a delay of "crisis," as has been observed for SV40 transformed human fibroblast strains (19). The effect of the original virus multiplicity on the amount of cells available for culture transfer during many subsequent passages, is not easily explained by the differences in the amount of transformed cells in the primary cultures at the time of the first culture transfer. It indicates that a more readily growing cell type results from high multiplicity of virus infection. It is unlikely that the distinction between R and T cells is related to this difference (3).

The present data represent part of a rather extensive study to characterize the SV40 human amnion cell relationships. Obviously, it has been necessary to employ cells cultured from many individuals in this investigation, and some minor differences in results can be related to this variation. However, SV40 transformation has been reproducible for all amnion preparations, and the differences, both in the initial response to SV40 infection and in the behavior of the subcultured strains, have been quantitative rather than qualitative.

*Summary.* SV40 transformation of primary cultures of human epithelial amnion cells depended on multiplicity of virus exposure, culture age, and medium. Under optimal conditions transformed foci were observed 12 days after infection. Morphological transformation frequency was $10^{-4}$. The percentage of cells containing T antigen early after infection was higher than reported for SV40 infected human fibroblasts. Early SV40 production was pronounced and similar virus

titers were observed after high and low virus multiplicity within 3 weeks. The amount of growth and the period of time before "crisis" depended on the transfer schedule, but also showed a direct relationship to the original multiplicity of virus exposure.

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