In vitro Transformation of Newborn-Hamster Kidney Cells by Simian Adenoviruses.* (32574)

JOHN L. RIGGS AND EDWIN H. LENNETTE
Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley

The *in vitro* transformation of newborn-hamster kidney cells by a number of viruses has been described(1-4). Such transformations have been characterized by the induction of new virus-specific complement-fixing antigens, an increased growth potential of the cells, a change in their morphological characteristics, and the ability to produce tumors when introduced into hamsters.

Hull *et al*(5) have shown that 6 of 18 simian adenoviruses produce tumors in newborn hamsters at varying times after subcutaneous inoculation. We have attempted to induce *in vitro* transformation of newborn-hamster kidney cells by these same oncogenic types of simian adenoviruses, and the result are described in this report.

Materials and methods. Viruses. The viruses used in this study were the prototype strains of SV20, SV33, SV34, SV37 and SV38 obtained from the American Type Culture Collection. The SA7 virus was obtained from Dr. Richard Heberling, National Institutes of Health. The seed viruses were passed at least once in the BSC-1 continuous passage line of African green monkey kidney cells, and the titers of these stocks, as determined by plaquing on BSC-1 cells, ranged in the order of 10^6 to 10^7 plaque forming units (PFU) per milliliter.

Cell culture. BSC-1 cells were grown in medium 199 in Earle's balanced salt solution with 10% fetal bovine serum. For maintenance of the cells for virus production, 0.4 mM of additional arginine was added and the fetal bovine serum concentration was reduced to 5%.

For transformation studies, cell cultures were prepared from the kidneys of hamsters which were less than 24 hours old. The kidneys were minced and trypsinized (using 0.25% trypsin in phosphate buffered saline (PBS), pH 7.5) by the usual procedures and the cells were planted in a 1:200 dilution of packed cell volume ($250 \times g$/10 min in Earle's minimum essential medium in Earle's balanced salt solution supplemented with 0.4 mM of additional arginine and 10% fetal bovine serum. Long-term maintenance of the cultures for the transformation studies was accomplished by feeding the cultures at weekly intervals with the same medium.

Complement-fixing antigens. The complement-fixing T-antigens were produced by making 10% suspensions of transformed cells in 0.01 M Tris buffered saline (pH 7.2) containing 0.5 mM Mg^{++} and 0.27 mM Ca^{++}. These cellular suspensions were frozen and thawed 3 times before being sonicated for 10 minutes in a Bronwill ultrasonic apparatus, and then stored at −70°C (mechanical refrigeration in rubber-stoppered tubes); before use, they were clarified by centrifugation at $300 \times g$/10 min.

Serological technics. The complement fixation (CF) test used in this study was our standard procedure adapted for use in the microtiter system(6).

Animal inoculation. Hamsters were inoculated subcutaneously between the scapulae with varying numbers of transformed cells contained in 0.05 ml of suspending fluid. Animals less than 24 hours of age and animals 3 to 5-days-old were used to determine the oncogenicity of the transformed cells.

Results. Virus inoculation. A 30 ml portion of the suspension of the 1:200 cell pack volume of newborn-hamster kidney cells prepared as described in *Materials and Methods* was inoculated with a final dilution of 1:20 of the various virus stocks. The infected cell suspension was then planted in 5 ml amounts in six 4-oz prescription bottles; these were then incubated at 36°C. Uninfected control cultures were processed in a like manner. The control and infected cells were fed with 7 ml of medium 3 days after establishment and at weekly intervals thereafter. The cultures were

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observed for morphological evidence of transformation for extended periods of time.

The following viruses failed to give any evidence of transformation: SV33, SV34, SV37 and SV38. After approximately one week in culture the cells inoculated with SV33, SV37 and SV38 showed some evidence of cytopathology. Many cells rounded and detached from the glass surface although the reaction was not generalized and many apparently normal cells remained. These remaining cells were maintained in the original culture for a period of approximately 90 days. No evidence of morphological transformation was noted in the cultures over this time period and they were discarded without further subculture.

The cells inoculated with SV34 did not show any evidence of cytopathologic change during the earlier time periods. No evidence of morphological transformation occurred and although the original cultures were subcultured at 90 days, the subcultured cells again remained relatively normal in appearance for an additional 30-day period, at which time they were discarded.

SA7 virus. Small colonies of epithelioid cells growing upon the monolayer of fibroblastic cells were noted at approximately 30 days post infection in the cultures infected with SA7 virus. Subculture of 2 of these cultures at this time failed to produce growth of any of the cells. The colonies of epithelial cells in the remaining infected cultures continued to increase in size and number. At approximately 120 days after infection, further attempts were made to subculture the cells. A 0.1% solution of trypsin in phosphate buffered saline (pH 7.5) was added to one culture and the trypsinizing procedure (room temperature) was followed visually at low power magnification on an inverted microscope. It was noted that the colonies of epithelial-like cells growing on the monolayer of fibroblast-like cells were more susceptible to the action of the trypsin than were the background cells. As soon as the cells in the colonies were released into the trypsin solution, it was removed and centrifuged to recover the suspended cells. These cells were then dispersed in 7 ml of fresh growth medium and 5 ml of the suspension was dispensed into a 2-oz prescription bottle and the remainder into two culture tubes. The fibroblast-like cell monolayer remaining in the original culture was fed with fresh medium for further cultivation.

Colonies of small epithelial-like cells appeared within 2 days in the cultures planted with cells derived from this light trypsinization procedure. Cells at the edge of the colony appeared as flattened epithelial-like growth, while the cells at the center of the colony often retracted and rounded and continued to grow in clumped aggregates of cells. The colonies of cells established in this manner were trypsinized (0.25% trypsin in phosphate buffered saline pH 7.5) and subcultured. It was evident after 3 further subcultures that a line of transformed cells had been established and the line was designated as 0-852. At the fifth passage, attempts were made to isolate virus from the transformed cultures. Supernatant fluids and frozen and thawed cell packs of the cells were inoculated into cultures of BSC-1 cells and held for 14 days. Blind passages of these cultures into fresh BSC-1 cultures failed to give evidence of the presence of infectious virus. Attempts were also made to isolate infectious virus by the sensitive Gerber technic(7), using the BSC-1 grivet monkey cell line as the indicator cell. No infectious virus was recovered in any of these attempts. The cell line is now routinely grown in 8-oz prescription bottles which are seeded with approximately 1×10^6 cells per bottle. After one feeding at approximately 4 days, the cells form a confluent monolayer within 7 days and are again ready for harvest for subculturing or preparation of CF antigen.

SV20 virus. Morphological transformation was not evident in the cultures inoculated with SV20 virus until approximately 60 days, when small colonies of cells were noted which were more refractile than the background fibroblast-like monolayer. These colonies failed to increase notably in size or number, and at 90 days subculture of the cells was attempted. A 0.25% trypsin solution in PBS pH 7.5 was added to three of the 4-oz bottle cultures. After removing the solution containing the detached cells, the cells were recovered by centrifugation, suspended in fresh medium, and planted in one 4-oz prescription bottle. Small colonies of cells soon appeared in this sub-

culture which had much the same appearance as that described for the SA7 transformation. The colonies continued to increase in size and were maintained a further 30 days, at which time subculturing was carried out; the resulting line of cells was designated as 0-851. At the fifth passage after establishment attempts were also made to isolate infectious virus from this cell line as described above for the SA7 transformation. No infectious virus was recovered in any of these attempts. The 0-851 line is now routinely subcultured at weekly intervals by seeding 1×10^6 cells into an 8-oz prescription bottle and feeding 4 days following establishment. A monolayer is then ready for harvest at 7 days for further subculturing or production of CF antigen.

Oncogenicity of transformed cells. To determine whether the cells transformed *in vitro* would produce tumors when inoculated into hamsters the cells were harvested by trypsinization, recovered by centrifugation, and suspended in PBS pH 7.5. The cells from the sixth passage transformed by SA7 virus were diluted and varying numbers of cells were inoculated into 3 to 5-day-old suckling hamsters. The results are shown in Table I. A total of 22 of 27 inoculated hamsters developed tumors by 120 days.

TABLE I. Occurrence of Tumors in 3- to 5-Day-Old Hamsters Inoculated with Varying Numbers of SA7 Transformed Cells (Sixth Passage Level).

No. of cells inoculated	No. animals with tumors* / No. animals inoculated
1×10^6	9/10
0.5×10^6	12/14
0.25×10^6	1/3

* 120 days post inoculation.

In the case of the cells transformed *in vitro* by SV20 virus, hamsters of varying ages (see Table II) were inoculated with two different dilutions of transformed cells suspended in PBS pH 7.5 from the fifth passage level. As shown in Table II, tumors developed by 120 days in 45 of 63 inoculated hamsters.

TABLE II. Occurrence of Tumors in Hamsters Inoculated with SV20 Transformed Cells (Fifth Passage Level).

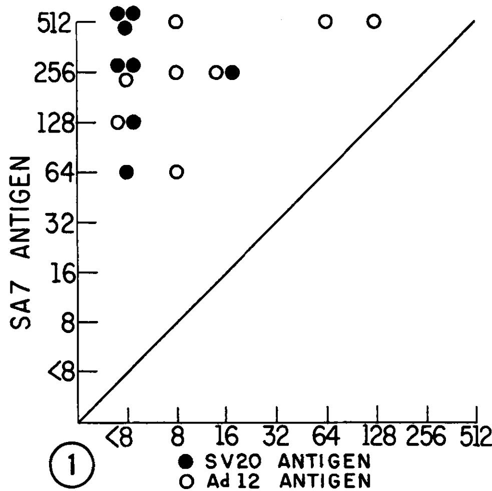
Age of hamsters	No. of cells inoculated	No. animals with tumors* / No. animals inoculated
≤24 hr	4×10^5	27/40
3–5 days	2.5×10^5	18/23

* 120 days post inoculation.

Presence of CF T-antigen in transformed cells. SA7 and SV20 transformed cells at various *in vitro* passage levels were prepared as CF antigens as described in *Materials and Methods* and tested for the presence of virus specific T-antigens. Sera from hamsters which had been inoculated with virus-free transformed cells and which produced large tumors in the oncogenicity-testing procedure were used in this reaction as the tumor-bearing hamster serum (TBHS). In addition, these sera and pools of serum from hamsters bearing tumors induced by inoculation of cells transformed by SV40 and/or by human adenovirus type 12 were tested in the CF reaction for the presence of any cross-reacting T-antigens. Complement-fixing T-antigens of SV40 and adenovirus type 12 were prepared from cells transformed *in vitro* and grown in culture in the same manner as described for the SV20 and SA7 transformed cells.

In each instance, T-antigen preparations made from cells transformed *in vitro* by SA7 and SV20 gave a positive CF reaction with their homologous serum. The serum pool from hamsters bearing tumors induced by cells transformed by SV40 virus reacted specifically in that a positive reaction was obtained only when homologous antigen was used in the test procedure.

Cross-reactions were noted, however, when heterologous antigens were tested with the SA7, SV20 and Adeno 12 TBHS. As can be seen from the diagram in Fig. 1, of the 8 individual sera from hamsters with tumors induced by SA7 transformed cells, only one gave fixation with the SV20 T-antigen preparation, while 6 of the 8 gave fixation with the Adeno 12 T-antigen. All of the sera which showed a heterologous cross-reaction reacted with the heterologous antigen to a lower titer than with the homologous antigen. Fig. 2 shows diagramatically the reactions of 12 sera from hamsters with tumors induced by Adeno 12 transformed cells. Only one of these sera gave fixation at a dilution of 1:8 or greater with the SV20 T-antigen, whereas 11 of the 12 sera gave fixation with the SA7 T-antigen



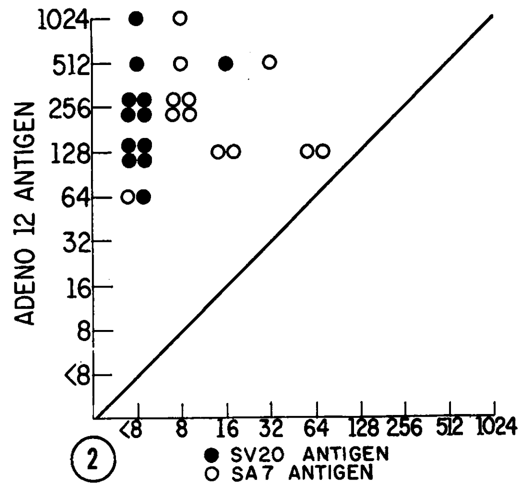


FIG. 1. Cross complement fixation between SV20 and Adeno 12 T-antigens and individual sera from 8 hamsters bearing tumors induced by cells transformed *in vitro* by SA7. The ordinate represents the CF titer of the sera against the homologous SA7 antigen, and the abscissa represents the heterologous CF titer against SV20 and Adeno 12 T-antigens.

FIG. 2. Cross complement fixation between SV20 and SA7 T-antigens and individual sera from 12 hamsters bearing tumors induced by cells transformed *in vitro* by Adeno 12. The ordinate represents the CF titer of the sera against the homologous Adeno 12 antigen, and the abscissa represents the heterologous CF titer, and the abscissa represents the heterologous CF titer against SA7 and SV20 antigens.

preparation. A more extensive cross-reaction was observed when sera from 14 hamsters with tumors induced by SV20 transformed cells were tested with the same heterologous antigens (Fig. 3). Five of the 14 sera reacted with the Adeno 12 T-antigen preparation, one serum reacting to the same titer as with the homologous antigen. All but one of these same 14 sera reacted with the SA7 T-antigen preparation; however, two of the sera gave fixation to a higher titer with this heterologous T-antigen than with the homologous antigen.

Discussion. The *in vitro* transformation of newborn-hamster kidney cells by two types of simian adenovirus was achieved by infecting the cells at the time the primary cultures were being established. Four additional types of simian adenovirus, shown to be tumorigenic in newborn hamsters by Hull *et al*(5), failed to induce transformation in these studies. It is significant that with 3 of these viruses which failed to transform the cells, an early cytopathic effect was noted, and although no attempts were made to determine if new infectious virus had been produced, it is possible a complete infectious cycle might have taken place. Such a situation would not preclude the possibility of transformation occurring concomitantly with such infectious cycles.

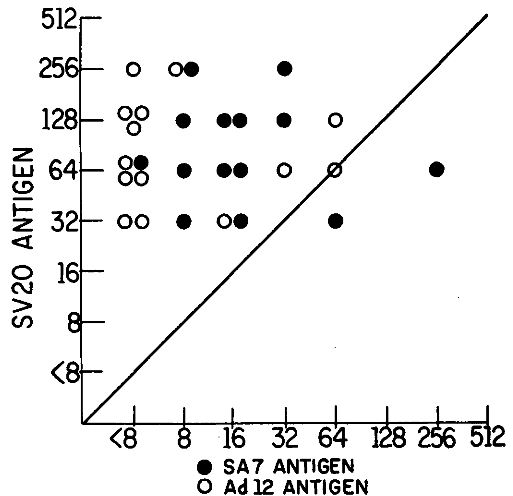


FIG. 3. Cross complement fixation between SA7 and Adeno 12 T-antigens and individual sera from 14 hamsters bearing tumors induced by cells transformed *in vitro* by SV20. The ordinate represents the CF titer of the sera against the homologous SV20 antigen, and the abscissa represents the heterologous CF titer against SA7 and Adeno 12 T--antigens.

Transformation which occurred following infection with SA7 and SV20 met established criteria for the transformation process, *i.e.*, (a) cells grew in colonies and had a more epithelial-like appearance rather than the usual fibroblast-like appearance of newborn hamster kidney cells; (b) upon subculture, the cells showed an increased growth rate, and after forming a monolayer continued to divide and grew in masses or aggregates of cells; (c) upon inoculation into hamsters tumors appeared in the majority of the inoculated animals; (d) extracts of the cells contained a new T-antigen which was virus specific in the complement fixation reaction; (e) the hamsters in which tumors were induced by these cells developed complement-fixing antibodies to these antigens.

The cross-reactions in the complement fixation tests were not totally unexpected. The two simian adenoviruses (SV20 and SA7) produced antigens in the transformed cells which would react with some of the sera derived from hamsters whose tumors had been induced by cells transformed by the human adenovirus type 12. This was most evident in the case of the SV20 transformation, with 11 of the 12 individual sera reacting to some degree. These results suggest that with transformation, a spectrum of antigens is induced in the transformed cells, of which one or more are shared by the different oncogenic adenoviruses.

Studies are under way in our laboratory to determine the transforming titer of these simian adenoviruses, and whether different clones of these cell lines vary in their ability to produce complement-fixing T-antigen(s).

Summary. Two simian adenoviruses, SA7 and SV20, induced transformation of newborn-hamster kidney cells *in vitro*. The transformation was characterized by an increased growth potential of the cells, a change in morphology of the cells, the production of a new T-antigen within the transformed cells, the production of tumors in hamsters by inoculation of the cells, and the appearance of complement-fixing antibodies to T-antigen in animals which developed tumors. These new T-antigens are virus specific.

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Pathogenic Properties of Encephalomyocarditis Virus Plaque Variants.* (32575)

JOHN E. CRAIGHEAD
Department of Pathology, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Mass.

Two variants (r^+ and r) of the encephalomyocarditis (EMC) virus were isolated and characterized by Takemoto and Liebhaber(1). After 4 days of incubation under agar, plaques of the r^+ variant were 1 mm or less in diameter whereas those of the r variant were 8 mm or greater. The small size of the r^+ plaques was shown to be due to a sulfated acid polysaccharide in the agar overlay which bound the virus electrostatically and prevented spread to neighboring cells(2). As might be expected, agar extracts, heparin and synthetic sulfated acid polysaccharides such

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