Adenovirus Transformation of Hamster Embryo Cells

BRUCE C. CASTO

*Institute for Biomedical Research, Education and Research Foundation, American Medical Association,
Chicago, Illinois 60610*

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Inoculation of hamster embryo cell cultures with human adenovirus type 12 (Ad12) or simian adenovirus (SA7) resulted in the formation of foci of morphologically transformed cells within 12 days. The rapid appearance of well-defined foci was dependent upon the transfer of cells into new plates, with sufficient dilution after virus adsorption, and was independent of virus dose. Dose-response studies showed linearity of focus formation with dilution of Ad12 or SA7. Results averaged from several experiments show plaque-forming unit to focus-forming unit ratios of approximately 1.8×10^6 for Ad12 and 2.6×10^5 for SA7. Other experiments showed that most of the adenovirus involved in transformation was adsorbed by 3 hr. Cell lines derived from SA7 transformed cells produced tumors within 19 days when inoculated intradermally into young adult hamsters. Such cell-induced tumors histologically resembled SA7 virus-induced hamster tumors. Formation of tumors with SA7 transformed cells was inhibited by prior immunization of test animals with SA7 or Ad12 virus.

Since the observation by McBride and Wiener (6) of the *in vitro* transformation of hamster kidney cells by human adenovirus type 12 (Ad12), several additional reports (2, 3, 5, 8, 9, 11; A. E. Freeman et al., *Federation Proc.* **25**:1051, 1966; J. D. Levinthal and W. Peterson, *Federation Proc.* **24**:174, 1965; W. A. Strohl et al., *Bacteriol. Proc.*, p. 136, 1966; G. L. Van Hoosier et al., *Federation Proc.* **24**:308, 1965) have appeared substantiating these findings and extending them to other cell types. Few reports contain data pertaining to quantitation of the transformation event. In addition, the time required for the appearance of cell transformation in the various cell cultures generally ranges from 1 to 2 months or longer. For example, Freeman et al. (2) have reported reproducible transformation of rat embryo cells by Ad12 in 18 to 36 days; however, in certain experiments, final focus counts were recorded 73 days postinfection.

This report has a dual purpose. One is to describe a method for the rapid demonstration (10 to 12 days) of transformed cell foci induced by Ad12 and simian adenovirus, SA7, in hamster embryo cells. The second is to present the results of several experiments designed to elucidate some of the biological parameters of adenovirus transformation of hamster embryo cells.

MATERIALS AND METHODS

Viruses. Simian adenovirus, SA7, was obtained from Klaus Schell (Microbiological Associates, Inc.), and was passaged two times in LLC-MK₂ cell cultures in this laboratory. The virus pool used in these studies had a titer of 1.2×10^8 plaque-forming units (PFU)/ml in LLC-MK₂ and 1.9×10^8 PFU/ml in African green monkey kidney cells (AGMK).

Ad12, Huie strain, was obtained from Clyde Goodheart of these laboratories. This virus had been plaque-purified in human kidney cells (HuK) and was shown previously to be oncogenic for newborn hamsters (4). The two virus stocks used for most of the transformation experiments were prepared in secondary HuK cells and had a titer of 3.2×10^8 and 2.0×10^9 PFU/ml in these cells.

Cell cultures. LLC-MK₂ cells were cultured in 100-mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.) in a 5% CO₂ atmosphere. Growth medium consisted of Eagle's minimal essential medium (MEM; General Biochemicals, Inc., Chagrin Falls, Ohio) supplemented with 10% fetal calf serum (FCS). HuK cells, used for the preparation and assay of Ad12, were cultivated in 100-mm plastic petri dishes in a 5% CO₂ environment using Eagle's medium, enriched with additional amino acids and vitamins (ERM) supplemented with 10% FCS. AGMK cells used for assay of the SA7 were cultured as above for HuK.

For plaque assay, cells were removed from 100-mm dishes either with ethylenediaminetetraacetate

(EDTA) buffer or with 0.2% trypsin; 1.5×10^6 to 2.0×10^6 cells were seeded into 60-mm plastic dishes containing the above medium. Overlay medium consisted of the respective basic medium for each cell type supplemented with 2 to 5% heat-inactivated FCS, 1% agar (Difco), and 100 μ g of diethylaminoethyl (DEAE)-dextran per ml to suppress viral inhibitors in the agar. After 5 days, a second overlay of 2.5 ml was added, and after 9 to 10 days the plates were stained with a third overlay containing 6% neutral red (1:1,000 stock). Final plaque counts were made 11 to 14 days after inoculation.

Hamster embryo cultures (HEC) were prepared by trypsinization of 12- to 13-day-old whole embryos. Ten to twelve 60-mm petri dishes were usually obtained from one embryo. HEC were grown in a 5% CO₂ atmosphere in ERM with 10% fresh FCS. The cell cultures were used within 3 to 6 days after seeding, at which time each plate usually contained 3×10^6 to 5×10^6 cells.

Transformation procedure. Four plates of drained HEC were inoculated with 0.2 to 0.3 ml per plate of test adenovirus contained in medium without serum. Virus was adsorbed for 4 hr at 37°C. Immediately after the adsorption period, 2.5 ml of 0.2% trypsin in ERM (serum and calcium omitted) was added to each plate. The cells from each dish were placed in individual tubes and centrifuged at 1,000 rev/min for 4 to 5 min. The sedimented cells were resuspended in ERM with 10% FCS and distributed so that each new plate contained approximately 2.5×10^5 to 3.5×10^5 cells. After 1 to 2 days, the medium was replaced with ERM containing 0.1 mM CaCl₂ (2) and 10% FCS. Medium changes were made every 3 to 4 days thereafter, and plates were examined for transformed cell foci at 25 times magnification 10 to 14 days after virus inoculation.

An alternate method for counting foci was used in certain experiments. An agar overlay consisting of the low calcium medium, 10% FCS, and 0.5% agar was added 10 to 12 days after virus inoculation, and final focus counts were made 7 to 10 days later. At this time, foci were clearly visible as circumscribed colonies with dense brownish centers. The use of the overlay facilitated the picking of cells from isolated colonies for establishment of cell lines. The agar overlay also permitted plates to be held for longer periods to verify that no significant increase in focus counts occurred after the first 2 weeks.

Propagation of adenovirus-transformed cells. Clonally derived cells were obtained without trypsinization from transformed cell foci maintained under agar, by removing a plug of agar with a pasteur pipette from directly above a colony of transformed cells. The agar plug with its adherent cells was transferred into a 60-mm petri dish containing 3 ml of the low calcium medium. Additional cells were then removed by flushing the exposed colony with medium. After approximately 7 to 10 days, colonies resulting from this transfer were passed to 2 or 3 new plates. Once confluent cultures were obtained, cells could be transferred every 3 to 4 days by dividing the cultures 1:10 at each transfer.

RESULTS

Effect of cell numbers on the development of adenovirus-transformed cell foci. These experiments were designed to test the influence of the number of cells transferred into new plates after virus adsorption on the time of appearance and numbers of adenovirus-transformed cell foci.

Primary HEC were inoculated with 0.3 ml of Ad12 per dish ($M = 37$). After a 4-hr adsorption period, the cultures were split so that five groups of new dishes contained cells ranging from 200,000 to 3,200,000 per dish in twofold increments. Adenovirus-type transformed cell colonies (as shown in Fig. 1-3) were counted 12 days after virus inoculation and cell transfer.

As can be seen from Table 1, dishes into which 3.2×10^6 cells were seeded contained no visible evidence of colony formation. The highest numbers of foci were obtained when new plates were seeded with 2×10^5 to 4×10^5 adenovirus-inoculated cells. If the efficiency of transformation is expressed as a ratio between the number of transformed cell foci and the total number of cells inoculated with adenovirus (t/cell), then the 296 foci, which resulted from seeding plates with 2×10^5 cells, would give a t/cell ratio of 2.3×10^{-5} . Plates seeded with 4×10^5 cells gave rise to 390 foci or a t/cell ratio of 3.0×10^{-5} . The smaller numbers of foci obtained from plates seeded with 2×10^5 cells may have been due to a diminished "feeder effect" between cells at this concentration as contrasted to those plates receiving 4×10^5 cells.

These results suggest that several cell divisions may be required for the early expression of adeno-

FIG. 1. Macroscopic appearance of foci in 60-mm petri dish 12 days after inoculation of hamster embryo cell cultures with SA7. Giemsa-stained.

FIG. 2. Single focus of transformed cells 12 days after inoculation of hamster embryo cell cultures with SA7. Giemsa-stained.

virus transformation. Further evidence for this interpretation was obtained by the following experiment. Four plates of HEC inoculated with SA7 were trypsinized, and 3×10^5 cells were transferred onto confluent cultures of hamster embryo cells or onto blank plates. SA7 inoculated cells, transferred to blank plates, gave rise to a total of 474 foci after 14 days, whereas no visible foci appeared when inoculated cells were transferred to those plates already containing dense cultures of hamster embryo cells.

Correlation between virus dose and the number of transformed cell foci. Four 60-mm dishes of HEC were inoculated with 0.2 ml each of one of a series of twofold dilutions of SA7 or Ad12. After virus adsorption, secondary cultures were established by seeding approximately 3×10^5 cells in each new plate. Final focus counts were made 12 days after virus inoculation and cell transfer.

Cultures inoculated with undiluted SA7 ($M = 12$) developed an average of 84 foci per plate (Fig. 4). Each twofold dilution of the virus resulted in an approximate twofold decline in the number of countable foci.

Two similar experiments with Ad12 are also shown in Fig. 4. Linearity of focus formation with virus dilution was maintained until the virus multiplicity per cell (M) rose to greater than 100; at $M = 115$ there appeared to be a slight depression in the number of expected transformants, and at $M = 230$ less than 10% of the foci were recovered. With SA7 virus, multiplicities of 0.8 per cell still resulted in formation of transformed cell foci within 12 days. There appeared to be no

correlation between virus dose and the time of appearance of transformed cell colonies.

Relationship between plaque-forming units (PFU) and focus-forming units (FFU). In Tables 2 and 3, data are shown from several transformation experiments with Ad12 and SA7. With Ad12 (Table 2), the average ratio of PFU to FFU, as calculated from seven different experiments with virus multiplicities per cell ranging from 5 to 115, was found to be 1.78×10^6 . In two experiments (Table 2, experiments 2 and 8) where the virus multiplicity per cell was excessive ($M = 231$, $M = 157$), an artificially high value was obtained (1.1×10^7 and 8.3×10^6 PFU/FFU, respectively), presumably as a result of killing of potential transformants. This interpretation was substantiated by the observation that there was a general decrease in the total cells per plate in these experiments 48 hr after virus inoculation.

With SA7 (Table 3), when the data from all eight experiments were used (virus multiplicities of 0.4 to 14 per cell), the average PFU to FFU ratio was found to be 2.5×10^5 .

In those experiments where maximal amounts of virus were inoculated onto HEC without resulting in a noticeable cell death (Table 2, experiments 3 and 4), the frequency of transformation was found to be approximately 1 per 10^4 and 1 per $10^{4.2}$ cells, respectively.

Effect of virus adsorption time on the numbers of transformed cell foci. A 0.2-ml amount of Ad12 (10^8 PFU; $M = 19$) was added to each of four plates of HEC at 4, 3, 2, 1, and 0 hr prior to removal of the cells with trypsin and distribution into new plates.

Figure 5 shows that most of the virus responsible for transformation was adsorbed by 3 hr. Approximately 27% of the FFU appeared to be adsorbed at time-zero. This seemingly high adsorption at this time most likely can be attributed to the dose of virus inoculated (10^8 PFU) and the omission of any washing of the cell sheet after virus adsorption, except that which was done coincidentally with the trypsinization procedure. Other experiments (*data not presented*) have shown that further incubation (up to 6 hr) did not result in increased numbers of transformed cell foci over that found at 3 hr.

Tumor induction in hamsters by adenovirus-transformed hamster embryo cells. Ad12 and SA7 transformed cells could readily be established in culture by use of ERM with 0.1 mM CaCl_2 and 10% fresh FCS. One such cell line, induced by SA7 in HEC, was passaged seven times and tested in young adult hamsters for its ability to induce tumors. Also included among the test animals were hamsters immunized with Ad12 by intramuscular injection (0.25 ml of virus sus-

FIG. 3. Appearance of SA7-inoculated hamster embryo cell cultures 12 days after virus inoculation. Giemsa-stained. (top) Periphery of focus showing appearance of transformed cells (arrow 1) as contrasted to primary hamster embryo cells (arrows 2). $\times 250$. (bottom) Cells nearer the center of a focus, showing typical appearance of adenovirus transformed cells. $\times 250$.

TABLE 1. *Effect of cell numbers on the appearance of adenovirus (Ad12)-transformed cell foci, 12 days after virus inoculation^a*

Ratio of ^b new plates to original plate	No. of cells seeded/plate	Total no. of transformed cell foci arising from original four plates
1:1	3.2×10^6	0
2:1	1.6×10^6	4
4:1	8×10^5	13
8:1	4×10^5	390
16:1	2×10^5	296

^a Each of four plates of hamster embryo cell culture was inoculated at an input multiplicity of 30 PFU/cell.

^b Cells were transferred 4 hr after virus inoculation.

TABLE 2. *Relationship between plaque-forming units (PFU) and focus-forming units (FFU) in the Ad12-hamster embryo transformation system*

Expt no.	Virus dose (PFU) ^a	Virus multiplicity	No. of transformed foci ^b	PFU/FFU
1	1.0×10^8	35.7	25	4×10^6
2	6.0×10^8	231.0	54	1.1×10^7
3	3.0×10^8	115.5	268	1.1×10^6
4	1.5×10^8	57.7	178	8.4×10^5
5	2.4×10^7	5.0	29	8.3×10^5
6	1.0×10^8	33.3	98	1.0×10^6
7	5.4×10^7	13.5	19	2.8×10^6
8	3×10^8	157.0	36	8.3×10^6
9	2×10^8	40.8	105	1.9×10^6
Avg ^c				1.78×10^6

^a Each of four plates was inoculated with 0.2 or 0.3 ml of virus suspension.

^b Total number of foci arising from inoculation of a single plate. Each figure represents the mean of four plates.

^c Data from experiments 2 and 8 are excluded from the calculation because of demonstrable cell death at these virus multiplicities.

TABLE 3. *Relationship between plaque-forming units (PFU) and focus-forming units (FFU) in the SA7-hamster embryo transformation system*

Expt no.	Virus dose ^a (PFU)	Virus multiplicity	No. of transformed foci ^b	PFU/FFU
1	3.8×10^7	13.8	53	7.2×10^5
2	3.8×10^7	11.9	113	3.4×10^5
3	1.0×10^6	0.45	5	2.0×10^5
4	1.5×10^6	0.4	11	1.4×10^5
5	2.0×10^7	3.7	89	2.3×10^5
6	1.0×10^6	0.45	9	1.1×10^5
7	1.5×10^6	0.4	15	1.0×10^5
8	1.0×10^6	0.5	6	1.7×10^5
Avg ^c				2.5×10^5

^a Each of four plates was inoculated with 0.2 or 0.3 ml of virus suspension.

^b Total number of foci arising from inoculation of a single plate. Each figure represents the mean of four plates.

^c Average of all eight experiments.

FIG. 4. *Linear relationship between the number of transformed cell foci and the concentration of adenovirus (normalized values). Symbols: X, SA7 ($M = 12$ at undilute); O, Ad12 ($M = 83$ at undilute); ●, Ad12 ($M = 231$ at undilute). Each point represents the total foci originating from one 60-mm petri dish. Calculations were based on the following mean counts per plate: SA7 (X), 84 at 0; Ad12 (O), 100 at 1:2; Ad12 (●), 268 at 1:2.*

pension, once a week for 3 weeks) and hamsters similarly immunized with SA7.

Six control, five Ad12-immunized, and five SA7-immunized hamsters were inoculated intra-

dermally with 1.5×10^6 cells (total count) in 0.2 ml of medium.

All of six control animals developed tumors at the site of inoculation within 19 days after introduction of the transformed cells (Table 4). Three of five and two of five animals in the Ad12- and SA7-immunized groups, respectively, also developed tumors within the same 19-day period. All immunized animals were sacrificed 33 days after inoculation of the SA7 transformed cells.

FIG. 5. Attachment of Ad12 focus-forming units to primary hamster embryo cell cultures. Symbols: ×, total foci originating from one 60-mm petri dish; ●, mean of four plates at each time; ⊗, excluded from calculation of mean.

TABLE 4. Tumorigenicity of the adenovirus-transformed hamster embryo cells

Treatment ^a	No. of animals with tumor ^b	Tumor size (cm ³)
None	6/6	0.5, 3.6, 11.2, 4.4, 1.2, 1.6
Ad12-immunized	3/5	0.05, 0.05, 0.25
SA7-immunized	2/5	0.8, 1.0

^a Animals were immunized by intramuscular injection of 0.25 ml of virus each week for 3 weeks.

^b At 19 days after intradermal inoculation of 1.5×10^6 SA7-transformed hamster embryo cells (seventh passage).

At this time, no more tumors appeared among the SA7 immunized animals; however, one additional animal in the Ad12-immunized group had developed a small tumor ($0.8 \times 0.4 \times 0.2$ cm). With but one exception, all tumors in the immunized animals were considerably smaller than the smallest of the control group. Histological examination of these presumably cell-induced

tumors showed them to be similar to those induced by SA7 virus inoculated into newborn hamsters.

DISCUSSION

This report describes a method whereby the transformation of cells by adenovirus can be assessed rapidly and quantitatively. Cultures of hamster embryo cells, inoculated with Ad12 or SA7, underwent transformation leading to the appearance of well-developed, dense areas of morphologically altered, rapidly growing cells, within 10 to 12 days after inoculation.

The rapid appearance of transformed cell foci, as described in this report, is in contrast to the extended times reported by Freeman et al. (2), and by others (*see* introduction), but is in agreement with the 14 to 23 days for detection of Ad12-transformed hamster embryo cultures as shown by Rafajko (9). One feature in common between experiments performed by Rafajko (9) and those presented in this report was the subculturing of cells after virus inoculation. Transfer of inoculated cells appears to be neces-

sary for the demonstration of well-defined adenovirus-transformed cell foci within 2 weeks, presumably by allowing the inoculated cells to undergo several cycles of cell division. Restricting the number of cell divisions, by transferring progressively larger numbers of inoculated cells, eventually resulted in complete inhibition of the appearance of transformed cell foci within the 12-day experimental period. Maximal numbers of transformed cell foci were found when approximately 3×10^5 to 4×10^5 hamster embryo cells were transferred to each new plate after the adenovirus adsorption period.

Other experiments have shown that distribution of adenovirus-inoculated cells (at optimal concentrations) onto confluent cultures of hamster embryo cells prevents the appearance of foci of transformed cells as compared with cells transferred to blank plates. Therefore, the initial infection of a cell with adenovirus does not release that cell from the inhibition imposed by other cells in the culture. The requirement for cell division for early expression of transformation, reported here for adenovirus and hamster embryo cells, is similar to that reported by Todaro and Green (13) for the SV40-3T3 transformation system.

When serial twofold dilutions of SA7 or Ad12 were added to HEC, the number of transformed cell foci decreased proportionally, thus providing a basis for the establishment of a relationship between PFU and FFU. By use of the data obtained from several experiments, it was found for SA7 and Ad12 that approximately 1 FFU can be related to 2.5×10^5 PFU and 1.8×10^6 PFU, respectively. The Ad12 PFU/FFU ratio reported here is slightly higher than that reported by Freeman et al. (2), but it is in agreement with that found by McAllister and MacPherson (5), who used rat embryo cells or a clonal line of hamster embryo cells. However, the PFU/FFU ratios for both SA7 and Ad12 are still much higher than the 10^4 and 10^3 reported for polyoma (7, 10, 14) and SV40 (1, 12), respectively.

With multiplicities of Ad12 virus per cell of approximately 57 and 115, the number of cells in culture developing into foci was found to be 1 per $10^{4.2}$ and 1 per 10^4 , respectively. Higher doses of virus ($M = 157$ and $M = 230$) caused extensive cell death, resulting in fewer foci than expected. For example, with $M = 157$ there was an 80% decrease in the expected number of foci, and with $M = 230$ more than 90% of the expected foci failed to develop. Therefore, under the present experimental conditions, the transformation frequency does not plateau with increased virus dose, as has been observed with polyoma (14) and SV40 (1); rather, it declines.

Adsorption experiments in confluent cell cultures of hamster embryo, with the use of optimal amounts of Ad12, have shown that the minimal contact time between the virus inoculum and the cells, to obtain maximal focus formation, was approximately 3 hr, with 70% of the virus in the inoculum being adsorbed during the first hour. Incubation of virus and cells for as long as 6 hr did not result in an increased number of foci over that found at 3 hr.

Cultures established from selected SA7 transformed foci were shown to produce tumors within 19 days in adult syrian hamsters when inoculated intradermally with 1.5×10^6 cells (total cell count). The SA7-induced transformation of the implanted cells (ruling out the possibility of either spontaneous transformation or transformation caused by some extraneous agent) was confirmed by the following: (i) in contrast to nonimmunized animals which developed large tumors within 19 days, animals immunized with homologous virus failed to develop tumors within 33 days or gave rise to tumors greatly reduced in size when challenged with the above cells (J. J. Trentin and E. Bryan, Proc. Am. Assoc. Cancer Res. 5:64, 1964); (ii) tumors formed by inoculation of SA7 transformed cells were histologically similar to tumors induced in hamsters by inoculation of newborn animals with SA7 virus (11); and (iii) serum from hamsters bearing cell-induced tumors had complement-fixing antibody to antigen extracted from virus-induced tumor passaged in vivo (*unpublished data*). Of interest was the finding that animals immunized with either SA7 or Ad12 virus were protected when challenged with SA7-transformed cells.

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