Characterization of Defectiveness of Human Adenoviruses in Green Monkey Kidney Cells.* (32569)

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Human adenoviruses undergo an abortive infection in simian cells. Following adsorption and penetration, they induce the formation of the adenovirus tumor antigen(1,2) and the synthesis of virus-specific DNA(3). However, no viral capsid antigen can be detected(1,2). If the monkey cells are coinfected with the simian papovavirus SV40, the human adenoviruses undergo a productive replication cycle with the production of virus capsid antigen(1,2) and infectious virus progeny(1,4-11). Similarly, coinfection of monkey cells with simian adenovirus SV15 (12), with a defective SV40 genome(5,14, 15) or with a defective monkey cell-adapting component(16) leads to a productive replicative cycle for the human adenovirus.

Further analysis of the abortive cycle of two human adenoviruses, types 2 and 7, in simian cells has revealed differences in the degree of their defectiveness.

Materials and methods. Cells. Primary African green monkey kidney (GMK) cells were grown in Melnick's lactalbumin hydrolysate medium (M-H) with 2% calf serum and 0.08% $NaHCO_3$. All media used contained 100 units of penicillin and 100 μg of streptomycin per ml. The cells were grown in 1- and 16-oz prescription bottles and in 60 × 15 mm plastic petri dishes and were generally used after incubation at 37°C for one week.

Human embryonic kidney (HEK) cells were supplied by the Human Tissue Procurement Program, National Cancer Institute; they were grown in Melnick's lactalbumin hydrolysate medium (M-E) with 10% fetal calf serum and 0.23% $NaHCO_3$ in 35 × 10 mm plastic petri dishes.

Viruses. Adenovirus type 7 (H) and adenovirus type 2 were obtained from Dr. Benyesh-Melnick, who had isolated them from clinical specimens. The viruses were used after 2 to 5 passages in HEK cells. Adenovirus type 7 (#19) was obtained by plaque-purification of the PARA-adenovirus 7 (stock SP2) population in HEK cells(17). The contaminating defective SV40 genome was removed by this procedure and the resulting population behaved as a typical human adenovirus 7(17). This virus was used after 5-6 passages in HEK cells.

Virus assay. Adenovirus yields were determined by plaque assay in HEK cells growing in 35 × 10 mm plastic petri dishes(17). Ten-fold dilutions of the virus suspension were made in tris-buffered saline (pH 7.4). The virus was added to the cells in 0.1 ml amounts; replicate plates were used for each dilution. The virus was allowed to adsorb to the cell sheet for at least one hour at 37°C with occasional manual rotation. Then, 1.5 ml of overlay containing 1% agar, 10% fetal calf serum, and 0.23% $NaHCO_3$ in Eagle's medium was added. A second overlay with a 1/20,000 dilution of neutral red was added one week later. Plaques were counted on the tenth day after inoculation.

Growth analysis. To analyze the replication of the viruses, 0.1 ml of the human adenovirus preparation was added to GMK cells growing in 1-oz prescription bottles. After adsorption at 37°C for one hour, the cell sheet was washed twice with tris-buffered saline (pH 7.4). Five ml of a fluid consisting of 2% fetal calf serum and 0.08% $NaHCO_3$ in Eagle's medium were added and the cultures were incubated at 37°C. At each designated time, the contents of two culture bottles were harvested by quick-freezing them in an alcohol-dry ice bath followed by thawing in a 37°C water bath. This process was repeated once more. Cell debris was removed

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by low speed centrifugation and the supernatant was assayed for virus.

For the inhibitor studies, the same experimental procedure was followed but the culture medium contained 10 μg/ml of fluorodeoxyuridine (FUdR), 10 μg/ml of cytosine arabinoside (ara-C), or 20μg/ml of bromodeoxyuridine (BUdR). Fresh medium (with the drugs) was added at 48 and 96 hours post-inoculation. The medium containing the inhibitor was removed at the time of harvest and an equal volume of tris-buffered saline was added to the cultures. The virus was released into the fluid and assayed as described above.

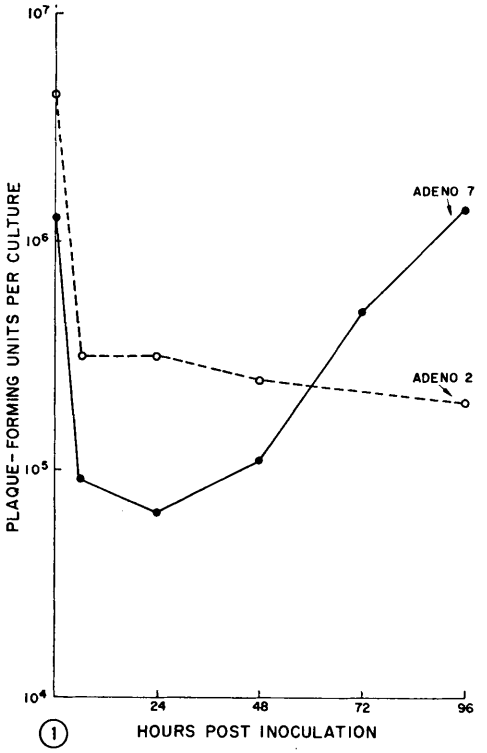
Results. Replication of adenovirus types 2 and 7 in GMK cells. The adenoviruses were added in 0.1 ml amounts to GMK cell monolayers in 1-oz prescription bottles at a multiplicity of approximately 1-5 plaque-forming units (PFU) per cell. The results of typical experiments performed with adenovirus 2 and 7 (H) are graphed in Fig. 1. Both viruses underwent an initial eclipse although residual virus titers remained fairly high. With adenovirus type 2, there was no increase in virus titer and a slight loss of infectious virus with time. With adenovirus type 7 (H), after a latent period of about 48 hours, there was a steady rise in the amount of infectious virus recovered for the following 24-48 hours. This experiment was terminated at 96 hours, but numerous experiments (see also Fig. 2) have indicated that there is no significant increase in virus titer after this time.

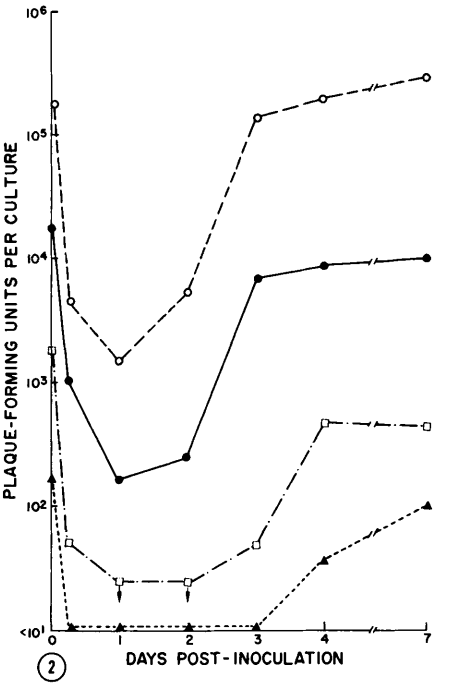
With adenovirus type 7 (H), the total virus recovered at 96 hours was almost identical to the titer of the virus inoculum. In this experiment, 1.3×10^6 PFU of adenovirus 7 were added to the cultures and 1.4×10^6 PFU were recovered at 96 hours following inoculation of the cultures. Similar results were obtained with adenovirus 7 (#19).

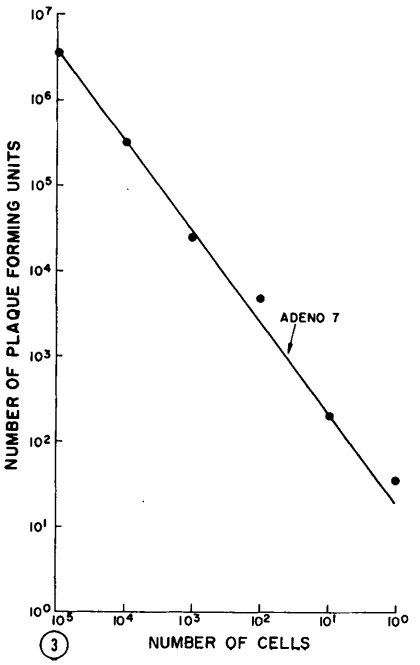
Relation between inoculum titer and virus recovery. Experiments were designed to determine whether the amount of virus recovered from GMK cell cultures inoculated with adenovirus 7 was dependent upon the amount of virus present in the inoculum. Therefore, GMK cell cultures were inoculated with tenfold increments of an adenovirus 7 (H) preparation and the total virus in the cultures calculated at various intervals. The results are presented in Fig. 2. When the inoculum contained about 10^5 PFU of adenovirus 7, 10^5 PFU could be recovered. When 10^4 PFU were added to the cultures, 10^4 PFU were recovered. Similar results were obtained with the other inocula. In each case, there was a latent period of 1 to 2 days followed by an increase in virus between 2 and 4 days. Titers generally remained constant between 4 and 7 days after inoculation of the cultures. Therefore, the amount of adenovirus 7 that can be recovered at this time from GMK cells appears to depend upon the amount of virus originally added to the cultures.

This type of replicative cycle in simian cells appears to be characteristic for adenovirus 7; several strains of adenovirus 7 that were tested had similar patterns of replication in simian cells.

Relation between virus titer and number of cells releasing virus. Experiments were designed to determine if the observed increase in infectious virus detected with adenovirus 7 in GMK cells was due to the presence of a few susceptible cells in the population that were releasing large amounts of virus or if each cell in the population was releasing a small amount of virus. A modified fluctuation test was therefore carried out. GMK cells were inoculated with adenovirus 7 and after incubation for 48 hours, the cells were dispersed with trypsin and suspended in tris-buffered saline. Ten-fold dilutions of the cells were made and virus was released from the cells by two cycles of quick-freezing and thawing. The amount of virus present in each dilution of cells was determined by plaque assay in HEK cells. The results are shown in Fig. 3. When the amount of virus detected is plotted against the number of cells, a straight line is obtained. Since the amount of virus detected is directly dependent upon the number of cells and varies directly with the dilution of cells, each cell in the population is presumably releasing an equal (though small) amount of virus. The increase in infectious titer is therefore not due to the increased susceptibility of a few


PLAQUE-FORMING UNITS PER CULTURE
10^7
10^6
10^5
10^4
ADENO 7
ADENO 2
24
48
72
96
HOURS POST INOCULATION
1


PLAQUE-FORMING UNITS PER CULTURE
10^6
10^5
10^4
10^3
10^2
$<10^1$
0
1
2
3
4
7
DAYS POST-INOCULATION
2


NUMBER OF PLAQUE FORMING UNITS
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
ADENO 7
10^5
10^4
10^3
10^2
10^1
10^0
NUMBER OF CELLS
3

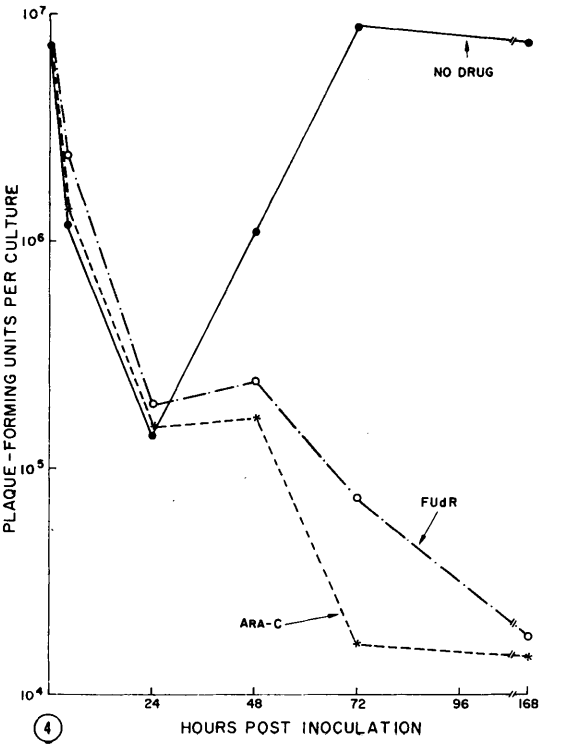

PLAQUE-FORMING UNITS PER CULTURE
10^7
10^6
10^5
10^4
NO DRUG
FUdR
ARA-C
24
48
72
96
168
HOURS POST INOCULATION
4

FIG. 1. Total number of plaque-forming units of adenovirus type 2 or 7 (H) at various times following inoculation of cultures of green monkey kidney cells. The viruses were assayed in human embryonic kidney cells.

FIG. 2. Total number of plaque-forming units of adenovirus type 7 (H) at various times following inoculation of cultures of green monkey kidney cells with 10-fold increments of the virus. The virus was assayed in human embryonic kidney cells.

FIG. 3. Total number of plaque-forming units of adenovirus type 7 (H) per number of green monkey kidney cells 48 hours after inoculation of the cultures. The virus was assayed in human embryonic kidney cells following disruption of measured numbers of cells.

FIG. 4. Total number of plaque-forming units of adenovirus type 7 (H) at various times following inoculation of cultures of green monkey kidney cells maintained in the presence of 10 μg/ml of FUdR or ara-C. The virus was assayed in human embryonic kidney cells.

cells but is due to a property shared by all the cells in the population.

Effect of inhibitors of DNA synthesis on the replication of adenoviruses in GMK cells. Since the amount of virus recovered from adenovirus 7-infected GMK cells depends upon the virus titer of the inoculum and since each cell in the population appears to contribute an equal amount of virus, it was possible that the recovered virus represents input genomes within new capsids. Studies were performed in the presence of inhibitors of DNA synthesis to determine whether the increase in virus obtained required synthesis of progeny DNA. Adenovirus 7-infected cultures were incubated in culture media containing 10 μg/ml of FUdR, 10 μg/ml of ara-C, or no drug and virus yields were calculated as before. The results are presented in Fig. 4. In the presence of the drugs, adenovirus 7 eclipsed normally. However, no increase in titer was detected between 24 and 48 hours and there was a sharp decline in the titer of infectious virus after 48 hours in the presence of either drug. A normal replicative cycle was obtained in the absence of the drugs, with the titer of recovered virus similar to the titer of the inoculum. Therefore, DNA synthesis appears to be required for the detection of the observed increase in infectious virus.

Similar experiments were carried out with adenovirus 7 grown in GMK cells in the presence of medium containing 20 μg/ml of BUdR. The results of these experiments are presented in Table I. In the presence of BUdR, no increase in the titer of infectious virus was detected. In the absence of the drug, the expected increase in the titer of infectious virus was noted. In another series of experiments, adenovirus 7 labeled in the DNA with H^3-thymidine was used as the inoculum. Repeated attempts to recover the label in the virus harvests failed. These results also suggest that the progeny virus contains newly-synthesized DNA.

TABLE I. Yields of Adenovirus 7 from Green Monkey Kidney Cells in Presence and Absence of BUdR.

Hr post-inoculation	Plaque-forming units/culture	
	No drug	BUdR
Input	3.5×10^6	3.5×10^6
24	1.7×10^4	6.5×10^6
48	4.5×10^5	7.0×10^6
72	8.0×10^5	4.0×10^6
96	6.0×10^5	2.0×10^6

Discussion. It has been well established that human adenoviruses are defective and replicate poorly if at all in simian cells(1-3) although a productive replicative cycle ensues if the cells are co-infected with other viruses(1,2,4-16). In this report we have described differences in the defectiveness shown by adenoviruses 2 and 7 in simian cells. Adenovirus 2 appears to enter the eclipse stage normally but no increase in infectious titer can be detected. Adenovirus 7 appears to have an eclipse period of about 24 hours. After that time, there is a small rise in the amount of infectious virus; however the amount of virus recovered does not exceed the input level. This was found to be true over a 10,000-fold range of input virus.

Further analysis has revealed that each cell in the population is releasing a similar amount of virus. Thus, the detected increase is not due to the presence of a few highly susceptible cells in the population. DNA synthesis appears to be required for the increase in infectious virus noted since the

amount of virus that can be recovered from inoculated cultures drops sharply in the presence of the DNA inhibitors, BUdR, FUdR, and ara-C.

Studies with BUdR and labeled input virus also suggest that the virus recovered from adenovirus 7-inoculated GMK cells contains newly-synthesized DNA rather than parental DNA strands. It has been previously shown that virus-specific DNA is formed in the abortive cycle and at the same rate as in a productive infection(3). Therefore, it appears that the block in the replicative cycle comes after DNA synthesis but before viral capsid antigen synthesis. Since a small amount of new virus is formed, however, the simian cell appears able to synthesize all components required for the manufacture and assembly of human adenoviruses. This would suggest that these viruses are produced in small quantity either because of inefficient transcription of viral DNA or because of inefficient translation by the simian cells of viral mRNA. It is possible that differences in the abortive cycle of the human adenovirus serotypes in GMK cells represent relative efficiency of the cells to accomplish one or both of these events. This condition may be analogous to that of some "leaky" bacterial virus mutants(18), in which the activity of a gene is not shut off completely. Since some residual expression of gene function remains, a limited quantity of virus is produced.

Summary. In the absence of foreign helper viruses, human adenoviruses are defective in simian cells, but the degree of defectiveness varies with the adenovirus. Adenovirus 2 initiates an infection in the simian cells but there is no increase in virus titer. When simian cells are infected with adenovirus 7, a small amount of infectious virus is produced but the amount never exceeds the input level. Each cell in the population produces a small amount of virus. The increase in infectious virus is dependent upon DNA synthesis. The simian cells therefore appear to be "leaky" for some human adenovirus serotypes.

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