Enhancement of Adenovirus Growth in African Green Monkey Kidney Cell Cultures by SV40. (29197)

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Mixed infection with simian virus 40 (SV40) and adenovirus 12 can occur in African green monkey kidney (AGMK) cells *in vitro,* and electron micrographs demonstrating both viruses within the nucleus of a single cell have been presented(1). Variable degrees of partial exclusion were produced by altering the time interval between inoculation of SV40 and adenovirus 12, and approximately 40% of the cells contained both viruses when the adenovirus was inoculated 24 hours after SV40(1). Further studies of AGMK cultures infected with both viruses compared with cultures infected with adenovirus 12 only have shown that SV40 enhances the growth of the adenovirus. A similar enhancement of growth of adenovirus 5 has been found when AGMK cultures were infected with SV40 and adenovirus 5.

Materials and methods. Cell Cultures: Primary AGMK cell cultures were obtained from Microbiological Associates, Inc., Bethesda, Md., and primary human embryo kidney cell cultures (HEK) from the Virology Research Resources Branch, National Cancer Institute, through the kindness of Drs. Robert E. Stevenson and Theodore Malinin. Roller tube cultures maintained in a medium composed of 2% fetal bovine serum and 98% mixture 199 and incubated at 36.5°C were used in all experiments.

Viruses: SV40 and adenovirus 12 strains were the same as those previously described (1). Adenovirus 5 was obtained from the American Type Culture Collection. SV40 was grown and titrated in AGMK cell cultures, and adenoviruses 12 and 5 were grown and titrated in HEK cultures. Viruses were titrated by serial 10-fold dilutions with 3 to 5 tubes per dilution. Titrations were observed for 21 days and titers were calculated by the Reed-Muench method.

The amount of adenovirus in the AGMK cultures after infection with both SV40 and adenovirus 12 or adenovirus 5 was determined by titration in HEK cell cultures in which a typical adenovirus-type CPE was produced. Although Shein and Enders have shown that SV40 can produce an incomplete CPE in HEK cell cultures after more than 17 days(2), the cellular degeneration described by them did not resemble the CPE produced by adenovirus 12 and 5, and the inocula they used contained considerably more SV40 than could have been present in the terminal dilutions in our titrations.

Experiments and results. In the first experiment, AGMK cell cultures infected with adenovirus 12 only were compared with similar cultures infected simultaneously with both SV40 and adenovirus 12 (Table I). After 48 hours' incubation, clusters of rounded refractile cells were observed in both the singly and doubly infected cultures although the changes were more extensive in the doubly infected cultures. Uninfected control cultures showed no changes. At 72 and 122 hours, the CPE had progressed in all infected tubes; however, it continued to be more severe and extensive in the doubly infected cultures.

Electron microscopic studies at 72 and 122 hours showed a significant difference between the singly and doubly infected cultures. In the singly infected cultures, although most of the cells manifested morphological changes associated with adenovirus infection such as nuclear enlargement with margination and clumping of chromatin, only a single cell among more than 500 examined contained intranuclear adenovirus particles. In contrast, in the doubly infected cultures, approximately 70% of the cells contained adenovirus particles only, 5% contained SV40 only, and 10% contained both types of virus particles.

Seventy-two hours after inoculation, 3 tubes from each group were frozen and thawed 3 times, pooled and titrated in HEK cultures to determine the amounts of adeno-

TABLE I. Growth of Adenovirus 12 in AGMK Cell Cultures After Single (Adenovirus 12) and Double (Adenovirus 12 + SV40) Infection.

Inoculum	Titer of adenovirus 12 per tube ($TCID_{50}$) Inoculated	72 hr	Cells with adenovirus by electron microscopy* 72 hr	122 hr
Adenovirus 12	$10^{6.5}$	$10^{5.5}$	<1%	0
Adenovirus 12 + SV40 ($10^{7.0}$ $TCID_{50}$)	$10^{6.5}$	$10^{7.2}$	75%	80%

* Includes cells with mixed infection.

virus 12 present. The results of the titrations were consistent with the electron microscopic observations. Tubes infected with $10^{6.5}$ $TCID_{50}$ of adenovirus 12 contained only $10^{5.5}$ $TCID_{50}$ while the tubes infected with the same amount of adenovirus 12 plus $10^{7.0}$ $TCID_{50}$ of SV40 contained $10^{7.2}$ $TCID_{50}$ of adenovirus 12.

In the previously reported experiments with mixed infection, the highest percentage of cells containing both SV40 and adenovirus 12 was found when the inoculation of adenovirus 12 was delayed for 24 hours after inoculation of SV40. A series of AGMK cultures infected with adenovirus 12 was therefore compared with a doubly infected series using this same time sequence for inoculation of the 2 viruses. Both the singly and doubly infected cultures were examined by electron microscopy 22, 24, 44.5, 48, and 65 hours after inoculation of the adenovirus 12 (Table II). No cells containing virus particles were found in the cultures infected with adenovirus 12 only, while, in the doubly infected cultures, intranuclear adenovirus particles were easily identified in 24 hours and, at 44.5 hours, approximately half of the cells had nuclei filled with adenovirus particles. There were also many cells with mixed infection. Although no virus particles were found in the cells of cultures infected with adenovirus 12 only, nuclear enlargement with margination and clumping of chromatin similar to that seen in cells containing virus in the doubly infected cultures was again noted. The number of cells showing these changes and the severity of the changes increased progressively during the 65 hour period of study.

TABLE II. Percent of Cells Containing Adenovirus Particles After Single (Adenovirus 12) and Double (SV40 Followed 24 Hours Later by Adenovirus 12) Infection.

Hr after adenovirus infection	Single infection ($10^{6.5}$ $TCID_{50}$ adenovirus 12)	Double infection ($10^{7.0}$ $TCID_{50}$ SV40 + $10^{6.5}$ $TCID_{50}$ adenovirus 12)
	(%)	(%)
22	0	0
24	0	5
44.5	0	50
48	0	40
65	0	60

Titrations of adenovirus 12 in the singly and doubly infected cultures with the 24 hour interval between inoculation of SV40 and adenovirus 12 again substantiated the electron microscopic observations (Table III).

TABLE III. Growth of Adenovirus 12 in AGMK Cultures After Single (Adenovirus 12) and Double (SV40 Followed 24 Hours Later by Adenovirus 12) Infection.

Inoculum	Titer of adenovirus 12 per tube ($TCID_{50}$) Inoculated	24 hr*	48 hr*
Adenovirus 12	$10^{6.5}$	$10^{4.1}$	$10^{4.4}$
Adenovirus 12 + SV40 ($10^{7.0}$ $TCID_{50}$)	$10^{6.5}$	$10^{4.2}$	$10^{7.5}$
Adenovirus 12	$10^{5.5}$	—	$10^{4.6}$
Adenovirus 12 + SV40 ($10^{7.0}$ $TCID_{50}$)	$10^{5.5}$	—	$10^{7.0}$

* After inoculation of adenovirus 12.

When $10^{6.5}$ $TCID_{50}$ per tube were inoculated, after 24 hours the singly infected tubes contained $10^{4.1}$ $TCID_{50}$ and the doubly infected tubes contained $10^{4.2}$ $TCID_{50}$. After 48 hours the singly infected tubes contained only $10^{4.4}$ $TCID_{50}$ while the doubly infected tubes contained $10^{7.5}$ $TCID_{50}$. Similar enhancement of growth of adenovirus 12 in the presence of SV40 was noted when $10^{5.5}$ $TCID_{50}$ of adenovirus 12 were used as inocula.

TABLE IV. Growth of Adenovirus 5 in AGMK Cultures After Single (Adenovirus 5) and Double (SV40 + Adenovirus) Infection.

Inoculum	Titer of adenovirus 5/tube ($TCID_{50}$) Inoculated	48 hr after adenovirus	Cells with adenovirus by electron microscopy* 48 hr after adenovirus
			(%)
Adenovirus 5	$10^{7.2}$	$10^{7.5}$	0
Adenovirus 5 + SV40 ($10^{7.0}$)	$10^{7.2}$	$10^{9.3}$	60
Adenovirus 5	$10^{7.2}$	$10^{7.2}$	0
SV40 ($10^{7.0}$) followed in 24 hr by adenovirus 5	$10^{7.2}$	$10^{9.3}$	70

* Includes cells with mixed infection.

When adenovirus 5 was substituted for adenovirus 12 in single and double infection experiments, results were similar to those obtained with adenovirus 12 (Table IV). No cells containing adenovirus particles could be found in the singly infected cultures 48 and 72 hours after inoculation. In the doubly infected cultures examined 48 hours after inoculation of the adenovirus, 60 to 70% of the cells contained adenovirus particles either alone or with SV40 particles. As had been observed with adenovirus 12, when the 2 viruses were inoculated simultaneously, most of the cells contained only adenovirus particles while, when the adenovirus 5 was added 24 hours after SV40, mixed infection was present in more than 35% of the cells.

Titrations again showed enhancement of growth of the adenovirus in presence of SV40. In 2 experiments, cultures infected with $10^{7.2}$ $TCID_{50}$ of adenovirus 5 alone contained $10^{7.5}$ and $10^{7.2}$ $TCID_{50}$ after 48 hours. When SV40 was added simultaneously, the cultures contained $10^{9.3}$ $TCID_{50}$ of adenovirus 5 after 48 hours. When the SV40 was inoculated 24 hours before the adenovirus, they also contained $10^{9.3}$ $TCID_{50}$ of adenovirus, 48 hours after inoculation of the adenovirus.

Discussion. Cell-virus systems in which one virus favors the growth of another virus have rarely been described. Kumagai and his associates found that Newcastle Disease Virus (NDV) multiplied faster in cultures infected with hog cholera virus than in normal cultures(3). Hermodsson showed that parainfluenza virus type 3 (PIV-3) increased the growth of NDV in calf kidney cell cultures (4). Our studies have shown that SV40 enhances the growth of adenovirus 12 and adenovirus 5 in AGMK cell cultures.

The mechanisms involved in the enhancement have not been determined. The development of adenovirus-type CPE in the cultures infected with adenovirus only, with electron microscopic changes in the cells suggestive of adenovirus infection but with no morphologically identifiable adenovirus particles, suggests some type of incomplete virus formation. It is possible that infection with SV40 in some way permits incomplete virus formation to go to completion.

Hermodsson has presented evidence that PIV-3 increases the growth of NDV by inhibiting the production and antiviral action of interferon(4). A similar mechanism may also play a role in the effects of SV40 on adenovirus multiplication in AGMK cells.

That mixed infection and enhancement occur with SV40 and adenovirus 5 as well as with SV40 and adenovirus 12 is of interest since there is no evidence that adenovirus 5 is oncogenic. This would suggest that the ability of SV40 and adenovirus 12 to grow together in AGMK cells is not related to their common oncogenic properties.

The possibility that SV40 may have enhanced or interfered with the growth of adenovirus in the HEK cultures used for titration of the progeny of the doubly infected AGMK cultures must be considered. In preliminary experiments, we have found no increase in titer of an adenovirus 12 pool when SV40 was added to it and the mixture was titrated in HEK cultures. Further studies of double infection with SV40 and adenoviruses in human cell cultures and in hamster cell cultures are in progress.

Summary. SV40 enhances the growth of adenovirus 12 in African green monkey kidney (AGMK) cell cultures. In the absence of SV40, adenovirus 12 produces CPE; however, after 72 hours, less than 1% of the cells contain adenovirus particles by electron microscopy, and titrations show no increase in virus.

When similar cultures are infected with both SV40 and adenovirus 12, after 72 hours 75% of the cells contain adenovirus particles and there is an increase in virus by titration. A similar enhancement of the growth of adenovirus 5 in AGMK cultures by SV40 has been observed. The development of progressive adenovirus-type cytopathic changes without demonstrable adenovirus particles in cells of AGMK cultures infected with adenoviruses alone suggests the possibility of some type of incomplete virus formation which is able to go to completion in the presence of SV40.

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Ability of a Fish Cell Line to Support the Growth of Mammalian Viruses. (29198)

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Venezuelan and Eastern equine encephalitis (VEE and EEE) viruses have been propagated in a wide variety of tissue cultures(1, 2,3). Most of these were derived from mammalian or avian sources, although Soret and Sanders(4) reported the growth of EEE virus in aquatic embryonic hosts maintained *in vitro*. Wolf and Quimby(5) recently established a continuous cell culture from tissues isolated from rainbow trout gonads (RTG). RTG cells are markedly different physiologically from mammalian or avian cells in that they (1) are incapable of growing above 25°C, (2) grow optimally at 22°C, and (3) are still capable of proliferating or maintaining themselves at temperatures as low as 4°C. This report deals with the growth and behavior of VEE and EEE viruses in the RTG cell line as compared with that in chick fibroblasts (CF) or L cells under the same cultural conditions.

Materials and methods. Virus. The Trinidad strain of VEE virus(6) and the Louisiana strain of EEE virus(7) were used in these investigations. The virus seeds were prepared from infected chick embryos homogenized as a 10% suspension in heart infusion broth.

Tissue culture. The RTG cell cultures, obtained through the courtesy of Dr. K. Wolf, were grown at 22°C in Eagle's medium supplemented with 10% fetal calf serum. The CF cell cultures were prepared from 10-day-old chick embryos by the trypsinization method of McClain and Hackett(8). The CF and L cell monolayers were propagated at 37°C in the same medium as the RTG cells.

Inoculation of culture. The cell cultures, grown in 4-ounce prescription bottles, were infected with varying doses of VEE or EEE virus that had been diluted in growth medium. Other diluents such as phosphate-buffered saline were toxic to the RTG cultures. After inoculation with virus and an adsorption period of 15 minutes at 22°C, the cultures were washed 3 times, fed with growth medium, and incubated at 22°C unless otherwise specified. Samples were collected after 2 hours of incubation and daily thereafter.

Staining. RTG cell monolayers on 11 × 22 mm coverslips were infected with a virus multiplicity of approximately 30 plaque-forming-units (pfu) per cell. The representative cultures were removed at daily intervals, fixed in absolute methanol, and stained for 20 minutes in a 1:10 dilution of Giemsa.

Virus assay. All samples were assayed on CF cells by the suspended plaque technique described by Brown and Officer(9), with the following modification: An inoculum of 0.05