ADENO-ASSOCIATED SATELLITE VIRUS INTERFERENCE WITH THE REPLICATION OF ITS HELPER ADENOVIRUS

By WADE P. PARKS, Ph.D., ANNA M. CASAZZA,* Sc.D., JUDITH ALCOTT, and JOSEPH L. MELNICK, Ph.D.

(*From the Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas 77025*)

(Received for publication 17 August 1967)

Although all viruses are dependent upon suitable cells for their growth, it has recently come to light that some viruses—called *satellites*—are totally dependent upon other viruses—called *helpers*—for their replication. In a system originally described for tobacco necrosis virus and its satellite, there was both enhancement of the defective virus and interference with the replication of the helper virus by the satellite (1). A similar relationship among animal viruses has recently been reported where, in addition to the enhancement of the defective adeno-associated satellite virus by its helper adenovirus, reduced yields of adenovirus have been noted in cultures coinfected with both viruses (2–5). In our preliminary studies, we noted that this interference was characterized neither by a delayed eclipse period for the helper virus nor by an increased adenovirus particle-to-infectivity ratio (4). This suggested that satellite interference with adenovirus replication was not simply the result of an increased proportion of noninfective adenovirions. A decreased amount of adenovirus complement-fixing antigens noted in cultures coinfected with adenovirus and satellite virus type 1 (2) supported this concept. Further studies of this interference have been carried out in an attempt to characterize the particular interaction in the satellite-adenovirus system, and to determine what relation, if any, interference has with enhancement.

Materials and Methods

Viruses and Sera.—The sources of simian adenovirus SV15 and type 4 satellite virus have been described (4). SV15(0) indicates adenovirus without satellite and SV15(4) indicates a stock containing a mixture of SV15 and type 4 satellite virus. Human adenovirus types 2 and 7 were passages of stock strains from the American Type Culture Collection, Rockville, Md. Serum against type 4 satellite virus was prepared in rabbits by intramuscular inoculation of an emulsion of equal volumes of type 4 satellite purified by twice banding in CsCl (10^{11} particles per milliliter) and complete Freund's adjuvant. On day 21, animals were reinoculated

* Fellow of Consiglio Nazionale delle Richerche on leave from Farmitalia Research Laboratories, Milan, Italy.

intramuscularly with purified virus alone and then were exsanguinated on day 28. The complement-fixing (CF) antibody titer of sera obtained in this manner was 1:1280 against 4–8 CF antigen units of type 4 satellite (6). Antiserum to CsCl-purified SV15(0) was prepared in a similar manner and had a neutralizing antibody titer of 1:20,000 against 100 plaque-forming units (PFU) of SV15 adenovirus. Vesicular stomatitis virus (Indiana), herpes simplex virus (Black), vaccinia (WR strain), and poliovirus (LSc) were also used. The sources of these stocks in our laboratory have been reported (7).

Cell Cultures.—Primary green monkey kidney (GMK) cultures were prepared and maintained as previously described (8). Human fetal kidney (HFK) cells[1] were prepared in a similar fashion except the cells were not used until the third or fourth tissue culture passage at which time the cells still appeared epithelial. Growth medium for HFK was Eagle's medium (9) with 10% fetal bovine serum, antibiotics, and sodium bicarbonate. Maintenance medium was 2% fetal bovine serum in Eagle's medium.

KB IIIA cells are a clonal line kindly supplied by Dr. M. Green, St. Louis University School of Medicine. These cells could either be grown in spinner cultures or as monolayers. 5% fetal bovine serum plus bicarbonate and antibiotics in Eagle's medium was used for growing these cells. BSC-1 cells (stable line of green monkey kidney cells) were supplied from laboratory stocks and maintained as previously described (10).

Virus Assays.—Adenovirus plaque assays in either GMK or HFK were performed as previously described (4, 11). Assays for poliovirus, vaccinia, vesicular stomatitis, and herpes simplex virus were all performed in GMK cell cultures using the same procedures as were used for adenovirus assays in GMK.

Type 4 satellite was assayed by electron microscopy (4) or by hemagglutination (12). Hemagglutination of satellite was found to be optimal at 4°C with human type O erythrocytes. Titrations were made by the microtiter system. A final erythrocyte concentration of 0.33% was employed with an incubation period of 60 min.

Density Gradient Purification of Satellite Virus.—Procedures for concentrating and purifying type 4 satellite virus and SV15 by equilibrium density gradient centrifugation in cesium chloride have been described (4). Purified virus preparations were assayed and used as soon as possible, since on standing in 0.15 M saline a precipitate would often form. All type 4 satellite preparations had a buoyant density of 1.43 g cm^{-3} in CsCl and had a physical particle-to-infectivity ratio of less than 10 when assayed using procedures already reported (4, 13).

Single Cell Isolation Technique.—Procedures were essentially those of Itoh and Melnick (14).

RESULTS

Specificity of Satellite Virus Interference.—Experiments were performed to determine whether type 4 satellite virus interfered with viruses other than adenoviruses. Two DNA-containing viruses (vaccinia and herpes simplex) and two RNA-containing viruses (poliovirus type 1 and vesicular stomatitis virus) were coinfected with type 4 satellite virus in an attempt to demonstrate interference. For this purpose, either satellite virus purified by cesium chloride density gradient banding, or heated (60°C for 15 min) tissue culture preparations containing adenovirus alone, or adenovirus plus satellite were used. The heating

[1] Obtained in part through the Human Tissue Procurement Program, National Cancer Institute.

of the mixed adenovirus and satellite virus preparations completely destroyed adenovirus infectivity and only slightly decreased the satellite virus infectivity as previously reported by Hoggan et al. (2). Adenovirus without satellite, SV15(0), was included as a positive control for satellite-mediated interference.

Experiment.—Virus stocks were diluted to contain between 1 and 20 plaque-forming units per GMK cell. Interfering preparations were SV15(4) and SV15(0) which were heated at 60°C for 15 min. Type 4 satellite virus purified by twice banding in CsCl density gradients was heated at 60°C for 10 min before use. The input multiplicity of type 4 satellite in the SV15(4) preparation was about 1000 virus particles per cell and in the purified stocks was between

TABLE I

Specificity of Type 4 Satellite Virus Interference

Preparation		Virus assayed									
		Poliovirus type 1		Vesicular stomatitis		Vaccinia		Herpes simplex		Adenovirus (SV15)	
		PFU/ml	Fold decrease	PFU/ml	Fold decrease	PFU/ml	Fold decrease	PFU/ml	Fold decrease	PFU/ml	Fold decrease
Satellite* + SV15	Satellite	2.5×10^7	0	4.8×10^7	0	2.7×10^6	2	2.5×10^6	6	6.5×10^6	25
Purified Type 4 Satellite‡	Satellite	—	—	—	—	—	—	1.8×10^7	0	9.0×10^6	17
SV15§	None	—	—	4.3×10^7	0	2.6×10^6	2	1.1×10^7	<2	1.6×10^8	0
None	None	2.5×10^7	—	4.5×10^7	—	7.3×10^6	—	1.5×10^7	—	1.5×10^8	—

* A preparation containing both SV15 and type 4 satellite referred to as SV15 (4) was heated at 60°C for 10 min.

‡ Type 4 satellite purified by twice banding to equilibrium in CsCl and then heated at 60°C for 10 min.

§ Preparation containing an adenovirus SV15 referred to as SV15(0) heated at 60°C for 10 min.

50 and 100 particles per cell. Equal volumes of the virus stocks and the interfering preparation were simultaneously inoculated onto GMK monolayers. After 1 hr adsorption at 37°C, medium was added and cultures were incubated at 37°C for 24 hr. Cultures were then harvested by freezing and thawing, and virus assays were determined by plaque formation in GMK bottle cultures. Suitable controls were included.

From the results shown in Table I, both heated preparations of adenovirus plus satellite, SV15(4), and purified type 4 satellite virus resulted in 17- and 25-fold decreases in adenovirus yields. Heated adenovirus which did not contain satellite, SV15(0), had no interfering effect on the replication of unheated adenovirus. The replication of poliovirus and vesicular stomatitis virus was unaffected by the presence of satellite virus. Vaccinia virus replication was only 3-fold less in the presence of heated SV15(4), the same decrease noted when heated SV15(0) was used. The yield of herpes simplex virus (HSV) was 6-fold

less in the presence of heated SV15(4) than when tested either alone or with heated SV15(0). However, when purified type 4 satellite was employed, no interference with HSV replication was noted. This suggested that the satellite particles with a density of 1.43 g cm^{-3} did not mediate the interference with HSV. As noted above, the same preparation of purified satellite reduced adenovirus yields by 25 times.

Attempts to Detect a Soluble Inhibitor (Interferon) of Adenovirus Replication.—Although type 4 satellite virus failed to significantly inhibit the replication of vesicular stomatitis virus (VSV) and HSV, viruses known to be sensitive to interferon (15), the possibility was considered that satellite virus by itself (nonreplicating cycle) induced a soluble inhibitor specific for adenovirus. Conditions optimal for the production of interferon in other virus-cell systems (16) were employed.

TABLE II

Attempt to Demonstrate a Satellite Virus-Induced Soluble Interfering Substance Specific for Adenovirus

Treatment of cells prior to adenovirus inoculation	Adenovirus titer (PFU/ml) at 48 hr postinoculation
24 hr supernate from satellite infected culture	1.7×10^7
24 hr supernate from control cell culture	1.1×10^7
48 hr supernate from adenovirus infected culture*	2.2×10^7
48 hr supernate from adenovirus plus satellite infected culture*	2.0×10^7
None	3.0×10^7

* Tissue culture supernate centrifuged at 78,000 × *g* for 3 hr.

Experiment.—BSC-1 cultures were infected with type 4 satellite virus purified by cesium chloride density gradient centrifugation (about 100 particles per cell). After 1 hr adsorption at 37°C, medium was added to the culture, and incubation was continued for 24 hr at 37°C. The supernatant fluid from the infected and uninfected cultures was then transferred to other BSC-1 cultures without dilution and incubated for an additional 24 hr. Cultures were drained and infected with adenovirus SV15(0) at a multiplicity of 20 per cell, and after adsorption for 1 hr at 37°C medium was then added. Cultures were harvested 48 hr after adenovirus infection and adenovirus was titrated in GMK cell cultures. Tissue culture fluids from monkey kidney cell cultures infected 48 hr previously with either adenovirus or with adenovirus plus satellite were centrifuged at 74,000 *g* for 3 hr and the supernatant fluid tested as above for interference with adenovirus replication.

The results of such an experiment, presented in Table II, failed to demonstrate any significant reduction in adenovirus production in cultures pretreated with supernatant fluids from satellite virus–infected cultures. When adenovirus was added to the same satellite-infected cultures from which the original supernatant fluids had been removed, the 24 hr adenovirus yields were reduced by

more than 95% when compared with the yields from cells which had not been infected with satellite virus. Adenovirus replication was also not significantly reduced by pretreatment with supernatant fluids from cultures infected with either adenovirus or adenovirus plus satellite virus. These data strongly suggested that a soluble inhibitor produced in the course of satellite virus interaction with cells was not responsible for interference with adenovirus replication.

Effect of Treatment of Satellite Virus on Interference.—Although we could demonstrate interference with adenovirus using satellite particles with a buoyant density of 1.43 g cm^{-3}, it was of interest to determine whether this interference could be effected by virus subjected to inactivation by physical or immunologic means.

Experiment.—Type 4 satellite virus purified by banding to equilibrium in CsCl density gradients was diluted to contain approximately 10^8 particles per milliliter. Aliquots of this preparation were then subjected to various treatments. Virus was mixed with specific type 4 rabbit antiserum that had been diluted 1:10, and incubated 1 hr at 37°C. Another aliquot was exposed to ultraviolet light irradiation (12 inches from a Westinghouse germicidal lamp) for 5 min. A third aliquot was heated in a water bath at 80°C for 30 min. Each aliquot was then mixed with a dilution of adenovirus calculated to contain 59 plaque-forming units per 0.1 ml and 0.2 ml was inoculated onto monolayer cultures of GMK cells. All inocula were adsorbed 1 hr and then overlaid with 1.5% agar containing 2% fetal bovine serum in Eagle's medium. Plaques were counted after 7 days of incubation.

TABLE III

Effect of Various Pretreatments of Satellite Virus Preparations on Their Capacity to Reduce Adenovirus Plaque Formation

Pretreatment of satellite	No. adenovirus plaques formed	Percentage Reduction
Antiserum (1 hr at 37°C)	64	9
UV irradiation (5 min)	70	2
Heating (80°C for 30 min)	76	0
None	2	96
Control	**No. adenovirus plaques formed**	**Percentage Reduction**
Adenovirus alone	71	—

As shown in Table III, satellite preparations which inhibited greater than 90% of plaque formation by adenovirus could be rendered noninhibitory by treatment with specific type 4 satellite antiserum which had no plaque-reducing effect on the helper adenovirus (but had a complement fixation titer of 1280 against four antigen units of purified type 4 satellite). Other treatments which effectively abolished the interfering capacity of satellite preparations included ultraviolet irradiation and heating at 80°C for 30 min. Heating the same prep-

aration at 60°C for 15 min did not significantly alter the interfering potency or the infectivity. These data suggest that (*a*) the interference is mediated directly through the satellite particle and (*b*) the interference requires infectious satellite virus.

Quantitation of Adenovirus Required for Interference.—Although satellite virus

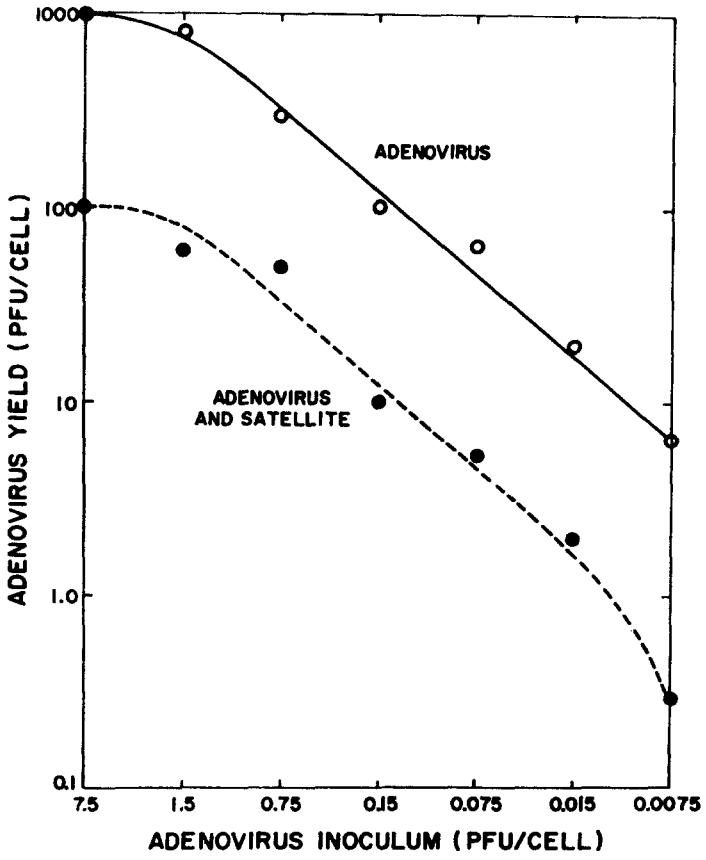

FIG. 1. Effect of adenovirus input on interference in monkey kidney cells.

particles seemed responsible for the interference, it was also possible that the degree of interference could be altered by varying the input of adenovirus.

Experiment.—Adenovirus was diluted to contain 7.5 to 0.0075 plaque-forming units per cell and replicate cultures of GMK were infected in the presence and absence of type 4 satellite virus (200 particles per cell). To allow for only one cycle of replication, cultures were frozen 24 hr after infection, and the harvests assayed for adenovirus plaque formation on monkey kidney monolayers. Since adenovirus production approaches a plateau 24–36 hr after infection (4), adenovirus yields were somewhat reduced per cell when frozen at 24 hr.

24 hr yields of adenovirus from cultures infected with varying dilutions of SV15(0) in the presence of a constant amount of satellite virus are shown in Fig. 1. A constant degree of interference was noted when adenovirus yields in the presence of satellite virus were compared to adenovirus single-cycle yields of satellite-free cultures. Although maximal yields of SV15 are reached by 24–36 hours (4, 17), experiments were performed in which cultures coinfected

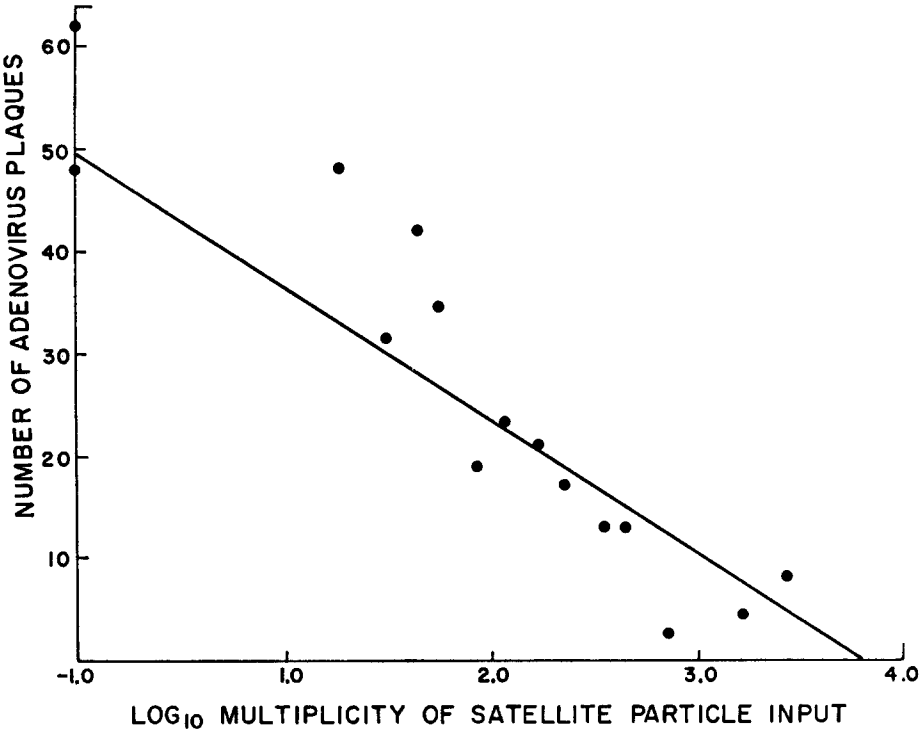

FIG. 2. Effect of satellite virus concentration on adenovirus plaque count.

with adenovirus and satellite were held for varying periods up to 120 hr after inoculation, and the same degree of interference was noted at each time interval tested after 24 hr. Under conditions where all cells were coinfected with adenovirus and satellite virus at the time of inoculation, reduction in adenovirus cytopathic effect was usually not noted. Titrations of such harvests for adenovirus properties revealed a proportionate, approximately 90%, reduction in plaque-forming units (Table I), adenovirus particles, and complement-fixing antigen. When low concentrations of adenovirus inoculum (0.01 PFU per cell) were used, conditions for multiple cycles of adenovirus replication existed; in the presence of high satellite virus concentration, a reduction in adenovirus

cytopathic effect was noted. However, if such cultures were not harvested until the adenovirus cytopathic effect was complete (usually 48–72 hr after the adenovirus cultures without satellite), approximately the same reduction in adenovirus infectivity was found when compared to the satellite-free adenovirus multiple cycle yields. The yields of adenovirus per cell in multiple cycle replication experiments were less than those obtained in single cycle experiments, presumably in part because of heat inactivation of released virus, but also because of a reduced quantity of adenovirus produced per cell in older GMK cultures (Parks, unpublished observations).

Amount of Satellite Virus Required for Interference.—It was shown above that in green monkey kidney cultures the major determinant of interference was the satellite virus itself. The kinetics of satellite interference were examined to determine the relative number of satellite particles capable of producing interference.

Experiment.—Various dilutions of SV15(4) heated at 60°C for 15 min were inoculated onto GMK monolayers in 1 oz bottle cultures together with a constant dose of adenovirus (48 PFU). After 1 hr incubation at 37°C, the cultures were overlaid. After 7 days, adenovirus plaques were recorded. The line in Fig. 2 represents the theoretical curve for a linear fit relating satellite concentration and adenovirus plaque count. Each point represents the average of four separate experiments.

As shown in Fig. 2,[2] in the presence of a constant amount of adenovirus inoculum, variation in the multiplicity of satellite input was found to alter the production of adenovirus plaque formation. When low concentrations of satellite virus (<40 physical particles per cell) were inoculated, the number of adenovirus plaques was equal to the counts in cultures without satellite. Increasing the satellite concentration by 100–1000 times resulted in 90–95% reduction in plaque formation. The line in the figure represents theoretical values if a linear fit was operable. Theoretical curves relating satellite concentration and adenovirus plaque counts using Poisson or exponential distributions gave a statistically poor fit with the observed data. The linear relationship for four separate experiments had a correlation coefficient of 0.8596 with the observed data. Therefore, the degree of interference seems to be directly proportional to satellite concentration over the range tested.

Eclipsing of Adenovirus in Presence of Its Satellite.—In order to determine the event during the adenovirus replication cycle at which the satellite exerts its interfering effect, experiments were performed to determine if satellite virus interfered with adenovirus adsorption, penetration, or eclipse. The first two of these steps have been shown to be the sites of interference in other virus systems (18, 19). In order to test viral eclipsing, it is important that cells be infected simultaneously. One method of initiating simultaneous infection was to expose cells to a high multiplicity of adenovirus for a short time and then to repeatedly

[2] We are grateful to Dr. Allan Levy and his associates at the Data Processing Facility of the Baylor University College of Medicine for statistical evaluation of these experiments.

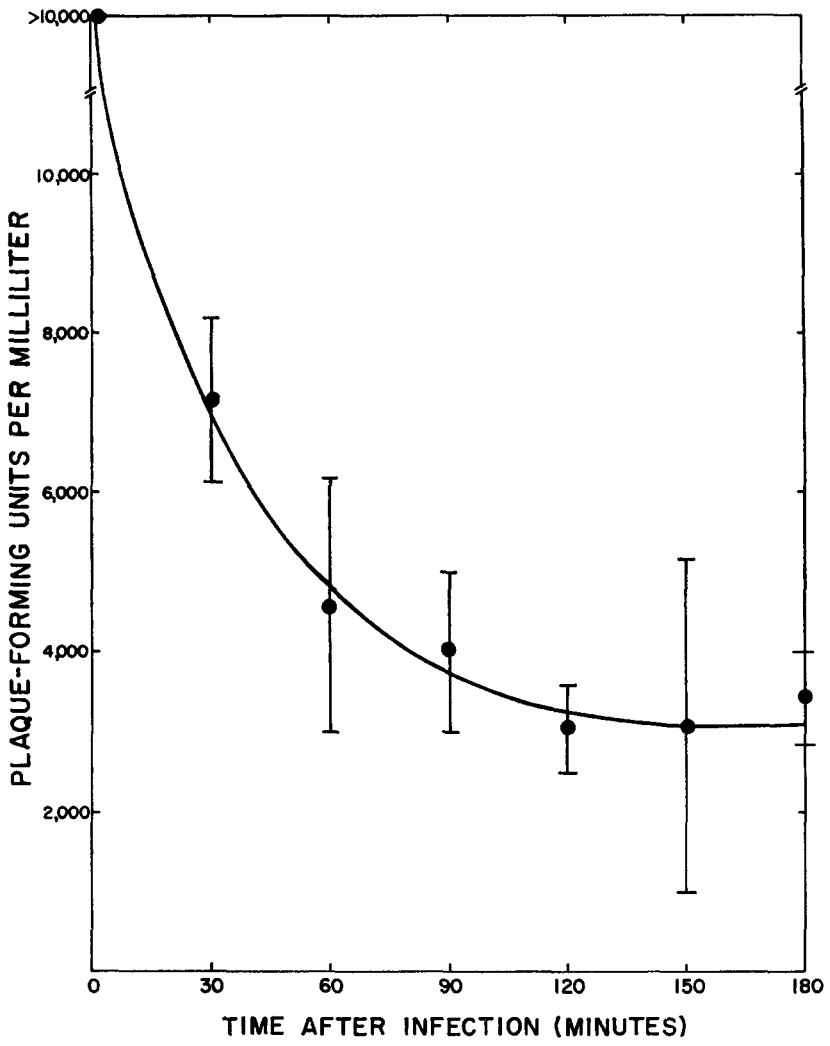

FIG. 3. Cultures were infected with type 4 satellite virus 2 and 24 hr before adenovirus infection. Control cultures were not infected with satellite. All three sets of curves were similar and the bars represent the ranges of values for all sets. Only a curve of the mean is shown. See text for experimental details.

dilute the virus-cell mixture in order to reduce the likelihood of subsequent viral attachment. To eliminate errors of interpretation involving simultaneous adsorption of satellite and adenovirus to the same cells, cultures were preinfected with satellite 2 and 24 hr prior to adenovirus infection, conditions which resulted in the same degree of interference noted when cultures were infected simultaneously (4).

Experiment.—Primary green monkey kidney cells (10^7 cells) were infected with CsCl-banded type 4 satellite virus (1000 particles per cell) and dispersed by trypsinization 2 and 24 hr later. The satellite-infected cells and uninfected cells in suspension were then concentrated by low-speed centrifugation at 1000 *g* for 10 min. After being resuspended in a small volume (1 ml), the cells were infected with CsCl-banded SV15(0) at a multiplicity of 100 PFU per cell, adsorbed for 5 min in a 37°C water bath, diluted to 10 ml with growth medium and then recentrifuged. The cell pellets were again resuspended in 10 ml and recentrifuged. After repeating the cycle once more, the cells were distributed in seven equal samples and placed in a 37°C water bath to allow eclipsing to proceed. Aliquots were frozen at the various times indicated in Fig. 3, and all three sets were assayed for adenovirus plaque-forming units in GMK cultures. The bars represent the absolute variation of adenovirus plaque counts during the eclipse period both in the presence and in the absence of type 4 satellite virus. Because all three curves were similar, only a curve of the mean is shown.

The kinetics of adenovirus eclipsing are shown in Fig. 3. The eclipsing of adenovirus in the absence of satellite virus was the same as noted in cells coinfected with satellite virus. The reduction in plaque counts is presented in absolute counts to eliminate the possibility that the degree of eclipsing and not the kinetics might be involved. The demonstration that adenovirus eclipsing occurs similarly in satellite-infected or satellite-free cultures indicated that an intracellular event occurring after the eclipse of adenovirus infectivity is involved in the interference phenomenon.

Temporal Pattern of Satellite Virus Interference with Adenovirus Replication.—Experiments were carried out to determine if the addition of satellite virus at different times after adenovirus infection would affect the degree of interference.

Experiment.—Type 4 satellite virus purified by equilibrium density gradient centrifugation in CsCl was added (700 particles per cell) at different times after adenovirus infection, adsorbed 1 hr at 37°C on drained monolayers, washed three times with medium, and then incubated at 37°C. All cultures were washed three additional times 12 hr after adenovirus infection and were frozen 24 hr after adenovirus infection. The titer of the adenovirus in the absence of satellite virus was 5.5×10^7 plaque-forming units per milliliter.

From the data shown in Fig. 4, it was observed that between 6 and 8 hr after adenovirus inoculation, adenovirus infectivity was no longer reduced by satellite virus. The yields of satellite virus harvested at 36 hr after adenovirus infection were unchanged in the presence and absence of adenovirus interference; the satellite hemagglutination titers were between 1280 and 2560 units per 0.05 ml. This experiment provided additional evidence for the previous conclusion that the interfering effect was occurring after the initiation of the eclipse period, since by 6 hr adenovirus infectivity had decreased to minimum.

The development of resistance of adenovirus to satellite interference occurs at the time in the adenovirus infection cycle when viral deoxyribonucleic acid biosynthesis commences (17). Further studies will be necessary to determine whether this step or subsequent ones in the adenovirus replication cycle are interfered with by satellite virus. Nevertheless, in these experiments and in

previously reported experiments (4), interference with adenovirus replication could be separated from satellite virus enhancement by adding satellite virus after the course of adenovirus infection was well along in time.

Single Cell Analyses of Adenovirus Yields in the Presence of Satellite Virus.—

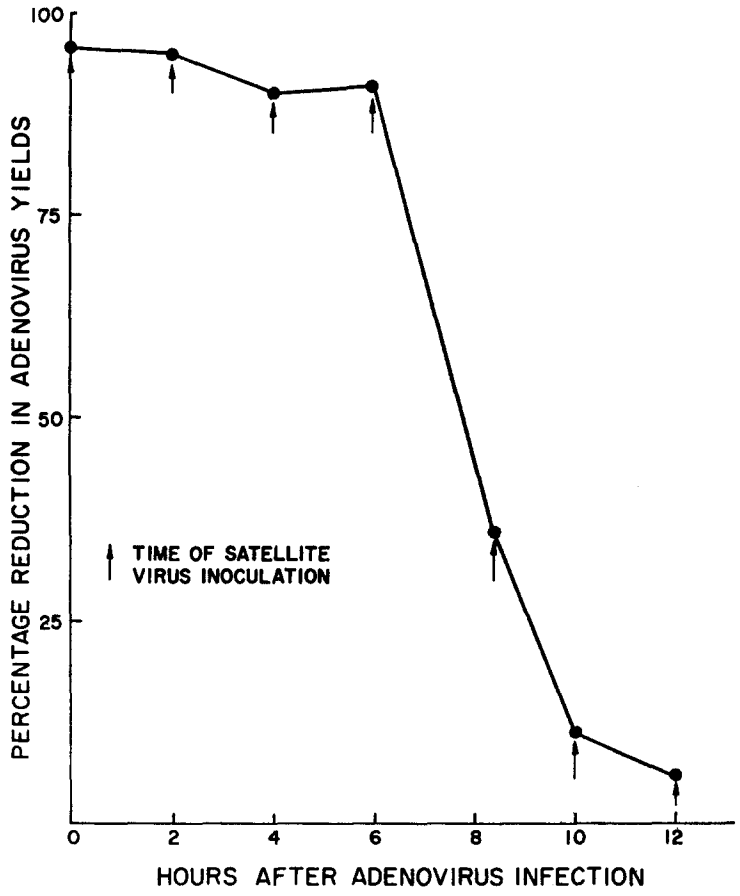

FIG. 4. Escape of adenovirus from interfering effect of satellite. Reduction in adenovirus yield failed to occur if the satellite was added 8 hr after its helper adenovirus.

To determine whether the approximately 90% reduction in adenovirus yields in the presence of satellite virus was the result of a uniform reduction of virus synthesis in the great majority of infected cells or the net result of many cells producing no adenovirus and only a few producing normal or increased amounts, single cell analyses were done. Two types of experiments were employed for these studies. First, we attempted to determine the proportion of cells plating

as infectious centers in cultures coinfected with both adenovirus and satellite virus. This proportion was compared to the results from cultures infected with adenovirus alone.

Experiment.—Primary GMK monolayers were inoculated with simian adenovirus SV15(0) at an input muliplicity of 10 PFU per cell in the absence and presence of type 4 satellite virus (100 particles per cell). After 1 hr adsorption at 37°C, the cells were washed three times and dispersed with 0.25% trypsin. After 15 min trypsinization, the cells were suspended in 10% fetal bovine serum in Eagle's medium and then counted in a hemacytometer. The cell suspension was then mixed with an equal volume of rabbit antiserum containing 20 antibody units to SV15(0). This mixture was incubated 30 min at 37°C. Dilutions were made to contain various numbers of cells per inoculum. For the first method—agar overlay—cells were inocu-

TABLE IV

Three Types of Assays to Demonstrate the Proportion of Adenovirus-Infected Cells Plating as Infectious Centers

A. Agar overlay.

Inoculum	Calculated cell input	Plaque count	Percentage plating
SV15 only	48	22	46
SV15 plus type 4 satellite	46	19	41

B. Cell isolation—fluid overlay and isolated cell harvest.

Inoculum	Cell isolation procedure			
	Fluid overlay		Isolated cell harvest	
	ID_{50}	SV15(4)/SV15(0)	ID_{50}	SV15(4)/SV15(0)
SV15 only	2.5		1.6	
SV15 plus type 4 satellite	2.5	1.0	6.0	3.7

lated onto drained monolayers of 1 oz bottle cultures of GMK cells, adsorbed for 1 hr at 37°C, and then overlaid with agar. The number of plaques counted at 7 days as compared to the calculated number of cells per inoculum provided the percentage of cells plaquing as infectious centers. For the second method—fluid overlay—various dilutions of infected cells were inoculated into tubes with monolayers of GMK cells. These tubes were then scored for adenovirus cytopathic effect for the next 14 days. The ID_{50} was calculated by the Reed and Muench formula (20) as described (21). Similarly in the third method, infected cells were first isolated in single drops (0.05 ml) in Petri dishes, covered with oil, and incubated at 37°C for 48 hr. The drops were then harvested by freezing and thawing and assayed for adenovirus infectivity in GMK monolayers. ID_{50} was calculated as above.

As indicated in Table IV, where three different methods of determining the number of infected cells were used, the proportion of cells producing adenovirus was similar in the presence and absence of satellite virus. The values presented are from representative experiments. As indicated in Fig. 1, where adenovirus

yields from monolayer cultures are shown, alteration of adenovirus multiplicity of input had no significant effect on the number of cells capable of producing adenovirus (infectious centers).

The second set of experiments involved measuring the production of adenovirus in isolated cells in the presence and absence of satellite virus.

Experiment.—Cultures were infected with SV15(0) at a multiplicity of 7 PFU per cell in the presence and absence of type 4 satellite virus heated at 60°C for 15 min. After 1 hr adsorption at 37°C, the cells were dispersed by trypsinization, counted in a hemacytometer, and diluted in medium to contain a calculated 1.5–3.3 cells per 0.05 ml (1 drop). Each drop was placed in the bottom of a plastic Petri dish, covered with saline-saturated sterile mineral oil, and incubated at 37°C. The dish was frozen 24 hr postinoculation and the drops collected after thawing. Plaque-forming titers of 1:20 dilutions of individual drops were determined in GMK monolayers.

TABLE V

Adenovirus Production in Isolated Cells with and without Satellite Virus

Inoculum	Exp	Adenovirus yield from single cells		
		Average PFU per virus-positive drop	Range of PFU	Percentage reduction
SV15(0)	1	400	20–1420	—
	2	1100	60–3520	—
SV15(0) plus type 4 satellite	1	40	20–100	90
	2	90	20–200	92

Table V indicates the adenovirus plaque-forming units (PFU) produced per drop in two separate experiments. The actual counts per cell are somewhat lower than yields from cells in a monolayer culture where yields of 7000 PFU per cell were noted (4). Nevertheless, the percentage reduction noted (90% and 92%) was very similar to the magnitude of adenovirus reduction noted in monolayer cultures. This strongly indicates that, in the present cell-virus system, the mechanism of satellite virus interference was a relatively uniform and marked depression of formation of infectious adenovirions in the majority of cells in the culture.

Interference with Other Adenoviruses in Different Cell Systems.—In attempting to study interference induced by satellite type 4 with other adenovirus "helpers," we tested two other cell systems which allow the replication of human adenoviruses types 2 and 7.

Experiment.—Type 4 satellite virus purified by equilibrium centrifugation in cesium chloride was diluted to contain about 150 particles per cell. The satellite virus inoculum was mixed with an equal volume of 5–20 PFU per cell of adenoviruses types 2 and 7 (human) and SV15 (simian). Sodium chloride (0.15 M) was the diluent for cultures inoculated with adenovirus in the absence of satellite. After 2 hr adsorption in HFK, KB, and GMK at 37°C, 2% fetal

bovine serum in Eagle's medium was added to the cultures. After 48 hr incubation at 37°C, the cultures were frozen and assayed for adenovirus infectivity by plaque formation.

These studies are presented in Table VI. Adenovirus types 2 and 7 as well as SV15 were relatively insensitive to the satellite interference when tested in human fetal kidney cells. Simultaneous control experiments in GMK cells with SV15 and satellite revealed a 98% reduction in adenovirus production. When tested in KB cells, adenovirus type 7 production was reduced by 65% and SV15 was decreased by 96%. Since little or no replication of human adenovirus types 2 and 7 occurs in GMK cells (22, 23), it was of interest that the 72 hr harvests of these viruses revealed no difference in titers in the presence and

TABLE VI

Effect of Cell Systems on Satellite Virus Interference with Three Different Adenoviruses

Virus tested		Cell system tested for interference					
		Human fetal kidney		KB III A		GMK	
		PFU/ml	Reduction	PFU/ml	Reduction	PFU/ml	Reduction
			%		%		%
Adenovirus type 2	No satellite	2.8×10^8		N.D.*		6.5×10^6	
	With satellite	4.6×10^8	—	N.D.	—	6.0×10^6	8
Adenovirus type 7	No satellite	7.5×10^8		1.5×10^7		3.3×10^6	
	With satellite	6.0×10^8	20	5.2×10^6	65	2.8×10^6	15
SV 15(0)	No satellite	4.1×10^7		9.0×10^7		1.5×10^8	
	With satellite	3.5×10^7	15	3.5×10^6	96	2.6×10^6	98

* N.D., not done.

absence of satellite. These data suggest that *cell type* is a definite determinant of satellite-induced interference and that the *adenovirus serotype* may also be of importance.

DISCUSSION

Several virus-virus interactions have been described which are characterized by noninterferon-mediated interference (24–28). The findings reported in the present work are in agreement with those of Casto et al. (5) in that the interference noted with adenovirus replication is not mediated by interferon. It was previously reported that adenovirus SV15 particle-to-infectivity ratios were not significantly altered in cultures containing type 4 satellite virus (4). Hoggan et al. (2) also reported a reduced production of adenovirus type 7 complement-fixing antigens in human kidney cells infected with satellite virus type 1. Smith

et al. (3) suggested that an altered particle-to-infectivity ratio may have accounted for the interference noted, but their adenovirus assays were performed using the tube dilution technique in the presence of satellite virus, a procedure which may give erroneously low adenovirus infectivity titers (5).

Casto et al. (5) proposed that the interference noted was the result of a greatly reduced number of cells producing adenovirus, but that the producing cells yielded normal amounts of adenovirus. Their findings are not inconsistent with electron microscopic studies of doubly infected cells which were interpreted as having predominantly adenovirus or satellite particles (29–31). However, both the linearity of interference with decreasing inputs of adenovirus and the single cell analyses reported in the present study indicate a different mechanism. Specifically, cells infected with either adenovirus alone or with adenovirus and satellite virus produced a comparable percentage of infectious centers, yet only 10% as much adenovirus was produced in cells infected with satellite virus as in cells infected only with adenovirus. This strongly suggests that in our system, interference resulted in infected cells producing fewer adenovirions.

The relative specificity of satellite virus interference for adenovirus, the inability to demonstrate a soluble adenovirus inhibitor in the present study and the absence of a prolonged or altered lag period (4) suggest that interferon was not involved in satellite virus interference. Other examples of noninterferon-mediated interference between DNA-containing viruses include those reported by Khoobyarian and Fischinger (32) and by Roizman (24). The former authors reported that heated adenovirus type 2 interfered with vaccinia virus replication; they presented quantitative data similar to those in the present paper. Roizman (24) analyzed a system involving herpes simplex mutants in dog kidney cells and concluded that interference with an extended host range mutant might be mediated by the nonfunctional aggregation of proteins specified by the defective mutant. He reported that maximal enhancement of the defective virus resulted when there was minimal interference with the helper virus, and vice versa. This hypothesis was suggested as a mechanism for viral interference between related viruses.

When interference with the "helper" virus is accompanied by enhancement of the interfering virus, as in the herpes model and in the adeno-associated satellite system, it seems logical to consider some form of competition as the probable mechanism. Although we have been able clearly to separate enhancement from interference (4), this does not exclude the possibility of competition for adenovirus-specified products as the mechanism of interference, since the products might be at relatively low concentrations early in the replicative cycle of adenovirus and in excess later. Whether interference occurs at the stage of formation of progeny DNA or of the subsequent formation of viral capsid protein, or both, is not known. The temporal pattern of interference suggests that a relatively late event in adenovirus replication is involved both in inter-

ference and enhancement. Precisely how closely the two phenomena are related as to mechanism will require further study.

SUMMARY

Adeno-associated satellite virus type 4 interferes with the replication of its helper adenovirus. No interferon-like soluble substance could be detected in satellite-infected cultures and other DNA- and RNA-containing viruses were not inhibited by coinfection with satellite virus under conditions which reduced adenovirus yields by more than 90% in monkey cells. Altering the concentration of adenovirus in the presence of constant amounts of satellite resulted in a constant degree of interference over a wide range of adenovirus inocula and suggested that adenovirus concentration was not a significant factor in the observed interference. The interference with adenovirus replication was abolished by pretreating satellite preparations with specific antiserum, ultraviolet light or heating at 80°C for 30 min. This suggested that infectious satellite virus mediated the interference. Satellite virus concentration was found to be a determinant of interference and studies indicated that the amount of interference with adenovirus was directly proportional to the concentration of satellite virus.

8 hr after adenovirus infection, the replication of adenovirus was no longer sensitive to satellite interference. This was true even though the satellite virus was enhanced as effectively as if the cells were infected simultaneously with both viruses.

Interference with adenovirus infectivity was accompanied by reduced yields of complement-fixing antigen and of virus particles which suggested that satellite virus interfered with the formation and not the function of adenovirus products. When cells were infected either with adenovirus alone or with adenovirus plus satellite, the same proportion of cells plated as adenovirus infectious centers. However, the number of plaque-forming units of adenovirus formed per cell in the satellite-infected cultures was reduced by approximately 90%, the same magnitude of reduction noted in whole cultures coinfected with satellite and adenovirus. This suggested that all cells infected with the two viruses were producing a reduced quantity of adenovirus.

Supported in part by Public Health Service grants AI 05382 and 5 T1 AI 74, from the National Institute of Allergy and Infectious Diseases, and by grant CA 04600, from the National Cancer Institute, National Institutes of Health, Bethesda, Md.

BIBLIOGRAPHY

1. Kassanis, B. 1962. Properties and behavior of a virus depending for its multiplication on another. *J. Gen. Microbiol.* **27**:477,
2. Hoggan, M. D., N. R. Blacklow, and W. P. Rowe. 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. *Proc. Natl. Acad. Sci. U.S.* **55**:1467.

3. Smith, K. O., W. P. Gehle, and J. F. Thiel. 1966. Properties of a small virus associated with adenovirus type 4. *J. Immunol.* **97**:754.
4. Parks, W. P., J. L. Melnick, R. Rongey, and H. D. Mayor. 1967. Physical assay and growth cycle studies of a defective adeno-satellite virus. *J. Virol.* **1**:171.
5. Casto, B. C., R. W. Atchison, and W. McD. Hammon. 1967. Studies on the relationship between adeno-associated virus type 1 (AAV-1) and adenoviruses. I. Replication of AAV-1 in certain cell cultures and its effect on helper adenovirus. *Virology.* **32**:52.
6. Melnick, J. L. 1968. Serology of viruses. *In* Analytical Serology of Microorganisms. J. B. G. Kwapinski, editor. J. Wiley & Sons, Inc., New York. In press.
7. Wallis, C., J. L. Melnick, and F. Rapp. 1965. Different effects of $MgCl_2$ and $MgSO_4$ on the thermostability of viruses. *Virology.* **26**:694.
8. Melnick, J. L. 1956. Tissue culture methods for the cultivation of poliomyelitis and other viruses. *In* Diagnostic Procedures for Virus and Rickettsial Diseases. T. Francis, chairman of editorial committee. American Public Health Assn., New York. 97.
9. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science.* **130**:432.
10. Hopps, H. E., E. L. Bernheim, A. Nisalak, J. H. Tjio, and J. E. Smadel. 1963. Biologic characteristics of a continuous kidney cell line derived from African green monkey. *J. Immunol.* **91**:416.
11. Boeyé, A., J. L. Melnick, and F. Rapp. 1966. SV40-adenovirus "hybrids": presence of two genotypes and the requirement of their complementation for viral replication. *Virology.* **28**:56.
12. Ito, M., and H. D. Mayor. 1967. Hemagglutinin of type 4 adeno-associated satellite virus. *J. Immunol.* In press.
13. Ito, M., J. L. Melnick, and H. D. Mayor, 1967. An immunofluorescence assay for studying replication of adeno-satellite virus. *J. Gen. Virol.* **1**:199.
14. Itoh, H., and J. L. Melnick, 1959. Double infections of single cells with echo 7 and coxsackie A9 viruses. *J. Exptl. Med.* **109**:393.
15. Wagner, R. R. 1965. Interferon. A review and analysis of recent observations. *Am. J. Med.* **38**:726.
16. Merigan, T. C., D. F. Gregory, and J. K. Petralli. 1966. Physical properties of human interferon prepared *in vitro* and *in vivo*. *Virology.* **29**:515.
17. Kit, S., L. J. Piekarski, D. R. Dubbs, R. A. de Torres, and M. Anken. 1967. Enzyme induction in green monkey kidney cultures infected with simian adenovirus. *J. Virol.* **1**:10.
18. Crowell, R. L. 1963. Specific viral interference in HeLa cell cultures chronically infected with coxsackie B5 virus. *J. Bacteriol.* **86**:517.
19. Steck, F. T., and H. Rubin. 1965. The mechanism of interference between an avian leukosis virus and Rous sarcoma virus. II. Early steps in infection by RSV of cells under conditions of interference. *Virology.* **29**:642.
20. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent end-points. *Am. J. Hyg.* **27**:493.
21. Rawls, W. E., and J. L. Melnick. 1966. Rubella virus carrier cultures derived from congenitally infected infants. *J. Exptl. Med.* **123**:795.

22. O'Conor, G. T., A. S. Rabson, I. K. Berezesky, and F. J. Paul. 1963. Mixed infection with simian virus 40 and adenovirus 12. *J. Natl. Cancer Inst.* **31**:903.
23. Feldman, L. A., J. L. Melnick, and F. Rapp. 1965. Influence of SV40 genome on the replication of an adenovirus-SV40 "hybrid" population. J. Bacteriol. **90**: 778.
24. Roizman, B. 1965. Abortive infection of canine cells by herpes simplex virus. III. The interference of conditional lethal virus with an extended host range mutant. *Virology.* **27**:113.
25. Pereira, H. G. 1960. A virus inhibitor produced in HeLa cells infected with adenovirus. *Virology.* **11**:590.
26. Galasso, G. J., and D. G. Sharp. 1964. Relative plaque-forming, cell-infecting and interfering qualities of vaccinia virus. *J. Bacteriol.* **88**:433.
27. Huang, A. S., and R. R. Wagner. 1966. Defective T particles of vesicular stomatitis virus. II. Biologic role in homologous interference. *Virology.* **30**:173.
28. Sreevalsan, T., and R. Z. Lockhart. 1966. Interference not mediated by interferon. *Nature.* **212**:540.
29. Atchison, R. W., B. C. Casto, and W. McD. Hammon. 1966. Electron microscopy of adenovirus-associated virus (AAV) in cell cultures. *Virology.* **29**:353.
30. Archetti, I., E. Bereczky, and D. S. Bocciarelli. 1966. A small virus associated with the simian adenovirus SV11. *Virology.* **29**:671.
31. Mayor, H. D., M. Ito, L. E. Jordan, and J. L. Melnick. 1967. Morphological studies on the replication of a defective satellite virus and its helper adenovirus. *J. Natl. Cancer Inst.* **38**:805.
32. Khoobyarian, N., and P. J. Fischinger. 1965. Role of heated adenovirus 2 in viral interference. *Proc. Soc. Exptl. Biol. Med.* **120**:533.