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Interference Between Two Adeno-associated Satellite Viruses: a Three-Component System

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Adenovirus-associated satellite viruses interfere with the replication of their helper adenoviruses. According to a previous report, this interference is not mediated by interferon. A three-component system comprising simian adenovirus SV15 and satellites types 1 and 4 was studied to determine whether satellite viruses also interfere with one another. Satellite type 1 interfered with the replication of type 4 and vice versa. The degree of interference was directly proportional to the dose of interfering satellite. The events leading to mutual satellite interference were operative during the first 12 hr of replication, the period associated with active synthesis of viral deoxyribonucleic acid.

Adeno-associated satellite viruses are dependent on the presence of replicating adenoviruses in order to complete their growth cycles in susceptible cells (1, 6, 14). Casto, Atchison, and Hammon (3) showed that, during the course of type 1 satellite virus replication in primary cell cultures of swine, human, mouse, hamster, monkey, and calf kidney, the yield of helper adenovirus was decreased. The reduction in adenovirus yield was directly related to the amount of satellite virus present and inversely related to the amount of adenovirus used as challenge. Parks et al. (13) also reported that type 4 satellite virus interfered with the replication of its helper adenovirus. It was shown that the interference was not mediated through an interferon-like mechanism but that potentially infective satellite particles were necessary. In addition, they showed that the extent of interference was directly proportional to the concentration of satellite virus and that 8 hr after adenovirus infection the cycle was no longer sensitive to satellite interference. Currently there are four antigenically distinct serotypes of satellite viruses associated with adenoviruses of man and monkeys (2, 11, 15). All four types can replicate efficiently in cultured cells from a variety of hosts, providing that fully competent adenovirions are also present. In recent studies, we noted that cells inoculated with type 4 satellite sometimes produced less than optimal yields. Subsequently, it was found that these cells were already harboring a satellite virus of another serotype. These observations suggested that satellite viruses might interfere not only with adenovirus replication, but also with one another. The following experimental approach developed from these preliminary observations.

MATERIALS AND METHODS

Cells. Primary African green monkey kidney (GMK) cells were grown in Melnick's medium A supplemented with 2% fetal bovine serum and were maintained in medium B.

Viruses and assays. The source of our stock of satellite-free adenovirus SV15 and adeno-associated satellite viruses types 1 and 4 has been described (8). Infectivity titers of SV15 stock prepared in an established cell line (BSC-1) derived from GMK ranged from $10^{5.5}$ to $10^{7.5}$ TCD_{50} per 0.1 ml in primary GMK tube cultures. Satellite types 1 and 4 were prepared in BSC-1 cells in the presence of helper SV15. Before assay, adenovirus activity was destroyed by heating for 15 min at 60 C. The infectivity titer of type 1 was $10^{6.4}$ complement-fixing antigen-producing units (CFU) per 0.1 ml. The infectivity titer of type 4 ranged from $10^{5.5}$ to $10^{6.8}$ hemagglutinin-producing units (HAU) per 0.1 ml (8).

Infectivity of SV15 adenovirus was assayed by plaque titration on GMK monolayer cultures as described previously (14). The hemagglutination procedure was carried out as previously described (8), except that type B human red blood cells were used instead of type O. CF tests were carried out with the micro technique (12). Antiserum against type 1 satellite was kindly provided by Wade Parks.

Type 4 satellite infectivity was assayed by its HA-producing capacity (8). Primary GMK tube cultures were inoculated simultaneously with 0.1 ml of 10-fold dilutions of type 4 satellite and 0.1 ml of adequately diluted helper SV15. Satellite fluid was heated at 60 C for 15 min before inoculation to inactivate the contaminating adenovirus. The cultures were held at 37 C in 1.0 ml of fresh maintenance medium and were harvested about 5 days later, when complete

cytopathic effects (CPE) of SV15 appeared. The viruses were released by three cycles of freezing and thawing. The viral fluids were tested for newly synthesized heat-stable type 4 satellite HA as described above. The culture inoculated with the highest dilution of type 4 satellite which showed newly produced HA was designated as 1 HA-producing unit. Three to five tubes were used for each dilution. The 50% end point was calculated by the method of Reed and Muench. (16).

Similar methods were used for type 1 satellite, except that infectivity was assayed by CF-producing activity. The highest dilution of satellite which produced newly synthesized CF antigen was designated 1 CF-producing unit. No cross-reactions were detected between satellites types 1 and 4 with either the CF or HA tests.

RESULTS

Experiments on the effects of varying the dose of satellite type 4 (or type 1) on the yield of satellite type 1 (or type 4). Primary GMK monolayer cultures in 1-oz (ca. 30 ml) stoppered bottles were inoculated simultaneously with 0.1 ml of undiluted SV15 at a multiplicity of infection (MOI) of 0.4 plaque-forming units (PFU) per cell, 0.1 ml of undiluted type 1 satellite (MOI, 3 CFU/cell), and 0.1 ml of type 4 satellite (MOI, 0.4 HAU/cell) at various dilutions. All satellite virus fluids were heated at 60 C for 15 min before inoculation to inactivate contaminating SV15. The cultures were incubated for adsorption at 37 C for 1 hr, and then they were washed three times with maintenance medium. A 4-ml amount of fresh medium was added to the bottles. The cultures were again placed at 37 C and harvested at 12 to 48 hr after inoculation. The viruses were released by three cycles of freezing and thawing, and the clarified viral fluids were stored at −20 C before titration (Fig. 1). The yield of type 4 satellite was markedly depressed in all cultures which had been simultaneously inoculated with high concentrations of type 1 satellite (Fig. 1A). In the same cultures, the yield of type 1 satellite was reduced when undiluted type 4 satellite was added to the inoculum, as compared with the yield produced when cultures were inoculated with SV15 and type 1 satellite alone. Type 4 satellite at dilutions of 10^{-2} and 10^{-4} had no significant effect; however, the yield of type 1 tended to be inversely related to the amount of type 4 (Fig. 1B).

Other experiments were performed with undiluted SV15 (MOI, 20 PFU/cell), undiluted type 4 satellite (MOI, 4 HAU/cell), and type 1 satellite (MOI, 2 CFU/cell) at various dilutions (Fig. 2). Similar effects were observed, and the yield of type 1 satellite was depressed in cultures to which type 4 satellite had been added (Fig. 2A). In addition, the yield of type 4 satellite was inversely related to the amount of type 1 in the inoculum (Fig. 2B). Clearly, satellite viruses are capable of interfering with one another's replication, and the larger the dose of the interfering satellite, the greater the degree of interference produced.

In this series of experiments, the harvests at both 36 and 48 hr after simultaneous inoculation were also monitored for adenovirus activity. Typical results are shown in Table 1. When undiluted satellite was present in the inoculum (experiments 1, 2, 3, 4), about a 1-log drop in

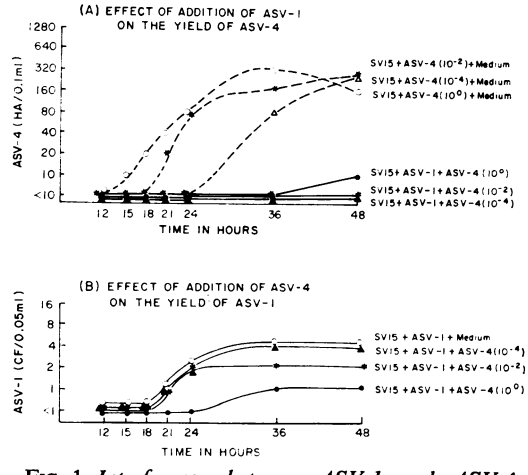

FIG. 1. *Interference between ASV-1 and ASV-4 (varying dose of ASV-4).*

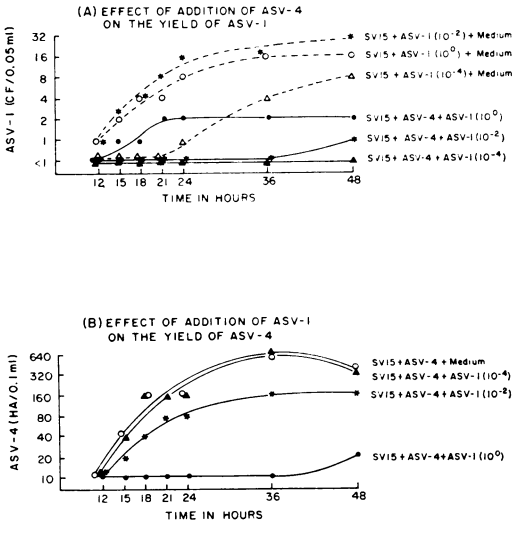

FIG. 2. *Interference between ASV-1 and ASV-4 (varying dose of ASV-1).*

TABLE 1. *Effect of satellites on the yield of adenovirus*

Expt no.	Inoculum	Adenovirus titer of SV15 (log 10 PFU/0.1 ml)	
		36 hr	48 hr
1	ASV-1 10^0 + ASV4 10^0 + SV15	4.9	5.1
2	ASV-1 10^0 + ASV4 10^{-2} + SV15	4.8	5.1
3	ASV-1 10^0 + ASV4 10^{-4} + SV15	5.0	4.9
4	ASV4 10^0 + SV15	5.1	5.1
5	ASV4 10^{-2} + SV15	ND[a]	6.1
6	ASV4 10^{-4} + SV15	6.3	6.4
7	SV15	6.0	6.0

[a] Not done.

adenovirus titer was observed. These results indicate that the total content of satellite in the inoculum is crucial for demonstrating inhibition of adenovirus. Dilution of total satellite content through omission of one satellite and dilution of the other leads to the disappearance of inhibition in adenovirus titer.

Experiments on the effects of varying the time of addition of satellite type 1 (or type 4) on the yield of satellite type 4 (or type 1). Primary GMK monolayer cultures in 1-oz (ca. 30 ml) stoppered bottles were inoculated simultaneously with 0.1 ml each of undiluted SV15 (MOI, 4 PFU/cell) and type 4 satellite (MOI, 2 HAU/cell). Viral adsorption was carried out at 37 C for 1 hr. After three washings, the cultures were cultivated at 37 C in 4 ml of maintenance medium.

Three bottles were removed at each indicated time, from 24 hr before to 36 hr after the inoculation of SV15 and type 4 satellite. Two of them were superinfected with 0.1 ml of undiluted type 1 satellite (MOI, 2 CFU/cell; after three washings with maintenance medium), whereas the remaining control bottle was inoculated with an equal volume (0.1 ml) of maintenance medium. After an adsorption period of 1 hr and three repeated washings, the cultures were held at 37 C in 4 ml of fresh maintenance medium.

The cultures were harvested 48 hr after the inoculation of SV15 and type 4 satellite. After three cycles of freezing and thawing and removal of cell debris by low-speed centrifugation, the viral fluids were stored at −20 C before assay (Fig. 3).

When type 1 satellite was added within 8 hr after simultaneous inoculation of type 4 satellite and SV15, the 48-hr yield of type 4 satellite was from 32- to 64-fold lower than that obtained in control cultures to which no type 1 satellite had been added. However, when type 1 satellite was added 12 or more hr after simultaneous inoculation of type 4 satellite and SV15, very little reduction in the yield of type 4 satellite was noted. These results indicate that events leading to mutual satellite interference occur during the time associated with the active period of viral deoxyribonucleic acid (DNA) synthesis (10), and that the mandatory functions of adenovirus are essentially completed within 12 hr, before the time when satellite type 4 progeny virus first appears (about 16 hr after inoculation; 13). The 48-hr yield of type 1 satellite, as measured by CF activity, remained relatively constant until type 1 satellite was added, 12 or more hr after simultaneous inoculation of SV15 and type 4 satellite, at which time CF activity could no longer be detected.

The effects of varying the time of addition of type 4 satellite on the inhibition of production of type 1 satellite were similar. Primary GMK monolayer cultures were inoculated with 0.1 ml each of undiluted SV15 (MOI, 3 PFU/cell) and type 1 satellite (MOI, 3 CFU/cell). They were then superinfected with 0.1 ml of undiluted type 4 satellite (MOI, 2 HAU/cell) at time intervals ranging from time zero to 36 hr after the inoculation of SV15 and type 1 satellite, by the same method as in the previous experiment. The control cultures were inoculated with equal volumes (0.1 ml) of maintenance medium. The cultures were harvested 48 hr after the inoculation of SV15 and type 1 satellite and were stored at −20 C. In addition, type 1 satellite was barely detectable by CF activity until type 4 was added, 12 hr or more after simultaneous infection with type 1 satellite and SV15. The 48-hr yield of type 4 satellite was decreased when type 4 was added 24 or more hr after inoculation of type 1 satellite and SV15 (Fig. 4). This decrease may

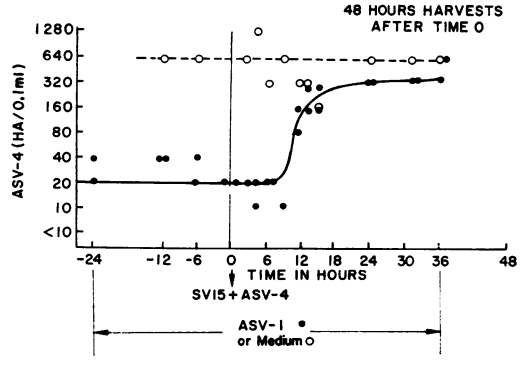

FIG. 3. *Effect of the time of addition of ASV-1 on the inhibition of ASV-4.*

result from the CPE of adenovirus, from decreased enhancement due to culture age, or from the shorter cultivation period for the superinfecting satellite before harvest and assay. The next experiment was carried out in order to clarify which was the case (Fig. 5). Primary GMK monolayer cultures were infected with 0.1 ml of undiluted SV15 (MOI, 2 PFU/cell) and type 1 satellite (MOI, 3 CFU/cell). Thereafter, they were superinfected with 0.1 ml of undiluted type 4 satellite (MOI, 2 HAU/cell) by the same method as described in the previous experiments. An equal volume (0.1 ml) of maintenance medium was added to the control cultures instead of type 4 satellite. The cultures were harvested 48 hr after superinfection with type 4 satellite (not after the previous infection of SV15 and type 1 satellite). Viral fluids were stored at −20 C before assay.

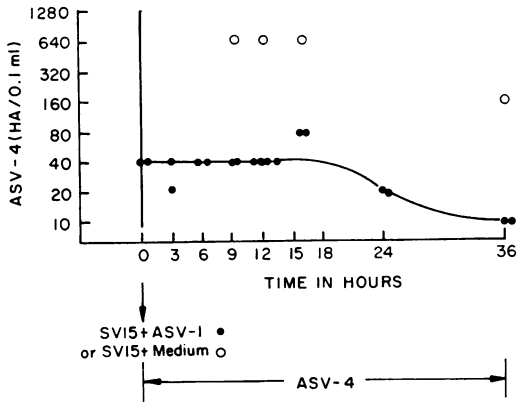

FIG. 4. *Effect of ASV-1 on the yield of ASV-4 added at different time intervals (harvests 48 hr after time zero).*

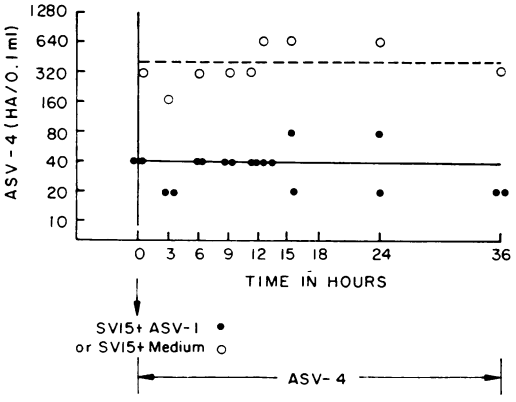

FIG. 5. *Effect of ASV-1 on the yield of ASV-4 added at different time intervals (harvests 48 hr after superinfection with ASV-4).*

Results obtained showed that the yield of the superinfecting satellite (in this experiment, type 4) remained essentially constant when harvest time was postponed until 48 hr after superinfection, indicating that sufficient time for replication of the superinfecting satellite should be allowed before assay. It also appears that CPE of adenovirus had little influence. CF data also showed that the yield of type 1 satellite was inhibited until type 4 was added, 12 or more hr after simultaneous inoculation of SV15 and type 1 satellite, evidence for interference with type 1 satellite production by early or previous inoculation of type 4 satellite.

Experiments on the effects of varying the time interval between the satellite viruses before addition of helper SV15. Primary GMK monolayer cultures in 1-oz (ca. 30 ml) stoppered bottles were infected with 0.1 ml of undiluted satellite type 4 (MOI, 2 HAU/cell) and cultivated at 37 C. The cultures were superinfected with 0.1 ml of undiluted type 1 satellite (MOI, 0.8 CFU/cell) at time intervals ranging from 12 hr before to 30 hr after inoculation of type 4 satellite and were incubated at 37 C in 4 ml of maintenance medium without helper SV15. The cultures were inoculated with undiluted SV15 (MOI, 2 PFU/cell) 48 hr after inoculation of type 4 satellite and were harvested 60 hr later. Viral fluids were stored at −20 C. An equal volume (0.1 ml) of maintenance medium was added to the control cultures in place of satellite viruses.

In these experiments, the yield of type 4 satellite remained 8- to 16-fold lower than that of the control cultures to which no type 1 satellite had been added, regardless of the time of addition of type 1 satellite (Fig. 6). Yields of type 1 satellite were barely detectable by the CF test in the cultures to which type 4 satellite had been added, indicating that interference between satellites occurred in this three-component system. This also indicates that the previously

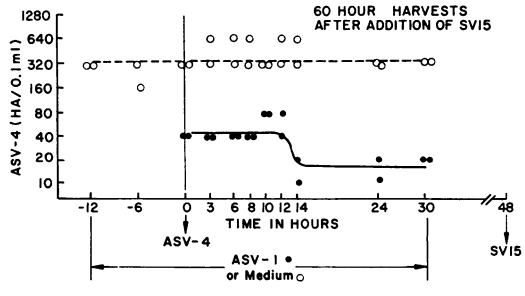

FIG. 6. *Effect of varying the time interval between adding types 1 and 4 satellite viruses before addition of helper SV15.*

inoculated type 4 satellite was unable to escape from the interfering activity of the superinfecting type 1 satellite unless helper adenovirus was present in the system and replication of type 4 satellite had begun to proceed (Fig. 3).

DISCUSSION

The experiments described here indicate the existence of a currently unique viral interference system made up of three interacting components, the simian adenovirus SV15 and the antigenically distinct satellite viruses, types 1 and 4. The products of this system can be monitored by different biological properties. Adenovirus activity can be destroyed by heat, and the remaining stable satellite type 4 can be detected by hemagglutinating activity. Type 1 satellite is detectable by CF activity, but in general the levels reached are too low to make significant estimates of changes in titer. In the presence of viable satellites, adenovirus can be monitored by plaque-forming ability. The three components are similar in that they all contain DNA.

There have been a number of reports on two-component viral interference systems in which satellites or defective viruses were involved. Again, in each system both components contained the same type of nucleic acid. Examples are the ribonucleic acid (RNA) system composed of tobacco necrosis virus and its satellite (9), the DNA system composed of adenovirus and a single satellite (13), and the interfering action of defective, physically distinguishable particles of the RNA-containing vesicular stomatitis virus on the production of infective vesicular stomatitis virions (5, 7).

Recently, a two-component interfering system comprised of an RNA- and a DNA-containing virion was described. This is the reovirus-vaccinia system studied by Dales and Silverberg (4). However, although each component contained a different type of nucleic acid, the major sites of viral synthesis and assembly were the same, namely, cytoplasmic in both cases. In the three-component system described in this paper, the sites involved in viral replication or assembly, and hence the sites of competition between viral genomes, are presumably nuclear.

Our results demonstrate that satellite types 1 and 4 are capable of interfering with one another's replication, and that larger doses of interfering satellite result in greater interference in the yield of the second satellite (Fig. 1, 2). In agreement with the previous studies of Parks et al. (13) on interference between a single satellite virus and adenovirus, they also show that interfering activity between mutual satellites is manifested during the 12-hr period of active adenoviral DNA synthesis. We suggest that satellite replication with necessary participation of adenovirus must be initiated before mutual interference between satellites can be demonstrated. The finding that total satellite content in the inoculum is of great importance in demonstrating a decrease in adenovirus yield is in agreement with Park's studies on the two-component adenovirus-satellite system (13). The necessity for allowing sufficient time for potentially viable satellite to complete a full replicative cycle is clearly demonstrated by the results in Fig. 3 and 4. The finding that the interference phenomenon was demonstrable before progeny virus from the previously inoculated satellite was detectable (i.e., within the limits of a single cycle of growth) is indicative that an explanation should be sought in terms of events occurring at the level of the individual cell. However, it is also possible that infection of all cells in the culture with satellites and adenovirus was not achieved. If this were so, the decrease in yield of superinfecting satellite shown in the 48-hr harvest would not occur. However, the higher multiplicities used in our experiments would militate against this possibility.

The members of our three-component system are conducive to independent identification and assay. This fortunate set of circumstances introduces a note of caution, in that the presence of adventitious and possibly unidentifiable satellite viruses in tissue culture or inocula may markedly affect the yields of known satellite agents under study and seriously interfere with interpretion of results.

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