Studies of the Enhancement of an Adenovirus-associated Virus by Herpes Simplex Virus

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SUMMARY

The defective adenovirus-associated viruses produce infectious progeny only in cells co-infected with an unrelated helper adenovirus. However, the adenovirus-associated viruses produce antigen detectable by immunofluorescence but not infectious virus in cells co-infected with herpes simplex virus. This incomplete helper effect provided by herpes simplex virus was studied in Hep-2 cells by quantitative kinetic procedures. Dose response studies showed that a single infectious adenovirus-associated virus-1 particle and a single infectious herpes simplex virus particle were sufficient to initiate adenovirus-associated virus antigen synthesis. Adenovirus-associated virus-1 antigen was formed 6 hr after infection with both viruses, and at a time before the production of infectious herpes simplex virus. Co-infected cells, deprived of arginine, formed adenovirus-associated virus-1 antigen while undergoing an abortive herpes simplex virus infection in which infectious herpes simplex virus was not produced.

Sequential inoculation studies showed that the 6 hr latent period for adenovirus-associated virus antigen formation was lengthened, rather than shortened, by pre-infection with herpes simplex virus. Cytosine arabinoside treatment of co-infected cells within 4 to 6 hr after infection prevented adenovirus-associated virus antigen formation.

INTRODUCTION

The adenovirus-associated viruses (AAV) are small, defective DNA viruses which produce infectious progeny only in cells co-infected with an unrelated helper virus, adenovirus (Atchison, Casto & Hammon, 1965; Hoggan, Blacklow & Rowe, 1966; Parks et al. 1967; Smith, Gehle & Thiel, 1966). This unique association of AAV with adenovirus also occurs in AAV infections of a natural host, man. Although serological surveys show that man is commonly infected with AAV, these viruses have been isolated only from adenovirus-infected children (Blacklow, Hoggan & Rowe, 1967a).

Studies on the mechanism by which adenovirus infection stimulates AAV growth indicate that a relatively late event in the adenovirus replicative cycle renders a cell competent to synthesize infectious AAV (Blacklow, Hoggan & Rowe, 1967b; Ito, Melnick & Mayor, 1967; Parks et al. 1967). This critical event is temporally associated with the replication of adenovirus DNA. With rare exception, it has not been possible to detect cells producing AAV antigens or particles in the absence of late adenovirus structural antigens or particles (Blacklow et al. 1967b; Parks et al. 1967).
Recent studies show that several herpes group viruses are able to prime cells for the production of AAV antigen detectable by immunofluorescence, but not for infectious AAV (Atchison, 1970; Blacklow, Hoggan & McClanahan, 1970). Thus, it appears that herpes viruses provide incomplete helper function(s) for the potentiation of AAV as contrasted with adenoviruses which provide all the required functions for a complete helper effect. The mechanism of the herpes virus-AAV interaction, and its possible contrasts with the mechanism of the adenovirus-AAV relationship is unknown. This report is a study of the interaction between herpes simplex virus type 1 and AAV type 1 and utilizes quantitative kinetic procedures to examine herpes virus replicative events that render cells competent to form AAV antigen detectable by immunofluorescence.

**METHODS**

*Viruses.* The AAV-1 (H) strain was employed; this was the same strain used in previous studies of the enhancement of AAV-1 by adenovirus (Blacklow et al. 1967b). Stock pools of AAV-1 were grown in human embryonic kidney (HEK) cells with adenovirus type 7 as helper, as previously described (Blacklow et al. 1967b). The virus stocks were heated at 56°C for 15 min. immediately before use. This procedure inactivates adenovirus infectivity with minimal inactivation of AAV, and therefore makes it possible to use AAV, free of infectious adenovirus, in quantitative and temporal studies of AAV-herpes virus interactions. AAV-1 infectivity titres (Hoggan et al. 1966), after heating, were $10^{7.5}$ TCID$_{50}$/0.1 ml.

Herpes simplex virus (HSV) type 1 was obtained from Dr J. Rose (National Institutes of Health, U.S.A.), and a stock pool was grown in Hep-2 cells as follows. Infected Hep-2 cells, maintained at 34°C with Eagle's basal medium No. 2 with penicillin, streptomycin and glutamine (BME) were scraped into the medium with a rubber policeman at 28 hr post-infection. The infected cells were frozen and thawed once, then treated for 25 sec. in a Raytheon sonic oscillator (10 kc., model DF 101). The virus was then diluted in an equal volume of skim milk and the material then distributed into screw-capped vials for storage at -60°C. The HSV pool was titrated in tube cultures of Hep-2 cells which were observed for 9 days at 36°C, scraped, and then blind passaged to fresh Hep-2 cells which were also observed for 9 days. The HSV stock titred $10^{7.8}$ TCID$_{50}$/0.1 ml.

*Tissue cultures.* Hep-2 cell cultures were obtained from Flow Laboratories Inc., Rockville, Maryland, U.S.A. For use in immuno-fluorescence tests, cells were grown on coverslips in 50 mm. plastic Petri dishes by methods described previously for HEK cells (Blacklow et al. 1967b). All cultures were kept at 36°C in 5% CO$_2$ atmosphere. At confluence, cultures were rinsed once with BME and renewed with 4 ml. of maintenance medium consisting of BME without added serum. Unless stated otherwise, cultures were then inoculated with 0.1 ml. of the appropriate virus dilution. At various times, coverslips were removed, rinsed in isotonic saline, pH 7.4, and fixed in cold acetone.

*Immunofluorescence tests.* AAV-1 specific antigen was assayed quantitatively by the indirect method as employed previously (Blacklow et al. 1967b). The percentage of cells with specific staining was determined by counting 1000 to 4000 cells; for very low percentages the number of positive cells on the entire coverslip was determined. The specificity of the tests for AAV-1 antigen produced with adenovirus or HSV helpers has been described elsewhere (Blacklow et al. 1967b; Blacklow et al. 1970).

*Arginine-deprivation tests.* Hep-2 coverslip cultures maintained with BME will be referred to as Arg+ cultures; washed Hep-2 coverslip cultures that were renewed with BME devoid
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of arginine will be termed Arg- cultures. Neither the Arg+ nor the Arg- cultures contained serum. Eighteen hr before infection, cultures were placed under Arg+ or Arg- conditions. Viruses were inoculated in a volume of 0.8 ml of the appropriate Arg+ or Arg- media. After 1½ hr, the cells were washed four times with isotonic saline and then renewed with their respective Arg+ and Arg- media.

HSV infectivity assays of inoculated Arg+ and Arg- cultures were performed at designated time intervals as follows. Cells were scraped into the medium, frozen and thawed once, then treated sonically for 25 sec as above. The inoculae were then titrated in Hep-2 tube cultures as above.

Cytosine arabinoside inhibition tests. Cytosine arabinoside (Ben Venue Laboratories, Bedford, Ohio, U.S.A.) was kindly supplied by Dr C. Crumpacker (National Institutes of Health, U.S.A.). The stock was initially dissolved in sterile water and then used at a final concentration of 20 μg/ml. in BME. At this concentration the cytosine arabinoside stock inhibited by 99% the incorporation of [14C]thymidine into cellular DNA.

RESULTS

Characteristics of AAV antigen produced with herpes virus helper

Hep-2 cells inoculated with adenovirus-associated virus-1 alone failed to produce immunofluorescent antigen, but when co-infected with AAV-1 and herpes simplex virus, the cells showed AAV specific antigen. As will be shown (Fig. 3, 5), AAV antigen was first detected 6 hr after inoculation, before the appearance of infectious HSV, and rapidly reached a plateau by 12 hr. The morphology of the AAV-1 antigen produced with HSV was similar to that produced with adenovirus (Blacklow et al. 1967b). Nuclei were filled with bright, homogenous, granular material. All five serial serum specimens collected over a 1½ year period from a guinea-pig hyperimmunized with AAV-1 (Hoggan et al. 1966) detected typical AAV-1 fluorescence on infected Hep-a cells, beginning with the initial postimmunization serum obtained 3 weeks after inoculation. Similar findings were found with AAV-1 antigen produced with adenovirus 7.

The suitability of the antigen assay for quantitative studies of the HSV-AAV interaction was shown by dose-response studies employing varying dilutions of AAV-1 in the presence of a constant dosage of HSV. Cultures were infected simultaneously with 10 ID 50 of HSV/cell plus serial dilutions of AAV-1; coverslips were harvested 24 hr after infection, and the percentage of AAV-1 FA-reactive cells determined. As shown in Fig. 1, there was a linear relationship in the region where the dosage of AAV was approximately one infectious particle or less/cell. This proportionality between percentage of staining and dilution factor indicates that one AAV particle is capable of initiating synthesis of antigen. Similar findings have been noted with AAV-1 antigen produced with adenovirus 7 (Blacklow et al. 1967b).

Conversely, cells were infected simultaneously with 1 ID 50 of AAV-1/cell plus serial dilutions of HSV. Coverslips were harvested 24 hr after infection and the percentage of AAV-1 staining determined. As shown in Fig. 2, there was a linear relationship in the region where the dosage of HSV was roughly one infectious particle or less/cell. This relationship suggests that one infectious herpes virus particle can serve as a helper for the production of AAV antigen.

Growth cycle studies of AAV and herpes virus in the presence or absence of arginine

Infectious HSV is not synthesized when arginine is omitted from the medium of cells in continuous cultivation (Becker, Olshevsky & Levitt, 1967; Courtney, McCombs & Benyesh-
Melnick, 1970; Roizman, Spring & Roane, 1967; Tankersley, 1964). The precise mechanism of this block in complete virus replication is not clear, but it is known that HSV-DNA (Becker et al. 1967), HSV-specific antigens (Courtney et al. 1970), and HSV structural proteins (Olshevsky & Becker, 1970) are synthesized under conditions of arginine deprivation. It has been suggested (Courtney et al. 1970; Olshevsky & Becker, 1970) that arginine may be necessary for the expression of relatively late HSV functions concerned with the encapsidation of virus DNA by an internal protein.

Fig. 1. Dose-response relationship of AAV-1 immunofluorescent antigen. Hep-2 cells were simultaneously inoculated with AAV-1 and herpes simplex virus, and the percentage of AAV-1 reactive cells determined 24 hr after infection. The m.o.i. of herpes was 10.

Fig. 2. Effect of herpes virus multiplicity on AAV-1 immunofluorescent antigen 24 hr after infection with both viruses. The m.o.i. of AAV-1 was 1.

Hep-2 cells were placed under either Arg⁺ or Arg⁻ conditions for 18 hr and then co-infected with HSV and AAV-1, each at a multiplicity of 10 TCID₅₀/cell. Assays were made at designated time intervals for AAV-1 antigen and infectious HSV; the results are depicted in Fig. 3. Infectious HSV was produced under Arg⁺ but not Arg⁻; conditions as shown in Fig. 3a. Further, the time of appearance of infectious HSV was between the sixth and the twelfth hr after infection, a finding that has been noted elsewhere (Roizman & Spear, 1968; Roizman, 1969) under similar experimental conditions.

Under Arg⁺ conditions, AAV-1 antigen was detected at the 6th hr (Fig. 3b), apparently at a time before the production of infectious HSV. This finding contrasts with the striking simultaneous formation of adenovirus and AAV particles, as well as adenovirus and AAV late antigens, that has been reported under a variety of experimental conditions (Blacklow et al. 1967b; Parks et al. 1967).

Arg⁻ cultures inoculated with HSV supported the synthesis of AAV-1 antigen, although there was some delay in its formation (Fig. 3b). These cultures therefore clearly supported the enhancement of AAV-1 under conditions in which HSV-DNA synthesis has been reported to occur, but under which infectious HSV was not produced.
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Time course of AAV antigen formation in cells sequentially infected with helper herpes virus and AAV

Attempts were made to define a period during the HSV growth cycle when the cells were competent to potentiate AAV. Accordingly, cultures were inoculated with AAV-1 at different times in relation to HSV, and the time of appearance of AAV-1 antigen determined. Hep-2 cells received HSV (multiplicity of 10 TCID 50/cell) 0, 2, 4, 6, 8, 10 and 12 hr before inoculation with AAV (multiplicity of 10 TCID 50/cell); HSV was also inoculated 12 hr following infection with AAV-1. Coverslips were harvested at 2 hr intervals after the final virus inoculation. The percentage of cells containing AAV antigen was determined.

As shown in Fig. 4, cells simultaneously inoculated with HSV and AAV-1 produced AAV staining by 6 hr, and a growth curve was determined that was similar to that shown in Fig. 3. In addition, cells pre-infected for 12 hr with AAV-1 also showed AAV antigen 6 hr following infection with HSV. However, with increasing intervals of pre-infection with HSV, AAV antigen correspondingly required a longer interval to appear following AAV inoculation. Indeed, with 12 hr of pre-infection with HSV, there was no AAV staining obtained by 10 hr after AAV inoculation; by contrast, 5% of cells simultaneously infected with both viruses showed AAV staining by 10 hr. It is apparent that pre-infection with HSV does not shorten the eclipse phase for AAV antigen production; rather, it lengthens the eclipse phase.

These data are in sharp contrast to those obtained from similarly designed kinetic studies of adenovirus-AAV interactions in which the eclipse period for AAV antigen formation can be shortened considerably by pre-infection with helper adenovirus for ten or more hr (Blacklow et al. 1967b; Ito et al. 1967). In the case of the HSV-AAV interaction, it is possible to speculate that the HSV-infected cell transiently becomes competent for AAV synthesis.
during the first 6 hr after infection, and remains competent for a limited period of time after which AAV synthesis is not achieved. This possibility suggests that HSV and AAV compete for a HSV-induced product that is required for the replication of both viruses; this product could be produced in excess early in the HSV cycle or be available in lesser amounts late in the cycle. As a result, with a long pre-infection with HSV, this product may be extensively utilized by HSV before AAV inoculation.

Effect of cytosine arabinoside on the enhancement of AAV by herpes virus

The preceding growth cycle studies have shown that AAV antigen is formed before the production of infectious herpes virus and at a time (6 hr) after herpes virus DNA synthesis is known to begin under similar experimental conditions (Roizman, 1969). In addition, HSV DNA synthesis proceeds in Arg− cells (Becker et al. 1967), in which we have shown

![Graph](image)

**Fig. 5.** Effect of cytosine arabinoside (CA) on the development of AAV-1 immunofluorescent antigen in cells co-infected with herpes virus and AAV-1, each at a m.o.i. of 10. Cytosine arabinoside, used at a final concentration of 20 µg./ml., was added to individual infected cultures at times indicated by arrows. Growth curve for AAV-1-infected cultures that lacked CA treatment is shown by continuous line plot. AAV-1 growth curves subsequent to additions of CA are indicated by broken line plots.

AAV is enhanced. It was therefore of interest to determine if HSV would enhance AAV under conditions in which HSV-DNA synthesis was blocked. The metabolic inhibitor, cytosine arabinoside (CA), is known to prevent HSV-DNA synthesis at a concentration of 20 µg./ml. (Levitt & Becker, 1967); under such conditions of inhibition, infectious HSV is not produced. Treatment of HSV-infected cells with CA offered an approach to the question whether AAV was enhanced when HSV-DNA synthesis was blocked. This approach was considered useful, even though it was subject to the limitation that CA treatment of cells co-infected with HSV and AAV should inhibit not only HSV-DNA synthesis but probably that of AAV as well. However, treatment with CA would determine whether or not de novo DNA synthesis in co-infected cells was required for AAV antigen formation.

Hep-2 cells were co-infected with HSV and AAV-1, each at multiplicities of 10 TCID50/cell. Coverslips were harvested at designated intervals and an AAV growth curve was obtained, shown in Fig. 5. At 2, 4, 6 and 8 hr after infection, CA was added to the medium and coverslips subsequently harvested 16 hr after the cells originally received HSV and AAV. As depicted in Fig. 5, cells treated with CA at 2 and 4 hr after infection failed to show AAV antigen at 16 hr. By contrast, cells treated with CA at 6 and 8 hr after infection produced
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approximately the same percentage of AAV-reactive cells at 16 hr as cells which did not receive CA.

These findings in part confirm the preceding time course studies in that events in the HSV growth cycle that occurred before 6 hr were unable to enhance subsequent AAV antigen formation when CA was added before 6 hr. In addition, the helper effect of HSV was unaltered by the activity of CA added after 6 hr. It is clear that inhibition of de novo DNA synthesis in co-infected cells within 4 to 6 hr after infection also prevented AAV antigen formation. The data are consistent with the hypothesis that replication of HSV DNA is required for AAV antigen formation; however, the hypothesis cannot be proven because of the probable effects of CA on AAV DNA synthesis as well as that of HSV.

DISCUSSION

The studies reported here on the incomplete helper effect on AAV-I by herpes virus can be compared with previous studies on the complete helper effect on AAV-I by adenovirus 7 (Blacklow et al. 1967b). Both similarities and differences between the mechanisms of enhancement in the two systems are apparent. The systems are similar in (t) the morphology of the AAV immunofluorescent antigen produced, (z) the detection of the antigen by all five serially collected sera from a guinea-pig hyperimmunized with AAV-I, and (3) the dose response studies which indicate that a single infectious AAV particle and a single infectious helper virus particle suffice to initiate AAV antigen synthesis.

The mechanisms of enhancement by the two helper systems differ sharply in two respects. First, AAV antigen synthesis is completed in the absence of newly synthesized infectious herpes virus, as evidenced by both the growth cycle and arginine deprivation studies. With the potentiation of AAV by adenovirus, both adenovirus and AAV particles, as well as adenovirus and AAV late antigens, show a striking simultaneity of formation under a variety of experimental conditions (Blacklow et al. 1967b; Parks et al. 1967). Second, the eclipse period for AAV antigen synthesis is not shortened by pre-infection with herpes virus; rather, such pre-infection lengthens the AAV eclipse phase. By contrast, pre-infection with adenovirus considerably shortens the latent period for AAV (Blacklow et al. 1967b; Ito et al. 1967). One may speculate that the herpes virus-infected cell is primed only transiently for AAV synthesis, and that, unless AAV is present at that time, the helper effect is not exerted. This hypothesis suggests that HSV and AAV compete for a HSV-induced product, and that a long pre-infection with HSV depletes the cell of this product that is also needed for AAV. An alternative explanation is that herpes virus-primed cells are more extensively damaged than are adenovirus-primed cells, and hence pre-infection with herpes virus damages cellular machinery needed for AAV synthesis. In this regard, however, no cytopathology was observed during the shorter periods of pre-infection with herpes virus; these periods of pre-infection indeed lengthened AAV eclipse, and the fact that AAV antigen was produced at a later time indicates that the cells were not damaged as regards their competence to synthesize AAV.

The data presented are consistent with the hypothesis that the helper effect of herpes virus on AAV-I is temporally and perhaps mechanistically associated with the replication of herpes virus DNA. First, AAV antigen synthesis occurs at 6 hr, a time when herpes virus DNA synthesis has already commenced (Roizman, 1969). Second, AAV is enhanced in herpes virus-infected Arg- cells in which herpes virus DNA synthesis occurs (Becker et al. 1967). Third, AAV-enhancing events that occur before 6 hr in co-infected cells can be shut off by the presence of the DNA inhibitor, cytosine arabinoside, whereas AAV enhance-
ment is unaltered by the action of cytosine arabinoside on later events in co-infected cells. The interpretation of these latter data, although consistent with the stated hypothesis, is subject to the experimental limitation that cytosine arabinoside would be expected to inhibit AAV-DNA synthesis as well as that of herpes virus. These data do indicate, however, that de novo DNA synthesis in co-infected cells is required for AAV antigen formation.

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REFERENCES


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