Immunological Reactivity of Antisera Prepared Against the Sodium Dodecyl Sulfate-Treated Structural Polypeptides of Adenovirus-Associated Virus

F. BRENT JOHNSON, NEIL R. BLACKLOW, AND M. DAVID HOGGAN

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014, and Division of Adult Infectious Diseases, University Hospital, Boston, Massachusetts 02118

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The preparation of antisera to the three purified sodium dodecyl sulfate (SDS)-treated polypeptide components (VP1, VP2, VP3) of adenovirus-associated virus (AAV) type 3H is described. In immunofluorescence tests (FA), these antisera stained heat-stable antigens with distinct morphologies in cells co-infected with either adenovirus or herpes simplex virus. Kinetic studies of antigen formation showed that VP1 antiserum first stained the cytoplasm (14 hr) and later (by 18 hr) stained both cytoplasmic and intranuclear areas. VP2 antiserum stained only discrete intranuclear areas, and VP3 antiserum stained nearly the entire nucleus. All three VP antigens appeared at about the 14th hr postinfection, about 2 hr prior to the appearance of whole virion antigen. The VP antisera cross-reacted in FA with AAV types 1 and 2 (all at one-eighth of the homologous titer), but did not react with other parvoviruses, i.e., rat virus, hemadsorbing enteric virus of calves, minute virus of mice, or H-1 virus. These non-neutralizing antisera reacted specifically with SDS-treated AAV virion antigens in complement fixation and immunodiffusion tests, and antiserum prepared against SDS-treated helper adenovirus structural polypeptides reacted with adenovirus polypeptide antigens. All antisera to SDS-treated polypeptides were specific for new antigens revealed on the dissociated peptides and did not react with whole virions, whereas whole-virion antisera did not cross-react with the polypeptide antigens. These findings suggest that antigens unique to the polypeptides of AAV are revealed by SDS treatment and that these antigens can be detected in cells prior to the folding of the polypeptides into the molecular configuration they possess as virion subunits. These results also indicate that at least one AAV polypeptide component is synthesized in the cell cytoplasm.

The adenovirus-associated viruses (AAV) are small, defective parvoviruses which require co-infection with a serologically unrelated helper adenovirus for production of infectious progeny (3, 14, 22, 28). Herpes simplex virus (HSV) acts as a partial helper, potentiating the formation of immunofluorescent (FA)-detectable virus antigen and infectious AAV deoxyribonucleic acid (DNA) without the assembly of these AAV products into infectious virions (2, 5, 6, 9). AAV thus appears to be defective in at least two functions, the ability to initiate synthesis of viral-specific products and the assembly of the products into mature virions.

It has been reported that the AAV virion contains three structural polypeptide components based upon molecular weight separations in polyacrylamide gels (17, 24). The combined molecular weight of the three structural polypeptides considerably exceeds the coding capacity of the AAV genome. This study was undertaken to produce immunological reagents against each of the structural polypeptides, to study antigenic relationships among the three polypeptides and their relationship to the polypeptide antigens of other parvoviruses, and to study the kinetics and intracellular locations of their biosynthesis or accumulation, or both.

MATERIALS AND METHODS

Viruses. The isolation and characterization of the AAV-1H, AAV-2H, and AAV-3H strains of AAV have been previously described (8, 14, 23), as have the adenoid 6 strain of adenovirus type 2 (Ad2; reference 26), the E-46- strain of adenovirus type 7 (Ad7; reference 25) and a field strain of herpes simplex virus (6). Other parvoviruses used included the Kilham strain of rat virus (RV; reference 18) obtained from W. P. Rowe, the Toolan strain of H-1 virus (30) from the American Type Culture Collection, the minute virus of mice (MVM; reference 10) obtained from John Parker, and the original strain of hemadsorbing enteric virus (HADEN; reference 1) obtained from Francis R. Abinanti.

Cell cultures. The growth of AAV in KB suspension culture with Ad2 helper and its subsequent purification have been previously described (17). Human embryonic kidney (HEK) cell monolayers were obtained from HEM Research, Inc., or Flow Laboratories, Inc., Rockville, Md., as were Hep-2 cell monolayers. Primary monolayer cultures from germfree rats were obtained from John Parker, and the rat nephroma cell line (4) was obtained from Lois Salzman; primary cultures of bovine embryonic kidney were obtained from Grand Island Biological Co., Grand Island, N.Y.

Polyacrylamide gel electrophoresis. Virus purified on isopycnic CsCl gradients and dialyzed in 0.01 M sodium phosphate buffer, pH 7.2, was disrupted in 1% sodium dodecyl sulfate (SDS) and 0.01 M dithiothreitol at 100 C for 1 to 2 min. Protein concentrations were estimated by the method of Lowry et al. (20) with bovine serum albumin as the standard protein. The polypeptide constituents of the disrupted virus were separated on 7.5% SDS-polyacrylamide gels by the method of Maizel (21) and as previously described (17).

Antiserum production. Antisera were prepared in Hartley strain guinea pigs found free from pre-existing antibody to adenovirus and AAV when tested by complement fixation (CF). Production of antibody against whole virus was previously described (14). Such antibody was obtained by foot pad inoculation of the virus in complete Freund's adjuvant.

Antisera to the polypeptide constituents of AAV-3 and AD2 were prepared by immunizing guinea pigs via foot pad inoculation with SDS-dissociated virus. Antiserum to the mixture of AAV polypeptides, that is, purified virus disrupted in 1% SDS with no electrophoretic separation, was prepared by inoculating guinea pigs each with 50 μ g of viral protein in Freund's complete adjuvant. This mixture was distributed between the two rear foot pads. The animals were bled at 2-week intervals. At 96 days after the primary inoculation, the animals were boosted by intramuscular injection of 50 μ g of SDS-dissociated viral proteins contained in incomplete Freund's adjuvant. Similarly, antiserum to SDS-disrupted Ad2 was prepared.

It was reported earlier that AAV-3H contains three structural polypeptides (17). The major one was designated VP1, and the other two were desig-

nated VP2 and VP3 in order of increasing molecular weight. Guinea pig antisera were prepared against each of these polypeptides. Purified virus was disrupted by the SDS procedure and separated on 10-cm 7.5% acrylamide gels. Of the 12 gels run in parallel, one was stained with Coomassie Brilliant Blue to locate the positions of the bands in the gels. The unstained bands were then dissected with a razor blade from the remaining gels. Figure 1 shows the three polypeptide-densitometric profiles on the gel which was stained, and the arrows indicate the positions at which the gels were cut. Three small segments of acrylamide gel were thus obtained from each column, each containing one of the separated viral polypeptides. That the gel pieces actually contained the polypeptide bands and that there was no overlap of a band in a neighboring gel piece was further verified by staining the three gel pieces obtained from such a gel column.

For immunization, unstained gel pieces were broken up by forcing them through a syringe and 19-gauge needle. This gel pulp was suspended in Freund's complete adjuvant and inoculated into the foot pads of guinea pigs. Two gel pieces were used for each immunization, with the animals receiving about 19 μ g of VP1 and 3 μ g each of VP2 and VP3. The antigen quantities were estimated by determining the quantity of protein put on the gels (Lowry method) and knowing the relative percentage of each polypeptide species (17). This dosage was repeated 46 days after primary injection, at which time the antigen was contained in incomplete adjuvant and was distributed among the foot pads and 2 to 3 intramuscular sites. The animals were bled 9 days after the secondary injections.

CF tests. The CF test was carried out as previously described (27). Antivirion sera (4-8 units) were tested against crude and purified (14) virus preparations containing mature virions and against SDS-

FIG. 1. Densitometric scan of the Coomassie Blue-stained acrylamide gel, which was a replicate of those used for preparation of purified VP antigens. The individual polypeptide bands were removed at the points indicated by the arrows.

dissociated virus or polypeptide antigens. The purified viral polypeptide antigens were recovered from acrylamide gel pieces by smashing the gel and eluting for 18 hr in 0.01 M phosphate buffer, pH 7.2. Furthermore, the polypeptide antisera (4–8 units) were tested against the variety of AAV and adenovirus antigens as well. These polypeptide antigens were all prepared by SDS dissociation, but since SDS is hemolytic it was necessary to remove it to concentrations below the sensitivity of the erythrocytes in the CF test. This was achieved by dialyzing the antigens at 37°C for 24 hr against several changes of 0.01 M phosphate buffer.

FA techniques. The indirect method of FA was used to detect AAV virion and polypeptide antigens. Cover slips with monolayers of Hep-2 cells infected with AAV and adenovirus helper or HSV partial helper were stained and observed as previously described (7).

Cover-slip preparations of RV, MVM, and H-1 viruses in secondary cultures of rat embryo or in the rat nephroma cell line were similarly tested, as was HADEN virus in secondary cultures of bovine embryonic kidney.

Neutralization of AAV. The neutralization assay of AAV based upon the suppression of CF antigen formation has been previously described (8). Neutralization experiments were performed in culture tubes containing HEK cell monolayers. The cultures were inoculated with 3×10^6 median tissue culture infective dose (TCID₅₀) of Ad2 and pre-incubated for 6 hr. Dilutions of heated serum were mixed with 100 to 1,000 TCID₅₀ AAV and allowed to react at room temperature for 20 min. The tubes were then inoculated with 0.1 ml of this mixture and incubated at 37°C for 5 days until 4+ cytopathic effects had developed. The cultures were freeze-thawed and tested by CF for AAV antigen induction.

Immunodiffusion. The method used for immunodiffusion was similar to that of Kuff et al. (19). The medium consisted of 0.75% agarose in 0.02 M sodium phosphate buffer (pH 7.2), 0.1 M NaCl, 0.02 M iodoacetamide, and 0.1% SDS. A 4-ml amount of this medium was added to 60-mm petri dishes. The wells were cut 3 mm apart and contained 5 μ liters of sample. Diffusion was allowed to proceed overnight at room temperature in a moist atmosphere.

RESULTS

FA staining of AAV-infected cells. Monolayers of Hep-2 cells were simultaneously infected with AAV-3H and Ad2 or HSV helper viruses. AAV-3 and Ad2-infected cells fixed 18 hr postinfection and stained with anti-AAV-3 whole-virion antiserum are shown in Fig. 2. As previously reported (7), the stain is localized in the nuclei and has a granular appearance. No cytoplasmic staining occurred. In contrast, when the cells were stained with anti-VP1 serum, marked fluorescence was localized in the cytoplasm at early time points (14 hr) (Fig. 3) and also included the nucleus at later time points (18 hr) (Fig. 4). The cytoplasmic

staining was characterized by a fairly homogeneous or a netlike appearance. Cells stained with anti-VP2 showed discrete, sharply demarcated intranuclear areas of fluorescence (Fig. 5). This pattern was typical of the VP2 antiserum and did not change with time. The staining observed with anti-VP3 serum (Fig. 6 and 7) was primarily limited to the nucleus; most of the nucleus was stained, with some areas clearly defined by clear halo-like zones. Thus, three distinct staining morphologies were noted with antisera to the three individual polypeptides of AAV-3, and each was different from the morphology seen with virion antiserum.

Experiments with HSV as helper virus showed that the same staining morphologies developed with this virus as those described for Ad2 helper. Uninfected cells, cells infected with AAV-3 alone, HSV alone, or adenovirus alone did not stain with any of the AAV polypeptide antisera.

Studies on the kinetics of formation showed that all the polypeptide antigens appeared about 14 hr after infection, and the virion antigen appeared at 16 hr.

Heat stability of the AAV polypeptide antigens. Monolayers of Hep-2 cells on cover slips were co-infected with AAV-3 and Ad2 and fixed in acetone 24 hr after infection. Such cover slips were heated at 56°C for 30 min in pH 7.4 phosphate-buffered saline, examined by indirect immunofluorescence, and compared to nonheated control cover slips. As an additional control, heated and unheated cells infected with Ad2 alone were stained with a serum specific for the heat-labile adenovirus T antigen (kindly provided by A. M. Lewis, Jr.). Even though the adenovirus T antigen was destroyed by heating, the AAV antigens which stain with antisera to VP1, VP2, and VP3 were not inactivated by heat under these conditions.

Specificity of anti-AAV-3H polypeptide antisera in FA. To determine if the anti-AAV-3 polypeptide sera would cross-react with FA antigens produced in cell cultures infected with other AAV serotypes, they were tested with AAV-1- and AAV-2-infected cover-slip preparations. Since AAV-3 and AAV-2 have been previously shown to cross-react by CF and FA (6, 12), it was not surprising to find similar staining patterns for AAV-2-infected cover slips. However, since cross-reactivity between AAV-1 and AAV-3 is rarely, if ever seen, it was quite unexpected to find similar patterns of FA staining of AAV-1-infected cells when stained by the AAV-3 polypeptide sera. The FA titers of these antisera against each of the AAV types studied can be seen in Table 1. It is interesting that in each case the heterologous AAV serotypes reacted at one-eighth of the

FIG. 2-4. Immunofluorescence photomicrographs of AAV-3H antigens prepared with adenovirus type 2 helper in Hep-2 cells. Fig. 2, Cells stained with anti-whole-virion serum. Fig. 3, Cytoplasmic staining with VP1 antiserum (14 hr after infection). Fig. 4, Cytoplasmic and intranuclear staining with VP1 antiserum (18 hr).

FIG. 5-7. Immunofluorescence photomicrographs of AAV-3H antigens prepared with adenovirus type 2 helper in Hep-2 cells. Fig. 5, Intranuclear staining with VP2 antiserum. Fig. 6 and 7, Nuclear staining with VP3 antiserum.

homologous AAV-3 reaction. These findings suggest that common antigens on the polypeptide level exist in these three AAV types. Furthermore, when the polypeptides are folded and oriented as structural units in the virions, their reactive antigens are distinct in each serotype, and the polypeptide antigens are not expressed.

To determine if the antipolypeptide antisera would cross-react with other members of the parvovirus group, cells infected with RV, HADEN virus, MVM, and H-1 virus were tested

TABLE 1. *Reactivity of anti-virus polypeptide (VP) antisera in immunofluorescence with adenovirus-associated viruses (AAV) and adenovirus-infected Hep-2 cells*

Virus	Serum titer ^a		
	Anti-VP1	Anti-VP2	Anti-VP3
AAV-1H	80	5	40
AAV-2H	80	5	40
AAV-3H	640	40	320
Ad7 ^b	0 ^c	0	0

^a Reciprocal serum dilution that gave 4+ staining of virus-infected cells in the indirect immunofluorescence test.

^b Adenovirus type 7.

^c No immunofluorescence at lowest dilution tested (1:5).

in FA with the various sera as well. Even though these infected cells showed the previously described positive fluorescence with their homologous antiviral sera (13), they did not stain with any of the three anti-AAV-3 polypeptide antisera.

Specificity of the antipolypeptide antisera in CF.

The CF reactions of virion and polypeptide antigens with their respective antisera are shown in Table 2. VP2 and VP3 are the minor constituents of AAV, and these two purified components were not detected in CF probably because insufficient quantities of antigen could be recovered from the acrylamide gels. Also, they may be poor antigens.

It can be seen in this table, as previously reported (8, 12), that the antivirion sera are distinct for their homologous AAV serotypes, with slight crossing between AAV-2 and AAV-3. In the unpurified preparations, both AAV and its helper adenovirus are present. None of these virion sera reacted in CF against the polypeptide antigens. In contrast, the antipolypeptide sera reacted with the polypeptide antigens and not the virion antigens, except that some cross-reactivity was noted in the adenovirus system, possibly indicating the presence of degenerated or spontaneously dissociated adenovirions in the virion antigen preparation or incompletely dissociated virions in the immunogen preparation.

From this table it can be further noted that on the polypeptide level AAV and adenovirus do not cross-react. This is significant because the amount

TABLE 2. *Comparative antigen titers of virion and polypeptide antigens when tested by complement fixation with antivirion and antipolypeptide sera^a*

Antigens	Titers of antivirion sera					Titers of antipolypeptide sera			
	Ad2	AAV-1	AAV-2	AAV-3H	AAV-3H mixed polypeptides	VP1	VP2	VP3	Ad2 polypeptides
Virion antigens									
Ad2, crude	64	0 ^b	0	0	0	0	0	0	2
purified	64	0	0	0	0	0	0	0	8
AAV-1, crude ^c	16	64	0	0	0	0	0	0	0
purified	0	32	0	0	0	0	0	0	0
AAV-2, crude ^c	4	0	16	0	0	0	0	0	0
purified	0	0	32	0	0	0	0	0	0
AAV-3H, crude ^c	4	0	0 ^d	4	0	0	0	0	0
purified	0	0	0 ^d	32	0	0	0	0	0
Polypeptide antigens									
AAV-3H mixed polypeptides	0	0	0	0	256	64	64	64	0
AAV-3H VP1	0	0	0	0	16	16	4-8	0	0
Ad2 polypeptides	0	0	0	0	0	0	0	0	128

^a All antisera were used at a concentration of 4 to 8 units. Abbreviations: Ad2, adenovirus type 2; AAV, adenovirus-associated virus; VP, virus polypeptide.

^b No detectable complement fixation at lowest concentration tested (1:2).

^c The crude preparations contained adenovirus helper.

^d A trace reaction occurred from 1:4 (crude) to 1:16 (purified).

of protein found in the AAV virion exceeds the coding capacity of its genome, and this observation suggests that the helper adenovirus does not share any structural polypeptides with the AAV virion.

In addition to these findings, the results in Table 2 show that anti-VP2 cross-reacts with VP1 but anti-VP3 does not cross-react in this manner. In this regard, it should also be noted that there were no detectable antibodies in these sera against extractable constituents of the acrylamide gel.

Reactivity of antipolypeptide antisera in immunodiffusion tests. Other attempts to define the reactivities and specificities of the antisera were made by experiments involving immunoprecipitation in agarose gels. SDS and iodoacetamide were included in the medium to block aggregation and complexing and refolding of the polypeptide antigens. Results of such an immunodiffusion test can be seen on the diffusion plate shown in Fig. 8a. A schematic drawing of this plate is shown in Fig. 8b. It was noted that antiserum to the mixture of AAV-3 polypeptides (A, B, C) formed immunoprecipitin lines with SDS-degraded AAV-3 (F), VP1 (G), VP2 (H), and VP3 (I). Anti-VP1 (D) reacted with an antigen in the mixture (F) and with VP1 (G, J). Well E contained anti-Ad2 polypeptides which did not react with any AAV polypeptide antigen. Acrylamide gel pieces were tested, and no antibody could be demonstrated against the gel constituents.

The entire spectrum of antisera and antigens tested by this method is shown in Table 3. It was again observed that antipolypeptide sera only reacted with polypeptide antigens and not with virion antigens. Similarly, antivirion antisera reacted only with homologous virion antigens, with the exception of a nonspecific reaction with Ad2 polypeptide antigens. This antigen preparation reacted with every guinea pig serum tested, including nonimmune pre-serum. When tested with nonimmune sera from other species of animals, it was found that the Ad2 polypeptide antigen reacted with every serum tested, as well as a 7% bovine serum albumin preparation. The sera tested included African green monkey, rhesus monkey, rat, hamster, mouse, ferret, rabbit, human, chicken, horse, and fetal calf serum. Since all of these unrelated sera, as well as the non-antibody-containing bovine serum albumin, gave an immunoprecipitin-like line, it was concluded that the reaction was not due to an antigen-antibody reaction. Consequently, Ad2 polypeptides, in our hands, did not lend themselves to study in this system. However, AAV polypeptides reacted with the typical specificity expected in antigen-antibody reactions. It should be noted

that anti-VP2 and VP3 sera did not react with their homologous antigens, probably because of their low antibody titers (seen both in CF and FA), and because of the small amounts of VP2 and VP3 polypeptides utilized as antigens. Very slight cross-reactivity was noted between anti-VP1 serum and VP2 and VP3 antigens, and this was only observed when the diffusion plates were rinsed, dried, and stained with amido schwarz.

Neutralization studies. Neutralization of AAV-3 was attempted with both anti-whole-virion serum and the antipolypeptide sera. Even though antivirion serum neutralized at a titer greater than 1,280, no neutralizing activity was observed in the antisera prepared against the purified AAV components.

DISCUSSION

The studies reported here utilized immunological reagents prepared against the purified components of virus which was disrupted in SDS to study time and intracellular location of biosynthesis or accumulation of virus constituents. We have found that antiserum to the major structural polypeptide of AAV-3H (VP1) in indirect FA tests stains the cytoplasm of cells actively infected with AAV and a helper adenovirus or herpes virus. The antigen first appears in the cytoplasm and later also accumulates in clearly demarcated areas in the nucleus. This observation is consistent with the suggestion that this polypeptide is synthesized in the cytoplasm and is transported to the nucleus. Velicer and Ginsberg (31, 32) and Henry et al. (11) observed the transport of newly synthesized adenovirus proteins into the nucleus using radiolabeling and immunologic techniques with antivirion serum. They suggest that immunological reactivity was probably not acquired until after transport. In the case of AAV, no reproducible cytoplasmic fluorescence is seen with antivirion serum. Since we have shown that the VP1 serum does not react with the antigens on whole virus, but rather with new antigens revealed on the unfolded polypeptide, it seems logical to conclude that VP1, or a molecule having VP1 antigens, is synthesized in the cytoplasm and transported to the nucleus before it receives its tertiary structure. Indeed, with the general approach employed here, it may be possible to demonstrate cytoplasmic synthesis of viral constituents of many DNA viruses with antisera to their dissociated viral polypeptides.

It was observed that antisera to the two minor constituents of AAV-3 (VP2 and VP3) stained primarily nuclear areas. We cannot conclude, however, that these two components are synthesized in the nucleus. It is possible that they are synthesized in the cytoplasm and that their con-

FIG. 8. *a*, Formation of immunoprecipitin lines in SDS-containing agarose gel with antipeptide antisera. Antiserum to SDS-disrupted AAV-3H was placed in wells A, B, and C. Well D contained VP1 antiserum and well E contained antiserum to Ad2 polypeptides. The antigens were placed as follows: well F, SDS-disrupted AAV-3H; well G, VP1; well H, VP2; well I, VP3, and well J, VP1. A schematic drawing of the precipitin lines that developed is shown in *b*.

centrations do not reach detectable levels due to a slow rate of synthesis or a high rate of transport into the nucleus. Our data do not definitively clarify the initial site of synthesis of VP2 and VP3.

The combined molecular weight of the three AAV structural polypeptides exceeds the coding capacity of the AAV genome (17, 24). The studies reported here indicate no cross-reactivity between AAV and Ad2 dissociated polypeptides, and thus

TABLE 3. Reactions between polypeptide and virion antisera and the polypeptide and virion antigens when tested by immunodiffusion^a

Antigen	Antipolypeptide sera					Antivirion and control sera		
	AAV-3H mixed poly-peptides	VP1	VP2	VP3	Ad2 mixed poly-peptides	AAV-3H	Ad2	PGPS ^a
AAV-3H mixed polypeptides	+	+	± ^b	+	0 ^c	0	0	0
AAV-3H VP1	+	+	0	0	0	0	0	0
AAV-3H VP2	+	±	0	0	0	0	0	0
AAV-3H VP3	+	±	0	0	0	0	0	0
AAV-3H virions	0	0	0	0	0	+	0	0
Ad2 virions	0	0	0	0	0	0	+	0
Uninfected cells, media	0	0	0	0	0	0	0	0

^a Abbreviations: Ad2, adenovirus type 2; AAV, adenovirus-associated virus; VP, virus polypeptide; PGPS, preimmunized guinea pig serum.

^b Indicates formation of a barely discernable immunoprecipitin line.

^c Indicates formation of no visible immunoprecipitin line.

adenovirus does not appear to be the source of any AAV structural components. It is possible that the AAV components could arise by cleavage of a precursor protein resulting in peptides with overlapping segments, similar to the poliovirus model (15, 16, 29). Our data are incomplete regarding the potential antigenic homologies among the three polypeptides because of the lack of recoverable VP2 and VP3 antigens. But we did observe a cross-reaction between anti-VP2 and VP1 antigen in CF, but not between anti-VP3 and VP1. It is not yet clear whether this cross-reaction derives from common amino acid sequences in VP1 and VP2, or that simply the VP2 immunogen was contaminated with VP1. Further studies are being carried out to resolve this question. In the immunofluorescence studies, even though each antiserum stained with a distinct staining morphology, a common characteristic of each stain was the round ball-like appearance of areas within the nuclei. These sites could be accumulations of VP2 polypeptides having common amino acid sequences with VP1 and VP3.

All antisera prepared against the individual purified polypeptides did not neutralize AAV-3. It should be noted, however, that the antiserum against the mixture of AAV polypeptides, i.e., SDS-degraded virus having undergone no electrophoresis, had slight neutralizing activity. Its neutralization titer was 40, and it had a CF titer of 1,280. This compares with a neutralization titer of >1,280 and a CF titer of 1,280 for antivirion serum. The primary antibody activity of this antipolypeptide serum, then, is clearly not directed toward the intact virion. The weak neutralizing activity of this serum probably is an indication that a few intact virions escaped degradation by the SDS procedure and were immunogenic when injected into guinea pigs. This ob-

servation may be corroborated by the fact that a small portion of radioactivity does not enter the gel when tritiated amino acid-labeled virus is analyzed on polyacrylamide gels (*unpublished data*).

In the absence of helper virus, AAV does not initiate detectable DNA replication (J. A. Rose, *personal communication*), or the synthesis of viral FA antigen (5), or infectious virus (3, 14, 22, 28). Adenoviruses provide a complete helper effect, leading to production of infectious AAV. HSV acts as a partial helper with production of infectious DNA (9) and antigen stainable with antivirion serum (5, 6). The AAV, then, may be defective in at least two functions, a function initiating synthesis or transport of viral DNA and protein, and a later function involving assembly of structural components. The studies reported here indicate that in the sequence of cellular events the earlier block is prior to production of viral polypeptides and does not involve the transport of these polypeptides to the nucleus nor the folding of the polypeptides into the tertiary structures they have as virion subunits.

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