STUDIES OF SMALL DNA VIRUSES FOUND IN VARIOUS ADENOVIRUS PREPARATIONS: PHYSICAL, BIOLOGICAL, AND IMMUNOLOGICAL CHARACTERISTICS*

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Electron microscopic studies of adenovirus preparations have revealed the presence of small "virus-like" particles approximately 22 mμ in diameter in a number of human and simian adenovirus types.[1-5] Some workers have interpreted these particles as being adenovirus subunits,[4-6] while others[1-3] have indicated that they represent a contaminating virus. Our early studies[1] suggested that the 22-mμ particles were viruses which replicated poorly, if at all, in human cells in the absence of adenovirus replication. Atchison *et al.*[2] also presented evidence that the small particles were infectious, that they were defective, and that they were immunologically distinct from any known adenovirus antigen. They tentatively referred to this virus as adeno-associated virus (AAV) and we shall continue to use this name.

The purpose of the present report is to describe some of the physical, biological, and immunological characteristics of the small particles found in various adenovirus preparations with further evidence that they are defective viruses and not adenovirus subunits.

Materials and Methods.—Viruses: Two stock pools of adenovirus type 7 (Ad. 7), strain LLE46$^+$, were prepared after five passages in African green monkey kidney (AGMK) cell culture[7-9] and one human embryonic kidney (HEK) cell culture passage. These pools had adenovirus infectivity titers in HEK cells of $10^{4.5}$ to $10^{5.5}$ $TCID_{50}$ per 0.1 ml and, when observed under the electron microscope (EM), contained 10^2 to 10^3 times as many small virus particles as typical adenovirus particles. These pools also had the capacity to induce SV40 T antigen in HEK and AGMK cells.[8, 9] This substrain shall be called E46$^+$ (+1), the symbol (+1) indicating presence of AAV-1 particles.

E46$^-$, a substrain of LLE46$^+$ obtained by limiting dilution passage in HEK cells,[8] was grown in HEK cells. This substrain had lost the capacity to induce SV40 T antigen in tissue culture, and was free of AAV. It will be referred to as E46$^-$ (0), the symbol (0) indicating absence of small particles. The E46$^-$ (0) pool had an infectivity titer of 10^8 $TCID_{50}$ per 0.1 ml in HEK cells. The presence or absence of SV40 T antigen was checked by immunofluorescence and AAV was checked by complement fixation (CF), immunofluorescence, and EM.

A pool of adenovirus type 12, strain 97838, was found contaminated with a serologically distinct AAV.[10] This AAV is referred to as AAV-2. AAV-2 was readily transferred to E46$^-$ (0) which then became E46$^-$ (+2).

A third AAV type (AAV-3) was recovered from a passage of E46$^+$ in AGMK cells in the presence of AAV-1 antiserum.

A pool of infectious canine hepatitis virus (ICH) made from a field strain[11] was examined and found free of small particles. This ICH (0) pool had an infectivity titer in Madin-Darby continuous canine kidney cells (MDCK),[12] of $10^{6.5}$ $TCID_{50}$ per 0.1 ml.

Assays of adenovirus infectivity were made in HEK cells using 2–3 tubes per dilution. AAV infectivity was measured by titration in HEK cells in the presence of 10^5 $TCID_{50}$ of E46$^-$ (0), allowing the cultures to progress to 4+ cytopathic effect (CPE) and then testing for AAV CF antigen and AAV particles by EM. Titers were estimated by the method of Reed and Muench.[13]

Adenovirus neutralization tests were carried out as previously described (procedure 2) in HEK cells.[14] AAV neutralization tests were performed in the presence of 1000 $TCID_{50}$ of Ad. 7 helper. After 4+ CPE the amount of AAV and Ad. CF antigens were assayed by CF.

All virus pools were passed in antibiotic-free cultures and found free of mycoplasma.

Cell cultures: HEK cells were obtained from Flow Laboratories, Rockville, Md., as 32-oz bottle or tube cultures. They were grown in Eagle's basal medium (BME) and 10% calf serum. Upon receipt in our laboratory they were washed 3× in BME and maintained in BME with 2% heated agammaglobulinic calf serum (agamma calf serum was obtained from Hyland Laboratories, Los Angeles, Calif.). The MDCK cells are currently carried in our laboratory in 199 medium[15] with 10% agamma calf serum and are maintained in BME with 2% agamma calf serum.

All media contained penicillin and streptomycin except when the pools were being checked for mycoplasma contamination.

Electron microscopy: Specimens were prepared for EM examination using the pseudoreplica technique of Sharp[16] as modified by Smith and Melnick.[17] A few drops of virus suspension were spread on a small 2% ionagar block and allowed to dry at room temperature. A few drops of 0.20% parlodion in amyl acetate were then placed on the blocks which were again allowed to dry. The membranes thus formed were floated in 2% phosphotungstic acid (pH 5). After a few minutes, copper grids were placed on the floating membranes and they were picked up on a sheet of parafilm. All specimens were examined on an RCA EMU 3G microscope, equipped with a high-magnification kit, at an accelerating voltage of 100 kv.

Separation of adenovirus from AAV in cesium chloride gradients: HEK bottle cultures were infected with the various substrains of E46 at a multiplicity of 0.01 $TCID_{50}$ per cell. All inocula were treated with 10% v/v chloroform for 10 min in the cold. The cultures were then incubated at 37°C and observed until 20% of the cells showed CPE (40–60 hr). The cells were scraped into the supernatant fluid and centrifuged at 75,000 × *g* for 90 min. The pellet was suspended in 3–5 ml of 0.05 *M* Tris buffer pH 8. After being frozen and thawed five times, the suspended pellet was extracted with Genetron 113 as described by Epstein and Powell.[18] The slightly opalescent aqueous phase was then adjusted to a density of 1.35 gm/cm^3 with crystalline CsCl, and centrifuged in a Spinco preparative ultracentrifuge (SW39 head in model L2) at 100,000 × *g* for 40 hr. Small 0.25-ml fractions were withdrawn from a hole in the bottom of the tube by forcing mineral oil in at the top with the aid of a microburet (Micro-Metric Instrument Co., Cleveland, Ohio). Appropriate fractions were pooled and rebanded at their own average density.

Preparation of antiserum: Antisera against Ad. 7, AAV-1, AAV-2, and AAV-3 were made by foot pad injection of pretested guinea pigs with purified virus fractions obtained by banding three times in CsCl. A single injection with complete Freund adjuvant was given and the animals bled approximately every 3 weeks. A reference serum prepared against a strain of AAV-1 from SV15[2] was kindly supplied by Dr. R. W. Atchison. In our hands, using a CF microtiter technique,[19] this serum had a CF titer of 1280 against 4–8 units of AAV-1 but no reactivity against 4–8 units of Ad. 7 virus. The titer of the various sera we have prepared will be described in the text.

Separation of adenovirus particles from AAV by ultrafiltration through Millipore filters: Crude AAV-containing fluids were prepared by centrifuging $E46^+$ (+1)-infected HEK cell extracts at 11,000 × *g* for 10 min. The supernatant fluid was then cleared of adenovirus particles by passing through pre, 220-mμ, 100-mμ, and 50-mμ Millipore filters arranged in tandem with the aid of Swinny adapters. Such preparations were found to contain observable AAV by EM but no adenovirus particles, and were free of adenovirus infectivity.

Results.—Transfer of AAV between adenovirus strains: The capacity to produce AAV particles was readily transferred to $E46^-$ (0) by mixed infection of HEK cells with 10^5 $TCID^{50}$ of $E46^-$ (0) and E46 (+1) preparations from which the adenovirus component had been eliminated by: (1) diluting 10- to 100-fold beyond the adenovirus endpoint; (2) passage through 50-mμ filters; or (3) heating at 60°C for 30 min. After a single passage, these $E46^-$ virus preparations showed abundant AAV particles by EM and reacted in CF with AAV antiserum, and are referred to as $E46^-$ (+1).

Transfer of AAV to heterologous adenoviruses: One of us previously reported[1] that an Ad. 2 and an Ad. 5 stock contained on occasional AAV-like particle when examined by EM. Attempts to increase the number of such particles and confirm

TABLE 1

TRANSFER OF AAV FROM E46$^+$ (+1) TO ICH (0)

Virus		Serum	CPE	Presence of Virus at Fifth MDCK Passage		
				AAV*	ICH*	Ad. 7†
ICH 10^{-3}		None	+	0	+	0
E46$^-$ (0)	10^{-2}	"	0	0	0	0
"	10^{-3}	"	0	0	0	0
E46$^+$ (+1)	10^{-2}	"	0	0	0	0
"	10^{-3}	"	0	0	0	0
"	"	Anti-AAV & Anti-Ad. 7	0	0	0	0
"	"	Anti-Ad. 7	0	0	0	0
ICH 10^{-3} + E46$^-$ (0)	10^{-3}	None	+	0	+	0
" + E46$^+$ (+1)	10^{-3}	"	+	+	+	0
" "	"	Anti-AAV & Anti-Ad. 7	+	0	+	0
" "	"	Anti-Ad. 7	+	+	+	0
Uninoculated control		None	0	0	0	0

* The second, third, and fifth passages checked for presence of AAV and ICH virus by EM and CF.
† The second and fifth passages were checked for Ad. 7 by two blind passages in HEK cells.

their presence by CF have failed. However, when E46$^+$ (+1) and Ad. 2 viruses are passed together in the presence of anti-Ad. 7 serum, many AAV particles are found which react with specific AAV antiserum. Ad. 2 (+1) thus obtained was passed five times, and AAV particles and antigen were found in high titer.

Perhaps even more significant is the fact that ICH (0) virus, which will not replicate in human cells, can serve as an AAV helper in dog cells (MDCK). AAV was easily transferred to ICH (0) virus by passaging in MDCK cells in the presence of 50-mμ filtrates of E46$^+$ (+1). In another type of experiment, MDCK cultures were inoculated with ICH (0), E46$^-$ (0), and E46$^+$ (+1) alone and in combination, with or without anti-AAV and anti-Ad. 7 serum (Table 1).

As can be seen, AAV was recovered with ICH virus at the fifth MDCK passage in the presence of anti-Ad. 7 serum but not with anti-AAV serum. It was found at approximately a 100–1 ratio of AAV particles to ICH. It is thus apparent that ICH can serve as an AAV helper in dog kidney cells.

Antigenic, immunogenic, and electron-microscopic characterization of the various fractions of E46$^+$ (+1) and E46$^-$ (0) separated by isopycnic CsCl gradients: After centrifuging for 40 hr, CsCl gradients of E46$^+$ (+1) usually showed 3–4 bands when observed by transverse illumination in a darkened room. Usually, the lowest visual band had an average density of 1.395 gm/cm^3. It contained the bulk of AAV particles but sometimes was contaminated with a few adenovirus particles which were subsequently eliminated during the second and third banding. The most prominent band occurred at a density range of 1.345–1.355. It contained most of the full adenovirus particles and was always contaminated with AAV particles. In 5 of 11 experiments, only one additional band was noted. In six other experiments this light band (density range, 1.30–1.33) appeared to be split into two or more bands. The bands in this density range were not well demarcated and tended to mix during collection. When such bands were examined by EM, they were found to contain many irregular, mostly empty adenovirus particles. Although a visible band was rarely seen in the density range of 1.40–1.45, AAV particles were regularly demonstrable in that density range by EM.

When E46$^-$ (0) preparations were spun for 40 hr, they usually showed two distinct bands, the heaviest at a density of 1.345–1.355. This very sharp band contained the bulk of the complete adenovirus particles but no AAV. The other band was at

TABLE 2

ANTIGENIC AND IMMUNOGENIC ANALYSIS OF CsCl FRACTIONATED E46$^+$ (+1) AND E46$^-$ (0)

Virus	Fraction pool	Density range	CF Antigen Titer* AAV	CF Antigen Titer* Adeno 7	Guinea pig no.	Maximum CF Antibody Titer† AAV	Maximum CF Antibody Titer† Ad. 7
E46$^+$ (+1) A Single banding	I	1.41–1.44	16	0	5	160	0‡
					6	320	0
					7	320	0
					8	640	0
	II	1.37–1.40	32	0	9	640	20
					10	80	10
					11	1280	0
					12	320	20
	III	1.34–1.36	16	16	1	80	640
					2	160	320
	IV	1.30–1.33	0	256	3	0	320
					4	0	320
E46$^+$ (+1) B Triple banding	I	1.41–1.44	32	0	—	—	—
					13	1280	0
					14	1280	0
	II	1.37–1.40	64	0	15	2560	0
					16	1280	0
	III	1.34–1.36	16	4	—	—	—
	IV	1.30–1.33	8	256	—	—	—
E46$^-$ (0) Double banding	I	1.41–1.43	0	0	25	0	0
					26	0	0
	II	1.37–1.40	0	0	29	0	0
					30	0	0
					33	0	1280
					34	0	1280
	III	1.34–1.36	0	64	35	0	1280
					36	0	1280
					27	0	320
	IV	1.30–1.33	0	32	28	0	320

* CF antigen titer expressed as the reciprocal dilution of the antigen which gave 3+ or > fixation with 8–16 units of guinea pig antibody.

† CF antibody titer expressed as the reciprocal dilution of serum which gave 3+ or > fixation with 4–8 units of antigen.

‡ 0 = No complement fixation at 1:10 dilution, the lowest dilution of serum tested.

a density of 1.30–1.33. It was quite diffuse and in some experiments seemed to be two overlapping bands. Again this lighter band contained many irregular adenovirus particles. All fractions were examined by EM from the first and second banding of E46$^-$ (0), and no AAV particles were ever seen.

In each experiment all fractions in the following density ranges 1.41–1.43, 1.37–1.40, 1.34–1.36, and 1.30–1.33, were pooled and labeled fraction pool I, II, III, and IV, respectively. After dialysis overnight against phosphate-buffered saline pH 7.2, they were titrated for CF antigen and then injected into pretested animals as described in *Materials and Methods*.

Table 2 gives the antigenic composition of the various fraction pools and their immunogenic capacity. The separation effected by a single 40-hr banding is shown in experiment E46$^+$ (+1) A. Noteworthy is the fact that even though no CF evidence of adeno antigen was noted in fraction pool II of the E46$^+$ (+1) A experiment, a few adenovirus particles were noted by EM. Further, there was a slight antigenic response to adenovirus in three of four animals immunized with this preparation.

As shown in experiment E46$^+$ (+1) B, when the standard procedure of rebanding two times in CsCl was employed, fraction II contained 64 units of AAV CF antigen but no adeno CF antigen and did not induce adeno CF antibody. When examined by

EM, this fraction contained many AAV particles but no adenovirus particles.

Thermal inactivation of AAV-1: AAV-1, either purified and suspended in 0.05 *M* Tris buffer or in crude adenovirus tissue culture harvests, was maintained for at least 6 months at 2–4°C and at least 30 days at 25°C without loss of CF antigen or infectivity. The results of 60°C inactivation of clarified crude preparations of E46$^+$ (+1) are shown in Figure 1. Initially, there was a slight drop in the adeno CF antigen(s), but the bulk of both AAV and adeno CF antigens were stable for 4 hr at 60°C. On the other hand, all measurable adenovirus infectivity was lost in less than 5 min, while the half life of AAV infectivity at 60°C was about $^1/_2$ hr.

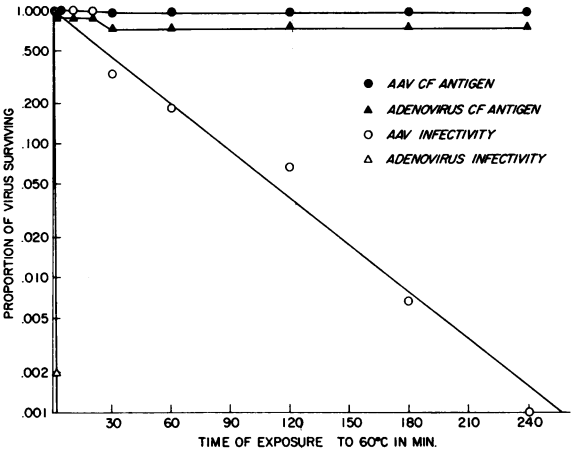

FIG. 1.—Heat inactivation of E46$^+$ (+1).

Demonstration of an inhibitory effect of AAV on adenovirus: AAV has been eliminated from various adenovirus stocks by limiting dilutions,[20] by specific AAV antiserum,[2] and by plaquing.[20] One observation which we have repeatedly made is that stocks containing AAV always seemed to have lower infectivity titers when titrated in HEK cells than similar non-AAV-containing stocks. Because this chance observation seemed so consistent, experiments were undertaken to investigate this effect under controlled conditions.

New passages of E46$^-$ (0) and E46$^-$ (+1) were prepared in the same lot of HEK cells at the same time and at the same adenovirus cell multiplicity (10 $TCID_{50}$ per cell). After 4+ CPE occurred in both series, the resulting stocks were then titrated in fresh HEK cell cultures. The E46$^-$ (0) had an infectivity titer of 10^8 $TCID_{50}$ per 0.1 ml and an Ad. CF titer 32 (0 against anti-AAV serum). The E46$^-$ (+1) had an infectivity titer of 10^7 with an Ad. CF titer of 2 and an AAV CF titer of 16.

A striking reverse effect was also noted when anti-AAV serum was used to reduce or eliminate AAV from E46$^+$ (+1) cultures. Pre- and postinoculation sera from AAV-immunized guinea pigs were tested for their ability to neutralize AAV-1. The neutralization procedure was repeated for three consecutive passages as shown

TABLE 3

INHIBITORY EFFECT OF AAV ON ADENOVIRUS

Serum	AAV CF serum titer	Serum dilution tested	CF Antigen Titer: Pass. 1 AAV	Pass. 1 Ad.	Pass. 2 AAV	Pass. 2 Ad.	Pass. 3 AAV	Pass. 3 Ad.
None	—	—	—	—	32	4	4	Tr.
G.P. 13 Pre	<10	1:20	8	2	32	4	8	4
G.P. 13 Post	640	1:20	0	64	0	32	0	32
G.P. 15 Pre	<10	1:20	16	4	16	4	16	8
G.P. 15 Post	1280	1:20	0	64	0	32	0	32
G.P. 22 Pre	<10	1:20	32	8	32	4	8	2
G.P. 22 Post	1280	1:20	0	64	0	64	0	32

in Table 3. It is clearly evident that as the CF titer against AAV was reduced, the Ad. CF titer increased 4–16-fold.

Preinoculation sera from all the animals tested had no effect either in decreasing the AAV titer or increasing the adenovirus titer. After the third passage, five additional passages were carried out without antiserum to see if the AAV had been eliminated or just reduced below the level of the testing procedure. The AAV was eliminated when anti-AAV serum was used at 1:20 dilution. Another observation not noted in Table 3 is that clear adenovirus CPE was evidenced by the second or third day after passage with anti-AAV serum, but not until the fourth or fifth day in the passages carried with control antiserum.

TABLE 4

DEMONSTRATION OF AAV SEROLOGICAL TYPES BY CF

Purified antigens	CF Antigen Titer When Tested against 8 Units of Antibody			
	Anti-AAV-1	Anti-AAV-2	Anti-AAV-3	Anti-Ad. 7
AAV-1	256	0	0	0
AAV-2	0	32	0	0
AAV-3	0	0	64	0
Ad. 7	0	0	0	64

Demonstration of AAV serological types: AAV-1, AAV-2, and AAV-3 were eliminated from adenovirus stocks during a single tissue culture passage in the presence of a 1:20 dilution of homologous AAV serum but not with heterologous sera. The various AAV's could also be distinguished by CF as shown in Table 4.

Discussion.—Two hypotheses have been suggested on the nature of the small particles in adenovirus stocks, i.e., that they are adenovirus subunits[4–6] or an extraneous virus.[1–3] In support of the first hypothesis, Melnick *et al.*[4] reported that SV15 and several human adenoviruses could apparently be made to yield small particles by treatment of the complete virion *in vitro*, and Mayor *et al.*[6] reported that the small particle could initiate an adenovirus infection in tissue culture. These findings suggested to them that the small polyhedral particle was an internal component of the mature adenovirion and was in all probability related to the adenovirus genome.

Smith *et al.*,[21] who studied the capsids of various adenoviruses after fragmentation with sodium lauryl sulfate, found many groups of 9 capsomeres but no groups of 6. They pointed out that such a 6 capsomere "missing piece" would be about the same size as the small particles found in various adenovirus stocks.[4] They emphasized, however, that they had no direct evidence that the reported 20-mμ particle[4] is a structural component of the adenoviruses they studied.

Our data and those of Atchison *et al.*[2] do not support the adenovirion subunit hypothesis. AAV was readily transferred from one human adenovirus preparation to others and even from a human adenovirus to ICH virus. Antisera prepared against three purified AAV types gave no cross-reactivity in CF or by neutralization when tested against adenovirus. In fact, such sera have proved valuable for freeing adenovirus stocks of serologically related AAV strains with resulting increased adenovirus yields. Conversely, serum prepared against purified clean adenovirus stocks does not cross-react with purified AAV in CF or prevent its replication in the presence of heterologous adenovirus types.

In unpublished experiments,[22] our attempts to obtain AAV from *purified* adeno-

virus type 7 by genetron treatment or degrading the virion were not successful; however, we were able to increase the apparent number of AAV particles in crude adenovirus preparations, known to contain AAV, by treatment with genetron, chloroform, or ether. This was not unexpected since the AAV particles are frequently found in large aggregates bound by lipid-like membranes and such treatment simply freed large aggregates from these membranes and cell debris.

It should be pointed out that additional data recently reported by Parks *et al.*[23] now support the second hypothesis.

The origin of the AAV contaminants in the various adenovirus preparations is not known. With one possible exception, field isolates of adenovirus have not been found to contain AAV. Many AAV-1-contaminated stocks have been passaged in monkey kidney cells, which are known to harbor many indigenous viruses, but AAV-2-contaminated stocks have been passed only in human cells.[26] The extreme stability and high titers are compatible with the possibility that some adenoviruses acquired the agent by cross contamination in the laboratory.

All AAV preparations we have examined appear to be morphologically similar to the stable DNA containing rat virus (RV)[24] and the H-1 virus.[25] However, the RV and H-1 viruses are serologically unrelated to the three distinct AAV types we have studied[26] and to other AAV strains studied by Atchison[27] who has found that some AAV strains from SV15 are related to each other by CF and precipitin but not by neutralization. Atchison *et al.*[2] using acridine orange staining concluded that the small particles from their SV15 preparations contained double-stranded DNA. Our three serologically defined AAV types incorporate tritiated thymidine.[28] The DNA from AAV-1 has been further characterized by physical and chemical techniques.[29] These studies indicate that the extracted DNA is double-stranded and differs significantly in base composition from Ad. 7 DNA. These findings, together with other data already discussed, make it obvious that AAV is not a subunit of the adenovirion. In the cell systems studied by us and by Atchison *et al.*,[2, 27] it has been shown that AAV can persist in cell culture but remain undetected until infectious adenovirus is added. The specific mechanism by which adenovirus stimulates AAV replication is not known. Inactivated adenovirus,[22] virus-free filtrates of Ad. 7[22] or infectious preparations of many other DNA or RNA viruses[2, 22] could not be substituted. Further, cells transformed by adenovirus, which contained early adenovirus T antigen, did not support AAV replication.[22] Thus, adenovirus replication, or at least some adenovirus genetic function occurring later than T antigen formation, is required for detectable AAV replication. AAV might be considered a defective virus in the same broad sense that the RNA-containing satellite virus(SV) is defective since SV is dependent on the serologically unrelated tobacco necrosis virus for its replication (Kassanis and Nixon[30, 31]). As does SV, AAV must code for its own protein coat but does not replicate to appreciable titers without its helper. The Rous sarcoma virus dependency on Rous-associated virus is well known,[32] as is the fact that certain human adenoviruses do not replicate well, if at all, in monkey kidney culture in the absence of SV40 virus.[33, 34] Brailovsky and Chany[35] have observed that certain DNA viruses (Ad. 12, polyoma, and SV40) stimulate the replication of partially inactivated RV. The various mechanisms whereby these various systems operate await further biochemical study. Such studies of this "virus helper" phenomenon may lead to a better basic understanding

of the replicative as well as other virus mediated changes, such as transformation, which occur in infected host cells.

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