References and Notes

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Adenovirus-Associated Defective Virus Particles

Abstract. *Small, DNA-containing particles were separated from preparations of a simian adenovirus. These particles differed antigenically from the adenovirus. Replication of the particles in cell cultures was obtained only when they were inoculated simultaneously with adenoviruses. This suggests that these adenovirus-associated particles behave as defective viruses.*

Electron microscopy of simian adenovirus type 15 (SV15) has disclosed that negatively stained preparations contained a high concentration of small, uniform, virus-like particles, together with typical adenovirus particles (*1*). Our present study was undertaken to determine whether the small particles represented an unrelated virus containing SV15 or were in some manner intrinsically associated with the adenovirus.

The small particles, now tentatively referred to as adeno-associated virus (AAV), were observed first in a pooled harvest of rhesus-monkey-kidney-cell (RMK) cultures infected with SV15. These cultures had been collected and frozen when essentially complete cytopathic effect was apparent. This particular pool had been stored since 1961 at −20°C and represented a third passage in the kidney-cell cultures after the virus had been received in this laboratory (*2*). Portions (10 ml) of this and subsequent preparations were subjected to five cycles of freezing and thawing. This treatment was followed by centrifugation at low speed (12,000*g*, 15 minutes, 4°C) to separate the crude debris. The supernatant was centrifuged at high speed (60,000*g*, 90 minutes, 4°C); the pellet contained slightly purified virus particles. The reconstituted pellets were negatively stained (*3*) by phosphotungstic acid as follows. A drop of the pellet suspension was placed in contact with a carbonized, formvar-coated, electron microscope grid, blotted after 1 minute, put in contact with a drop of the stain for 4 minutes, then blotted and air dried. Two percent aqueous phosphotungstic acid was used at *p*H 1.8 through 7.4, with *p*H 4.5 giving the best results. Observations were made with a Philips EM-200 electron microscope, single or double condenser illumination being used. Photographic plates were exposed at microscope magnifications of 41,000 and 71,000.

At the lower magnification, a large number of discrete particles were observed (Fig. 1). The particles have an average diameter of 240 Å and show hexagonal profiles in many instances (arrows). Both stain-penetrated and nonpenetrated forms are apparent and correspond in appearance with known viruses, with or without nucleic acid cores (*4*). At the higher magnification, the AAV particles (Fig. 2) also show a rough surface suggesting the presence of capsomeres (*4*).

The crude suspensions of SV15 were filtered through a 50-mμ Millipore membrane to separate AAV particles from SV15. The pooled filtrates, when concentrated tenfold by high-speed centrifugation, provided a more purified particle suspension. These suspensions did not elicit adenovirus cytopathic effect after inoculation onto RMK cultures, when observed for a 14-day period. During this period, the AAV particles present in the suspensions caused no cytopathic effect in the kidney cultures, nor did the inoculated cultures yield AAV particles by electron microscopy. Likewise, cytopathic effects were not manifest within a period of 7 to 11 days after inoculation of the particles on African-green-monkey-kidney, mouse-embryo, rabbit-embryo, calf-kidney, or hamster-kidney cultures, or on a continuous cell line of KB(B) (*5*). Newborn mice and hamsters were inoculated by various routes, but showed no apparent illness 2 months after inoculation.

Purified AAV concentrates were stained for DNA or RNA content with 0.01 percent acridine orange (*6*). The yellow-green fluorescence of these preparations indicated that AAV contains double-stranded DNA (*7*). Preparations of unfixed AAV digested with pepsin and treated with deoxyribonuclease served as stain controls (*7*).

Another preparation of SV15 (*8*), hereafter referred to as SV15(M), did not contain AAV particles when examined in the electron microscope. A first passage pool, made in RMK cultures, was used to find out whether AAV could replicate in the presence of the type of virus with which it was originally found. Two-tenths milliliter of a 1 : 10 dilution of the AAV pool was added simultaneously with 0.2 ml of a 1 : 10 dilution of SV15(M) to each of two bottle cultures of RMK. Kidney-cell cultures of the same lot were similarly inoculated with SV15(M) alone or with AAV alone. Bottles were incubated at 37°C until cytopathic effects (graded 2+ or 3+) were observed in all cultures inoculated with SV15(M). Fluids were then prepared for electron microscopy. The results of this and subsequent experiments have demonstrated that AAV can replicate in RMK cultures when SV15(M) is present. The AAV particles appeared in larger numbers than adenovirus particles. The AAV-inoculated kidney-cell cultures were consistently negative for AAV as were the SV15(M)-inoculated cultures, the latter having roughly the same numbers of adenovirus particles as those inoculated with SV15 and AAV simultaneously.

A newly isolated human adenovirus type 2 preparation (*9*) was found free of AAV when examined in the electron microscope. This adenovirus, referred to now as Ad2, also had the capacity to help AAV replicate. Control cultures inoculated with either AAV alone or Ad2 alone were devoid of detectable AAV particles.

Several other DNA and RNA viruses (*10*), which themselves replicated well in RMK cultures, were similarly tested with portions of the aforementioned AAV pool with similar controls. All culture fluids were processed for observation, when suitable viral cytopathic effect was observed. All failed to induce AAV replication. In each instance a DNA or RNA virus characteristic of that inoculated was observed and photographed with the electron microscope in those cultures developing specific cytopathic effect.

A titration of one lot of purified AAV suspension was performed by inoculation of a tenfold dilution onto RMK cultures in the presence of a con-

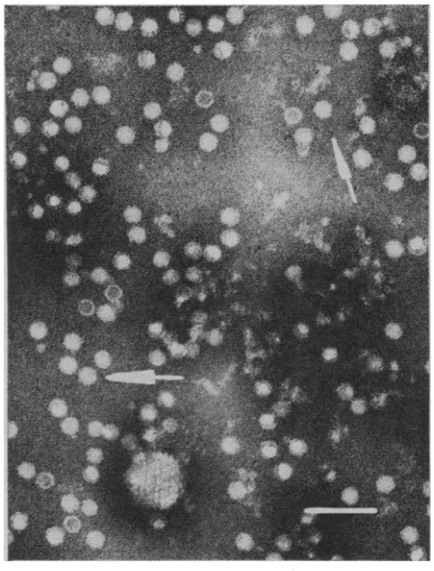

Fig. 1. Adenovirus cell culture preparation slightly purified by differential centrifugation. Smaller AAV particles (arrows) surround the typical adenovirus (SV15) present. Scale, 1000 Å.

stant amount of SV15(M). The AAV up to the sixth tenfold dilution proved capable of replicating to a degree recognizable by electron microscopy.

The AAV was tested for ether sensitivity by the addition of an equal part of diethyl ether and incubated at 4°C for 24 hours. A resistance to ether was demonstrated by the ability of treated AAV to replicate in the usual manner.

Antiserums (*11*) were prepared in guinea pigs by multiple inoculations of the purified AAV suspensions. Undiluted antiserum, added to an equal amount of 1:10 dilution of SV15 (containing AAV) and incubated for 1 hour at 25°C, prevented AAV replication in RMK cultures. Particles of AAV were present in expected numbers when normal serum from uninoculated guinea pigs was substituted for the antiserum to AAV, the same lot of virus and cultures being used. No neutralization of SV15 was observed by this antiserum to AAV. Furthermore, passage of SV15 three times with antiserum to the AAV in kidney cultures has apparently eliminated AAV from the preparation, since it did not reappear in five subsequent passages in the absence of serum. Furthermore, no antigenic relationship was found between AAV and SV15 particles by complement fixation and precipitation tests (*12*).

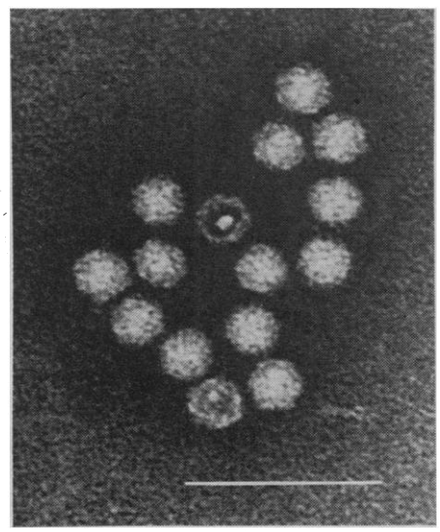

Fig. 2. The AAV particles obtained in a more purified preparation and separated from adenovirus by membrane filtration. The surface reveals units resembling capsomeres. Scale, 1000 Å.

Apparently then, AAV may be called an incomplete or defective virus in view of the evidence that it does not readily replicate alone. In a somewhat analogous manner, the defective RNA-containing Rous sarcoma virus requires the help of Rous-associated virus, or other viruses of the avian leukoses group, for replication of the sarcoma virus or for development of the sarcoma (*13*). Whether AAV can produce foci of transformed cells in suitable tissue cultures, as does the defective RSV in chick-embryo tissue culture, remains to be shown. Our studies (*12*) show no detectable viral antigen or antigens in the culture fluids and centrifuged pellets from RMK cultures 4 days after inoculation with AAV alone. Hoggan's work (*14*) implies that some small virus-like particles found in human adenovirus preparations need the presence of the adenovirus for their replication.

The possibility that these small particles (AAV) might be adenovirus antigen, adenovirus-infected cell particles as yet undescribed, or degraded adenovirus particles (*15*) was considered. These alternatives appear unlikely for the following reasons. First, the complement-fixing antigens, types 1, 2, and 3, are all smaller than AAV, having diameters of 100 Å, 170 Å, and 80 Å, respectively (*16*). Second, SV15(M) yielded no AAV particles when passed seven times in RMK cultures. Third, disrupted forms of adenovirus are frequently found in preparations for electron microscopy, particularly after fluids are centrifuged at high speed, but none of these particles resembles AAV.

Other small DNA-containing viruses of approximately this size include Toolan's H-1 and H-3 viruses (*17*), O'Malley's A-1 virus (*18*), Kilham's Rv virus (*19*), and perhaps the small infectious "unit" reported in Yaba virus preparations (*20*). Several of these are at least suspect or correlated with oncogenesis and others are "candidate" agents for infectious or serum hepatitis. Since adenoviruses of several types are under intensive study as oncogenic viruses and many adenoviruses have been isolated from persons and animals with hepatitis, the finding of these adenovirus-associated particles has potential interest in both of these areas of current intensive research on etiology. The fact that antiserum to AAV can specifically inhibit AAV formation may prove important in removing this or other similar agents from prospective seed virus for vaccine preparation.

ROBERT W. ATCHISON
BRUCE C. CASTO
WILLIAM MCD. HAMMON
Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania

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Isoenzymic Specificity of Impaired Clearance in Mice Infected with Riley Virus

Abstract. *The clearance from the plasma of purified lactate dehydrogenase isoenzyme No. 5, which is increased in the plasma of mice infected with Riley virus, is impaired during the infection. That the clearance of purified isoenzyme No. 1 is unchanged by infection with Riley virus provides an explanation of why only the lactate dehydrogenase-5 isoenzyme activity is elevated in the plasma of infected mice.*

A viral infection of mice, characterized by a five- to tenfold increase in lactate dehydrogenase (LDH) plasma in the absence of other pathological changes, was first described by Riley *et al.* (*1*). The activities of a number of other enzymes, including isocitrate dehydrogenase, are also raised in the plasma of mice infected with Riley virus, whereas those of others, such as alkaline phosphatase and alanine transaminase, are not (*2*).

We have suggested that the increased plasma-enzyme activities of infected mice might be due to impaired clearance of endogenous enzymes from the plasma (*3*). Plasma clearance of intravenously injected LDH and isocitrate dehydrogenase is impaired in mice infected with Riley virus, whereas clearance of alkaline phosphatase and alanine transaminase is unaltered (*4*).

Studies on the patterns of LDH isoenzyme in the plasma of normal and of infected mice have shown that, of the five electrophoretically distinct forms normally present, only the slowest migrating (LDH-5) is increased in amount during Riley virus infection (*5*). The concentration of this isoenzyme is highest in liver, spleen, and skeletal muscle; the fastest-migrating LDH isoenzyme, LDH-1, on the other hand, is found in high concentration in heart tissue (*6*). It was therefore of considerable interest to determine whether the impaired clearance of intravenously injected LDH in mice infected with Riley virus was specific for the LDH-5 isoenzyme.

Male mice of a randomly bred albino strain, either uninfected or infected with Riley virus 4 to 6 days before, were used. The rate of clearance of injected LDH is depressed, and remains so from 3 to at least 20 days after infection (*4*). Mice were injected by way of the dorsal vein of the penis, and heparin was added to the blood samples which were obtained by bleeding from the tail. Plasma LDH clearance was determined by measuring the plasma LDH activities spectrophotometrically (*7*) at 2 minutes and at 3 hours after intravenous injection of purified crystalline LDH-1 (pig heart, Boehringer) or LDH-5 (rabbit muscle, Boehringer) (both at 1 mg/100 g of body weight) containing approximately 0.1 mg enzyme protein per milliliter of phosphate-buffered saline. As the specific activity of the LDH-5 preparation was about 2.5 times that of the LDH-1, a further experiment was carried out in which the LDH-5 isoenzyme (0.04 mg per milliliter of saline) was injected. This gave an initial LDH activity in the plasma similar to that produced by 0.1 mg of the LDH-1 isoenzyme per milliliter. The purity of the LDH-1 isoenzyme was stated to be more than 98 percent, and that of the LDH-5 isoenzyme to be more than 99 percent, relative to the other bands (*8*). The level of LDH in plasma immediately prior to LDH injection was also determined for each mouse, to give the activity at the "steady state." Clearance was then referred to the increase above the steady state at any time.

Table 1. Clearance of plasma LDH-1 and LDH-5 (expressed as international units, IU) after intravenous injection into normal mice and mice infected with Riley virus.

Riley virus infection*	Plasma LDH (IU/ml × 10^{-3})			Clearance of injected LDH (%)	*P* between means (% clearance)
	Prior to injection	Excess over steady state† after			
		2 min	3 hr		
			LDH-1, 0.1 mg/100 g		
−	0.28 ± .03 ‡	2.10 ± .10	1.66 ± .10	21.0 ± 3.3	> .6
+	1.85 ± .09	1.85 ± .04	1.39 ± .14	25.1 ± 6.6	
			LDH-5, 0.04 mg/100 g		
−	0.39 ± .01	1.58 ± .13	0.57 ± .08	64.1 ± 5.0	< .001
+	2.00 ± .11	1.11 ± .06	1.40 ± .04	− 27.2 ± 4.5 §	
			LDH-5, 0.1 mg/100 g		
−	0.36 ± .03	5.60 ± .10	1.32 ± .17	76.2 ± 3.4	< .001
+	2.05 ± .06	6.19 ± .35	4.31 ± .13	29.6 ± 4.5	

* Mice were inoculated with Riley virus ($10^5 ID_{50}$), that is, 10^5 infective doses, 50 percent effective, 4 to 6 days before isoenzyme injection. † Obtained by subtracting the LDH activity of the steady state prior to injection from the observed activity at 2 minutes and at 3 hours. ‡ Values are means ± standard error of the mean of six observations in each case. § Percentage increase.

The clearance rate of LDH-1 was similar in the two groups of animals; thus there appears to be no inhibition of the clearance of this fast-moving isoenzyme in the infected mice. In the case of LDH-5, there was a highly significant inhibition of clearance in the infected animals. The uninfected mice cleared more than 60 percent of the injected LDH-5 isoenzyme, at either dose, within 3 hours, whereas the infected mice had cleared only 30 percent of the higher dose in this time, and none of the lower dose. Indeed, in the infected mice, the plasma LDH, instead of falling, had risen by 3 hours. This phenomenon of a rising LDH content, which we regularly observe in infected mice after small doses of LDH-5, must result from an increase in circulating endogenous enzyme. This could be due to an increased input resulting from some effect of the injected enzyme preparation such as cellular damage, or to a decreased clearance resulting from a blocking effect of the injected material. It might be expected that larger doses of injected enzyme preparation would produce a greater damaging or blocking effect than smaller doses. However, owing to the biphasic exponential nature of the rate of enzyme clearance (*4*), high doses of enzyme are cleared very much faster than small doses, and this high rate may mask small changes in endogenous enzyme input or clearance rate.

The rate of clearance of the LDH-1