

Adeno-Associated Virus in Adenovirus Type 3 Conjunctivitis

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Although human infection with adenovirus-associated virus (AAV) has been demonstrated, there is no evidence that disease results from such infections. The proportion of adenovirus infections which are dual infections with AAV is virtually unknown, since special methods are required to demonstrate infection with AAV. To search for AAV, we re-examined a collection of specimens from 40 persons involved in an epidemic of pharyngoconjunctival fever associated with a swimming pool. Virological and serological studies indicated that the etiological agent was adenovirus type 3. When the 91 original eye, throat, and fecal specimens were re-examined, using methods suitable for detection of adenovirus and AAV, 37 strains of adenovirus type 3 and 35 strains of AAV type 3 (AAV3) were isolated. Surprisingly, 19 AAV3 but only 11 adenovirus isolates were found in eye specimens, whereas adenovirus isolates were equally distributed in all types of specimens. Four AAV3 strains were isolated from adults. Significant (fourfold or greater) rises in AAV3 complement-fixing antibody titers were seen in six of 14 persons shedding AAV3, whereas nine of 10 persons shedding adenovirus type 3 showed significant rises in adenovirus complement-fixing antibody. These results raise the question whether AAV persists better in eyes than adenovirus or that a possible association with conjunctivitis might be present. In contrast to the results in the specimens from the swimming pool epidemic, only one of 36 adenovirus strains isolated in other Seattle-based studies yielded AAV. Complement fixation tests on serial sets of sera collected from 60 children not involved in the swimming pool episode revealed nine AAV2 and 12 AAV3 infections during a 4-year period.

Adenovirus-associated viruses (AAV), originally discovered as contaminants in adenovirus preparations, have become recognized as distinct viruses and are classified as parvoviruses. They require adenovirus helper activity for measurable replication in cell culture and presumably in vivo but are immunologically and structurally unrelated to their helpers (1, 15). Four serotypes are presently recognized. The presence of antibodies to AAV has been demonstrated in animals and humans, and AAV types 2 and 3 have been isolated from children (4, 21; R. W. Atchison, B. C. Casto, W. McD. Hammon, and N. P. Rapoza, *Fed. Proc.* 25:249, 1966). Further evidence for human infection with AAV2 and AAV3 was found by seroepidemiological surveys (3, 5, 6, 21, 25). Experimental evidence indicates that AAV can interfere with adenovirus replication and decrease oncogenicity of adenoviruses (7, 12, 13, 17, 22). In a study of cancer patients and controls, no significant difference in prevalence of antibodies for AAV was shown in the two groups

(18, 28, 29). So far, no disease has been attributed to AAV, and it is not known how pathogenesis of adenovirus infections in humans might be affected by these viruses.

In the hope of finding some answers to these questions, we investigated the possible role of AAV during an epidemic of pharyngoconjunctivitis associated with adenovirus type 3. The epidemic, which has been reported elsewhere (10), was characterized by a sharp outbreak of pharyngoconjunctival fever in children after exposure to unchlorinated water and by milder symptoms in secondarily infected adults, with conjunctivitis a predominating symptom. This report describes the results of AAV isolation attempts and serological tests on specimens collected from patients and contacts during this circumscribed outbreak. A surprisingly high isolation rate of AAV3 in conjunctivitis patients and serological evidence for AAV3 infections were found. Therefore, adenovirus isolates and serum specimens from other studies in Seattle were examined for comparison. Serological

studies were confined to AAV2 and AAV3, the two serotypes definitely known to infect man (6, 19, 21, 24).

MATERIALS AND METHODS

Viruses. Reference AAV2 and AAV3 with adenovirus type 7 as helper were received from M. D. Hoggan (National Institute of Allergy and Infectious Diseases), who also supplied reference AAV2 and AAV3 guinea pig antisera. A field strain of adenovirus type 7, free of AAV contamination, which had been isolated in our laboratory from a throat specimen (9) was used as helper in AAV isolation attempts.

Cell lines. Two human heteroploid cell lines were used in the study, HeLa-M and KB. Both cell lines were grown on glass in Eagle minimum essential medium with 10% fetal bovine serum. A concentration of 2% serum was used during infection. KB cells were grown in suspension culture in AUTO POW MEM (Flow Laboratories, Rockville, Md.), supplemented with 0.006 M L-glutamine and 10% heat-inactivated horse serum (14). A concentration of 5% heat-inactivated horse serum was used during infection.

Specimens for virus isolation attempts. Twenty-six adenovirus type 3 isolates (cell culture fluids) from persons associated with the swimming pool epidemic were tested for AAV (10). Adenovirus and AAV isolation was also attempted from 91 original specimens (eye, throat, and fecal specimens), including those which had previously yielded adenovirus type 3 in the original study. Twenty-five adenovirus type 3 and 11 adenovirus type 7 field strains which had been isolated in our laboratory from Seattle Virus Watch specimens (9) were tested for presence of AAV in a comparison study.

Serum specimens. Paired acute and convalescent serum specimens had been collected from 19 persons associated with the swimming pool epidemic. Also available from that study were 87 single serum specimens which had been obtained about 1 month after the swimming pool episode. All sera were tested for adenovirus, AAV2, and AAV3 complement-fixing antibodies. Sixty sets of five consecutive serum specimens each were tested in a comparison study for adenovirus, AAV2, and AAV3 complement-fixing antibodies. The serial specimens had been collected over a 4-year period from 60 school children ranging in age from 6 to 12 years.

AAV antigen preparation. AAV2 and AAV3 reference strains were initially propagated in HeLa-M cells with adenovirus type 7 as helper and were then grown in KB cell suspension culture for production of large amounts of virus as described by Johnson et al. (16). Adenovirus type 7 helper virus was used at a concentration of 1 to 10 plaque-forming units/cell and AAV at a multiplicity of about 100 to 1,000 physical particles/cell. AAV was purified by two isopycnic bandings in CsCl and one additional purification step on sucrose gradients. Briefly, 2-ml aliquots were layered over 30-ml preformed continuous sucrose gradients [30 to 10% sucrose, 0.03 M tris(hydroxymethyl)aminomethane, 1.0 M NaCl, pH 7.8] and centrifuged at 4 C for 5 h at $79,000 \times g$ in an SW 25.1

rotor. One-milliliter fractions were collected by bottom puncture, and optical densities were determined at 280 nm. Fractions containing virus were desalted using Amicon Centriflo 2100 CF 50 membrane ultrafilters (Amicon Corp., Lexington, Mass.) and checked for purity by electron microscopy after they had been found free of adenovirus infectivity in HeLa-M and KB cells and free of adenovirus and cell antigens by complement fixation (CF) test.

Preparation of antisera. Antisera against AAV2 and AAV3 were made by footpad injection of prebled guinea pigs. Approximately 10^{11} particles with complete Freund adjuvant were injected. Two weeks later a booster shot was given intramuscularly with incomplete Freund adjuvant. Animals were bled 7 days later.

Antiserum was also prepared in rabbits using the method of Cooney and Kenny (8), which employs an initial intramuscular injection of immunogen with incomplete Freund adjuvant followed by a series of intravenous injections.

Electron microscopy. Virus fluid was applied to 300-mesh copper grids previously coated with a thin film of parlodion. After 1 min, excess fluid was carefully drained on filter paper, and 2% phosphotungstic acid, pH 7.2, was applied for 10 s. Excess stain was drained on filter paper, and the grids were air dried and then examined in an RCA EMU 3G electron microscope.

Serological tests. (i). CF. Infected cell culture fluids were tested for CF antigen in twofold dilutions from undiluted through 1:8, using 4 U of monospecific human and type-specific hyperimmune animal sera. Serum specimens were tested in twofold dilutions from 1:4 through 1:64 with 4 U of type-specific CF antigens. The microtiter CF technique described by Sever (26) with overnight fixation at 4 C was employed.

(ii). Virus agglutination observed by electron microscopy. The technique has been described by Smith et al. (27). Briefly, antiserum and virus dilutions were mixed and incubated at room temperature for 30 min. Mixtures were then applied to copper grids and prepared for electron microscopy as described above. Normal guinea pig serum and saline were used in negative controls. Grids were read immediately after preparation and reactions were graded as follows: 1+, mostly single virions with antibody attached; 2+, 20 to 40 virions per immune complex; 3+, more than 50 but less than 100 virions per immune complex; 4+, more than 100 virions per immune complex. Immune complexes were checked in 20 fields at the lowest possible instrumental magnification.

Virus isolation. (i). Adenovirus isolation. Procedures for adenovirus isolation and identification have been described elsewhere (9).

(ii). AAV isolation. AAV isolation was attempted in two human heteroploid cell lines, HeLa-M and KB. For isolation from adenovirus-infected cell culture fluids, the indigenous adenovirus was employed as helper virus. For isolation from original specimens which had been stored at -70 C, the indigenous adenovirus was used as helper virus, if present. In addition, an adenovirus type 7 field strain, free of

AAV contamination, was employed as helper virus at a multiplicity of 1 to 2 plaque-forming units/cell. Fluids were passed three times in cell culture tubes and were then inoculated into monolayers of each type of cell prepared in 16-oz (0.473-liter) prescription bottles. When destruction of the cell sheet was almost complete (3 to 4 days after inoculation), the cells were scraped into the supernatant fluid, pelleted by centrifugation, resuspended in 2 ml of veronal-buffered saline, and ruptured by 6 freeze-thaw cycles to release virus. Cell debris was removed by centrifugation, and the supernatant fluids were treated with a fluorocarbon, Genesolv D.

Demonstration of AAV CF antigen and detection of virus by electron microscopy and immunoelectron microscopy were used as indicators for AAV isolation and identification.

RESULTS

Purity of AAV antigen. Satisfactory separation of AAV and adenovirus CF antigens was obtained by two isopycnic bandings in CsCl and one additional purification step on preformed continuous sucrose gradients as described above. The final purification step on a sucrose gradient of one AAV preparation (previously banded twice in CsCl) is shown in Fig. 1. The bottom AAV peak was found to consist mainly of small virus aggregates and the main AAV peak mostly of single and intact AAV particles. Partially degraded AAV particles were most frequently observed in the top AAV peak. AAV and adenovirus CF antigens were found in the top fractions, but virus particles could not be observed, and adenovirus contamination was restricted to top fractions. Purified AAV3 is shown in Fig. 2.

Virus isolation and identification. Twenty-six of the 91 specimens which had been collected from 40 persons for the study of the swimming pool episode had yielded adenovirus type 3. These virus isolates were first examined for AAV and, surprisingly, 16 were shown to contain AAV3. An electron micrograph of one of the isolates is shown in Fig. 3. Serological

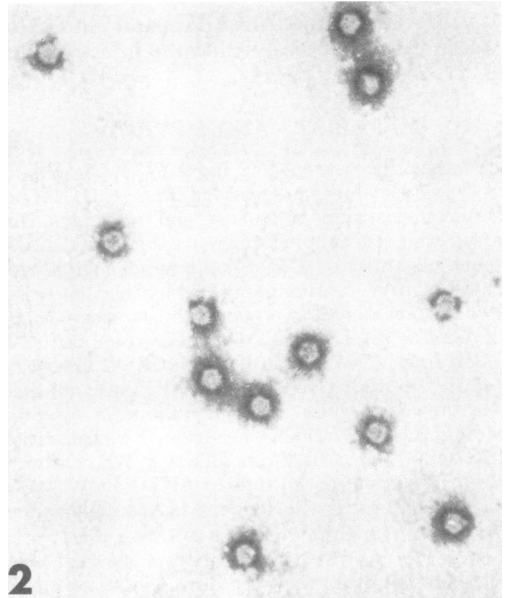


FIG. 2. AAV3, negatively stained. $\times 144,400$.

methods used in identification and differentiation of AAV isolates are shown in Tables 1 and 2. Positive results were obtained with homologous antisera in both CF and agglutination tests, whereas negative reactions were seen with AAV2 antisera under the test conditions employed. An example of a 4+ AAV3-anti-AAV3 immune complex, observed in the electron microscope, is shown in Fig. 4a and b. Identification of four AAV isolates as AAV3 was further substantiated by density determinations in CsCl. The average densities of AAV reference strains and isolates propagated and banded under identical conditions, as described in Materials and Methods, were found to be: 1.380 g/cm for AAV2, 1.389 g/cm for AAV3 reference strains, and 1.390, 1.391, 1.391, and 1.390 g/cm for AAV3 isolates.

Since so many of the adenovirus type 3 isolates also yielded AAV3 (61.5%), all 91 original specimens were re-examined for both adenovirus and AAV. Results of isolation attempts are summarized in Table 3. AAV3 was isolated from 35 (38.5%) specimens as compared to 37 (40.7%) adenovirus type 3 isolations. More than one-half (19/35) of the AAV3 isolates were obtained from eye specimens, whereas adenovirus type 3 was distributed equally in eye, throat, and fecal specimens. Specimens from two persons, designated throat/eye, were counted separately because throat and eye swabs had been placed into the same specimen collection vial. In one of these persons, a throat swab collected after convalescence was negative for both vi-

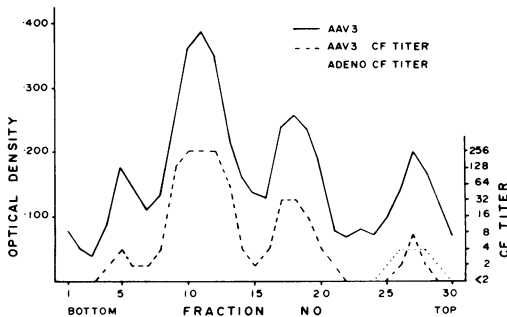


FIG. 1. Purification of AAV on continuous sucrose gradient.

ruses; in the other person, throat and fecal specimens collected after convalescence were

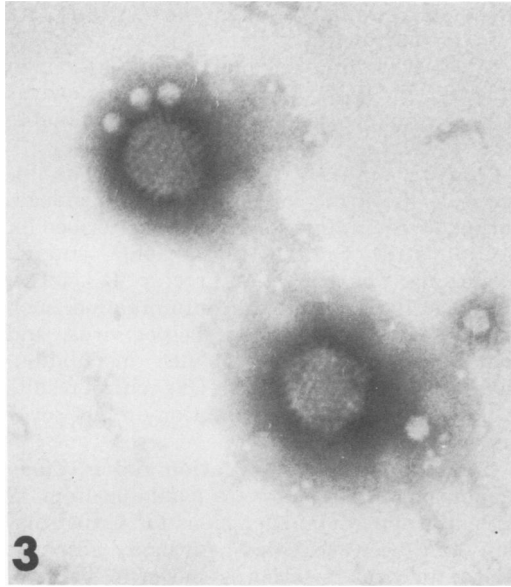


FIG. 3. AAV3 and adenovirus type 3, negatively stained. $\times 104,000$.

negative for AAV3, and the fecal swab was positive for adenovirus type 3.

Isolation results from specimens collected from persons are shown in Table 4. Adenovirus type 3 and AAV 3 were isolated from 14 persons in the same specimen. AAV3 isolation from one specimen and adenovirus type 3 from another were found in two persons. In one of these, AAV was isolated from the eye and adenovirus from the throat specimen; in the other, AAV was isolated from the eye and adenovirus from the fecal specimen. AAV3 only was isolated from seven persons. In five of these only the eye specimens yielded AAV, and both eye and throat specimens from the two other persons yielded AAV. Adenovirus type 3 only was found in four persons. Overall, adenovirus type 3 was isolated from 20 persons and AAV3 from 23 persons.

Association of AAV with adenovirus isolates from other Seattle-based studies. Because of the apparent association of AAV3 and adenovirus type 3 in the swimming pool outbreak, it was of interest to explore possible AAV3 occurrence with other adenovirus type 3 isolates from the Seattle community. Therefore, 25 adenovirus type 3 and 11 adenovirus type 7

TABLE 1. AAV type identification by demonstration of CF antigen

Virus	Source	CF with antiserum				
		Patient F.C. conval. serum (1:16) ^a	Anti-AAV3 G.P. serum ^b (1:300)	Anti-AAV2 G.P. serum (1:300)	Anti-AAV3 human serum (1:30)	Anti-AAV2 human serum (1:30)
AAV	Patient F.C.	4+ ^c	4+	Neg	4+	Neg
AAV3H	Reference virus	4+	4+	Neg	4+	Neg
AAV2H	Reference virus	Neg	Neg	4+	Neg	4+

^a Ratio in parentheses indicates serum dilution. Neg, Negative.

^b G.P., Guinea pig.

^c Complete fixation.

TABLE 2. AAV type identification by agglutination observed by electron microscopy

Virus	Source	Degree of agglutination				
		Patient F.C. conval. (1:40) ^a	Anti-AAV3 G.P. (1:100)	Anti-AAV2 G.P. (1:100)	G.P. ^b (1:20)	Saline (control)
AAV	Patient F.C.	4+	4+	Neg	Neg	Neg
AAV3H	Reference virus	4+	4+	Neg	Neg	Neg
AAV2H	Reference virus	Neg	Neg	4+	Neg	Neg

^a Ratio in parentheses indicates antiserum dilution.

^b Preimmune guinea pig (G.P.) serum.

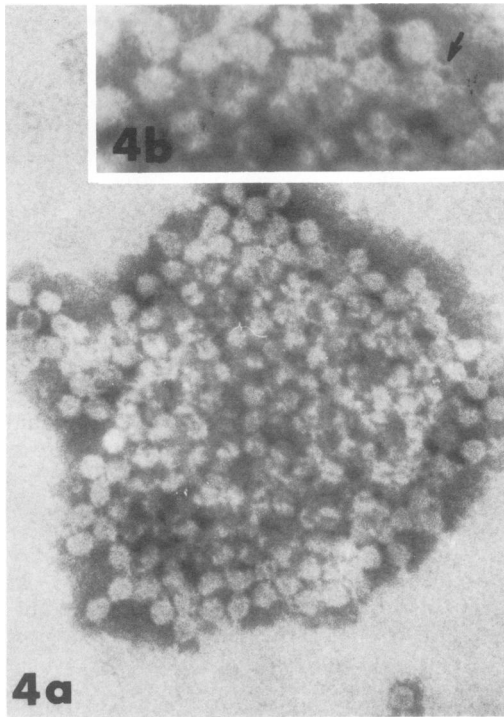


FIG. 4. (a) AAV3 immune complex, negatively stained. $\times 104,000$. (b) AAV3 immune complex with thread-like structures (arrow) presumably antibody, negatively stained. $\times 258,000$.

TABLE 3. Adenovirus and AAV isolates from specimens of swimmers and contacts

Type of specimen	No. of specimens	AAV3 positive		Adenovirus 3 positive	
		No.	%	No.	%
Eye	29	19	65.5	11	37.9
Throat	34	8	23.5	13	38.2
Throat/eye ^a	2	2	100.	2	100.
Fecal	26	6	23.1	11	42.3

^a Swab from throat and eyes was placed in the same collection vial.

TABLE 4. Association of adenovirus and AAV isolated from swimmers and contacts

Virus isolated	Persons	
	No.	%
AAV3 positive/adenovirus type 3 positive	16	40.0
AAV3 positive/adenovirus type 3 negative	7	17.5
AAV3 negative/adenovirus type 3 positive	4	10.0
No virus isolated	13	32.5

field strains, isolated from throat and fecal specimens (9), were examined for AAV. Only one AAV isolate was obtained from the 36 adenovirus strains examined, an AAV3 isolate from an adenovirus type 7 strain which had been isolated from a child's throat specimen collected in 1967. Unfortunately, adenovirus isolates from eye specimens were not available for study.

Controls for AAV contamination. The importance of controls for extraneous and endogenous AAV contamination has been described (4, 14, 15; K. O. Smith, W. D. Gehle, and H. Montes des Oca, *Bacteriol. Proc.*, p. 153, 1968). The most likely sources of contamination, such as environment, cell lines, helper virus, and contamination during specimen inoculation, were consistently monitored. Our control results indicate that AAV contamination did not occur in our study.

Correlation of virus isolation and antibody response. Paired sera were available from 19 patients from whom specimens for virus isolation had been collected. Antibody response related to virus isolation is shown in Table 5. Thirteen of 17 persons with adenovirus type 3 and/or AAV3 isolates had significant (fourfold or greater) rises in adenovirus CF antibody titer, whereas only six of the 17 persons had significant rises in AAV3 CF antibody titers. Two persons from whom neither virus could be isolated showed no rise in antibody titers to either virus. Rises in CF antibody titers to AAV2 were not observed.

Distribution of AAV2, AAV3, and adenovirus CF antibody titers in serum specimens from persons associated with the swimming pool epidemic. Single serum specimens collected 1 month after the outbreak were available from 87 swimmers and contacts. The distribution of CF antibody titers is shown in Table 6. CF antibody titers of ≥ 4 to AAV2 were found in 11 sera and to AAV3 in 44 sera, and CF antibody titers of ≥ 8 to adenovirus were found in 46

TABLE 5. Correlation of adenovirus type 3 and AAV3 isolation results with CF antibody response in 19 persons from whom paired sera were available

Virus isolated	No. of persons	\geq Fourfold rise in CF titer			
		Both	Only AAV3	Only adenovirus	Neither
Both	7	3	0	4	0
Only AAV3	7	1	2	4	0
Only adenovirus	3	0	0	2	1
Neither	2	0	0	0	2

TABLE 6. *Distribution of AAV2, AAV3, and adenovirus CF titers in 87 single serum specimens collected from swimmers and contacts about 1 month after the swimming pool episode*

Antibody	CF titer							% Positive
	<4	4	<8	8	16	32	>64	
AAV3	43	11		11	5	7	10	50.7
AAV2	76	1		5	2	1	2	12.6
Adenovirus			41	16	11	9	10	52.7

sera. The association of CF antibody titers to AAV2, AAV3 (>4), and adenovirus (>8) is shown in Table 7. CF antibodies to both adenovirus and AAV3 were found in 26 persons, whereas CF antibodies to both adenovirus and AAV2 were found in only 8 persons. In addition, 17 persons had CF antibody to adenovirus only, 16 to AAV3 only, and one to AAV2 only.

Seropidemiological findings in 60 school children followed over a 4-year period. CF tests for antibodies to AAV2, AAV3, and adenovirus were performed on 60 sets of five consecutive serum specimens which had been collected over a 4-year period from school children from various areas in Seattle from 1968 to 1973. A 3-month interval exists between 1968 and 1969 specimens and 1- to 2-year intervals thereafter. In addition to a comparison with the serological findings in the swimming pool study, presumably this long-term study might also give information as to occurrence of infections with these viruses as well as prevalence and persistence of antibody. Prevalence of CF antibodies in 1968, at the beginning of the study, was found to be 41.7, 28.3, and 21.7% for adenovirus, AAV2, and AAV3, respectively.

Virus infections, detected by significant (fourfold or greater) CF antibody rises, are shown in Table 8. The highest rates of infection were observed in the first interval, 1968 to 1969. In contrast to the swimming pool study, evidence for both AAV2 and AAV3 infections was observed, and frequencies of infection were similar for both viruses. In the five persons with significant AAV2 CF antibody rises in 1969, three of whom had concurrent adenovirus CF antibody rises, one person had lower antibody levels in 1970 and all persons had lower antibody titers in 1973. In the five persons with significant AAV3 CF antibody rises, three of whom also had concurrent adenovirus CF antibody rises, decreased antibody levels were observed in two persons in 1970 and all persons had lower antibody levels in 1973. Concurrent significant CF antibody rises to AAV2 and AAV3 were observed in one person. Significant CF antibody rises to one type in 1 year and

significant rises to the other type in another year were observed in two persons.

DISCUSSION

AAV infections in cell culture or in human or animal hosts by necessity involve two virus systems. Pure and specific reagents are therefore mandatory to distinguish between the effects of the two systems. In our hands, CsCl gradients did not completely separate AAV and adenovirus antigens. This initial difficulty was overcome by one additional purification step on sucrose gradients. Immunogens consisting of purified AAV preparations elicited antibody with low CF activity but high and specific agglutination activity (as shown in electron microscopy). This may be a reflection of our immunogen preparation, method of immunization, or both.

The importance of purified virus preparations as antigens and of potent type-specific antisera and precautions against inadvertent AAV contamination cannot be over-emphasized (4, 15; Smith et al., *Bacteriol. Proc.*, p. 153, 1968). Since type-specific AAV reference sera were not available at the beginning of our study, human sera were screened for type-specific CF antibodies to AAV2 and AAV3. Monovalent human sera to both types were found and used in the study. Results were subsequently confirmed

TABLE 7. *Association of CF antibodies for AAV2, AAV3, and adenovirus in single serum specimens of swimmers and contacts collected about 1 month after the epidemic*

CF antibody present	No. of persons
Adenovirus, AAV2, AAV3	5
Adenovirus, AAV2	3
Adenovirus, AAV3	21
AAV2, AAV3	2
Adenovirus only	17
AAV2 only	1
AAV3 only	16
All absent	22

TABLE 8. *Demonstration of AAV2, AAV3, and adenovirus infections by significant CF antibody rises in 60 children followed over a 4-year period*

Year	> Fourfold CF antibody rises		
	AAV2	AAV3	Adenovirus
1969 ^a	5 ^b	5	13
1970	2	3	5
1972	2	4	3
1973	0	0	1

^a Four months after initial specimen collection.

^b Number of persons.

with type-specific hyperimmune animal sera and guinea pig reference sera. Cell lines and adenovirus helper used in the study were found free of AAV contamination by all testing procedures employed. Experimental control data indicate that environmental factors or other possible sources of AAV contamination were not a factor in our results.

Adenovirus infections usually occur as sporadic infections in the community and in closed populations, although several epidemics of adenovirus type 3 in special settings have been reported. A similar pattern might occur in AAV infections as might be inferred from the studies of Blacklow et al. in a receiving home for young children (6), Rosenbaum et al. in military recruits (25), and Parks et al. in a hospital population (21). The collection of specimens from the swimming pool epidemic gave us a unique opportunity to look for possible association of AAV with adenovirus infection in this situation. Few studies of swimming pool conjunctivitis associated with adenovirus have been reported, and association of AAV has not been investigated in any of these studies. Other workers have seen both AAV2 and AAV3 circulating in a population simultaneously (4), but in our studies only AAV3 was isolated and a high isolation rate of AAV3 from eye specimens was observed. It is probable that this result reflects a "point" epidemic outbreak in which most cases were infected at the same time in the swimming pool, that the infecting source contained both adenovirus and AAV3, and that secondary cases contracted the same dual infection.

Isolation of AAV from adults has not been previously reported, but in our studies three adults shed AAV3. Two of these adults were the parents of a family with eight members, all of whom had AAV3 eye isolates. Both infected parents represented secondary cases. In one parent, AAV3 was also isolated from a throat specimen and a fourfold rise in AAV3 antibody was observed. The other parent had a negative throat culture and low AAV3 CF antibody titers in both acute and convalescent serum specimens. Both parents had a fourfold rise in adenovirus antibody titer, but adenovirus could not be isolated. The third adult case was a swimmer from whom AAV3 was isolated from an eye specimen. No antibody response to either AAV or adenovirus was seen.

Blacklow et al. reported seroconversion in all children from whom AAV2 and AAV3 had been isolated (5). In our study, seven of nine persons from whom AAV3 was isolated showed no antibody response to AAV3 in paired sera. Only

convalescent sera were available from the remaining two persons, both of which were negative for AAV CF antibody. The small number of cases might be inadequate for proper analysis. However, two contributing factors could explain such results. First, six of these AAV3 isolates were obtained from eye specimens, a poor immunogenic site. Secondly, timing of blood sample collection might have been unfavorable for detection of antibody rises in some cases.

AAV3 isolates without concurrent adenovirus isolation were obtained from seven persons. In five cases, AAV3 was isolated from eye swabs only and in two from eye and throat swabs. Concurrent adenovirus infections were indicated in five of these persons by significant rises in antibody titers. One person had no adenovirus antibody, and serum specimens were not available from another person. Conceivably, these results might simply reflect lesser stability of adenovirus on handling and storage, a less sensitive adenovirus isolation system, or longer persistence of AAV at the site of infection. Since adenovirus 3 was re-isolated from all previously positive specimens and, in addition, from 11 specimens which had not previously yielded virus, it appears more likely that AAV persists longer, particularly in the eye.

The prevalence of AAV3 and AAV2 CF antibodies in acute serum specimens from 19 swimming pool subjects was found to be 26 and 10.6%, respectively. The prevalence of AAV3 and AAV2 CF antibodies in 87 sera collected about 1 month after the epidemic was found to be 50.7 and 12.6%, respectively. In contrast, AAV3 and AAV2 CF antibody prevalence in 60 school children in 1968 was found to be about the same for both viruses, 21.7 and 28.3%, respectively. Six fourfold rises in AAV3 CF antibodies and none in AAV2 were observed in 19 swimming pool subjects. In contrast a maximum of five fourfold or greater AAV3 and an equal number of AAV2 CF antibody rises during 1 year were seen in the 60 school children studied. These results indicate that special conditions existed in the swimming pool epidemic.

Isolation of AAV3 from swimming pool subjects with subsequent fourfold CF antibody rises and significant CF antibody rises to AAV3 and AAV2 in school children show that these viruses infect humans, as has been reported by others (3, 4, 6, 21, 25). AAV have been shown experimentally to depress oncogenicity of adenoviruses (12, 13, 17). Sprecher-Goldberger et al. (28, 29) examined sera of human cancer patients and controls for CF antibodies to AAV and adenoviruses looking for a negative associa-

tion. In our studies, CF antibody responses of only short duration or no CF antibody response at all was observed in a significant number of persons known to be shedding virus. Furthermore, in our follow-up study of school children, disappearance of CF antibody as well as a general decrease in antibody frequencies and levels were observed during a 4-year observation period. These results and those of other workers mentioned indicate that more studies are needed to evaluate the possible influence of AAV infections on cancer, using CF antibody titers as a measure of past infection.

The surprisingly high isolation rate of AAV3 in conjunctivitis patients may be just an incidental association and merely indicate that all cases in this epidemic were infected from a common source. If so, AAV3 may be viewed as a marker for the adenovirus type 3 involved in the swimming pool episode. On the other hand, since so many AAV3 strains were isolated from eye specimens in an epidemic in which conjunctivitis was a distinguishing feature, further investigation of possible AAV infections in cases of conjunctivitis is warranted.

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