ISOLATION OF ADENOVIRUS-ASSOCIATED VIRUSES FROM MAN

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The adenovirus-associated viruses (AAV) are a recently described group of DNA viruses that are unique in that they require an unrelated virus (adenovirus) for their replication.[1-5] They have heretofore been described only as contaminants of laboratory stocks of human and simian adenoviruses; their natural history is unknown. However, antibodies to the various AAV serotypes have frequently been found in humans and monkeys,[6, 7] and seroconversions to AAV types 1, 2, and 3 have been detected[7] in the nursery population at Junior Village, Washington, D.C.[8] This population, comprised of infants six months to three years of age institutionalized for sociological reasons, has been shown by longitudinal surveillance studies to experience frequent infection with various adenovirus serotypes. It was felt that the children in this population offered an optimal opportunity for the isolation and characterization of AAV strains of known human origin. This report describes the isolation of strains of AAV types 2 and 3 from anal and throat specimens from six children who developed antibodies to these AAV's.

Materials and Methods.—Tissue culture: Primary human embryonic kidney (HEK) cell cultures were obtained from Flow Labs., Inc., Rockville, Maryland; HEM Research, Inc., Rockville, Maryland; and the Viral Carcinogenesis Branch, National Cancer Institute. All cells were maintained in 2% heated agammaglobulinic calf serum in Eagle's basal medium no. 2 (MEM) with penicillin and streptomycin.

Viruses: A 10-fold concentrated preparation of Adenovirus 12 (Ad. 12) (Huie strain) was supplied by Dr. Kendall O. Smith; it titered $10^{7.0}$ $TCID_{50}$/0.1 ml in HEK cells. It was free of detectable AAV by the criteria of immunofluorescence (FA), complement-fixation (CF), and electron microscopy (EM), and has remained free of AAV for six serial passages in four different lots of HEK cells. Dr. Smith has found Ad. 12 a more efficient helper for AAV than other adenovirus types tested.[9]

The Ad. 7 was the E46$^-$ (0) strain,[2] which is free of detectable AAV by FA, CF, and EM. It was grown in HEK cells, and titered $10^{7.5}$ $TCID_{50}$/0.1 ml in HEK cells. It was used as helper in a few AAV isolation attempts.

The reference strains of AAV 1, 2, and 3 were the previously described laboratory strains,[2] which have been designated for identification purposes as the 1(H), 2(H), and 3(H) strains, respectively.

Clinical specimens: Detailed descriptions of the institutionalized pediatric population at Junior Village, as well as the specimen collection techniques used, have been given elsewhere.[8] We have previously reported[7] that 5 of 12 children in 1957–8 and 7 of 28 children in 1965, all of whom had documented adenovirus infections, developed antibodies to multiple AAV serotypes during the course of 1 year of residence in Junior Village. Serially collected sera from all but one of these children were tested for AAV and adenovirus CF antibodies and AAV neutralizing antibody by the techniques described previously.[7]

Clinical specimens for virus isolation studies, stored at −20°C, were available from all but one of the children who showed seroconversions to AAV. These included 9 anal and 8 throat swab specimens from five children in 1957–8 and a total of 174 anal swab specimens, collected weekly, from six children in 1965. The specimens from 1957–8 had been saved because they had been found to contain adenoviruses.[8] The specimens from 1965 had not been opened or thawed prior to the experiments reported here.

In addition, 40 anal specimens were examined from six children who did not show seroconversions. These specimens were collected during the same 5-week period in 1965 during which the majority of AAV isolates were obtained.

Virus isolation procedure: For AAV and adenovirus isolation, 0.1 ml of each anal or throat specimen was inoculated into four HEK tubes, two of which had been preinfected for 1 hr with Ad. 12 at a virus/cell multiplicity of 1. These Ad. 12-preinfected tubes were frozen at complete adenovirus cytopathic effect (CPE), which occurred at 5 days. One-tenth ml of the fluid was then passed to two fresh HEK tubes; these were also frozen at complete CPE. Both first and second passage fluids were tested for AAV CF antigen by reaction against guinea pig reference antisera[2] to AAV 1, 2, and 3.

The other two HEK tubes, inoculated with clinical specimens without helper, were observed for adenovirus CPE for at least 21 days. Positive fluids were passed, and both first and second passage fluids were tested for AAV CF antigen. Adenovirus isolates were typed by neutralization tests as previously described.[10]

All AAV-positive specimens were retested; in 15 of 18 instances AAV of the same type was reisolated. The 3 instances where AAV could not be reisolated are not considered as isolates here.

All isolation work was done in a closed laboratory cubicle in which no other adenovirus or AAV experimentation was performed. However, because of the remarkable stability of AAV, there is a high risk of laboratory contamination of tissue cultures and virus pools. Therefore, special control measures were included in all isolation experiments. Twenty to fifty per cent of cell cultures in each test received Ad. 12 alone; as with the tubes inoculated with clinical specimens, each Ad. 12-inoculated control tube was passed to a fresh HEK tube, which was assayed for AAV CF antigen. Also, representative Ad. 12 control tubes from each experiment were passed a total of six times and assayed for AAV at each passage level. These controls, consisting of 152 tubes receiving adenovirus alone, were all negative for AAV. Thus, the three possible sources of AAV contamination—laboratory environment, HEK cells, and adenovirus helper—were effectively ruled out as spurious sources of isolations.

Typing of AAV isolates: Most of the serologic characterization of AAV strains has been performed in CF, and the four known laboratory serotypes of AAV have been established primarily on this basis.[2, 11] The guinea pig sera used in this report were prepared from the reference strains of AAV, and were type-specific in CF, neutralization, and FA tests, except for low-level cross-reactions between AAV 2 and 3.[12]

The isolates reported here were classified as to serotype primarily by their reactivity in CF; they were further characterized using the following AAV neutralization test. Two-tenths ml of a 1:3 dilution of the second passage fluid was heated at 56°C for 15 min (to eliminate the infectious Ad. 12) and then was reacted for 45 min at room temperature with 0.2 ml of various dilutions of control and AAV type-specific guinea pig neutralizing antibody. The reaction mixtures were inoculated onto HEK cells that had been preinfected for 4 hr with Ad. 7 at a virus/cell multiplicity of 3. These tubes were frozen at complete CPE and examined for the presence of AAV in CF.

Results.—Virus isolations from Junior Village children with AAV seroconversions: AAV isolates were obtained from two of the five 1957–8 and four of the six 1965 children who showed seroconversions to AAV (Table 1); the isolation and antibody data from the four AAV-positive children in 1965 are outlined in Figure 1. All 15 strains were of AAV types 2 or 3. In no instance was any reactivity with the AAV-1 guinea pig serum encountered. It is noteworthy that all but one of the AAV isolates were accompanied by an adenovirus isolate from the same specimen, and that the AAV strains were isolated in association with five different adenovirus types. The one AAV isolate without adenovirus was from a child (Da. T.) infected with Ad. 2 for the prior two weeks and with Ad. 3 for the subsequent three weeks. None of the isolates was accompanied by serious illness.

On the basis of AAV isolation data, the low-level CF and neutralizing antibody responses to AAV 1 seen in Figure 1 appear to be due to immunologic cross-reactivity between AAV serotypes. Further, the AAV 2 and 3 antibody response to AAV 2 infection in Figure 1*C* and *D* presumably reflects the immunologic relatedness of AAV 2 and 3.

TABLE 1

AAV ISOLATES

Child	Date	Source of specimen	AAV type	Ad. type	AAV isolated with added helper	AAV isolated without added helper
G. A.	4/2/58	Throat	2	1	+	−
"	"	Anal	2	1	+	N.A.
"	5/14/58	Throat	2	3	+	−
"	"	Anal	2	3	+	−
L. E.	7/14/57	Throat	2	9	+	N.A.
D. C.	3/18/65	Anal	2	2	+	+
"	3/25/65	Anal	2	2	+	+
Da. T.	4/1/65	Anal	2	2	+	+
"	4/8/65	Anal	3	2	+	−
"	4/15/65	Anal	3	−	+	−
"	4/22/65	Anal	3*	3	+	+
De. T.	4/1/65	Anal	2	2	+	+
"	4/15/65	Anal	3	3	+	+
"	4/22/65	Anal	3	3	−	+
R. L.	11/12/65	Anal	2†	7	+	+

N.A., no adenovirus isolated in 1967. Original adenovirus isolate made in 1957–8 not available for testing.
* Reaction primarily with AAV 3 antiserum in CF as described in text (slight cross-reaction with AAV 2 antiserum); neutralized by both AAV 2 and 3 antibodies.
† Reaction primarily with AAV 2 antiserum in CF as described in text (slight cross-reaction with AAV 3 antiserum); neutralized by both AAV 2 and 3 antibodies.

Nine of the ten AAV isolates obtained in 1965 were clustered in a five-week period in March and April. Because of this finding, 40 anal swab specimens from six children without seroconversion were examined for AAV throughout this five-week period. Three of these children had antibody to AAV 2 upon admission to Junior Village, while the other three children remained free of AAV antibody throughout 1965. Although six adenovirus isolations (four Ad. 1, one Ad. 2, and one Ad. 26) were obtained from these children, no AAV isolations were made. Thus, we were able to isolate AAV only from antibody-free children who subsequently seroconverted to AAV.

Certain characteristics of the AAV virus isolation system are worthy of note. Six of the isolates were positive in CF at the first passage, while the remaining nine isolates required a second passage. Two clinical specimens (one containing AAV 2 and one with AAV 3) that were positive at the first passage were titered for AAV by the standard two-passage procedure; both were positive through dilutions of 10^{-3}. On the assumption that the original swab, which had been diluted in 3 ml of fluid, contained 0.1 gm of feces, this result would indicate an AAV titer of roughly 10^5 to 10^6 infectious doses per gram of feces. Without added adenovirus helper, the adenovirus present in the clinical specimen provided the helper for AAV in eight of the ten 1965 isolates (Table 1). On the other hand, AAV could be isolated from the 1957–8 specimens (probably having less adenovirus as a result of prolonged storage and multiple thawings) only in the presence of added adenovirus helper. With these 1957–8 specimens, Ad. 7 was also found to function as helper.

Characterization of AAV isolates: The AAV isolates from Junior Village showed the basic characteristics of the previously described[2] laboratory strains of AAV types 2 and 3. All 15 isolates replicated only in the presence of adenovirus helper, as detected both by CF and by reaction with homotypic guinea pig antiserum in immunofluorescence. All isolates were found to be infectious after heating at 56°C for 15 minutes. Further, they were examined in EM and appeared morphologically indistinguishable from the reference strains of AAV 2 and 3.

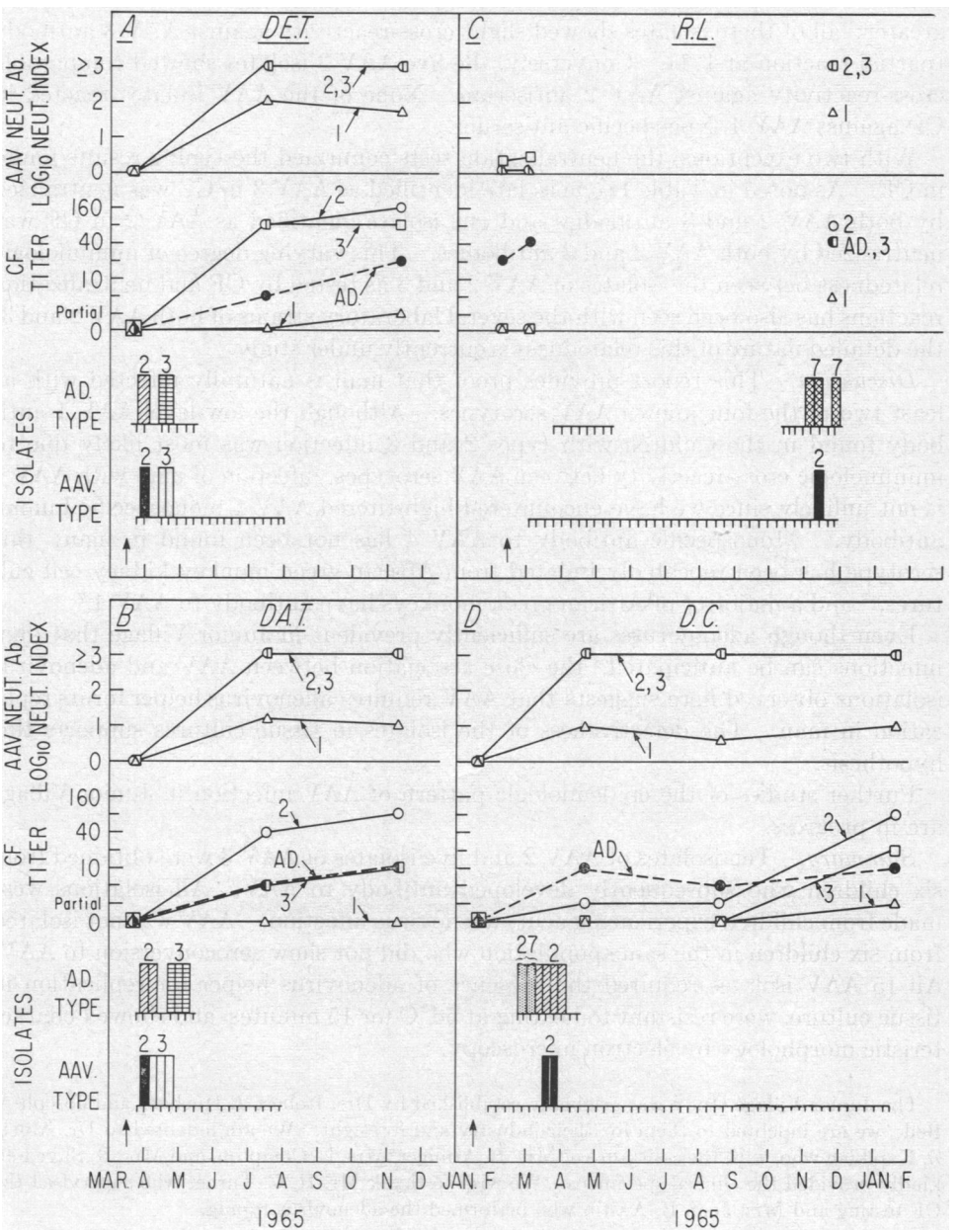

FIG. 1.—AAV and adenovirus isolations from four Junior Village children in 1965. Serotypes are indicated by the numbers above the bars; negative specimens are designated by vertical marks without a bar. CF and neutralizing antibody data for each child are shown above the isolation data (the AAV serotype is indicated by the number). AAV neutralizing antibody, detected by a reduction in the percentage of AAV fluorescent-staining cells, is expressed as the $\log_{10}$ neutralization index obtained with a 1:5 serum dilution. The arrow at the bottom left of each of the 4 graphs indicates the time of entry into Junior Village. It should be noted that there were no sera available from R. L. in *C* between February and November.

The ten AAV 2 isolates reacted in CF with homotypic antibody to titers of 1:4 or greater; all of these isolates showed slight cross-reactivity against AAV 3 antibody (partial reaction at 1:1). Conversely, the five AAV 3 isolates showed comparable cross-reactivity against AAV 2 antiserum. None of the AAV isolates reacted in CF against AAV 1 type-specific antiserum.

With two exceptions, the neutralization tests confirmed the typing results found in CF. As noted in Table 1, one isolate identified as AAV 3 in CF was neutralized by both AAV 2 and 3 antibodies and one isolate identified as AAV 2 in CF was neutralized by both AAV 2 and 3 antibodies. This varying degree of immunologic relatedness between the isolates of AAV 2 and 3 as tested by CF and neutralization reactions has also been seen with the several laboratory strains of both AAV 2 and 3; the detailed nature of this relatedness is currently under study.

Discussion.—This report provides proof that man is naturally infected with at least two of the four known AAV serotypes. Although the low-level AAV 1 antibody found in the children with types 2 and 3 infection was most likely due to immunologic cross-reactivity between AAV serotypes, infection of man with AAV 1 is not unlikely since we have encountered high-titered AAV 1 monospecific human antibody.[7] Monospecific antibody to AAV 4 has not been found in man; this serotype has been repeatedly isolated from African green monkey kidney cell cultures,[13] and a majority of African green monkeys have antibody to AAV 4.[7]

Even though adenoviruses are sufficiently prevalent in Junior Village that dual infections can be anticipated,[8] the close association between AAV and adenovirus isolations observed here suggests that AAV requires adenovirus helper for its replication in man. The defectiveness of the isolates in tissue cultures supports this hypothesis.

Further studies of the epidemiologic pattern of AAV infection in Junior Village are in progress.

Summary.—Ten isolates of AAV 2 and five isolates of AAV 3 were obtained from six children who subsequently developed antibody to AAV. All isolations were made from children experiencing acute adenovirus infection. AAV was not isolated from six children in the same population who did not show seroconversion to AAV. All 15 AAV isolates required the presence of adenovirus helper for replication in tissue culture, were resistant to heating at 56°C for 15 minutes, and showed characteristic morphology by electron microscopy.

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[1] Atchison, R. W., B. C. Casto, and W. McD. Hammon, *Science*, **149**, 754 (1965).

[2] Hoggan, M. D., N. R. Blacklow, and W. P. Rowe, these PROCEEDINGS, **55**, 1467 (1966).

[3] Mayor, H. D., and J. L. Melnick, *Nature*, **210**, 331 (1966).

[4] Archetti, I., E. Bereczky, and D. S. Bocciarelli, *Virology*, **29**, 671 (1966).

[5] Smith, K. O., W. D. Gehle, and J. F. Thiel, *J. Immunol.*, **97**, 754 (1966).

[6] Atchison, R. W., B. C. Casto, W. McD. Hammon, and N. P. Rapoza, *Federation Proc.*, **25**, 249 (1966).

[7] Blacklow, N. R., M. D. Hoggan, and W. P. Rowe, *J. Natl. Cancer Inst.*, in press.

[8] Bell, J. A., R. J. Huebner, L. Rosen, W. P. Rowe, R. M. Cole, F. M. Mastrota, T. M. Floyd, R. M. Chanock, and R. A. Shvedoff, *Am. J. Hyg.*, **74**, 267 (1961).

[9] Smith, K. O., and J. F. Thiel, *Proc. Soc. Exptl. Biol. Med.*, **125**, 887 (1967).

[10] Rowe, W. P., R. J. Huebner, J. W. Hartley, T. G. Ward, and R. H. Parrott, *Am. J. Hyg.*, **61**, 197 (1955).

[11] Parks, W. P., J. L. Melnick, R. Rongey, and H. D. Mayor, *J. Virol.*, **1**, 171 (1967).

[12] Hoggan, M. D., and N. R. Blacklow, unpublished data.

[13] Hoggan, M. D., and A. M. Lewis, unpublished data.