

## New Human Adenovirus (Candidate Adenovirus Type 35) Causing Fatal Disseminated Infection in a Renal Transplant Recipient

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An antigenically distinct adenovirus is described which was isolated in March 1973 from the lungs and kidney of a 61-year-old woman who died of diffuse interstitial adenovirus pneumonia 55 days after receiving a cadaveric renal allograft. Complement fixation, hemagglutination inhibition, and serum neutralization tests on sequential serum specimens from the patient confirmed that the adenovirus infection occurred in coincidence with her clinical illness and failed to document concomitant infection by any other common respiratory agent. Pathological and virological findings indicated that the pneumonia was only one manifestation of a disseminated adenovirus infection, the source of which may have been a latent infection pre-existing in the donor kidney. The adenovirus, purified by terminal dilution and plaque procedures, has antigenic, morphological, biological, biophysical, host susceptibility, and hemagglutinating properties characteristic of adenovirus group 1A. Buoyant densities in CsCl are 1.340 g/ml for the virion, 1.300 g/ml for the group complement-fixing (hexon) antigen, and 1.290 g/ml for the major soluble complete hemagglutinin (dodecon). The virus was serologically distinct from adenoviruses 1 to 34 in reciprocal serum neutralization tests with antisera to these viruses. We propose this virus as candidate adenovirus type 35 (Holden).

A case of fatal disseminated adenovirus (AV) infection in an immunosuppressed renal transplant recipient was recently described in which the causative virus was isolated from lung and kidney tissue obtained at autopsy (31). We report here the characterization of that virus isolate. Our studies confirm that the isolate is an AV and demonstrate that it is serologically distinct from the 34 human AV serotypes heretofore recognized. We therefore propose this virus as candidate AV type 35 (Holden). The subsequent isolation of this same AV from the urine of five other renal transplant recipients or immunosuppressed patients over the past few years (J. C. Hierholzer, unpublished data) suggests that AV 35 infection may represent an important problem for the recipients of renal allografts.

### MATERIALS AND METHODS

**Clinical summary.** The clinical and pathological features of the case have been reported in detail

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elsewhere (31). Briefly, the patient (Holden) was a 61-year-old white woman who received a cadaveric renal transplant on 31 January 1973. The donor was a previously healthy 27-year-old male auto accident victim who matched the patient in two of four HL-A antigens. The patient received postoperative immunosuppression with azathioprine and corticosteroids. After an early phase of anuria due to biopsy-proven acute tubular necrosis, her renal function gradually improved until shortly before death. Three weeks after transplantation, she developed superficial corneal ulcerations and an orolabial vesicular eruption. Herpes simplex virus (HSV) type 1 was isolated from both lesions. The corneal ulcerations resolved after topical treatment with 5'-iodo-deoxyuridine, and the orolabial lesions were gradually arrested. Early in March, the patient became febrile and developed a right middle lobe infiltrate. Despite antibiotic therapy, her pulmonary status worsened, and she developed bilateral diffuse interstitial infiltrates, which progressed in the face of empiric therapy with pentamidine isethionate and amphotericin B. The patient died of respiratory insufficiency on 27 March 1973, 55 days after renal transplantation. Autopsy revealed a diffuse necrotizing interstitial pneumonia with extensive hyaline membrane formation and a great many cells containing deeply basophilic intranuclear inclusion bodies. The renal allograft showed diffuse interstitial edema with numerous foci of tubular epithelial

degeneration and necrosis. In these foci, most of the tubular epithelial cells contained basophilic intranuclear inclusion bodies identical to those in the lungs. Similar inclusion bodies were also identified in the pancreas and ovary.

**Virus isolation.** Lung and kidney tissue obtained at autopsy were processed independently in two different laboratories at different times.

In laboratory 1 (H.S. and M.N.O., Virus Research Unit, Children's Hospital Medical Center, Boston), samples of lung and kidney were washed, minced, washed again, and suspended in Hanks balanced salt solution. Roller tube cultures of primary human embryonic kidney (HEK) cells, a human fibroblast cell strain (FS-9), and a continuous monkey kidney cell line (Vero) were each inoculated with 0.2 ml of suspension and rolled at 37°C. Samples of unprocessed lung and kidney and samples of lung and kidney suspension in Hanks balanced salt solution were stored at -70°C.

In laboratory 2 (J.C.H., Center for Disease Control [CDC], Atlanta), after an 18-month period of storage at -70°C, specimens of lung and kidney tissue obtained at autopsy were homogenized in tryptose phosphate broth containing 0.5% gelatin, 16,000 U of penicillin per ml, and 8,000 µg of streptomycin per ml. Tube cultures of HEK cells, diploid human embryonic lung fibroblasts (HELFL), a continuous line of human epidermoid carcinoma (HEP-2), and primary rhesus monkey kidney (MK) cells were each inoculated with 0.5 ml of clarified (1,000 × g, 10 min, 4°C) tissue suspensions and also with 0.5-ml samples of the lung and kidney tissue suspensions that had yielded the original isolates in laboratory 1. HELFL and MK cultures were rolled, and HEP-2 and HEK cultures were stationary, all at 35°C.

Maintenance medium consisted of Eagle minimal essential medium (MEM) with varying concentrations of bicarbonate (0.07 to 0.14%), 50 U of penicillin per ml, 50 µg of streptomycin per ml, and 1 µg of amphotericin B per ml and 2% bovine serum. In laboratory 2, MEM also contained 50 µg of chlortetracycline per ml. For FS-9 cells, MEM was replaced by Dulbecco's modified Eagle medium (no. H-16, Grand Island Biological Co., Grand Island, N.Y.). MK cells were maintained in MEM without serum.

All cultures were passaged and observed for cytopathic effects (CPE) for at least 4 weeks. CPE was read in unstained preparations and after the preparations were stained with hematoxylin and eosin, May-Grunwald-Giemsa, and Van Orden stains (12). In addition, MK cell cultures were hemadsorbed with guinea pig erythrocytes to detect myxoviruses before being discarded as negative (12).

Eleven-day-old embryonated eggs were inoculated amniotically and allantoically with 0.05 ml of the original tissue suspensions, the supernatants of HEK cell cultures showing 4+ CPE after inoculation with lung and kidney tissue suspensions, and the strain-purified virus stocks. They were examined 6 days later and serially passaged three times. One-day-old ICR white Swiss mice were inoculated intracerebrally and intraperitoneally with 0.02 ml of

these same preparations and observed for 10 days (23, 27, 47).

**Strain purification.** The AV was strain-purified from an early HEP-2 passage of the original lung isolate by triple terminal dilution passages in HEK followed by triple plaque purification on HEP-2 (12). The strain-purified virus was then passaged in 32-ounce (ca. 1-liter) glass prescription cultures of HEP-2 cells to produce a working virus stock: lung/HEK<sub>2</sub>HEP<sub>3</sub>HEK<sub>3</sub>(HEP<sub>1</sub>HEK<sub>1</sub>)<sub>3</sub>HEP<sub>1</sub>. The strain-purified virus had an infectivity titer of 10<sup>5.0</sup> mean tissue culture infective doses (TCID<sub>50</sub>)/ml in HEP-2 and 10<sup>6.2</sup> TCID<sub>50</sub>/ml in HEK. All serological and virus property tests were done with this strain-purified virus and with virus reisolated in laboratory 2 from the original autopsy lung (lung'/HEK<sub>1</sub>HEP<sub>3</sub>). In addition, all serological tests were also done with the virus originally isolated in laboratory 1 from the patient's kidney (kidney/HEK<sub>2</sub>HEP<sub>3</sub>) and with the viruses reisolated in laboratory 2 from the original kidney tissue and the suspensions of minced lung and kidney, which had been stored in -70°C.

Sterility checks on the final, strain-purified virus preparation and on reisolated virus stocks were carried out by inoculating various bacteriological, mycological, and mycoplasma media to reveal nonviral contaminants (16), by breakthrough neutralization tests to detect other complete viruses (10, 14), and by complement fixation (CF) tests for AV-associated virus (AAV) types 1 to 4 (21). These incomplete satellite viruses were also sought by electron microscopic examination of virus after multiple serial passages in HEK and after fractionation of this passaged virus in 30 to 50% linear CsCl gradients (12, 21, 24). AAV type 4 was also sought by hemagglutination (HA) tests with human type O erythrocytes (24).

Antiserum to the strain-purified virus was prepared in horses and in New Zealand white rabbits (14, 15) and stored at -30°C.

**Virus characterization.** Commonly accepted procedures for virus characterization were used to place the isolate in the proper virus group (23, 27, 35, 47). Nucleic acid type was determined by parallel titration of virus in HEK cells with regular medium, medium containing 10<sup>-4</sup> M 5'-iodo-2'-deoxyuridine, and medium containing 20 µg of cytosine arabinoside per ml. Infectivity end points were read at 7 days. Viral nucleic acid was localized in infected HELFL cells by acridine orange staining as previously described (28). The effects of chloroform, acid, temperature, and cationic strength on virus infectivity were determined as previously described (12).

Electron microscopy was performed on fresh lung and kidney tissue obtained at autopsy and on HEK passage supernatant fluids harvested at 3 to 4+ CPE. The former were fixed in 1.5% glutaraldehyde in collidine buffer, postfixed in 1.33% buffered OsO<sub>4</sub>, and embedded in Epon 812. Blocks were stained with uranyl acetate and lead citrate and examined with an RCA-EMV-36 electron microscope. Clarified (1,000 × g, 10 min, 4°C) supernatant fluids were concentrated by pelleting at 105,000 × g for 1 h and prepared for examination by the pseudoreplica tech-

nique of Sharp (42). The grids were stained with 2% potassium phosphotungstate at pH 7.0 and examined at 80 kV in a Philips EM-300 microscope.

The group-specific AV CF antigen (hexon) was assayed by block titration with mouse immune ascitic fluid prepared against purified AV 2 hexons in the standard CF test, with overnight fixation of 5 U of complement (2, 4). Hemagglutinating antigens were measured in standardized HA and hemagglutination inhibition (HI) tests with 0.01 M phosphate-buffered saline diluent, pH 7.2, and 0.4% mammalian or 0.5% avian erythrocyte suspensions (17, 18). HA tests were performed with cells from a variety of mammalian and avian species at 37 and 2°C to allow precise subgrouping of the virus (11). All virus isolates were identified by using standard HA, HI, CF, and serum neutralization (SN) tests (2, 15, 17, 18, 44) with reference equine (CDC, Atlanta) and rabbit (National Institutes of Health [NIH], Bethesda) antisera.

Counter-current immunoelectrophoresis tests with type-specific antipepton and antidodecon rabbit antisera were performed as previously described (13).

Prototype strains of AV 1 to 33 were originally obtained from the American Type Culture Collection and have subsequently been maintained as working stocks in our laboratory as described (11, 15). Prototype strains of candidate AV 34 (Compton) and candidate AV 35 (Holden) are now also available from the American Type Culture Collection (12301 Parklawn Dr., Rockville, MD 20852; catalog no. VR-716 and VR-717, respectively).

## RESULTS

**Virus isolation.** Original (laboratory 1) as well as frozen (laboratory 2) lung and kidney tissue yielded an agent that produced CPE in cells of human origin (primary human amnion, HEK, FS-9, HELF, WI-38, HEP-2, KB, RU-1, and HeLa), but not in monkey cells, within 3 days of inoculation. The CPE was typical of that produced by AVs (23, 27, 40); it consisted of cell rounding, enlargement, increased opacity, and aggregation into irregular cell clusters. Staining revealed the presence of basophilic Cowdry type B intranuclear inclusions. Histopathological examination of lung, kidney, pancreas, and ovary obtained from the patient at autopsy revealed numerous cells containing similar intranuclear inclusions.

No other cytopathic, hemadsorbing, or interference-inducing agent was isolated from the patient's lung or kidney in tissue culture, and no agent was detected when lung and kidney tissue suspensions were passaged in embryonated eggs or inoculated into 1-day-old mice.

Preliminary studies in laboratory 1 showed that the agents isolated from lung and kidney were acid and chloroform stable, passed through 100-nm but not 50-nm filters, and were

completely inhibited in HEK cells by  $10^{-4}$  M 5'-iodo-2'-deoxyuridine or 20  $\mu$ g of cytosine arabinoside per ml. Electron microscopic examination of lung and kidney tissue obtained at autopsy revealed numerous intranuclear inclusion bodies composed of 75- to 80-nm virus particles in crystalline array. These were typical of AV inclusions (3). CF tests with group-specific antibody to purified AV hexon antigen confirmed that both isolates were AV, and HA with rhesus monkey, rat, and human type O erythrocytes indicated that both isolates belonged to Rosen's HA group 1 (38). It was therefore concluded that the patient had suffered a disseminated AV infection (31). However, neither isolate could be identified by SN or HI tests with type-specific antisera to AV types 1 to 33.

The AV was reisolated more than 18 months later, in laboratory 2, from samples of lung and kidney that had been stored at  $-70^{\circ}\text{C}$  and from stored samples of the lung and kidney cell suspensions that had yielded the initial isolates in laboratory 1. In laboratory 2 the reisolated viruses, as well as the original lung and kidney isolates from laboratory 1, were all found by initial testing to be "typical" but "untypable" group 1A AVs (11). They produced AV-like CPE in cells of human origin, hemagglutinins with an HA titer of  $\geq 4,096$  with rhesus and vervet monkey erythrocytes, and no titer with other mammalian and avian cells (Table 1), and AV hexon antigen with a CF titer of 16 to 128 when tested with mouse immune ascitic fluid prepared against purified AV 2 hexons. The isolates did not react in CF tests with antisera to HSV or respiratory syncytial virus. In studies with reference equine (CDC) and rabbit (NIH) antisera to AV types 1 to 33, only antisera to AV 11 and 21 exhibited any HI titers with the isolates, and only AV 11 antisera exhibited any SN titer. All of these titers were low ( $\leq 10$ ), and the isolates were not inhibited in either HI or SN tests by any of the remaining group 1 antisera (i.e., to AV 3, 7, 14, and 16). We at first assumed that the isolates could not be typed because they were a mixture of AV group 1 types. However, various combinations of group 1 antisera, including an AV 11/21 serum pool, did not neutralize them beyond a 1:8 serum dilution.

**Virus characterization.** The virus was purified as described in Materials and Methods; all characterization and serological data were obtained with both strain-purified virus and virus reisolated from lung tissue in laboratory 2. Sterility tests for bacterial, fungal, and mycoplasma contamination were negative after 28 days of incubation at  $37^{\circ}\text{C}$ . Breakthrough SN tests

did not reveal contamination with any other complete virus. CF tests for AAV types 1 to 4 were negative, as were HA tests with human type O erythrocytes at 4°C for AAV 4 (24). As an additional test for AAV contamination, HEK passage supernatants were examined by electron microscopy before and after CsCl equilibrium density gradient centrifugation (12). No AAV-like structures were found in the passage supernatants or in gradient fractions with densities of 1.39 and 1.43 g/ml, the density in CsCl of AAV 1 to 3 and AAV 4, respectively (21, 24).

The strain-purified virus exhibited the same biological behavior as the original isolates, replicating to  $10^4$  to  $10^7$  TCID<sub>50</sub>/0.1 ml at 14 days in tissue cultures of human cells (see above). In all of these it produced AV-like CPE with basophilic Cowdry type B intranuclear inclusions (23, 31). The virus did not replicate in monkey cells in the absence of adventitious simian viruses, but it did replicate in MK cultures, with the production of AV-like CPE, in the presence of simian virus 40 (36, 37). The virus did not produce lesions in the chorioallantoic membrane of embryonated eggs or replicate in the amniotic or allantoic fluids, even after three serial passages. It did not appear to be pathogenic when inoculated intracerebrally and intraperitoneally into 1-day-old mice (23, 27, 47).

The virus was acid stable, chloroform stable, and, with the methods used (12), partially heat labile; there was a 0.8-log drop in titer during 1 h at 50°C in MEM at pH 7.0, and this was unaffected by the presence of 1 M MgCl<sub>2</sub>.

The virus produced yellow-green nuclear fluorescence in HELF cells stained with acridine orange and was inhibited by  $10^{-4}$  M 5'-iodo-2'-deoxyuridine, findings consistent with a double-stranded deoxyribonucleic acid genome (28, 47).

Supernatant fluids from virus-infected cells harvested 2 days after all of the cells exhibited CPE contained soluble CF and HA antigens characteristic of AVs. When tested with optimal dilutions (1:512 to 1:1,024) of mouse immune ascitic fluid prepared against purified AV 2 hexons, these supernatant fluids were found to contain the AV group-specific CF antigen (hexon) at titers of 16 to 128. In HA tests with a battery of avian and mammalian erythrocytes at 37 and 2°C, only rhesus and vervet monkey cells were agglutinated (Table 1). The supernatant fluids contained complete hemagglutinins detectable with these erythrocytes at titers of 4,096 to 8,192, placing the virus in Rosen's HA group 1 (38) and, more specifically, in subgroup 1A (11).

TABLE 1. HA properties of AV 35 (Holden)

Erythrocyte species	HA titers <sup>a</sup> at:	
	37°C	2°C
Vervet monkey . . . . .	1,024-8,192	1,024-8,192
Rhesus monkey . . . . .	1,024-8,192	1,024-8,192
Human O . . . . .	<2	<2
Rat, rat-HS <sup>b</sup> . . . . .	<2	<2
Mouse, gerbil, guinea pig . . . . .	<2	<2
Dog, cow, sheep . . . . .	<2	<2
Chicken, goose, turkey . . . . .	<2	<2

<sup>a</sup> The HA titer is the reciprocal of the highest dilution of antigen causing complete hemagglutination in 1 h at 37 or 2°C; range of titers listed includes data on three passages, each tested with erythrocytes from seven animals (11).

<sup>b</sup> Rat erythrocytes with virus diluted in heterotypic serum (HS) diluent (phosphate-buffered saline containing 1%, rat-erythrocyte-absorbed, AV 6 equine antiserum) (11, 38).

Electron microscopy of HEK cell passage supernatant fluids revealed AV-like particles with cubic icosahedral symmetry and an average diameter, excluding projections, of 75 nm (range, 71 to 77 nm) (22). Hexon and vertex capsomeres were discernible, and fiber projections were frequently seen at the vertexes. No envelope or limiting membrane was present.

To further characterize the AV isolate and its soluble antigens, we analyze supernatant fluids from infected HEK cell cultures by equilibrium density centrifugation in 30 to 50% CsCl gradients (12). Gradient fractions were assayed for virus-like structures by electron microscopy, for infectious virions by titration in HEK cell cultures, for hexon antigen by CF tests with anti-AV 2 hexon mouse immune ascitic fluid, for complete hemagglutinins by HA tests with rhesus monkey erythrocytes in phosphate-buffered saline, and for incomplete hemagglutinins by HA tests with rhesus monkey erythrocytes in phosphate-buffered saline with 1% rhesus erythrocyte-absorbed AV 16 equine antiserum (12). The AV virion was found to have a buoyant density of 1.34 g/ml, as judged by the presence in fractions with that density of particles with typical AV morphology and coincident peaks of infectivity, complete HA activity, and hexon CF activity. Only two soluble components were found: hexon, with group-specific CF activity and typical morphology by electron microscopy in fractions with a density of 1.30 g/ml, and dodecan, with complete HA activity and typical morphology by electron microscopy in fractions with a density of 1.29 g/ml. No soluble incomplete hemagglutinins were detectable in type 16 heterotypic serum diluent. No 20- to 24-nm particles resembling AAV were seen in fractions with densities of 1.39 g/ml (AAV types 1 to 3) or 1.43 g/ml (AAV 4).

AV 35 (Holden) was characterized antigenically by HI and SN tests with the virus (strain-purified virus and low-passage isolates) and hyperimmune reference equine antisera to prototype strains of AV 1 to 34 (15, 44) and hyperimmune rabbit antiserum to prototype AV 34 (12). Reciprocal HI and SN tests were carried out with hyperimmune equine and rabbit antisera to AV 35 (Holden) and prototype strains of AV 1 to 34. In the case of AV types 1 to 33, only reference antisera to AV 11 and AV 21 exhibited any HI activity with AV 35 (Holden), and only equine antiserum to AV 11 exhibited any SN activity (Tables 2 and 3). Conversely, rabbit and equine antisera prepared against strain-purified AV 35 (Holden) exhibited cross-reactions with AV 11, 14, and 21 by HI and with AV 11 by SN (Tables 2 and 3). All of these titers were low ( $\leq 20$ ). In addition, AV 35 (Holden) did not produce precipitin lines in countercurrent immunoelectrophoresis tests with rabbit antisera to purified AV 3, AV 7, and AV 11 penton and dodecan antigens (13).

AV 35 (Holden) and AV 34 exhibited bilat-

eral, but unequal, cross-reactivity in HI tests (Table 2) but little or no cross-reactivity in SN tests (Table 3).

**Serological study of the patient.** Ten serum specimens from the patient were available, including one obtained before renal transplantation. The HI and SN titers of these sera are shown in Table 4. Antibody to AV 35 (Holden) was first detectable 3 weeks after renal transplantation, before pneumonia developed. This seroconversion, with rising HI and SN titers to the AV 35 isolate, provides clear evidence that the patient was infected with this agent. At the same time, the HI tests with the other group 1 AV serotypes indicate that these viruses were not currently infecting the patient. Interestingly, the patient's sera do not exhibit the cross-reactivity with AV 34 that was demonstrable with hyperimmune rabbit and equine antisera (Tables 2 and 3).

The distinction between AV 35 (Holden) and AV 34 is further underlined by the results of HI and SN tests with six sequential sera from the patient (Compton) from whom prototype AV 34

TABLE 2. Relationship of AV type 35 (Holden) to AV types 1 to 34 by reciprocal HI titers<sup>a</sup>

AV type	Reference equine antisera to AV types:							Rabbit antisera to AV type:		Equine antisera to AV type:	
	1-10	11	12-13	14	15-20	21	22-33	34	35 (Holden)	34	35 (Holden)
1-10	- <sup>b</sup>	-	-	-	-	-	-	<5	<5	<5	<5
11	-	160	-	10	-	10	-	<5	<5	<5	5
12-13	-	-	-	-	-	-	-	<5	<5	<5	<5
14	-	10	-	40	-	10	-	<5	10	<5	5
15-20	-	-	-	-	-	-	-	<5	<5	<5	<5
21	-	<5	-	<5	-	40	-	<5	<5	<5	5
22-33	-	-	-	-	-	-	-	<5	<5	<5	<5
34	<5	<5	<5	<5	<5	5	<5	320	160	640	320
35 (Holden)	<5	5	<5	<5	<5	5	<5	20	640	160	1,280

<sup>a</sup> HI titer is defined as the reciprocal of the highest dilution of antiserum completely inhibiting hemagglutination by 4 HA units of antigen per 0.025 ml.

<sup>b</sup> -, See reference 15 for complete tables of homologous and heterologous titers.

TABLE 3. Relationship of AV type 35 (Holden) to AV types 1 to 34 by reciprocal SN titers<sup>a</sup>

AV type	Reference equine antisera to AV types:			Rabbit antisera to AV type:		Equine antisera to AV type:	
	1-10	11	12-33	34	35 (Holden)	34	35 (Holden)
1-10	- <sup>b</sup>	-	-	<5	<5	<5	<5
11	-	160	-	<5	20	<5	20
12-33	-	-	- <sup>c</sup>	<5	<5	<5	<5
34	<5	<5	<5	640	<5	160	5
35 (Holden)	<5	5	<5	<5	320	5	640

<sup>a</sup> SN titer is defined as the reciprocal of the highest dilution of antiserum causing a 2<sup>+</sup> (50%) reduction in CPE from that observed in the working dilution (virus control) in 3 or 4 days.

<sup>b</sup> -, See reference 15 for complete tables of homologous and heterologous titers.

<sup>c</sup> - -, Antiserum types 14 and 21 have homologous SN titers of 1:160 (15)

TABLE 4. Patient's serological response to group 1 AVs and to AV 35 (Holden)

Serum	Date	Comment	Serum antibody titer to AV type <sup>a</sup>										
			HI test						SN test				
			3	7	11	14	16	21	34	35 (Holden)	34	35 (Holden)	
S1	1-24-73		<8	64	8	<8	<8	<8	<8	<8	<8	<8	<8
S2	1-31-73	Renal transplantation	<8	64	8	<8	<8	<8	<8	<8	<8	<8	<8
S3	2-9-73		<8	64	8	<8	<8	<8	<8	<8	<8	<8	<8
S4	2-14-73		<8	64	8	<8	<8	<8	<8	<8	<8	<8	<8
S5	2-22-73	Isolation of HSV-1	<8	64	16	<8	<8	<8	<8	8	<8	<8	<8
S6	3-2-73	Febrile illness	<8	64	<8	<8	<8	<8	<8	32	<8	<8	8
S7	3-12-73	Febrile illness	<8	64	<8	<8	<8	<8	<8	64	<8	<8	16
S8	3-19-73	Febrile illness	<8	64	<8	<8	<8	<8	<8	128	<8	<8	32
S9	3-26-73	Febrile illness	<8	64	<8	<8	<8	<8	<8	256	<8	<8	64
S10	3-27-73	Death	<8	64	<8	<8	<8	<8	<8	256	<8	<8	128

<sup>a</sup> HI antibody is the reciprocal of the highest dilution of serum completely inhibiting hemagglutination by 4 HA units of virus per 0.025 ml in 1 h at 37°C. SN antibody titer is the reciprocal of the highest dilution of serum causing a 2<sup>+</sup> reduction in CPE from that observed in the virus controls at 3 days after inoculation.

was isolated. Although these sera had HI titers as high as 128 and SN titers as high as 64 against AV 34 (12), they consistently gave HI titers of <4 and SN titers of <8 with AV 35 (Holden) (data not shown).

All of the viruses isolated from the patient's tissues (i.e., the lung and kidney isolates originally obtained in laboratory 1 and the viruses reisolated in laboratory 2 from samples of frozen lung and kidney and from samples of the lung and kidney suspensions originally prepared in laboratory 1 are identical to each other and to the strain-purified AV 35 (Holden). They all have identical HA and CF properties, and they give identical HI and SN titers with rabbit antiserum to strain-purified AV 35 (Holden) and with the patient's sequential sera.

The patient's sera were also tested by CF against a battery of respiratory agents, by HI against coronavirus OC-43, and by microneutralization (43) against HSV-1 and -2 (Table 5). The results provide evidence of infection with HSV-1, influenza A, parainfluenza 3, cytomegalovirus, and coronavirus OC-43 at some (undetermined) time before 24 January 1973. They also suggest that the HSV-1 infection documented in February 1973 represented recurrent, rather than primary, infection. Cytomegalovirus was not isolated from the patient, either premortem or postmortem, and no cytopathology characteristic of this agent was observed in tissues examined at autopsy.

## DISCUSSION

The characteristics of the AV 35 (Holden) strain fit the accepted criteria for the AV group (6, 7, 22, 27, 35, 39-41, 47). It replicates only in cells of human origin, where it produces typical AV CPE with basophilic Cowdry type B intra-

nuclear inclusions and soluble CF and HA antigens typical of AVs (2, 4, 12, 23, 27, 40). It is chloroform and acid stable, but partially heat labile, with no cationic stabilization. Its genome is double-stranded deoxyribonucleic acid. The AV 35 (Holden) virions have a buoyant density in CsCl of 1.34 g/ml, and electron microscopy reveals AV-like particles with cubic icosahedral symmetry and an average diameter, excluding projections, of 75 nm. The virions have typical hexon and vertex capsomeres and fiber projections at the vertexes. They have no envelope or limiting membrane. The production, in tissue culture, of high titers of complete hemagglutinins detectable only with rhesus and vervet monkey erythrocytes places the AV 35 (Holden) in HA group 1A (11).

AV 35 (Holden) shows little or no serological cross-reactivity with AV types 1 to 33 in reciprocal HI and SN tests. AV 35 (Holden) and AV 34 (Compton) exhibit bilateral, but unequal, cross-reactivity in HI tests but essentially no cross-reactivity by SN. Moreover, sequential sera from patient Holden, which showed a seroconversion with rising HI and SN titers to AV 35 (Holden), did not cross-react with AV 34 (Compton), and sera from patient Compton, which had high levels of HI and SN antibodies to AV 34 (Compton), did not show significant cross-reactivity with AV 35 (Holden).

Concerning the possibility that the AV isolated from the patient might be a non-human AV (artificially introduced in the laboratory during the attempted virus isolation), we noted that neither primary monkey cells nor non-human AVs were being utilized in laboratory 1, either at the time that AV 35 was first isolated or during the period of its characterization. The continuous monkey cell lines that were in use

TABLE 5. Patient's serological response to common respiratory agents

Serum	Date	Comment	MN test		CF test										HI test (corona- virus (OC-43)			
			HSV-1	HSV-2	Influenza		Parainfluenza				Mumps	Ru- beola	RSV	M. pneu- moniae		AV <sup>a</sup>	CMV	Corona- virus 229E
					A	B	1	2	3	4								
S1	1-24-73		16		<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8
S2	1-31-73	Renal transplan- tation	8		<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8
S3	2-9-73		8		<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8
S4	2-14-73		8	10	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8
S5	2-22-73	Isolation of HSV-1	8		<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8
S6	3-2-73	Febrile illness	16		<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8
S7	3-12-73	Febrile illness	16		<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8
S8	3-19-73	Febrile illness	16		<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8
S9	3-26-73	Febrile illness	16		<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8
S10	3-27-73	Death	16	10	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	8

<sup>a</sup> MN antibody titer is the corrected microneutralization titer (t<sub>c</sub>) obtained with the HSV microneutralization test previously described (43); it corresponds to the reciprocal of the highest dilution of serum completely neutralizing the CPE-producing capacity of 100 TCID<sub>50</sub> of prototype HSV-1 or HSV-2. CF antibody titer is the reciprocal of the highest dilution of serum fixing 5 U of complement overnight in the standard CF test (2, 4). HI antibody titer is the reciprocal of the highest dilution of serum completely inhibiting hemagglutination by 4 HA units of virus per 0.025 ml in 1 h at 37°C. RSV, Respiratory syncytial virus; CMV, cytomegalovirus.

<sup>b</sup> Purified AV 2 hexon antigen (4) is used to measure antibody only to the AV group-specific antigen.

(Vero, CV-1, BSC-1) were derived from frozen stocks held in laboratory 1, and these had been repeatedly shown by biological and biochemical assays to be free of AVs and of AV genetic information. Furthermore, the rising titers of HI and SN antibodies to AV 35, which the patient developed in the month preceding her death, provide strong evidence for infection by the virus strain which was subsequently isolated from her tissues.

We therefore conclude that the Holden strain is a new human AV serotype, AV 35, which is antigenically distinct from the 34 previously recognized human AV serotypes.

The patient from whom this new AV was isolated was a renal transplant recipient who died of respiratory failure. Pathological and virological findings at autopsy revealed diffuse interstitial adenovirus pneumonia and indicated that the pneumonia was only one manifestation of a disseminated infection, which involved the transplanted kidney and other organs as well (31). Serological studies indicate that the patient suffered a primary infection with the AV and provide no evidence of coincident infection by other common respiratory agents. Cytomegalovirus was not isolated from lung or kidney tissue. Temporal and serological data, the extensive involvement of the transplanted kidney, and the pattern of pathology in the lung (which indicates that the route of pulmonary parenchymal infection may have been hematogenous rather than respiratory) suggest the possibility that disease in this patient resulted from reactivation and dissemination of a latent AV infection in the donor kidney (31). Thus, it is interesting that donor kidneys were also suspected of being the source of AV 34 (Compton) and a subsequent AV 34 isolate, both of which were recovered from urines of cadaveric renal allograft recipients (12, 25). Unfortunately, serum and other specimens from the donors were not available in any of these cases to help determine if the donors had had prior infection with the virus.

Immunologically compromised patients suffer an extraordinarily high incidence of opportunistic infections caused by bacteria, fungi, protozoa, and viruses. The increased vulnerability of these patients to virus infection extends to members of nearly every virus group, including AVs, herpesviruses, myxoviruses, papovaviruses, picornaviruses, and poxviruses. The source of infection is rarely documented, but at least in the case of the herpesviruses (HSV, cytomegalovirus, varicella-zoster virus, and Epstein-Barr virus) it often appears to be endogenous, resulting from the activation of a

latent virus infection in the patient. In the recipient of an allograft, the transplanted tissue itself can also be the site of such a latent infection (20), with subsequent reactivation favored by transplantation into a nonimmune host (45), allograft rejection (19, 34), and immunosuppressive therapy.

The capacity of AVs to infect the urinary tract in normal and immunosuppressed individuals is now well recognized (5, 8, 9, 12, 26, 29-33). Most urinary tract isolates belong to HA group 1 (38), the members of which now include AV types 3, 7, 11, 14, 16, 21, 34, and 35 (Holden). Perhaps the capacity of these particular AVs to agglutinate simian erythrocytes is related, in some way, to their apparent affinity for the tissues of the urinary tract. The capacity of these agents to infect the urinary tract and to establish latent infections (1, 40, 46) increases the likelihood that AVs of HA group 1 will be transmitted by renal transplantation. Perhaps their subsequent activation, dissemination, and tendency to produce disease will be modulated by the recipient's immunity to the particular AV in question. Our patient, however, lacked detectable antibody to AV 35 before transplantation.

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#### LITERATURE CITED

- Baum, S. G., and R. I. Fox. 1975. Human adenovirus infection in monkey cells: an example of host restriction at a step late in replication. *Cold Spring Harbor Symp. Quant. Biol.* 39:567-573.
- Casey, H. L. 1965. Standardized diagnostic complement fixation method and adaptation to micro test. *Public Health Monogr.* no. 74, Public Health Service, Washington, D.C.
- Chou, S. M., R. Roos, R. Burrell, L. Guttman, and J. B. Harley. 1973. Subacute focal adenovirus encephalitis. *J. Neuropathol. Exp. Neurol.* 32:34-50.
- Dowdle, W. R., M. Lambriex, and J. C. Hierholzer. 1971. Production and evaluation of a purified adenovirus group-specific (hexon) antigen for use in the diagnostic complement fixation test. *Appl. Microbiol.* 21:718-722.
- Fiala, M., J. E. Payne, T. V. Berne, S. Poolsawat, J. Schieble, and L. B. Guze. 1974. Role of adenovirus type 11 in hemorrhagic cystitis secondary to immunosuppression. *J. Urol.* 112:595-597.
- Ginsberg, H. S. 1962. Identification and classification of adenoviruses. *Virology* 18:312-319.
- Ginsberg, H. S. 1972. Adenoviruses. *Am. J. Clin. Pathol.* 57:771-776.
- Gresser, I., and S. Kibrick. 1961. Isolation of vaccinia virus and type 1 adenovirus from urine. *N. Engl. J. Med.* 265:743-744.
- Gutekunst, R. R., and A. D. Heggie. 1961. Viremia and viremia in adenovirus infections: detection in patients with rubella or rubelliform illness. *N. Engl. J. Med.* 264:374-378.
- Hampil, B., and J. L. Melnick. 1969. Method for testing virus stocks for viral contaminants. *Appl. Microbiol.* 17:17-20.
- Hierholzer, J. C. 1973. Further subgrouping of the human adenoviruses by differential hemagglutination. *J. Infect. Dis.* 128:541-550.
- Hierholzer, J. C., N. O. Atuk, and J. M. Gwaltney, Jr. 1975. New human adenovirus isolated from a renal transplant recipient: description and characterization of candidate adenovirus type 34. *J. Clin. Microbiol.* 1:366-376.
- Hierholzer, J. C., and M. Barne. 1974. Counterimmunoelectrophoresis with adenovirus type-specific anti-hemagglutinin sera as a rapid diagnostic method. *J. Immunol.* 112:987-995.
- Hierholzer, J. C., and W. R. Dowdle. 1970. Hemagglutination properties of adenovirus types 20, 25, and 28. *Proc. Soc. Exp. Biol. Med.* 134:482-488.
- Hierholzer, J. C., W. C. Gamble, and W. R. Dowdle. 1975. Reference equine antisera to 33 human adenovirus types: homologous and heterologous titers. *J. Clin. Microbiol.* 1:65-74.
- Hierholzer, J. C., W. C. Gamble, K. D. Quist, and W. A. Chappell. 1972. Comparison of immunization methods for producing reference adenovirus antisera in horses. *Appl. Microbiol.* 24:398-404.
- Hierholzer, J. C., and M. T. Suggs. 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. I. Standardization of erythrocyte suspensions. *Appl. Microbiol.* 18:816-823.
- Hierholzer, J. C., M. T. Suggs, and E. C. Hall. 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. II. Description and statistical evaluation. *Appl. Microbiol.* 18:824-833.
- Hirsch, M. S., D. A. Ellis, P. H. Black, A. P. Monaco, and M. L. Wood. 1973. Leukemia virus activation during homograft rejection. *Science* 180:500-502.
- Ho, M., S. Suwansirikul, J. N. Dowling, L. A. Youngblood, and J. A. Armstrong. 1975. The transplanted kidney as a source of cytomegalovirus infection. *N. Engl. J. Med.* 293:1109-1112.
- Hoggan, M. D., N. R. Blacklow, and W. P. Rowe. 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological and immunological characteristics. *Proc. Natl. Acad. Sci. U.S.A.* 55:1467-1474.
- Horne, R. W. 1973. The structure of adenovirus particles and their components, p. 67-82. *In* A. J. Dalton and F. Haguenu (ed.), *Ultrastructure of animal viruses and bacteriophages: an atlas*. Academic Press Inc., New York.
- Hsiung, G. D. 1973. *Diagnostic virology—an illustrated handbook*, 2nd ed. Yale University Press, New Haven, Conn.
- Ito, M., and H. D. Mayor. 1968. Hemagglutinin of type 4 adeno-associated satellite virus. *J. Immunol.* 100:61-68.
- Keller, E. W., J. C. Hierholzer, R. H. Rubin, P. H. Black, and M. S. Hirsch. 1977. Isolation of adenovirus type 34 from a renal transplant recipient with interstitial pneumonia. *Transplantation* 23:188-191.
- Lecatsas, G., O. W. Prozesky, and J. van Wyk. 1974. Adenovirus type 11 associated with haemorrhagic cystitis after renal transplantation. *S. Afr. Med. J.* 48:1932.
- Lennette, E. H., and N. J. Schmidt (ed.). 1969. *Diagnostic procedures for viral and rickettsial infection*, 4th ed. American Public Health Association, New York.
- Mayor, H. D. 1962. Biophysical studies on viruses using the fluorochrome acridine orange. *Prog. Med. Virol.* 4:70-86.



29. Mufson, M. A., R. B. Belshe, T. J. Horrigan, and L. M. Zollar. 1973. Cause of acute hemorrhagic cystitis in children. *Am. J. Dis. Child.* 126:605-609.
30. Mufson, M. A., L. M. Zollar, V. N. Mankad, and D. Manalo. 1971. Adenovirus infection in acute hemorrhagic cystitis. *Am. J. Dis. Child.* 121:281-285.
31. Myerowitz, R. L., H. Stalder, M. N. Oxman, M. J. Levin, M. Moore, J. D. Leith, N. M. Gantz, J. Pellegrini, and J. C. Hierholzer. 1975. Fatal disseminated adenovirus infection in a renal transplant recipient. *Am. J. Med.* 59:591-598.
32. Numazaki, Y., T. Kumasaka, N. Yano, M. Yamanaka, T. Miyazawa, S. Takai, and N. Ishida. 1973. Further study on acute hemorrhagic cystitis due to adenovirus type 11. *N. Engl. J. Med.* 289:344-347.
33. Numazaki, Y., S. Shigeta, T. Kumasaka, T. Miyazawa, M. Yamanaka, N. Yano, S. Takai, and N. Ishida. 1968. Acute hemorrhagic cystitis in children: isolation of adenovirus type 11. *N. Engl. J. Med.* 278:700-704.
34. Olding, L. B., F. C. Jensen, and M. B. A. Oldstone. 1975. Pathogenesis of cytomegalovirus infection. I. Activation of virus from bone marrow-derived lymphocytes by *in vitro* allogenic reaction. *J. Exp. Med.* 141:561-572.
35. Portnoy, B., and M. A. Salvatore. 1974. Adenoviruses, p. 695-702. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), *Manual of clinical microbiology*, 2nd ed. American Society for Microbiology, Washington, D.C.
36. Rabson, A. S., G. I. O'Connor, I. K. Berezsky, and F. J. Paul. 1964. Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV 40. *Proc. Soc. Exp. Biol. Med.* 116:187-190.
37. Rapp, F., M. Jerkofsky, and D. Vanderslice. 1967. Characterization of defectiveness of human adenoviruses in green monkey kidney cells. *Proc. Soc. Exp. Biol. Med.* 126:782-786.
38. Rosen, L. 1960. A hemagglutination-inhibition technique for typing adenoviruses. *Am. J. Hyg.* 71:120-128.
39. Rowe, W. P., J. W. Hartley, and R. J. Huebner. 1956. Additional serotypes of the APC virus group. *Proc. Soc. Exp. Biol. Med.* 91:260-262.
40. Rowe, W. P., R. J. Huebner, J. W. Hartley, T. G. Ward, and R. H. Parrott. 1955. Studies on the adenoidal-pharyngeal-conjunctival (APC) group of viruses. *Am. J. Hyg.* 61:197-218.
41. Schlesinger, R. W. 1969. Adenoviruses: the nature of the virion and of controlling factors in productive or abortive infection and tumorigenesis. *Adv. Virus Res.* 14:1-61.
42. Sharp, D. G. 1960. Sedimentation counting of particles via electron microscopy, p. 542-548. *In* Proceedings of the International Congress on Electron Microscopy, 4th Congress, 1958. Springer-Verlag, Berlin.
43. Stalder, H., M. N. Oxman, and K. L. Herrmann. 1975. Herpes simplex virus microneutralization: a simplification of the test. *J. Infect. Dis.* 131:423-430.
44. Stevens, D. A., M. Schaeffer, J. P. Fox, C. D. Brandt, and M. Romano. 1967. Standardization and certification of reference antigens and antisera for 30 human adenovirus serotypes. *Am. J. Epidemiol.* 86:617-633.
45. Stevens, J. G., and M. L. Cook. 1974. Maintenance of latent herpetic infection: an apparent role for antiviral IgG. *J. Immunol.* 113:1685-1693.
46. Strohl, W. A., and R. W. Schlesinger. 1965. Quantitative studies of natural and experimental adenovirus infections of human cells. II. Primary cultures and the possible role of asynchronous viral multiplication in the maintenance of infection. *Virology* 26:208-220.
47. Wilner, B. I. 1969. A classification of the major groups of human and other animal viruses, 4th ed. Burgess Publishing Co., Minneapolis.