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New Human Adenovirus Associated with Respiratory Illness: Candidate Adenovirus Type 39

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A new human adenovirus was isolated from a stool specimen from a 2-year-old El Salvadorian male hospitalized with severe respiratory illness. The virus, strain D335, is a typical adenovirus by routine classification tests and bears little resemblance to the other 38 adenovirus serotypes by standard hemagglutination inhibition and serum neutralization tests. The only significant cross-reaction observed was between adenovirus types 39 and 13, bilaterally, by the hemagglutination inhibition test. The virus was clearly differentiated from the other human adenoviruses by restriction enzyme analyses with *Sma*I, *Sac*I, and *Kpn*I enzymes. Strain D335 agglutinates human and rat erythrocytes to moderately high titers and is placed in hemagglutination subgroup 2B on the basis of differential hemagglutination with erythrocytes from 13 animal species. It has a density in cesium chloride of 1.339 g/ml and produces soluble components in human embryonic kidney culture that band at 1.303 g/ml (hexon), 1.283 g/ml (dodecon), and 1.212 g/ml (fiber), the last component being the only incomplete hemagglutinin found. The viral DNA is cleaved into 17 fragments by the *Sma*I restriction enzyme, indicating that strain D335 is a member of adenovirus subgenus D. Strain D335 is herein described as candidate adenovirus type 39 (*Mastadenovirus* h 39).

Adenovirus infections continue to be associated with a wide variety of illnesses in humans. Because this range of symptomatology and disease is so extensive and because a particular set of serotypes tends to predominate in each disease association, it is important to identify or to antigenically characterize isolates from clinical situations.

Since the first 33 serotypes were established (9, 11, 30, 36), new types have been found which have important clinical associations. These serotypes are reviewed here to place candidate adenovirus type 39 (Ad39) in proper perspective. Ad34 was first isolated from the urine of a 17-year-old male who was experiencing a lengthy episode of fever in March 1972 after a cadaveric kidney transplant in February 1972 (14). It has since been identified as a cause of pneumonia (26) and other illnesses in renal transplant recipients and other immunosuppressed patients and occasionally in non-immunosuppressed persons.

Ad35 was first recovered from the lung and kidney tissues of a 61-year-old female who died of interstitial pneumonia in March 1973, 2 months after receiving a cadaveric kidney transplant (32). Typical adenovirus inclusions were also found in the spleen and ovaries of this patient at autopsy, indicating a severe generalized infection, but the tissues were not saved for virus isolation (29). Ad35 has since been frequently identified, both by isolation and serology, in a variety of illnesses in immunosuppressed patients and occasionally in non-immunosuppressed persons.

Ad36 was isolated in February 1978 in a stool specimen from a 6-year-old girl with enteritis in Giessen, West Germany (40).

Ad37 was first identified in May 1976 in an eye swab from a 40-year-old male with epidemic keratoconjunctivitis in Amsterdam (6, 31). Shortly thereafter, Ad37 was isolated in The Netherlands in cervical swabs from four patients with cervicitis, in eye swabs from 21 patients with epidemic keratoconjunctivitis, and in eye, throat, and stool specimens from a 19-year-old male with pharyngoconjunctival fever (6, 31, 37). Ad37 has also been associated with conjunctivitis and urethritis in Sweden, Norway, and West Germany (6, 37), Italy (4), England (34, 35), Australia (2), and the United States (J. C. Hierholzer, unpublished data). In most of these cases the viruses remained untyped or were thought to be intermediate strains at the time they were isolated; they were later correctly identified after Ad37 antiserum became available.

Ad38 is the first of the "noncultivable" or enteric adenoviruses to be even partially successfully adapted to the laboratory (5, 10, 23–25, 27). It causes some 5 to 15% of infantile gastro-

enteritis cases in many parts of the world. Strain characterization of this subgroup of viruses is currently under way to delineate antigenic types.

During the course of our studies with the noncultivable adenoviruses—those specimens which were positive for adenovirus by direct electron microscopy (EM) examination of stools but from which a virus could not readily be cultivated in cell cultures—we isolated several viruses which were typable by routine procedures, including one which did not react with antisera to the established adenovirus serotypes. This virus was recovered from a stool specimen from a 2-year-old child with respiratory illness but no documented enteritis. The case happened to be part of the enteric adenovirus study only because the patient was hospitalized at the same time many other infants were hospitalized with diarrhea. The present report describes candidate Ad39 and its case of origin.

MATERIALS AND METHODS

Case report. The patient (case D335) was a 2.5-year-old male from Soyapango, El Salvador, who was hospitalized in San Salvador in February, May, and June 1977 with a diagnosis of bilateral bronchitis on all three occasions. The first two episodes appeared to be successfully treated with penicillin after hospital stays of 2 and 1 day, respectively. The third episode commenced on 8 June 1977. Upon admission, the patient's hematocrit was 37%, the hemoglobin was 10.6 g/100 ml, and the differential leukocyte count was 54% lymphocytes, 44% neutrophils, and 2% eosinophils. No improvement was noted after 5 days of treatment with penicillin. The child had bronchitis, pharyngitis, fever, cough, chest pain, and dysuria throughout his hospital stay. Diarrhea and vomiting were not reported. Throat and urine cultures were consistently negative for bacterial pathogens; virus cultures were not attempted in San Salvador. A stool specimen obtained on 13 June 1977 and shipped frozen to the Centers for Disease Control, Atlanta, Ga. was positive by EM for adenovirus. This virus, strain D335, is the subject of this report.

Virus isolation. The specimen was prepared for tissue culture inoculation as previously described (10). Briefly, 1 g of stool was mixed with 2.5 ml of phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and shaken vigorously with glass beads. After removal of the extract, the beads were rinsed with another 2.5 ml of buffer. The combined extract was clarified at 2,000 × *g* for 20 min. The clarified suspension was examined by EM (10) and inoculated onto primary human embryonic kidney (HEK), human epidermoid carcinoma (HEp-2), and primary rhesus monkey kidney (MK) cell cultures as described previously (14). Cultures were subpassaged several times. Cultures positive for cytopathology (CPE) were tested by standardized hemagglutination (HA), hemagglutination inhibition (HI), complement fixation (CF), and serum neutralization (SN) tests (3, 19, 20, 33).

Strain purification. The isolate was strain purified by triple terminal dilutions as previously described (14). Working stocks were checked for contamination with other complete viruses by the serum breakthrough neutralization test (12, 16), for contamination with the adenovirus-associated virus types 1 to 4 (AAV1 to AAV4) by CF tests (21), EM (1), and density gradient centrifugation (21, 22), and for contamination with bacteria, yeasts, and mycoplasmas by inoculation onto a variety of specialized media (18).

Antiserum production. Antiserum to the strain-purified virus was prepared in New Zealand white rabbits (16) and in a horse (17) and stored at −30°C.

Virus characterization. The adenovirus group-specific hexon component was assayed by CF with anti-Ad2 hexon mouse immune ascitic fluid with overnight fixation of 5 U of complement (3, 7), by fluorescent-antibody staining with antihexon conjugates (28), and by counterimmunoelectrophoresis tests with antihexon sera (15). HA antigens were assayed by the standardized tests with 0.01 M PBS diluent, pH 7.2, and 0.4% mammalian or 0.5% avian erythrocyte suspensions (19, 20). The virus was subgrouped by differential HA with a number of erythrocyte species (13). Serological relationships of the virus to prototype Ad1 to Ad37 were determined by HI tests (20) and SN tests (33) with reference horse antiserum (17). Reciprocally, Ad1 to Ad38 were tested by HI and SN against Ad39 rabbit and horse antisera. For Ad36 to Ad38, rabbit antisera were used in addition to the equine Ad36 and Ad37 antisera.

Analysis of viral DNA. Confluent 25-cm^2 monolayers of HEp-2 cells were infected with prototype adenovirus strains and harvested when all cells exhibited 4+ CPE, usually within 3 to 6 days at 36°C. After the growth cycle, monolayers were lysed in 0.5 ml of lysis buffer (1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 10 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA) at 37°C for 30 min. To facilitate purification of the DNA, 200 ng of proteinase K (Sigma Chemical Co.) was added to the cell lysate, and incubation was continued for 60 min. The cell lysate was then extracted with an equal volume of phenol saturated with TSE buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA). DNA obtained from the lysed cells was ethanol precipitated, suspended in TSE buffer, and treated with RNase for 60 min at 37°C. The DNA was extracted as described above and ethanol precipitated. The DNA, suspended in water, was quantitated spectrophotometrically; 2 μg of DNA from each infected cell lysate was then digested with the restriction enzyme *Sma*I in 15 mM Tris (pH 8.0)–6 mM $MgCl_2$–15 mM KCl at 37°C for 2 h. The restriction fragments were separated in a 1.2% Studier submerged gel as described previously (8).

RESULTS

Virus isolation. Strain D335 was readily visualized as an adenovirus by EM of the original stool extract and was estimated to have a titer of 10^8 mean tissue culture infective doses per ml. The virus was recovered at passage 1 in HEK and HEp-2 cells and showed typical adenovirus CPE and morphology by EM. No growth was observed in MK after four passages. Initial at-

tempts to type the isolate by HA subgrouping and HI tests were unsuccessful.

Strain purification. The virus was then strain purified by triple terminal dilution in HEK cells. The final passage (stool/$HEK_2HEp_4HEK_3HEp_2$; 18 December 1980) was produced in bulk for seed virus, working stock, and immunizing antigens and was comprehensively tested for purity. Serum breakthrough neutralization tests revealed only Ad39. CF tests for AAV1 to AAV4 were negative, as were EM and HA examinations of fractions of CsCl gradients at densities of 1.39 and 1.43. Sterility tests for bacterial, fungal, and mycoplasmal contaminants were negative after 28 days at 37°C.

Virus characterization. HEp-2 cultures harvested 2 days after 4+ CPE were tested for various proofs of adenovirus infection. In CF block titrations, the cultures had 1:8 optimal antigen titers against a 1:512 optimal dilution of Ad2 hexon mouse immune ascitic fluid. In direct fluorescent-antibody tests, the cultures exhibited 3+ to 4+ group-specific staining with a 1:120 dilution of rabbit Ad9 antihexon conjugate. In counterimmunoelectrophoresis, precipitin lines were consistently observed when the cultures were electrophoresed against Ad2 hexon mouse immune ascitic fluid and rabbit Ad8 and Ad9 hexon antisera.

In HA tests with a battery of mammalian and avian erythrocytes, human and rat erythrocytes were the predominant cells agglutinated (Table 1). The range of titers (1:128 to 1:8,192) for these cells plus the low-level agglutination of monkey and mouse cells places Ad39 in HA subgroup 2B, closest to Ad26 and Ad27 within that subgroup.

TABLE 1. HA properties of strain D335

Erythrocyte species	HA titers[a] at:	
	37°C	4°C
Vervet	4	2
Rhesus	4	2
Human O	2,048	1,024
Rat	2,048	2,048
Rat-HS[b]	1,024	512
Mouse	8	4
Gerbil	2	0
Guinea Pig	2	1
Dog	1	0
Chicken	1	0
Goose, turkey	0	0
Cow, sheep	1	0

[a] An HA titer is defined as the dilution factor of the highest dilution of antigen causing complete HA with 0.4% mammalian or 0.5% avian erythrocyte suspensions in 0.01 M PBS diluent, pH 7.2, in 1 h. The range of titers listed includes data on three passages tested with erythrocytes of at least three animals (13).

[b] Rat erythrocytes with virus diluted in heterotypic serum (HS) diluent (PBS containing 1% rat erythrocyte-absorbed Ad30 equine antiserum).

Infected HEK cultures were fractionated on 30 to 50% preformed CsCl gradients in PBS to identify the soluble components of Ad39 and to provide an additional search for AAV. The cultures were harvested 2 days after 4+ CPE, frozen and thawed three times, clarified by centrifugation, and layered onto 10-ml gradients. The gradients were centrifuged in an SW41 rotor at an average of 207,000 × *g* for 23 h. Fractions collected by hole puncture were assayed for refractive index by a Bausch & Lomb refractometer, for soluble antigens by HA and CF titrations, and for morphological structures by EM (Fig. 1). The virion, identified by all property tests and EM, banded at a density of 1.339 g/ml. The hexon component was identified by EM and by reactivity with Ad2 hexon mouse immune ascitic fluid in the CF test and had an equilibrium density of 1.303 g/ml. The major soluble complete hemagglutinin was identified by EM as the dodecon, with a density of 1.283 g/ml. The minor soluble hemagglutinin had incomplete HA activity and a density of 1.212 g/ml and was tentatively identified as the fiber component. No AAV particles were seen at densities of 1.39 (AAV1 to AAV3) or 1.43 g/ml (AAV4).

Serological relationships. Antigenic characterization was performed by repeated HI and SN tests with Ad39 and reference antisera to Ad1 to Ad38 and, reciprocally, by HI and SN tests with Ad39 rabbit and horse antisera versus Ad1 to Ad38 antigens. The HI titers showed low-level, one-way relationships with Ad20 and Ad36, low-level bilateral crosses with Ad9, Ad10, Ad26, and Ad29, and a high-level, bilateral cross with Ad13 (Table 2). The latter was 4-fold lower than homologous, and the others were ≥16-fold lower than homologous. Homologous HI titers of Ad39 rabbit and horse antisera were 1:2,560. The SN titers showed one-way crosses with Ad8 and Ad10 and two-way relationships with Ad29 and Ad36 (Table 3). All cross-reactive titers were ≥eightfold lower than homologous titers. Homologous SN titers of Ad39 rabbit and horse antisera were 1:640.

Restriction enzyme analyses. We carried out restriction endonuclease analyses on most of the human adenovirus prototype strains, including Ad2 to Ad4, Ad6 to Ad10, Ad13, Ad15, Ad17, Ad19, Ad19A, Ad20, Ad22 to Ad24, Ad26 to Ad30, Ad32, Ad33, Ad36, Ad37, and Ad39, with a variety of restriction enzymes, including *Sma*I, *Sac*I, and *Kpn*I enzymes. Although all subgenus D viruses exhibited somewhat similar patterns, i.e., a number of fragments were shared, distinguishable differences in the migration of the restriction fragments of each virus

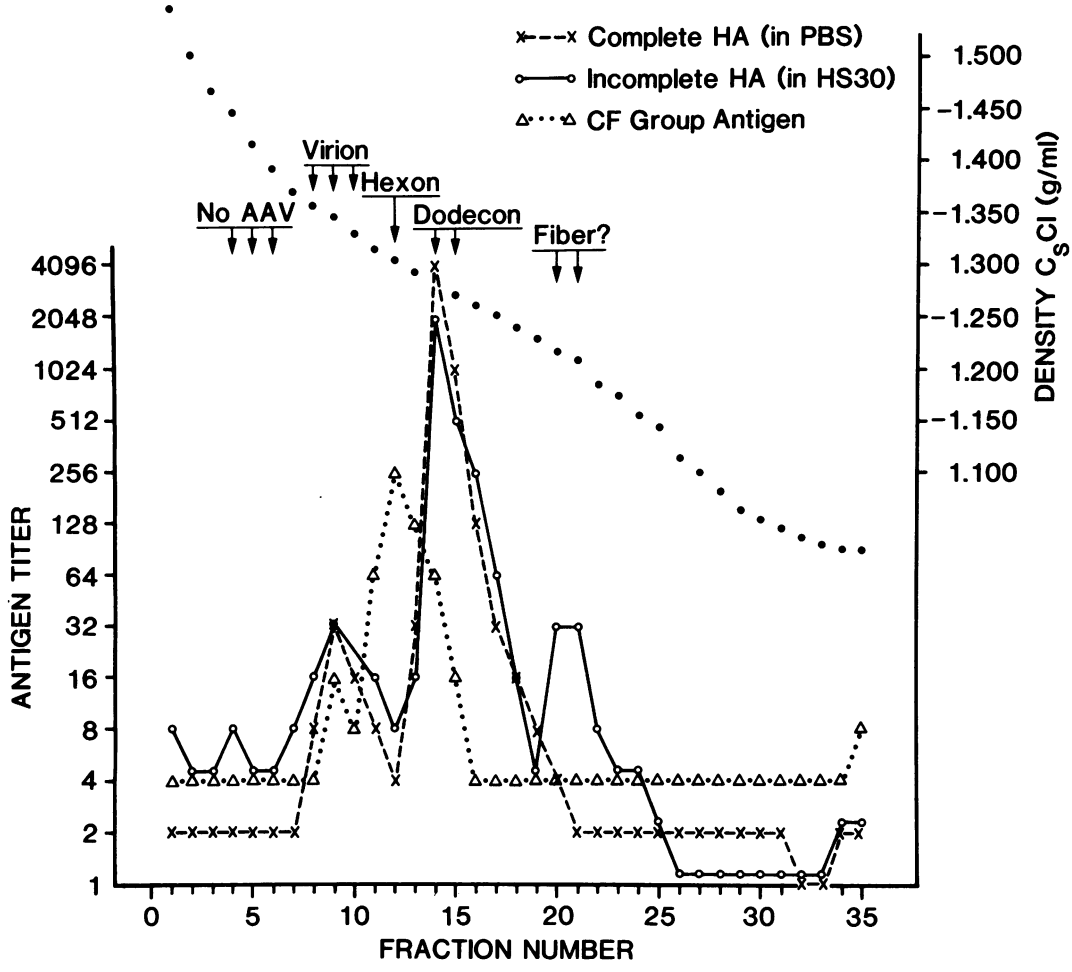

FIG. 1. Equilibrium cesium chloride gradient showing the buoyant densities of the Ad39 virion and soluble CF and HA components. Complete hemagglutinins were measured in PBS diluent, and incomplete hemagglutinins were titrated in Ad30 heterotypic serum diluent. Arrows indicate visualization by EM.

TABLE 2. Relationship of D335 strain to Ad1 to Ad38 by the HI test

Ad type	HI titer of antiserum to Ad type[a]:									
	9	10	13	20	26	29	36	All others	39	
									Rabbit	Horse
9	**320**	20	0	0	0	0	0	—[b]	0	5
10	20	**320**	0	0	0	0	10	—	0	5
13	0	0	**320**	20	10	0	10	—	80	80
20	0	0	0	**320**	0	0	0	—	0	0
26	40	20	0	0	**2,560**	0	0	—	0	20
29	0	0	0	0	0	**2,560**	5	—	0	5
36	0	0	0	5	0	20	**1,280**	0	0	5
All others	—	—	—	—	—	—	0	—	0	0
39	20	20	80	20	10	10	0	0	**2,560**	**2,560**

[a] HI titers are listed as dilution factors of the highest dilution of antiserum that completely inhibited the agglutination of 4 HA units of virus per 0.025 ml of antiserum incubated with 0.4% rat erythrocytes for 1 h at 37°C; 0 = <5.

[b] —, Additional heterologous titers are given in reference 17.

TABLE 3. Relationship of D335 strain to Ad1 to Ad38 by SN test

Ad type	SN titer of antiserum to Ad type[a]:						
	8	10	29	36	All others	39	
						Rabbit	Horse
8	**160**	0	0	0	—[b]	0	20
10	0	**160**	0	0	—	0	10
29	0	0	**80**	5	—	0	5
36	0	0	10	**160**	0	10	0
All others	—	—	—	0[c]	—	0	0
39	0	0	5	10	0	**640**	**640**

[a] SN titers are listed as dilution factors of the highest dilution of antiserum that caused a 2+ reduction in cytopathology from that observed in the working dilution (virus control) per 0.2 ml in MK cells in 3 days at 36°C; 0 = <5.

[b] —, Additional heterologous titers are given in reference 17.

[c] Except 1:5 with Ad24 and Ad37.

were apparent. The restriction pattern of Ad39 is easily differentiated from those of all other viruses tested, including Ad30, which has a similar number of restriction fragments. A number of the larger *Sac*I fragments of Ad30 and Ad39 have similar migration rates, but further analysis by *Sma*I and *Kpn*I enzymes showed that these viruses are clearly distinct (Fig. 2).

No relationship by restriction patterns was seen between Ad39 and any of the types which exhibited HI or SN relationships with Ad39 (Fig. 3). In fact, both the migration rates of the fragments and the total number of fragments are distinct between Ad39 and its serologically related viruses. The number of fragments generated by *Sma*I digestion of Ad39 is 17, which suggests that the virus belongs to oncogenic subgroup D (i.e., subgenus D) according to data presented by Wadell et al. (36). Compared with most subgenus D viruses, this number of *Sma*I fragments is rather high; most of the subgenus D viruses we have tested have 14 to 15 fragments.

DISCUSSION

Strain D335 was recovered from a child hospitalized with respiratory illness. The virus was present to approximately eight logarithmic dilutions in an extract of the stool specimen, suggesting a current and active infection. Further evidence of the association of this virus with the illness could not be obtained, however, because paired serum specimens were not available. It is possible that Ad39, although it was clearly infecting the patient, did not cause any or all of the symptoms listed, because (i) viruses are not isolated from approximately 40% of patients with respiratory illness even when comprehensive techniques are used for collection and isolation and (ii) HA subgroup 2 adenoviruses can often be isolated from stool specimens without any clear association with symptomatic illness.

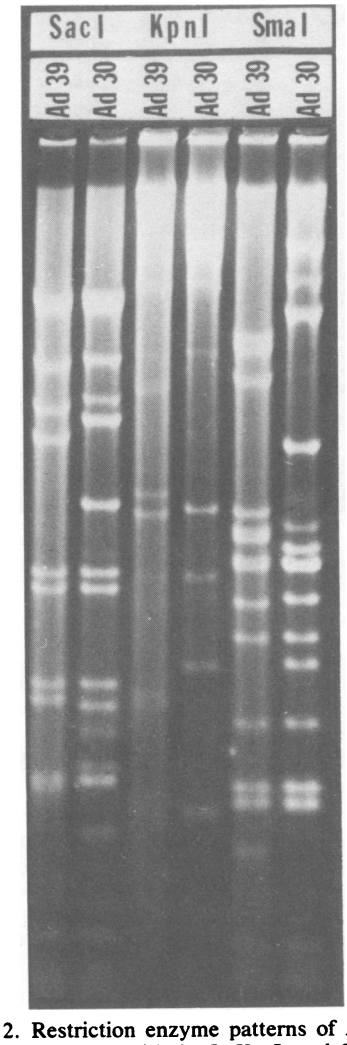

FIG. 2. Restriction enzyme patterns of Ad30 and Ad39 after digestion with *Sac*I, *Kpn*I, and *Sma*I.

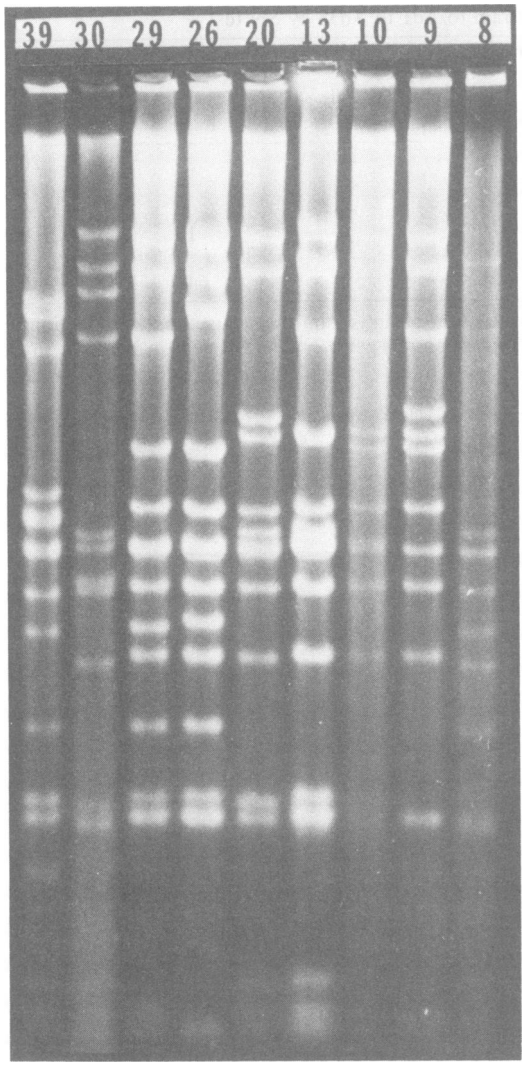

FIG. 3. *Sma*I restriction enzyme patterns of all adenovirus serotypes which serologically cross-react with Ad39.

Thus, a clear association of this virus with clinical symptoms must await additional cases and further documentation.

The serological relationships of Ad39 to other human adenoviruses pose the usual problem observed among HA subgroup 2 viruses (17, 38, 39). All of the relationships found were among HA subgroup 2 types, but many were found with the horse antiserum and not the rabbit antiserum. Thus, only Ad13 exhibited a definite relationship with Ad39 by HI, and no virus showed a significant relationship with Ad39 by SN. The low-level crosses observed in only one antiserum, whether by the HI or SN test, are probably the result of heterotypic anamnestic responses by that particular animal to the immunizing antigen, as discussed previously (17).

A close relationship of Ad39 to other subgenus D viruses was not observed by restriction enzyme analyses. As with other subgenus D serotypes, some fragments have migration rates that are similar within the entire subgenus. Ad39, however, is clearly distinct and has 17 fragments produced by *Sma*I digestion.

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