Isolation of an Adenovirus 32 Strain from Human Brain in a Case of Subacute Encephalitis (36204)

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(Introduced by J. A. Morris)

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This paper describes the isolation of an adenovirus from the brain of a 42-year-old male dying with subacute encephalitis and radiation-treated lymphosarcoma. Neuropathologic examination, prompting viral isolation studies, revealed a subacute focal encephalitis with neuronal intranuclear inclusion bodies which contained adenovirus-like particles seen by electron microscopy (1). The adenovirus isolated is related by neutralization test to candidate adenovirus type 32 (2) and by hemagglutination inhibition test to adenovirus type 27. The isolation of this unusual adenovirus from the brain suggests that the patient's reduced immunological defenses may have played a role in the pathogenesis of this aberrant infection.

Materials and Methods. Clinical history. The patient had lymphosarcoma for 5 years, during which time, he was treated with immunosuppressants and radiation. Four and one half weeks before his death, he complained of scotomata and diplopia. One week later he developed seizures and a diffusely slow EEG. His cerebrospinal fluid (CSF) had 7 mononuclear cells, 162 mg/100 ml of protein, and 86 mg/100 ml of glucose. Serum immunoelectrophoresis showed a marked reduction in all immunoglobulin fractions. A provisional clinical diagnosis of progressive multifocal leukoencephalopathy was made.

Specimens. Brain tissue, blood, and CSF were collected 2 hr after the patient's death and stored at −70° for 3 months before virus isolation studies were initiated. A 10% suspension of brain tissue was made in phosphate buffered saline (pH 7.4), clarified by centrifugation at 2500 rpm for 15 min, and used for inoculation into human embryonic kidney (HEK), HeLa, and WI-38 cells.[1] In an attempt to detect virus in the serum and CSF, the postmortem serum and CSF were inoculated into HEK and HeLa cells and then blind-passed twice with 2-week periods of observation. The isolated virus is referred to below as the JL strain adenovirus. Reisolation of the virus was accomplished in HEK cultures inoculated with a separate 10% suspension of previously unground brain tissue in phosphate buffered saline (pH 7.4).

Electron microscopy. Leighton tube cover slips of HEK cultures inoculated with the JL strain were washed in phosphate buffer, fixed for 2 hr in phosphate buffered 5% gluteraldehyde solution, and washed again in phosphate buffer. Cells were scraped off the cover slip, placed in 1% buffered osmium tetroxide, dehydrated in graded alcohol and embedded in Epon–Araldite mixture. Thin sections stained with uranyl acetate and lead citrate were examined with an RCA-EMU-3G electron microscope at 100 kV.

Neutralization (NT) tests. NT tests employing approximately 100 tissue culture 50% infective doses ($TCID_{50}$) of the virus were performed in HEK cells against 33 human type-specific adenovirus antisera (including adenovirus 32 and 33 antisera)[2] and the patient's serum and CSF. NT tests were also performed in primary rhesus monkey kidney

[1] The HEK cells were obtained from Microbiological Associates (MBA), Bethesda, MD; Flow Laboratories, Rockville, MD; and from HEM Research, Rockville. The HeLa and WI-38 cells were obtained from Flow Laboratories.

[2] Reference type-specific adenovirus antisera used:

Antisera	Source (NIAID, Bethesda, MD)
Ad. 1–19, 22–31	Reference Reagents Branch
Ad. 20, 21, and candidates Ad. 32, 33	Dr. Wallace Rowe

cells (MK), according to the method of Rowe *et al.* (3), using a dose of virus that produces 3+ cytopathic effect (CPE) in 48 hr.

Hemagglutination (HA) and hemagglutination inhibition (HI) tests. HA and HI tests were performed by the macromethod described by Rosen (4) employing rhesus monkey and rat erythrocytes. Sixteen Group 2 adenovirus antisera (including adenovirus 32 and 33)[2] and the patient's serum and CSF were tested for HI with the isolated virus.

Complement fixation (CF) tests. CF tests were performed in the conventional microtiter system (5, 6) employing 2 exact units of complement, 4 units of antigen, and a 1% suspension of sensitized sheep red cells. Adenovirus group CF antigen and adenovirus human reference antiserum were obtained from MBA.

Animal inoculations. HEK cultures infected with the third passage of the JL strain were frozen and thawed and inoculated: by the intracerebral (ic), intraperitoneal (ip), and intravenous (iv) routes into hamsters and mice; by the ic and iv routes into a spider monkey; and by the ic route into rhesus, cynomolgus, African green, and capuchin monkeys. Antiserum to the JL strain was prepared in rabbits by the intravenous inoculation of 1.0 ml of the HEK infected cultures followed by three intravenous inoculations at weekly intervals of 0.5 ml of the HEK infected cultures. Two weeks after the last inoculation, the rabbits were exsanguinated. Serial serum specimens from four inoculated monkeys and the rabbit sera were tested by NT, HI, and CF procedures for the presence of antibody response to the JL strain and for cross-reactivity to reference type-specific adenoviruses.[3] Attempts to demonstrate viremia in four monkeys were made by inoculation into HEK cultures of whole blood and plasma obtained on days 2, 3, 4, and 11 following ic injection of the JL strains; two blind passages were performed in HEK cultures at 2-week intervals.

Results. A strain (JL) of adenovirus related to candidate adenovirus type 32 (Ad. 32) by NT and to Ad. 27 by HI was isolated in HEK and HeLa cell cultures inoculated with 10% suspensions of human brain from a patient dying with subacute encephalitis and lymphosarcoma. On primary passage into HEK and HeLa cells, the virus induced a cytopathic effect (CPE) characteristic of adenovirus consisting of rounding, retraction, and formation of "grape-like" clusters of cells. When stained, infected cells contained enlarged nuclei and eosinophilic intranuclear inclusion bodies. Ultrastructural features of infected cell cultures were identical to those observed in neurons in the patient's brain and consisted of enlarged bizarre-shaped nuclei containing intranuclear inclusion bodies composed of symmetrical arrays of viral particles. The particles were approximately 70 to 75 nm in diameter and had an outer coat arranged in the form of an icosahedron. A light and electron microscopic study of the patient's neuropathological findings will be published separately (1).

Serial passage of the JL strain in HEK cell cultures was associated with an increase in infectivity titer and a shortening of incubation time for CPE to develop, *i.e.*, fifth passage JL virus in HEK cells had a $TCID_{50}$ of $10^{-6.2}$/ml with CPE beginning about 48 hr after inoculation of the cultures. Virus-infected HEK tissue culture fluids fixed complement in the presence of adenovirus human reference antiserum. The virus agglutinated rat erythrocytes and, to a lesser degree, rhesus erythrocytes, placing it in Rosen's Group 2 of adenoviruses (4). Reisolation of the virus was successful in HEK cells inoculated with a separately made suspension of the brain tissue that had been kept stored at −70° for an additional 3 months. In contrast, attempts to isolate virus in HEK cells inoculated with the patient's serum and CSF were unsuccessful.

When attempts were made to neutralize the JL strain employing 33 human type-specific adenovirus antisera, as well as the patient's postmortem serum and CSF, significant neutralizing antibodies were detected only in Ad. 32 antiserum. The results of selected cross-NT tests are shown in Table I. Ad. 32 antiserum had a titer of 1:320 and Ad. 27 antiserum had a titer of 1:10 against the JL strain in MK cells. Although antibody against the JL strain could not be detected in the patient's serum or CSF, the serum did have a neutralizing antibody titer of 1:20 against Ad. 32. The patient's

[3] Reference type-specific adenovirus used:

Antisera	Source (NIAID, Bethesda, MD)
Ad. 9, 27 candidates Ad. 32, 33	Reference Reagents Branch Dr. Wallace Rowe

TABLE I. Cross-Neutralization Tests of the JL Strain, Ad. 27 and Ad. 32.

Virus	Neutralizing antibody titers[a] in the serum of:				
		JL Strain		Type-specific antisera	
	Patient J.L.	Inoculated monkey[b]	Hyperimmunized rabbit[c]	Ad. 27	Ad. 32
JL Strain	*<10*[d]	*160*	*320*	10[d]	320
Ad. 27 (BP-4)	<10	<10	<10	*160*	<10
Ad. 32 (HH)	20	160	320	<10	*320*

[a] Titers are expressed as the reciprocal of the highest dilution of serum that neutralized the test dose of virus. Titers listed for the JL strain and Ad. 27 were results obtained using MK cells. Titers listed for Ad. 32 were obtained using HEK cells.

[b] Antibody titers in the serum of a capuchin monkey 120 days after inoculation of JL strain. Similar titers were obtained in sera from African green, cynomolgus and rhesus monkeys who had received the same inoculum.

[c] Antibody titers in the serum of a rabbit 2 weeks after the last of four weekly intravenous inoculations of the JL strain.

[d] When neutralization tests were performed in HEK cells, the patient's serum, Ad. 27 (and Ad. 9) antisera through a 1:80 dilution slowed the development of JL strain's CPE for 6 days, although, at the end of 2 weeks, these antisera, at a 1:10 dilution, failed to neutralize the test dose of 100 $TCID_{50}$ of virus.

serum and Ad. 9 and Ad. 27 antisera through a 1:80 dilution slowed the development of CPE for 6 days in HEK inoculated with a test dose of 100 $TCID_{50}$ of JL virus; however, at the end of 2 weeks, CPE broke through even at a 1:10 dilution and these sera thus failed to neutralize the virus completely.

Capuchin, African green, cynomolgus and rhesus monkeys inoculated with a single dose of the JL strain, as well as hyperimmunized rabbits, developed significant levels of neutralizing antibodies to the homologous virus and to Ad. 32, but not to Ad. 27 or Ad. 9. Illustrative data obtained in one hyperimmunized rabbit and the inoculated capuchin monkey are shown in Table I. In the rabbit, neutralizing antibodies directed against the JL strain and Ad. 32 had a titer of 1:320. In the capuchin monkey, neutralizing antibodies directed against the JL strain and Ad. 32 were present as early as 25 days after intracerebral inoculation of the virus and reached a titer of 1:160 which persisted for at least 120 days following inoculation.

When cross-HI tests were performed using the JL strain, Ad. 27 and Ad. 32, the isolate was more closely related to Ad. 27 than to Ad. 32. The results of selected cross-HI tests are shown in Table II. In tests of Group 2 antisera for HI antibodies to the JL strain, Ad. 27 antiserum had an HI titer of 1:1280, while all other Group 2 antisera, including Ad. 32 antiserum, had an HI titer of <1:20. HI antibody was present in the patient's serum against the JL strain, Ad. 27 and Ad. 32 with titers of 1:40, 1:20, and 1:20, respectively. In contrast, the patient's CSF had no HI antibodies to the JL strain, Ad. 27 or Ad. 32.

Capuchin, African green, cynomolgus and rhesus monkeys, as well as hyperimmunized rabbits, developed significant levels of HI antibodies to the homologous virus and Ad. 27. Illustrative data obtained in one hyperimmunized rabbit and the inoculated capuchin monkey are shown in Table II. Hyperimmunized rabbits had HI antibodies to the homologous virus and to Ad. 27 at a titer of 1:>2560. In the capuchin monkey, significant levels of HI antibodies to the homologous virus and Ad. 27 were present as early as 25 days after intracerebral inoculation of the virus and reached a titer of 1:1280 to the JL strain and 1:640 to Ad. 27 which persisted for at least 120 days following inoculation.

CF tests were run against adenovirus group CF antigen using the patient's postmortem CSF and serum, hyperimmunized rabbit sera, as well as capuchin, African green, cynomolgus

TABLE II. Cross-Hemagglutination-Inhibition Tests of JL Strain, Ad. 27 and Ad. 32.

Virus	Patient J.L.	Hemagglutination-inhibition antibody titers[a] in the serum of: JL strain: Inoculated monkey[b]	JL strain: Hyperimmunized rabbit[c]	Type-specific antisera: Ad. 27	Type-specific antisera: Ad. 32
JL strain	*40*	*1280*	*>2560*	1280	<20
Ad. 27 (BP-4)	20	640	>2560	*640*	<20
Ad. 32 (HH)	20	20	<20	<20	*640*

[a] Titers are expressed as the reciprocal of the highest dilution of serum that completely inhibited hemagglutination of rat erythrocytes by 4 units of antigen.

[b] Antibody titers in the serum of a capuchin monkey 120 days after inoculation of JL strain. Similar titers were obtained in sera from African green, cynomolgus and rhesus monkeys who had received the same inoculum.

[c] Antibody titers in the serum of a rabbit 2 weeks after the last of four weekly intravenous inoculations of the JL strain.

and rhesus monkey sera obtained on days 1, 25, and 120 after inoculation of JL strain. No CF antibody was demonstrable in the undiluted CSF or in the patient's serum, rabbit sera, and monkey sera when tested at 1:10 dilution.

The mice, hamsters, and monkeys inoculated with JL strain are still being observed, but have shown no signs of clinical disease or tumor formation during the 6 months since they were inoculated. Attempts to demonstrate a viremia in the capuchin, African green, cynomolgus and rhesus monkey whole blood and plasma obtained on day 2, 3, 4, and 11 following ic injection of the JL strain were unsuccessful.

Discussion. Ad. 32 (HH strain) was recently isolated by Blacklow *et al.* (2) from an anal swab of a 23-month-old asymptomatic child. Following the criterion of Blacklow *et al.* (2), the JL strain would be classified under the Ad. 32 serotype, since in cross-neutralizing antibody reactions, results between the JL strain and Ad. 32 were indistinguishable. However, Ad. 32 is related to HI to the homotypic strain, Ad. 24 and candidate Ad. 33; whereas the JL strain is related by HI to Ad. 27. The relationship of the JL strain to Ad. 32 by NT tests and to Ad. 27 by HI tests places this virus among the serologically intermediate strains, described by Wigand and Fliedner (7), and suggests that the JL strain may perhaps be considered a new serotype.

In a review of adenovirus infections of man, Sohier *et al.* (8) cite reports of isolations of adenoviruses from the CSF, lung, stool, and throat from cases of acute encephalitis. There are, however, only two previous reports (9, 10) of adenovirus isolations from brain tissue. The more detailed report describes the isolation of Ad. 7A from the brain, CSF and lung of a 1-year-old child who died after a 20-day illness manifested by diarrhea, fever, pneumonia, somnolence, and convulsions (9). In the second report, mention is made of the isolation of Ad. 7 from the thalamus and lung of a fatal case (10).

In our case, it is likely that the lymphosarcoma, immunosuppressants, and radiation therapy reduced the immunological defenses of the patient, resulting in a lowering of serum immunoglobulins and serum antibody levels. It is of interest to speculate whether the immunologically deficient status of the patient may have led to the activation of a latent adenovirus resulting in the subacute encephalitis observed in the brain at autopsy.

Summary. This report describes the isolation in tissue culture of an adenovirus from the brain of a 42-year-old man dying with radiation-treated lymphosarcoma and a 4.5-week history of neurological disease which, on histopathological evidence, was diagnosed as subacute encephalitis.

The isolate, designated the JL strain, was most closely related antigenically to Ad. 32 by NT test and to Ad. 27 by HI test. Type-specific antisera to 31 additional adenoviruses

failed to show significant neutralizing or hemagglutination-inhibiting relationships. Similarly, antisera prepared in rabbits and monkeys inoculated with the JL strain neutralized only homologous virus and Ad. 32 and inhibited hemagglutination of the homologous virus and Ad. 27 to a significant titer.

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