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Isolation and Characterization of a Papovavirus from Human Urine[1] (38131)

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Introduction. Gardner *et al.* (1) reported isolation in England of a human papovavirus (BK virus) from urine after renal transplantation. This observation was confirmed by Lecatsas *et al.* (2) who identified papovavirus particles by electron microscopy in urine from 8 renal transplant patients in South Africa. Morphologically similar viruses have been isolated from the brains of patients with progressive multifocal leukoencephalopathy in the United States (3, 4). This report describes the isolation and biological properties of another human papovavirus which was recovered from the urine of a renal transplant patient in Syracuse, NY.

Materials and Methods. Cell culture. Primary human embryo kidney (HEK) cells were purchased from Flow Laboratories and grown in MEM (5) with 10% tryptose phosphate broth and 10% fetal calf serum. Petri dish cultures, maintained at 37° in a humidified CO_2 incubator were used between 3rd and 6th passage for virus cultivation and assay.

Plaque assay. Monolayers of HEK cells in 60 mm Petri dishes were washed with Earle's BSS buffered at pH 7.2 with 0.01 *M* Tris (TBE), and infected for 2 hr at room temperature with 0.2 ml of virus diluted in TBE. The infected monolayers were overlaid with 8 ml of MEM containing 10% tryptose phosphate broth, 10% fetal calf serum and 0.8% agar, and incubated for 7 days. Cultures were stained with 2 ml of 1/10,000 neutral red for 6–8 hr, reincubated for 3 days and plaques counted.

Virus neutralization. Antisera diluted 1/5 in TBE were mixed with equal volumes of virus diluted in TBE to contain 2×10^3 or 2×10^2 PFU per 0.2 ml. These were incubated 1 hr at 37° and 2–3 hr at 4°. For controls, virus was mixed with nonimmune calf serum. Plaque assays for residual virus were done on each mixture as described above. Results were recorded as neutralization index (NI), which is the number of plaques in the control divided by the number of plaques in the treated preparation.

Microscopy. Light microscopy. Infected or control HEK grown on coverglasses were either fixed with Bouin's fluid and stained with hematoxylin and eosin or fixed with formol-alcohol and stained for DNA by the Feulgen method (6).

Electron microscopy. For direct observation of whole virus by negative staining, a drop of virus was mixed with a drop of 4% phosphotungstic acid, pH 6.9, and a carbon-film grid was floated on the drop for 10 min. The grid was blotted, dried, and examined. For examination of infected and control cells, HEK monolayers were fixed in glutaraldehyde, embedded in Maraglas, and thin sections were stained with uranyl acetate and basic lead citrate as described previously (7). To examine viral DNA, virus purified in a CsCl density gradient was made 5.4 *M* with urea and incubated at 25° for 1 hr. The solution was diluted with ammonium acetate and cytochrome C to give 1*M* ammonium acetate, 0.01% cytochrome C and 3.2 *M* urea. This was spread on a water hypophase and prepared for electron microscopy with a modification (8) of the Kleinschmidt method (9).

Density gradient analysis. CsCl was added to a virus suspension to a final concentration

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of 0.43 g/ml, and centrifuged at 40,000 RPM for 18 hr in a Beckman SW50.1 rotor. Fractions were collected from the bottom of the tube and tested for density, UV absorption and hemagglutinin.

Hemagglutination and hemagglutination-inhibition. Hemagglutination (HA) tests were done in plastic microtiter plates by making two-fold serial dilutions of virus in 0.05 ml of 0.01 *M* phosphate–buffered isotonic saline, pH 6.5 (PBS). An equal volume of 1.0% human type O erythrocytes in PBS was added, mixed, incubated 1 hr at room temperature, and results were read. Hemagglutination-inhibition (HI) tests were done by making serial, 2-fold dilutions of receptor-destroying enzyme (RDE)-treated serum (10) in 0.025 ml of PBS; 0.025 ml of virus diluted in PBS to contain 4 units of HA was added and incubated at 37° for 1 hr; 0.050 ml of washed 1% human type O erythrocytes in PBS was added; results were read after 1–3 hr at room temperature, as the highest dilution of serum that completely inhibited agglutination (1/10 was the lowest dilution tested).

Results and discussion. Identification of Papovavirus in Human Urine. Over a period of 3 mo, we examined 25 urine specimens from 7 patients who had received renal transplants 2–20 mo previously. Immnosuppression was maintained with 100 mg per day of azathioprine (Immuran) and 40 mg per day of methyl-prednisolone (Solumedrol). In each case, a 25 ml sample of urine was centrifuged at 79,000 × *g* for 2 hr, the pellets suspended in 0.25 ml of water and examined by negative staining in the electron microscope. Virus particles were found in urine of only 1 patient, R.F., a 38 year old male. The first sample was obtained from this patient 2 mo after transplantation, and additional samples were examined at approximately 2 week intervals. The first 2 contained no virus, but the third, collected 3 mo after transplantation contained large numbers of particles, with the capsomeric structure of papovaviruses (Fig. 1). Unfortunately, a week passed between collection and examination of the specimen. A sample of urine obtained as soon as possible thereafter contained small numbers of particles, and few or none were found in later samples. Apparently, the patient suffered a papovavirus infection, possibly of the kidney, which resolved. Immunologic data, presented below, supports this presumption.

Growth of Virus in Cell Structure. Four monolayers of HEK were infected with 0.05 ml each of pelleted virus from R.F. urine, and maintained with twice-weekly changes of medium. No pathology was observed and no virus was detected by electron microscopy for the first 4 weeks. Cytopathogenic effects (CPE) were noted in one culture after 5 weeks and the remaining cultures developed CPE during the folowing week. Papovavirus particles identical to those in the original urine were numerous in the culture medium (Fig. 1). The majority of particles in the original urine appeared "empty" while those from cell culture were mostly "full." The negatively stained particles from both urine and tissue culture measured 43 ± 0.2 nm which corresponds to the size of SV40 and polyoma viruses and is significantly less than the morphologically similar papilloma viruses (11). A second passage, initiated with homogenized infected cells and culture fluid, exhibited CPE in 9 days, and CPE was evident 3–5 days

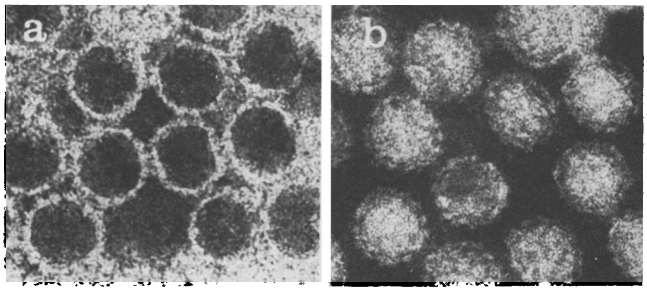

FIG. 1. Negatively stained whole mounts of human papovavirus particles. ×248,000. (a) Pelleted virus from urine of patient R.F. Most particles appeared empty. (b) Pelleted virus from HEK cell culture. Most particles appeared full.

after infection in later passages. Tests for Mycoplasma, generously performed by Dr. Bruce Calnek, were negative with 4th, 7th, and 11th passage virus. We designated this virus by the patient's initials, R.F. virus (RFV).

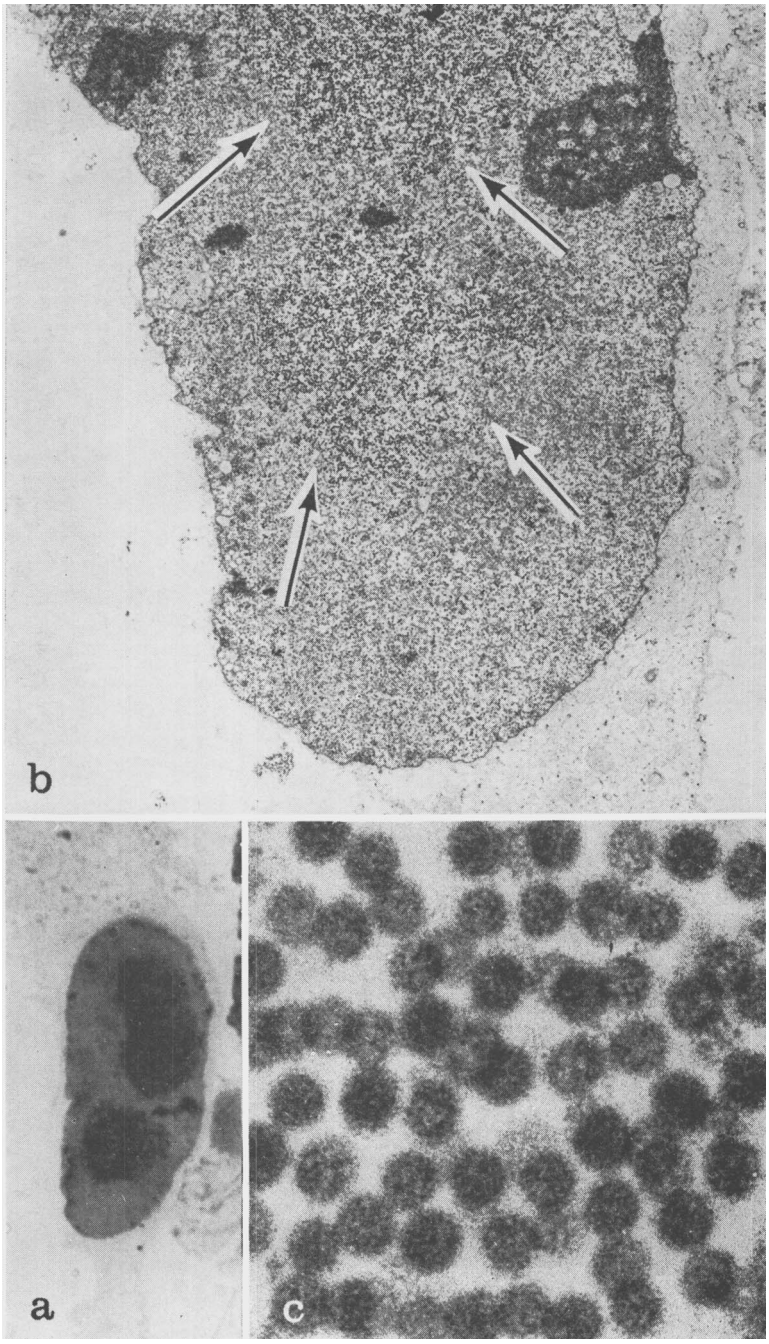

FIG. 2. Inclusion bodies of RFV. (a) Thick section of RFV-infected HEK cell nucleus. Note 2 inclusion bodies. ×1300. (b) Thin section of the same nucleus. Note inclusion bodies (arrows). ×5600. (c) Higher magnification of one of the inclusions, composed of virions. ×248,000.

Cytopathology. In HEK monolayers infected with 10^5–10^6 plaque-forming units (PFU) of RFV, detached rounded cells appeared in 3–4 days. Basophilic, Feulgen positive intranuclear inclusions were visible on day 3 and increased in numbers rapidly. To determine the composition of the inclusion bodies, alternate thick and thin sections of cell culture were examined by light and electron microscopy. Five days after infection with 7th HEK passage RFV, an HEK monolayer was fixed and embedded in Maraglas. One 0.5 μm section was cut, followed by a series of thin sections of approximately 50 nm. The thick section, mounted on a glass slide, was stained with Mallory's azure II-methylene blue; the thin sections were stained with uranyl acetate and basic lead citrate. Nuclei with inclusions identified in the thick sections by light microscopy were located and examined in thin sections by electron microscopy. The results in Fig. 2, demonstrate that the inclusion bodies are composed of virions. Crystalline arrays of virions which are sometimes present in SV40 and polyoma infected cells were not observed with RFV. Since the inclusion bodies were Feulgen positive, we concluded that the virus particles contained DNA. In thin sections, intranuclear virions measured 37 $\pm$ 0.3 nm, while extracellular virions were 42 $\pm$ 0.7 nm. As with negative stains the dimensions in thin sections are in the range of the polyoma-SV40 subgroup of papovaviruses, but are smaller than the papilloma subgroup (11). Tubular forms, characteristic of polyoma virus and wart virus, were not observed, either in thin sections or negative stains. As the CPE progressed, cytoplasmic vacuolization developed and eventually most cells detached from the monolayer leaving a sparse network of necrotic cells connected by thin cytoplasmic processes.

Plaque Assay. Cell cultures infected with suitable dilutions of RFV and overlaid with agar medium developed foci of CPE and were suitable for plaque assays. Virus yields from HEK cell cultures showing maximum CPE were 10^6–10^7 PFU/ml. Plaque-forming activity was resistant to treatment with chloroform (12).

Hemagglutination. Crude infected cell culture fluids agglutinated RBC at dilutions of 1/500–1/2000. Chloroform treatment did not affect HA titers. Untreated nonimmune human or animal sera inhibited hemagglutination at dilutions of 1/10–1/80. The inhibitory activity was abolished by treatment with RDE (10).

Cesium chloride density gradient analysis of RFV. RFV was isolated from 5 $\times$ 10^7 infected cells, which were frozen and thawed, ground in a glass homogenizer and treated overnight with 100 U of RDE. Cell debris was removed by centrifugation, and 1 ml of virus was obtained with an HA titer of 12,800. This was mixed with 2.37 gms CsCl, diluted to 5.5 ml and centrifuged at 40,000 RPM for 18 hr in a Beckman SW50.1 rotor. Fractions were collected and tested for density, UV absorption at 260 nm, and hemagglutinin. Samples were examined by negative staining with the electron microscope. The

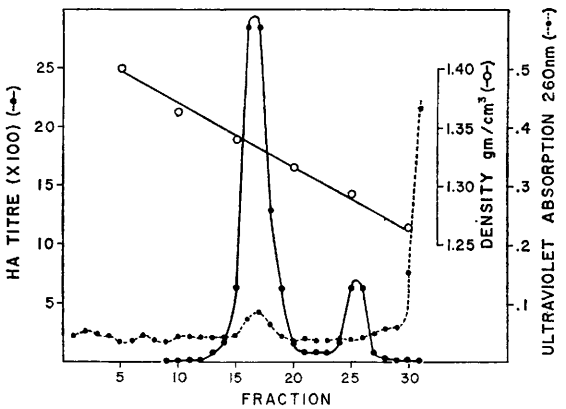

FIG. 3. Cesium chloride density gradient analysis of RFV.

results showed that RFV hemagglutinin was associated with two bands at densities of 1.34 and 1.29 g/cm^3 (Fig. 3). Similar results were reported with the BK strain of human papovavirus (13). Both bands contained large numbers of papovavirus particles but only the heavy band absorbed UV at 260 nm. The virions in the heavy band were complete and most appeared full while the empty particles in the light band were often broken and appeared fragile. Both full and empty particles agglutinated erythrocytes, indicating that the HA is the capsid. An infected cell extract, identical to that described in Fig. 3, but without RDE treatment, had an HA titer of 1280, and almost all activity was found at the top of a similar CsCl gradient. Thus, 90% of the virus from infected cell cultures was attached by RDE-sensitive bonds to structures of low density, probably membrane fragments.

Structure of Viral DNA. As noted above, Feulgen staining demonstrated that RFV inclusion bodies, which are composed of virions, contained DNA. The nucleic acid was extracted from purified full virions and examined by electron microscopy, with the generous assistance of Dr. Alix Bassel. The DNA molecules were mostly in the form of closed, double stranded, supercoiled circles (Form I) with smaller numbers of relaxed circles (Form II), which measured 1.6 ± 0.1 μm in length. The linear density of double stranded DNA spread on a water hypophase is 1.8×10^6 Daltons/μm (14), which gives a mol wt of 2.9×10^6 for the DNA of RFV. This corresponds to the mol wt of SV40 DNA and is significantly less than that of papilloma viruses (11).

Antigenicity of RFV. Purified RFV from the 1.34 g/cm^3 CsCl band was mixed with complete Freund's adjuvant and used to immunize a rabbit, which received two injections of virus, 3 weeks apart. Preimmune serum contained no significant antibody. An intermediate bleeding revealed a vigorous immune response, and large amounts of antibody detectible with hemagglutination-inhibition (HI) and virus neutralization tests were obtained after the second injection of virus (Table I). Paired, preimmune serum and immune calf-anti SV40 serum, purchased from Flow Laboratories, contained no HI activity against RFV. However the immune serum which, according

TABLE I. Hemagglutination-Inhibition and Neutralization Tests with RFV.

Serum	HI Titer	Neutralization Index
Rabbit anti-RFV		
8/7/73 (pre immune)	< 10	< 2
8/27/73	5120	nd[a]
9/18/73	160,000	> 950
Calf anti-SV40 (Flow Labs)		
Pre immune	< 10	< 2
Immune	< 10	4.2
Rabbit anti polyoma[b]		
608	< 10	< 2
611	< 10	nd
613	< 10	nd
Patient R.F.		
11/10/72	< 10	< 2
1/10/73	< 10	nd
1/16/73 (Transplant)	nd	nd
3/31/73	< 10	nd
4/12/73 (virus isolated)	nd	nd
5/13/73	20	nd
7/2/73	160	> 950
9/7/73	320	nd

[a] nd = not done.

[b] Generously supplied by Dr. Bernice Eddy.

to the supplier had a neutralization titer of 1/160 against 100 infectious units of SV40 virus, did show weak but significant neutralizing activity with RFV (Table I). This suggests an antigenic relationship between SV40 and RFV, and is consistent with the results Takemoto and Mullarkey (15) with the BK strain of human papovavirus. RFV has never been passaged in monkey cells, so there is no possibility that its relationship to SV40 derives from interaction with latent SV40 genetic material that might be present in monkey cell cultures. No previous work with papovaviruses has ever been done in this laboratory. Rabbit antipolyoma antisera contained no HI or neutralizing activity against RFV.

HI tests with serum from patient R.F. indicated that he had little or no HI antibody to RFV prior to or at the time of surgery and he remained free of antibody 2 mo later (Table I). As indicated above, virus was detected in the urine of this patient only once, 3 mo after surgery; urine specimens taken before and after that time contained no detectable virions. One month after virus was detected, HI antibody appeared, and increased in subsequent blood specimens, even though the patient was under immunosuppressive therapy. Neutralizing activity simultaneously rose significantly. It appears that R.F. was susceptible to the virus at the time of surgery, but the long interval between transplantation and the appearance of virus suggests the 2 events were not directly related. The data indicate that exposure to virus may have come later, perhaps from a source unrelated to the transplant. We did not have access to materials from the donor.

Incidence of RFV Antibodies in the Human Population. HI tests were done on 400 human sera, originally collected for Wasserman testing, most from young adults. Of the total, 375 (94%) were positive, with an HI titer of 10 or greater, and 325 (81%) had HI titers of 20 or greater. A similar, high incidence of antibodies was reported in England with the BK strain of human papovavirus by Gardner (16). These surprising results were supported by the finding that 6 different batches of commercially prepared human immune globulin each contained HI antibodies against RFV. These preparations, intended for passive immunization against poliomyelitis, are Cohn fraction II human globulins from pools of more than 1000 individual sera, concentrated about 10-fold with respect to the starting material. The results of HI tests (Table II) indicated that high levels of antibody against RFV have been present in the human population for many years. Furthermore, 3 batches were prepared with sera collected before the first field trials of poliomyelitis vaccines in 1954. This means that human antibodies against RFV could not have been induced by the SV40 virus that was present in some early batches of poliomyelitis vaccine (17). It also effectively eliminates the possibility that RFV represents a variant of SV40 originally introduced into humans with polio vaccine.

The size of RFV and the length of its DNA molecule place it in the SV40-polyoma subgroup of papovaviruses. RFV appears to share antigenic determinants with SV40 virus, and like that virus it appears to cause frequent inapparent infection in the natural host. Two properties distinguish RFV from SV40. The latter virus has never been shown to agglutinate erythrocytes (13) and it does not productively infect human cells in culture.

Of the known human papovaviruses, RFV most closely resembles the BK strain, which

TABLE II. Presence of Antibodies Against RFV in Pooled Human Globulin.

Manufacturer Batch no.	No. of sera in pool	Preparation date	HI Titer
Squibb 301-1	No record	July 1953	800
253-2	No record	March 1954	1600
253-1	No record	March 1954	800
341-2	32,400	October 1961	1600
Cutter G7634	> 1,000	September 1963	1600
G7780	> 1,000	October 1963	1600

was also isolated from human urine, has a hemagglutinin, grows and produces plaques in HEK, and is related antigenically to SV40 (1, 13, 15, 18). This report, together with previous studies from England (1) and South Africa (2) indicate worldwide distribution of 1 or more human renal papovaviruses. The viruses of renal origin appear to differ from papovaviruses isolated from progressive multifocal leukoencephalopathy (PML). The viruses isolated from PML by Weiner, *et al* (4, 19) were grown in monkey kidney or human fetal brain cell cultures and apparently are antigenically identical to SV40. The JC strain (3) was isolated and passaged in human glial cells and does not seem to be antigenically related to SV40. Comparative studies of the emerging family of human papovaviruses are contemplated.

Summary. A virus (RFV) isolated from the urine of a human renal transplant patient and grown in human embryo kidney (HEK) cell culture had properties associated with the polyoma-SV40 subgroup of papovaviruses. Virions with papovavirus capsomeric structure, 37–43 nm in diameter, contained circular, supercoiled DNA of 2.9×10^6 daltons. Density gradient analysis revealed 2 classes of particles in infected cell cultures; full virions at 1.34 g/cm^3 and empty virions at 1.29 g/cm^3. Both full and empty particles agglutinated human type O erythrocytes. Infectivity and hemagglutinin were resistant to chloroform. Hemagglutination-inhibition tests and plaque neutralization tests in HEK cells indicated that RFV was related to, but distinct from SV40 virus. Antibodies against RFV were present in at least 81% of 400 human adult sera and high titers of HI antibodies against RFV were present in pooled human immune globulin.

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