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Human Papovavirus, BK Strain: Biological Studies Including Antigenic Relationship to Simian Virus 40

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Some of the properties of a new human papovavirus, BK, have been examined. Host range studies of BK virus (BKV) showed human cells to be more sensitive to infection than monkey cells; human fetal brain cells appear to be highly sensitive to BKV, with the production of extensive cytopathology characterized by cytoplasmic vacuolization. The hemagglutinin of BKV is associated with the virion and is resistant to ether or heating at 56 C for 30 min. Fluorescent antibody as well as neutralization tests indicated antigenic similarities between simian virus 40 (SV40) and BKV. Cells undergoing lytic infection with BKV synthesized intranuclear T antigen(s) which reacted with SV40 T antibody demonstrable by immunofluorescence. However, BKV did not appear to induce SV40 transplantation antigens in transplantation-resistance tests. Evidence was obtained that BKV was present in humans prior to the widespread use of polio vaccines, thus ruling out the possibility that BKV is an SV40-related monkey virus, introduced into the human population by accidental contamination of poliovirus vaccines.

The isolation in cell culture of human papovaviruses has recently been reported by three different laboratories. Weiner et al. (18) isolated viruses from two cases of progressive multifocal leukoencephalopathy (PML), a rare demyelinating disease of the brain. Both viruses were antigenically very similar to the oncogenic simian virus 40 (SV40). From a case of PML, Padgett et al. (14) also isolated a papovavirus which they reported to be serologically unrelated to SV40, polyoma, or human wart viruses. Gardner et al. (4) isolated another papovavirus from the urine of a patient who had undergone a renal transplant. This virus, designated as BK virus (BKV) (the initials of the patient from whom the virus was isolated), appeared to have some cross-reactivity with SV40 by the immunoelectron microscopy technique. In studies reported here, we confirmed Gardner's observation of the relationship of BKV to SV40 by using fluorescent antibody (FA) as well as neutralization techniques; in addition, evidence that BK and SV40 induce the same intranuclear tumor antigen (T antigen) was obtained. These results together with other studies characterizing BKV are presented.

MATERIALS AND METHODS

Virus. A stock of BKV in its third passage was received from K. Shah. The virus had been propagated in Vero cells, the original host cell employed by Gardner for isolation of the virus.

Cells. Vero cells were obtained from the American Type Culture Collection at the 128th subculture level. These as well as the cells described below were grown in Eagle medium supplemented with 10% fetal bovine serum (FBS). After infection, the cells were maintained in medium containing 5% FBS.

WI-38 human diploid fibroblasts (10) were used from the 22nd passage and transferred to make new cultures at a 4:1 ratio until cell growth declined, generally after 30 passages.

Human fetal brain cell cultures were prepared by methods suggested to us by B. Padgett. Brains obtained from fetuses between 12 to 16 weeks were crudely minced and grown in 75-cm^2 plastic flasks (Falcon Plastics).

HA and HAI tests. Hemagglutination (HA) tests were performed by making serial twofold dilutions of virus in phosphate-buffered saline, pH 7.2 (PBS), in 0.25-ml volumes. An equal volume of 0.5% human type O erythrocytes were added and the tubes were shaken and allowed to settle at 4 C. The test was read after 2 h, and the highest dilution giving complete agglutination was considered the end point. Hemagglutination inibition (HAI) tests for BKV antibody were done by inactivating serum at 56 C for 20 min followed by adsorption with 10% type O erythrocytes to remove agglutinins which were present in some sera. The adsorbed sera were diluted twofold in PBS, followed by the addition of eight HA units of virus.

The mixtures were shaken and kept at room temperature for 30 min, and 0.5 ml of red cell suspension was added. After the tubes were again shaken, they were kept at 4 C and read after 2 h. The highest dilution giving no agglutination was taken as the titer of the serum.

FA tests. The indirect procedure was employed in all FA tests using fluorescein-conjugated anti-hamster and anti-rabbit globulin (BBL).

Antiserum to BKV. BKV antiserum was made in rabbits or hamsters by inoculating purified virus subcutaneously at 5-day intervals. Animals were bled 2 weeks after the third injection.

Neutralization test. Antibody against SV40 was measured by using a plaque reduction test in primary African green monkey kidney (AGMK) cells. Equal volumes of virus (50 PFU of large plaque SV40 per 0.2 ml) and serum were mixed and incubated at room temperature for 1 h, and 0.4 ml was inoculated onto confluent AGMK cultures. After 1 h of adsorption, overlay medium was added. Plates were stained by adding neutral red (1/40,000) in the overlay medium on the 11th day, and plaques were counted 24 h later.

Virus purification. Virus in supernatant fluids from infected cultures was pelleted through 5 ml of 30% sucrose in an SW25.1 rotor at 23,000 rpm for 2 h. The pellets were resuspended in PBS, and virus was banded in CsCl by isopycnic density gradient centrifugation.

RESULTS

General characteristics of the virus and virus-infected cells: host range. (i) Replication in Vero cells. The original isolation of BKV by Gardner et al. (4) was made in Vero cells, a continuous line of AGMK. When monolayer cultures of Vero cells were infected with BKV (third passage), cytopathic effects were slow in developing initially and difficult to distinguish from uninfected controls; they consisted of rounding of the cells and did not differ significantly from uninfected aging Vero cultures. Replication of virus could only be ascertained by the presnce of hemagglutinin in the supernatant fluid. Whereas the initial virus stock caused the production of hemagglutinin only after 2 to 3 weeks post-infection, later virus passages reduced this time interval to about 1 week. However, lysis of all the cells did not occur, and with medium changes, cultures could be maintained with continued virus production for over 6 weeks. FA studies revealed only 5 to 10% of the cells synthesizing viral antigen at any time. The infected cultures thus resembled typical carrier cultures, with high levels of hemagglutinins being produced (1:320 or greater HA titers).

(ii) Primary and continuous lines of AGMK. Although cytopathic effects were seen in cultures of primary AGMK, CV-1, or BSC-1 cells, there was no evidence of hemagglutinin production, nor could the growth of BKV be established in these cells.

(iii) Human cells. A variety of different types of human cells were tested for susceptibility to BKV. Both fibroblast and epithelial-like cells were found to be highly susceptible to lytic infection with BKV. Infection of WI-38 cells with BKV resulted in cytopathic effects which were observed as early as 5 to 6 days after infection, but most of the hemagglutinin remained cell associated. Human fetal brain cells which consist mainly of spongioblasts and astrocytes (16) were highly susceptible to infection with the production of cytoplasmic vacuolization (Fig. 1A and B) very similar to that produced by SV40 in AGMK. Human cells thus appear to be more sensitive to BKV than monkey cells, and further studies are being conducted to determine the optimal cell-virus system.

Properties of BKV hemagglutinin. Some of the properties of the BKV hemagglutinin have recently been described (13). We carried out similar studies and obtained results similar to those reported by Mantyjarvi et al. (13). Optimal temperature for HA, using PBS as the diluent, is at 4 C. HA also occurs at 25 C but the titer is two- to fourfold lower. At 37 C agglutination does not occur. The hemagglutinin is insensitive to exposure to 20% ether for 2 h, and heating to 56 C for 30 min results in no loss of HA titer. Finally, as proven in experiments described below on purified virus, the hemagglutinin is intimately associated with the virion.

Purification of BKV. Infected Vero cells which did not show marked cytopathic effects nevertheless released large amounts of hemagglutinin into the supernatant fluids. Purification of virus from the medium by pelleting through sucrose (as described in Materials and Methods) resulted in complete recovery of the hemagglutinin in the pellet; the supernatant fluid was devoid of hemagglutinin, suggesting that the hemagglutinin was virion associated. This was verified when the pellet was banded in CsCl gradients. The virus which banded at a density of 1.34 g/cm^3 was dialyzed against PBS and tested for hemagglutinating activity; the titer usually was in the range of 30,000 or greater. When examined by electron microscopy, particles typical of the SV40-polyoma subgroup were seen, measuring approximately 45 nm (Fig. 2). The association of HA with the virions of BKV has also been reported recently by Mantyjarvi et al. (13). "Empty" particles which banded at a density of 1.29 g/cm^3 also had high hemagglutinating titers.

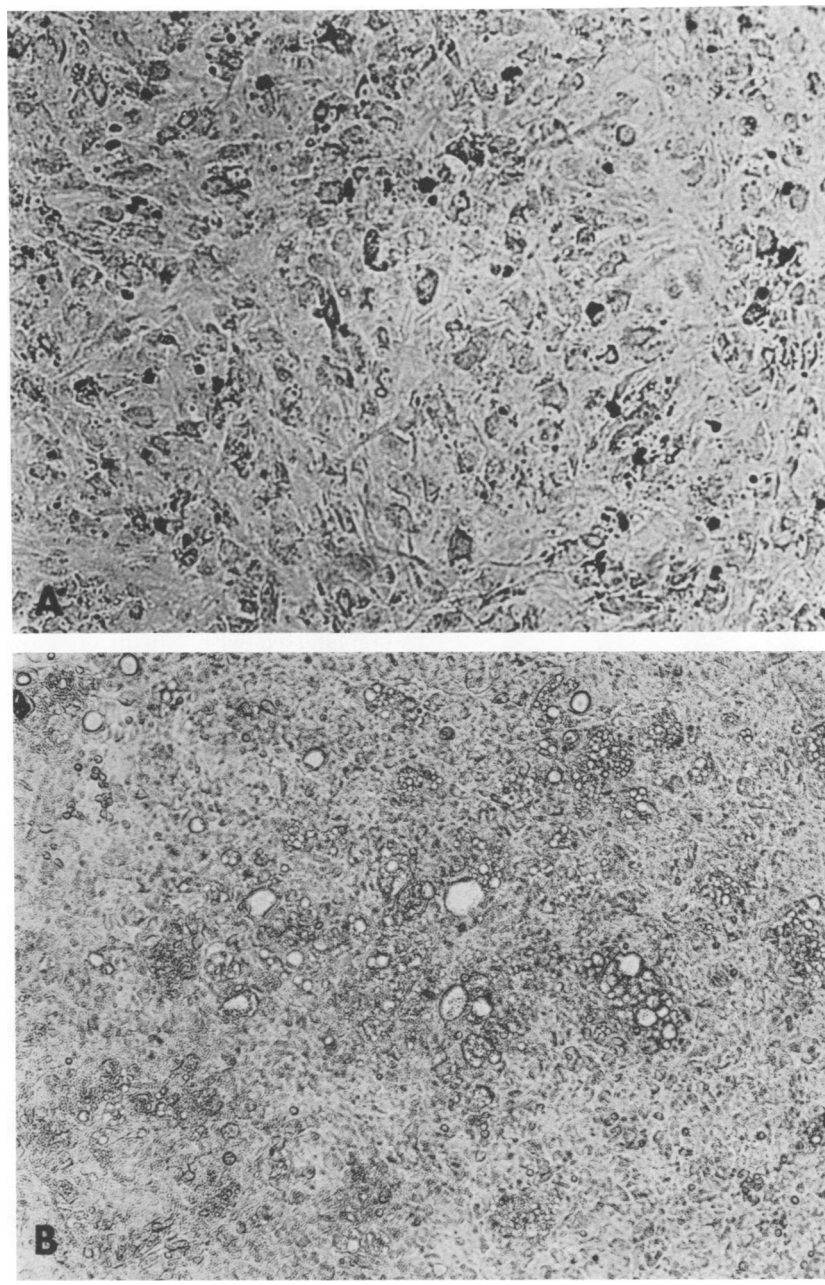

FIG. 1. *Uninfected (A) and BKV-infected (B) human fetal brain cell cultures. Note the cytoplasmic vacuolization and increased granularity of the infected cells. Magnification, ×100.*

Relationship of BKV to other papovaviruses. Gardner et al. (4) found no reaction of BKV with antiserum against human wart or polyoma virus but noted a weak reaction against SV40 by immunoelectron microscopy. We carried out further tests of possible cross-reactions between BKV and other members of the polyoma subgroup of papovaviruses by FA as well as HAI tests. The results are tabulated in Table 1. Antiserum prepared against the hemagglutinating polyoma, rabbit kidney vacuolating agent (9), or the mouse K-virus (11), did not inhibit or react with BKV by either the HAI or FA tests. On the other hand, anti-SV40

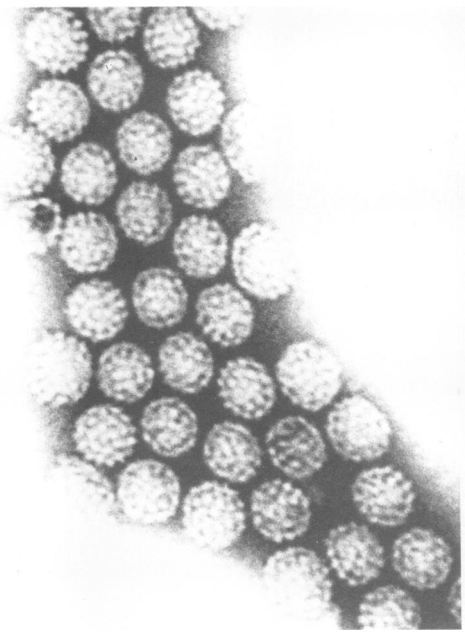

FIG. 2. *Purified BKV with typical papovavirus morphology and capsomeric structure. Magnification, ×250,000.*

TABLE 1. *FA and HAI tests between BKV and other papovaviruses*

Antibody[a]	Test	
	HAI[b]	FA[c]
BKV	1:640	+
SV40	<1:20	+
Polyoma	<1:20	-
Rabbit kidney vacuolating virus	<1:20	-
K-Virus	<1:20	-

[a] All antisera were prepared in rabbits except anti-K virus, which was mouse antiserum kindly provided by J. Parker.

[b] Serial two-fold dilutions of serum mixed with 8 U of BKV, incubated for 1 h at 37 C before addition of human type O erythrocytes.

[c] FA tests were performed by the indirect procedure, as described in text, by using BKV-infected Vero cells.

serum (negative for SV40 T antibody) reacted by the FA test in BK-infected cells, but the same serum failed to inhibit HA of BKV. The absence of HAI antibody in anti-SV40 serum was observed in other sera prepared in hamsters and rabbits; the reason for this is unknown. These sera also reacted well by the FA test, giving typical bright intranuclear fluorescence (Fig. 3) when reacted with BK-infected Vero cells.

The apparent sharing of cross-reacting antigens between SV40 and BKV as seen by the FA test was further confirmed by neutralization tests. Rabbit anti-BKV serum which had an HAI titer of 1:640 showed low neutralizing activity against SV40 in plaque-reduction tests; a 1:20 serum dilution neutralized 50 PFU of SV40. Since a plaque assay for BKV is still not available, its homologous titer is unknown. Antiserum against BKV also gave a weakly positive intranuclear fluorescence when tested by the FA procedure using AGMK cells which had been infected by SV40 for 48 h.

Studies on cross-reactions of BKV with SV40-induced nonvirion antigens. Since BKV and SV40 shared common structural antigens when tested by FA or by neutralization, it was important to determine whether BKV also induced two of the known SV40 virus-specific antigens, the intranuclear T antigen and the membrane-associated tumor-specific transplantation antigen (TSTA).

T antigen. WI-38 cells grown on cover slips were infected with 0.5 ml of undiluted BKV (HA titer of 1:640). After 1 h of adsorption, Eagle medium containing 1% FBS was added. Cover slips were examined by the indirect procedure at daily intervals by using SV40 hamster tumor antibody. Twenty-four hours after infection, approximately 25% of the cells showed bright intranuclear staining similar to that observed in SV40-transformed cells (Fig. 4). The percentage of fluorescent cells increased to about 50% by 48 h and by 5 days, when cytopathic effects were evident, over 75% of the cells were positive for T antigen. Several other hamster anti-T antisera, all negative for antiviral antibody, gave identical results. Polyoma hamster anti-T antibody did not react with BKV-infected cells. We conclude, therefore, that BKV induces T antigen(s) immunologically similar to or identical with the SV40 T antigen(s).

General characteristics of the virus and virus-infected cells: TSTA. SV40 virus induces resistance to homologous tumor cell challenge when animals are immunized with infectious virus (2, 7). To test whether BKV induced the same TSTA as SV40, weanling hamsters were immunized with SV40 or BKV. Animals were immunized by subcutaneous injection of 10^7 PFU of SV40 (small plaque virus) and 1 ml of BKV which had a hemagglutinating titer of 1:640. Animals received three doses of virus at 5-day intervals. Two weeks after the last inoculation, the animals were inoculated with 10-fold dilutions of virus-free SV40 hamster tumor cells previously demonstrated to have the transplan-

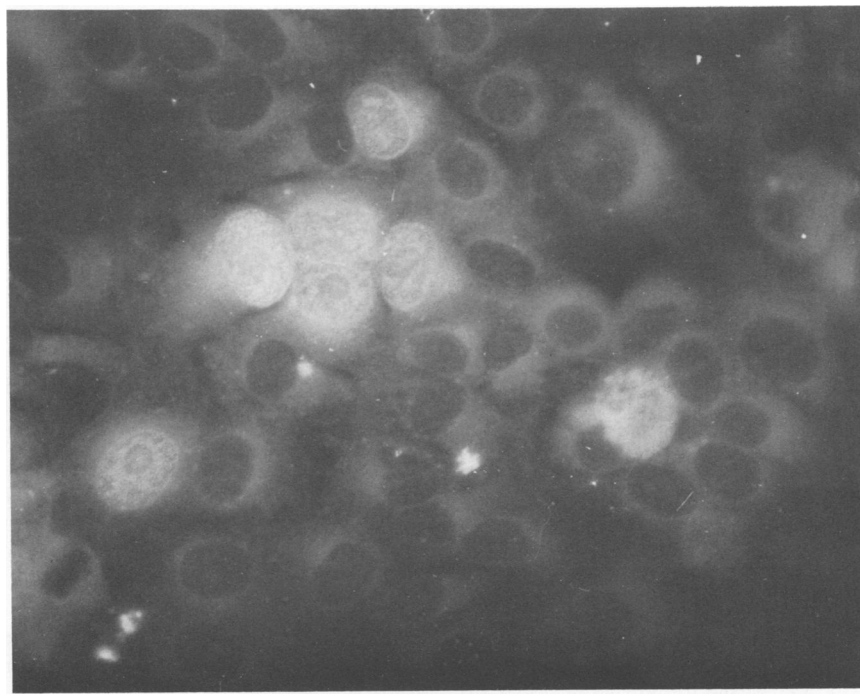

FIG. 3. *Vero cells 9 days after BKV infection stained with SV40 antiviral antibody, showing bright intranuclear fluorescence in a focus of infected cells. Magnification, ×300.*

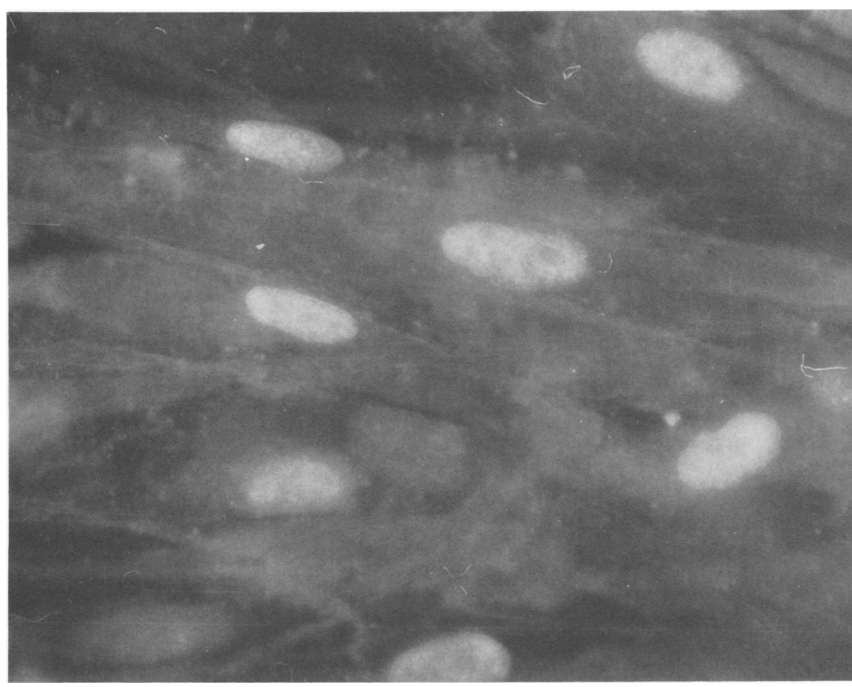

FIG. 4. *WI-38 human fibroblast cells infected for 72 h and stained with SV40 T antibody. Fluorescence is confined to the nuclei. Magnification, ×300.*

tation-rejection antigen (unpublished data). Control animals which did not receive any virus were also inoculated with tumor cells. Animals were observed over a period of 3 months for tumor formation, and the results of this test are summarized in Table 2. Animals immunized with SV40 showed a high degree of resistance to SV40 tumor cells requiring over 100-fold greater cell numbers to produce tumors than control animals. BKV-immune animals showed little or no resistance to SV40 tumor cells, and there was no delay or decrease in the size of tumors compared to controls. BKV, therefore, did not appear to induce the same transplantation antigen as SV40. However, since BKV-induced tumor cells are not available, it is not known whether the immunization procedure employed in this experiment was adequate to protect animals against homologous tumor cell challenge. However, serum from animals immunized with BKV obtained prior to cell challenge showed high antibody titers as measured by the HAI test (1:640 to 1:1,280).

BKV: human or monkey virus? Because of the sharing of common structural as well as nonstructural (T antigen) antigens between BKV and SV40, it became important to determine whether BKV is a human or monkey virus, possibly introduced into the human population as a result of contamination of polio virus vaccine. Two kinds of experiments were conducted to answer this question.

(i) Tests of other "wild" strains of SV40 for hemagglutinating capacity. Since standard SV40 strains have never been reported to possess hemagglutinating activity, a number of new isolates from rhesus monkey kidneys (kindly provided by Kendall Smith and Bernice Eddy) were tested for HA of human type O erythrocytes. Two of 15 "wild" strains were positive for agglutinins when crude virus suspensions were tested. After purification and banding in CsCl gradients, the hemagglutinating activity was no longer associated with the virions. It was concluded, therefore, that none of 15 newly isolated SV40 strains had hemagglutinating activity, and this is a property not generally associated with SV40.

TABLE 2. *Transplantation-resistance test of SV40- and BKV-immune hamsters to SV40 tumor cells*

Hamsters immunized against	No. of cells inoculated				
	10^2	10^3	10^4	10^5	10^6
SV40[a]	0/5[b]	0/5	0/5	3/5	4/5
BKV	1/5	4/5	5/5	5/5	5/5
None	3/5	5/5	5/5	5/5	5/5

[a] Animals were immunized subcutaneously with SV40 or BKV every 5 days. Two weeks after the third virus injection, animals were challenged wih SV40 tumor cells.

[b] No. of animals with tumors/no. of animals inoculated.

(ii) Tests of pre-polio vaccine sera for BK antibody. Ten sera from adults collected during the period 1949 to 1952 (provided by R. J. Huebner), prior to the widespread use of polio vaccines, were tested for HAI antibody to BKV. Seventy percent (7/10) were positive for BKV antibody at dilutions of 1:40 or greater, which is approximately the same as that found in recently collected sera. Some of the positive sera were also tested by FA on BK-infected Vero cells, and the presence of antibody was confirmed by this test also. BKV thus appears to be a common human virus which was not inadvertently introduced into the human population through contamination of poliovirus vaccines.

DISCUSSION

The studies reported here show an antigenic relationship between BKV and SV40. BKV was originally isolated by Gardner et al. (4) from the urine of a patient who had undergone renal transplantation. Recent studies by Lecatsas et al. (12) indicate that human papovaviruses may be common "latent" viruses in human kidneys which are activated after immunosuppression. These workers examined centrifuged pellets from urine of renal transplant patients by electron microscopy and found 8 of 12 samples positive for papovavirus-like particles. Since biological studies were not done, it is not certain whether these viruses are identical or related to BKV.

Our data show that not only are structural antigens common between BKV and SV40, but both viruses appear to induce the same or related nonvirion T antigen(s). However, they apparently do not induce the same transplantation antigens, although definitive answers to this question await the availability of BKV-induced tumors which can be used in transplantation-rejection tests. It should be pointed out that viruses which induce the same T antigens may not necessarily induce the same transplantation antigens. It may be recalled that polyoma mutants have been described by Hare (8) which are defective in inducing transplantation rejection although they induce the same T antigen. At least five or six structural proteins have been reported in the SV40 virion (1, 3, 5); additional studies should reveal exactly which of the structural proteins are shared between BKV and SV40.

Because of the antigenic similarity of BKV to SV40, it was important to determine whether this virus had been introduced and had become established in humans as a consequence of contamination of poliovirus vaccines with SV40. Hemagglutinating strains of SV40 do not appear to exist, but, more important, the antibody studies with sera obtained prior to the widespread use of poliovaccine prove that BKV or an antigenically similar virus was existent in humans before poliovirus vaccines.

Although BKV is apparently related to SV40, there are certain properties of the virus which readily distinguish it from SV40. (i) It is a hemagglutinating virus. (ii) It has a different host range. SV40 grows to limited extent without apparent cytopathology in human diploid cells (6, 15, 17). BKV, on the other hand, is highly lytic for human cells; it can cause cytopathic effects on initial passage in primary AGMK but, unlike SV40, the virus does not appear to replicate on serial passage.

The significance of BKV to human disease remains to be determined. From serological studies by Shah and Daniel (Fed. Proc., **32**:1019, 1973), it is clear that BKV infection is common in humans and the virus may perhaps be the counterpart of SV40 in rhesus monkeys. Its relationship to the other hemagglutinating papovavirus isolated by Padgett et al. (14) is not known at the present time.

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