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Rapid Detection of Polyomavirus BK by a Shell Vial Cell Culture Assay

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Polyomavirus BK (BKV) causes asymptomatic latent infection in the human host that is reactivated during periods of immune suppression. Detection by conventional tube cell culture is difficult and time consuming because BKV exhibits slow growth with late (14 to 28 days) and subtle cytopathic effects. We developed a shell vial cell culture assay (SVA) using a cross-reactive monoclonal antibody to the T antigen of simian virus 40 to detect BKV rapidly by indirect immunofluorescence. Nuclear fluorescence was seen in BKV-infected cells as early as 16 h postinoculation; 6 to 28 times more foci were present at 36 h postinoculation. Human embryonic kidney cells infected with BKV produced 7 to 42 times more fluorescent foci than MRC-5 or rhabdomyosarcoma cells did. Centrifugation enhanced the infectivity of BKV in the SVA. To define the clinical utility of SVA, urine specimens from organ transplant patients were tested. Of 27 patients, 4 (15%) were found to be positive by SVA. SVA offers a simple and rapid method for detection of BKV that can be of use in clinical studies of this virus.

Polyomavirus BK (BKV) is a small, nonenveloped DNA virus. Asymptomatic infection with BKV is common, and latent virus persists in the kidneys after primary infection (5, 7). In immunocompromised and pregnant patients, virus can be excreted in the urine (1, 8, 12, 16). BKV may be associated with ureteral stenosis after renal transplantation (12, 16). Although oncogenic in hamsters, no human tumors have definitely been associated with this virus (22).

BKV replicates in several human and monkey cell lines, but it grows slowly in conventional tube cell cultures and viral replication is inefficient (8, 20). Because of this problem with conventional culture, a number of alternative methods for detecting BKV have been described. These include urine cytology and electron microscopy (8), indirect immunofluorescence microscopy (17), enzyme-linked immunosorbent assay and DNA hybridization (2), and polymerase chain reaction (3). A simpler, more rapid culture method could complement these methods of detection. The shell vial cell culture assay (SVA) has been successfully used to detect a number of slowly replicating viruses. We report here the application of this technique for the detection of BKV.

MATERIALS AND METHODS

Virus. BKV (Gardner isolate, 1971) was kindly provided by E. O. Major of the National Institutes of Health (Bethesda, Md.). Stocks of BKV were prepared by inoculating flasks of Vero cells. After 2 weeks of incubation, cell layers were digested with trypsin and separated from the supernatant. Infected Vero cells were subjected to three cycles of rapid freezing and thawing to liberate viral particles. Viral inocula were measured by a hemagglutination (HA) assay with human type O erythrocytes as described previously (18).

Cells. Human embryonic kidney (HEK), MRC-5, Chinese hamster ovary (CHO), McCoy, and rhabdomyosarcoma (RD) cells were purchased from Viromed (Minnetonka, Minn.). Cells were seeded into 1-dram (ca. 3.7-ml) shell vials, each of which contained a round cover slip (diameter, 12 mm), and grown in Eagle minimum essential medium with 10% fetal bovine serum. When the cell layer became confluent, Eagle minimum essential medium with 5% fetal bovine serum was substituted.

Infection of cells. Shell vials containing secondary HEK, RD, CHO, McCoy, or MRC-5 cells were inoculated with 0.2 ml of serum-free Eagle minimum essential medium containing BKV. BKV inocula ranging from 0 to 25 HA units per vial were used to assess the effects of the inoculum size. A standard inoculum of 100 HA units per vial was used for assessing the susceptibilities of different cell lines to BKV infection. Shell vials were either centrifuged at 700 × *g* for 45 min or incubated without centrifugation for 90 min. One milliliter of Eagle minimum essential medium with 5% fetal bovine serum was then added, and the shell vials were incubated at 36°C.

Staining of infected cells. The T antigens of the polyomaviruses simian virus 40 (SV40) and BKV are functionally similar and share common epitopes (9). A commercially available monoclonal antibody to SV40 T antigen (PAb416; Oncogene Science, Inc., Mineola, N.Y.) cross-reacts with the T antigen of all strains of BKV (22). We used this antibody to stain infected cell monolayers by the indirect immunofluorescence technique. After incubation, medium was removed and cover slips were washed twice with phosphate-buffered saline and fixed with cold acetone for 10 min. A total of 150 μl of a 1:30 (vol/vol) dilution of SV40 T-antigen mouse monoclonal antibody (3.3 μg/ml) was applied and incubated at 36°C for 30 min. After washing with phosphate-buffered saline, 150 μl of a fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (IgG) conjugate (Cooper Biomedical, Malvern, Pa.) was applied to the cover slip and incubated for 30 min. Stained cover slips were inspected by fluorescence microscopy, and each fluorescent nucleus was counted as one focus.

Specimens from patients. Urine specimens were collected from 27 recent solid organ transplant patients (17 liver, 8 kidney, 2 heart). Specimens were centrifuged at 1,500 × *g*

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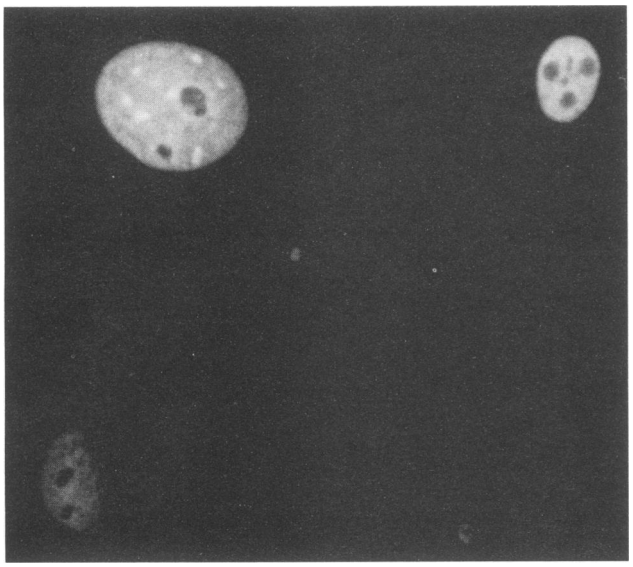

FIG. 1. Nuclear fluorescence in infected HEK cells after indirect immunofluorescence staining with a monoclonal antibody to SV40 T antigen and a fluorescein-labeled goat anti-mouse IgG conjugate. Magnification, ×400. The appearance of the nuclear fluorescence was similar in RD and MRC-5 cells.

for 20 min, and the supernatants were discarded. The sedimented cells were suspended in sterile water and subjected to three cycles of rapid freezing and thawing to liberate viral particles. Specimens were then inoculated into two HEK-seeded shell vials each and centrifuged at 700 × *g* for 45 min. Cover slips were stained at 36 h postinoculation as described above.

For patients with positive results, hospital records were reviewed to ascertain immunosuppressive regimens and urinary tract symptoms.

RESULTS

T antigen was detected by indirect immunofluorescence in the nuclei of BKV-infected HEK, MRC-5, and RD cells (Fig. 1). There was no nuclear fluorescence in CHO or McCoy cells when they were stained up to 4 days after infection. Uninfected cell monolayers did not show nuclear fluorescence in any of the cell types.

HEK cells were the most sensitive for the growth of BKV (Fig. 2). From 7 to 42 times more fluorescent foci were seen in BKV-infected HEK cells than were seen in MRC-5 or RD cells infected with identical inocula. Although nuclear fluorescence could be seen as early as 16 h after infection, 6 to 28 times more foci were present at 36 h (Fig. 2). Because of this finding, monolayers were stained at 36 h in all subsequent experiments.

Centrifugation enhanced the detection of BKV by SVA (Fig. 3), regardless of the inoculum titer. In order to determine the sensitivity in SVA, HEK cells were inoculated with serial dilutions of BKV. The smallest inoculum producing positive results was 0.03 HA units, which consistently produced one or two foci per cover slip when it was centrifuged. Without centrifugation, 1 HA unit was the lowest inoculum detected. With higher inocula, there was at least a fourfold greater number of foci present in centrifuged shell vials than in noncentrifuged shell vials.

Centrifugation force and duration were varied to determine the optimal conditions for detecting BKV in HEK-seeded shell vials. Centrifugation at 700 × *g* for 45 min produced the greatest degree of infectivity. Fewer foci were present when centrifugation was done at 200 × *g* or for 15 min. No additional enhancement occurred when centrifugation was increased to 1,000 × *g* or for 90 min.

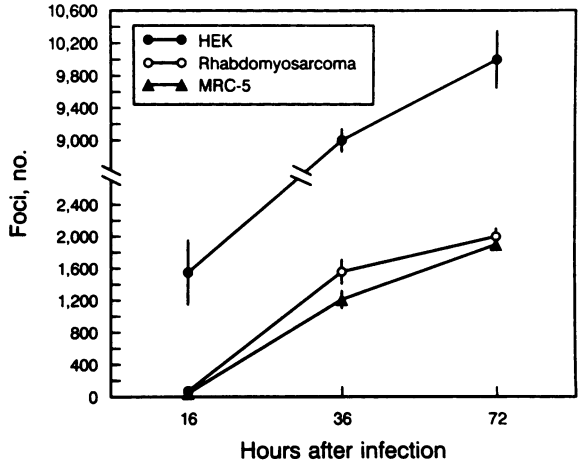

FIG. 2. Comparison of three cell types for detection of BKV T antigen by immunofluorescence. A total of 100 HA units of virus was used for inoculation of each shell vial. Shell vials were centrifuged at 700 × *g* for 45 min. Each fluorescent nucleus was counted as one focus.

Fifty-five urine specimens from 27 transplant recipients were tested by SVA. All specimens were obtained within 6 months of organ transplantation. Five specimens were toxic to the cell monolayer. Of the 50 evaluable specimens, 5 specimens (10%) from four patients (15%) were positive. All four patients were seropositive for BKV by HA inhibition (pretransplant serologic status was unknown). Three of the patients received liver transplants, and the fourth patient received a heart transplant. All were on immunosuppressive regimens consisting of cyclosporine, prednisone, and azathioprine. None of the four patients had symptoms of hemorrhagic cystitis or ureteral stenosis.

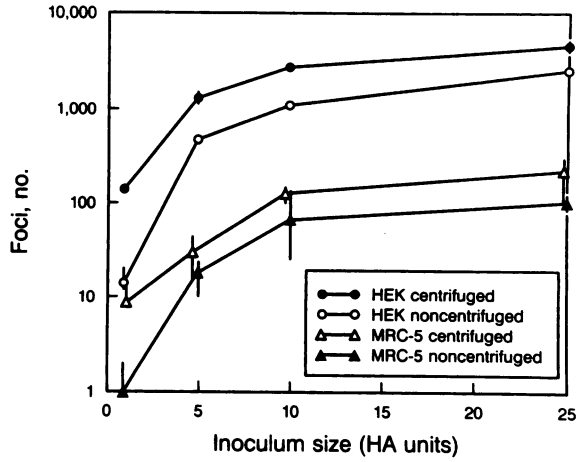

FIG. 3. Effects of centrifugation and inoculum titer on number of fluorescent nuclei in HEK and MRC-5 shell vials. Centrifugation was performed at 700 × *g* for 45 min. Indirect immunofluorescence staining for T antigen was done 36 h after infection.

DISCUSSION

BKV grows slowly in conventional tube cell cultures. The initial report of the isolation of BKV (12) noted that cytopathic effects were first observed in monkey kidney cells 18 days after inoculation. Cytopathic changes in Vero cell cultures were not apparent for 1 to 3 months. Even in HEK cells (the optimal cells for assay and propagation of BKV), plaques did not develop for 14 to 28 days, although a cytopathic effect was detected as early as 5 days after infection (8, 20).

SVA is a technique that has wide application for the rapid detection of viruses. SVAs for the detection of the herpesviruses, adenovirus, and influenza virus have been described previously (10, 11, 13–15). Low-speed centrifugation enhanced in vitro infection with all of these viruses as well as with the human immunodeficiency virus and rotavirus (6, 19). Our research shows that there was a similar enhancement for BKV with low-speed centrifugation. Besides increasing the infectivity by centrifugation, the main requirement for development of a given SVA is the availability of a monoclonal antibody directed against an early viral antigen. We used a cross-reactive monoclonal antibody against epitopes shared by the SV40 T antigen and the T antigen of BKV. This method can detect T antigen as early as 16 h after infection, but it is much more sensitive at 36 h.

Previous studies have examined clinical urine specimens for polyomaviruses. Using cytologic and virologic methods, Coleman et al. (8) found that 13.5% of renal transplant patients excreted polyomavirus. Twenty percent of renal transplant patients were positive for polyomavirus by cytology, immunofluorescence microscopy, or electron microscopy in the series of studies done by Hogan et al. (16). Of 55 bone marrow transplant patients, 20 were positive by DNA hybridization and 15 of 55 were positive by enzyme-linked immunosorbent assay in a series of studies from Johns Hopkins University (2). Although no direct comparison was made, results achieved by our SVA are comparable to those achieved by other methods.

SVA offers two major advantages over other techniques for BKV detection. The first one is the limited time required to perform and interpret the test. The second advantage is that most of the materials are commonly available in virology laboratories. The monoclonal antibody and cell lines can be readily obtained from commercial sources. No radioactive materials are used, and the technique is easy to learn.

An important consideration when using the SVA is the cross-reactivity of the monoclonal antibody. JC virus, the other human polyomavirus, can also appear in the urine (1, 8, 16) and produce a T antigen similar to those of BKV and SV40 (9). The antibody used in those experiments cross-reacted with the T antigen of JC virus. Although JC virus is reported to replicate only in primary human fetal glial, SV40 immortalized human fetal glial (SVG), and adult human brain cells, it produces T antigen in other cell lines (4, 21). Because of this, SVA should be considered as a screening tool for polyomaviruses rather than a BKV-specific assay.

We conclude that the SVA offers a rapid and reliable method for detection of BKV. As with other viruses, low-speed centrifugation enhances BKV infectivity. Clinical results obtained by SVA were similar to those obtained in previous studies by other techniques. This improved culture method can be used to assist in detecting BKV in clinical studies of organ transplant and other immunosuppressed patient populations.

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