INFECTION AND IMMUNITY, June 1975, p. 1375-1381
Vol. 11, No. 6

Printed in U.S.A.

Occurrence of BK Virus and BK Virus-Specific Antibodies in the Urine of Patients Receiving Chemotherapy for Malignancy

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Received for publication 30 December 1974

Urine specimens from 23 children and 9 adults who were undergoing treatment for malignancy as well as urines from 40 normal individuals were concentrated and examined for evidence of papovavirus infection. Papovavirus particles were detected in 6 of 64 urines examined by electron microscopy. Three of the particle-positive urines induced BK virus-specific immunofluorescence after inoculation of WI38 cells, and three isolations of BK virus were made by inoculation of urines from virus-excreting patients into Vero cells. BK virus-specific hemagglutination-inhibiting and immunofluorescence neutralizing antibodies were found in a majority of urines from adult patients, in about a fifth of pediatric patients, and less often in normal urines. Urines of virus-excreting patients generally had antibodies. In indirect fluorescent antibody tests, BK virus-specific antibodies of the immunoglobulin G class were found in five urine specimens from patients; immunoglobulin A antibodies were not detected in any urine. These data suggest that activation of BK virus is related to immunosuppression and not to transplantation itself and that the occurrence of virus-specific antibodies in urine may be indicative of virus multiplication in the urinary tract.

Since the original isolation of BK virus from the urine of a renal transplant patient with ureteric obstruction (7), several subsequent isolations of the virus have been made, all from the urine of individuals who were being treated with immunosuppressive agents after kidney transplantation. Coleman et al. (4) found that 10 of a series of 74 renal transplant patients excreted papovavirus in their urine. Virus particles were demonstrated in the urine of eight of these patients by electron microscopy (EM), and BK virus (BKV) was isolated from four patients in Vero cell cultures. In a significant number of those patients excreting virus, an increase in serum antibody titer to BKV was observed. Lecatsas et al. (10) found papovavirus-like particles in the urine of 8 out of 18 patients who had received renal transplants. It was suggested that the relatively high rate of virus excretion in this group of patients may have been related to the use of antilymphocyte serum in therapy. Three of a group of 17 renal transplant patients studied by Shah et al. (14) had serological evidence of BKV activity, and neutralizing antibodies to the virus were found in the urine of five. Attempts to demonstrate virus in the urine of these patients were unsuccessful. Recently, BKV has been isolated in the U.S.A. from the urine of a renal transplant recipient (5).

Serological data accumulated in England (6) and in the U.S.A. (13) suggest that infection with BKV is common during childhood. The prevalence of antibody to the virus rises rapidly after 1 year of age; by age 3, about 50% of children have BKV serum antibody, and the prevalence approaches 100% by age 10 to 11. Antibody prevalence is somewhat lower in adults. It is not known if primary BKV infection produces any illness.

All reports of urinary excretion of papovaviruses have been based upon observation of renal transplant patients. Papovavirus infection may, in these cases, be a problem peculiar to kidney transplantation in that the urinary tract of these patients may be particularly susceptible to either primary infection or to reactivation of a latent virus which may have been present in the tissue of the donor or recipient. Alternatively, the observed virus activity may be a result of immunosuppression, representing either reactivation of latent virus or new infection. If the latter explanation is correct, then one would expect to find evidence of infection in individuals who have reduced immunocompetence but are not renal transplant recipients. In

the present work, urines of children and adults who were undergoing chemotherapy for malignancy as well as of a matched control group of normal individuals were studied for evidence of papovavirus infection.

MATERIALS AND METHODS

Study groups. The patient group included 9 adults and 23 children who were undergoing chemotherapy for leukemia or solid tumors at Baltimore City Hospital (Baltimore, Md.) or the Johns Hopkins Hospital. In general, the adults were hospitalized while undergoing treatment and were considered to be "heavily immunosuppressed." They received cyclophosphamide, cytosine arabinoside, vincristine, daunomycin, and prednisone in various combinations. Two of these patients had received bone marrow transplants prior to the collection of specimens. The children studied were being followed as outpatients, and most were being treated according to the St. Jude's protocol for total therapy of acute lymphocytic leukemia (8) or a modification of this program. The control group, consisting of 17 adults and 23 children, matched the patient group both in age (Table 1) and sex distribution.

Collection and concentration of urine. Spot urine specimens were collected from each member of the study group, and were usually stored at −70 C prior to concentration. Several sequential specimens were collected from some patients without knowledge of the results of testing of earlier specimens. Single specimens were collected from controls. All tests were performed on concentrated urines. Specimens were concentrated either by ultrafiltration through an Amicon PM30 membrane (Amicon, Lexington, Mass.) or by dialysis against 0.01 M NH_4OH followed by lyophilization and resolubilization in phosphate-buffered saline. When necessary, sequential urine specimens from an individual patient were pooled to obtain a minimum volume of 50 ml for concentration. The degree of concentration achieved ranged from 10- to 100-fold, with a mean of 35-fold. The concentrated urines were stored at −70 C until used.

Collection of serum specimens. A single serum specimen, collected at the same time as the first urine specimen, was available for each of eight adult and two pediatric patients.

TABLE 1. *Age distribution of patient and control groups*

Age (yr)	No. in age groups	
	Patients	Controls
3–5	5	6
6–8	5	4
9–11	5	8
12–14	7	3
15–16	1	2
20–29	2	4
30–39	1	2
40–49	2	1
50–59	2	7
60–69	2	3

Tests for virus. Attempts to demonstrate the presence of BKV in urine specimens were made by several different methods. The cytopathic effect of BKV may not be seen until 2 to 4 months after inoculation of Vero cells with virus-containing urine. In an effort to develop a quicker method to identify BKV in urine, inoculated cells were examined for BKV-specific immunofluorescence (IF). One-tenth milliliter of concentrated urine was inoculated onto WI38 cell cultures on Lab-Tek slides (Miles Laboratories, Westmont, Ill.) and allowed to adsorb for 1 h at 37 C. Each well was then washed 3 times with Hanks balanced salt solution and medium (minimal essential medium with 5% fetal calf serum) was added. At 4, 11, and 14 days after inoculation, the slides were harvested, fixed in cold (−20 C) acetone, and then stained for BKV antigen by the indirect fluorescent antibody (FA) method.

Urine specimens selected from those concentrated by ultrafiltration were screened for virus particles by EM after negative staining. Preliminary studies with stock BKV suggested that small amounts of virus might be more readily detected if the material to be examined was incubated with viral antiserum prior to observation. Thus 0.4 ml of concentrated urine was mixed with 0.1 ml of a 1:40 dilution of rabbit anti-BK serum (hemagglutination inhibition [HI] titer 1:640) and incubated for 1 h at 37 C. The mixture was then centrifuged for 1 h at 30,000 rpm in a type 40 Beckman rotor. The supernatant fluid was decanted, and the pellet was drained thoroughly and then resuspended in 4 drops of distilled water. Two drops of this material were then mixed with 5 drops of 4% phosphotungstic acid containing 0.02% crystallized bovine serum albumin and latex spheres (88 nm in diameter) at a final concentration of approximately $10^{9.0}$ spheres per ml of the resuspended pellet. The mixture was applied to carbon-evaporated Formvar-coated grids by using a finely drawn Pasteur pipette, and the excess was carefully withdrawn with filter paper. The grids were examined with a Siemens Elmiskop I at a magnification ranging from ×5,000 to ×40,000. To insure that equal volumes of suspension were screened for all samples, virus particles were searched for until a total of 500 latex particles were counted before a specimen was considered negative. This required an examination of a minimum of seven squares of a grid of 300-squares/inch mesh.

Urine specimens in which virus particles were demonstrated by EM were inoculated into Vero culture tubes and observed for 12 weeks for BKV cytopathic effect. Isolated strains were identified by HI tests with reference rabbit antisera prepared with the original BKV strain.

Tests for antibodies. BKV hemagglutination inhibition tests were carried out as previously described (13), except that the hemagglutinating antigen used was obtained by freezing and thawing three times of supernatant fluid and cells from WI38 cell cultures infected with BKV. Acetone-extracted urines or sera (9), after adsorption with type O human erythrocytes, were tested in twofold dilutions with 4 to 8 hemagglutination units of BKV. The lowest dilution tested

was 1:2 for urine and 1:20 for serum specimens. An HI titer of 1:8 or above was interpreted as indicative of HI antibodies in the urine.

Indirect FA tests were conducted as previously described (13) except that the BKV antigen used consisted of WI38 cell cultures grown on Lab-Tek chamber slides which had been harvested five days after inoculation with BKV. Tests were conducted with fluorescein-labeled rabbit anti-human immunoglobulin G (IgG) and anti-human immunoglobulin A (IgA) sera (Meloy Laboratories, Springfield, Va.). Immunofluorescence neutralization (IFN) tests with BKV were carried out as previously described (13) except that WI38 cell cultures were used.

All specimens were tested for antibodies to SV40 virus by indirect FA tests with antigens in African green monkey kidney cultures acutely infected with SV40 (12). Likewise, 28 urines were screened for SV40 neutralizing antibodies by IFN tests performed in secondary African green monkey kidney cultures.

RESULTS

Demonstration of virus in urine. Urines concentrated by lyophilization were unsuitable for EM examination for virus particles. In tests of 64 specimens concentrated by ultrafiltration and treated as described in Materials and Methods, aggregates of papovavirus particles (Fig. 1) were observed in 6 of 43 specimens from 21 patients and in none of 21 from matched controls (Table 2). Particles were readily recognizable as papovaviruses by their size and sub-unit structure. Most particles appeared as "empty" shells, which may have been due to actual lack of cores or to stain penetration of damaged particles. Over 90% of the particles found in each of the six positive samples were aggregated in clusters. Although the prior incubation of the urine with BK virus antiserum appeared to aggregate particles and facilitate identification, some aggregation of particles was also seen in the absence of the antiserum. Treatment with antiserum was therefore not useful for specific identification of the virus.

The six positive specimens were collected from three patients, two adults and one child. Of the 27 urines from 15 patients which were

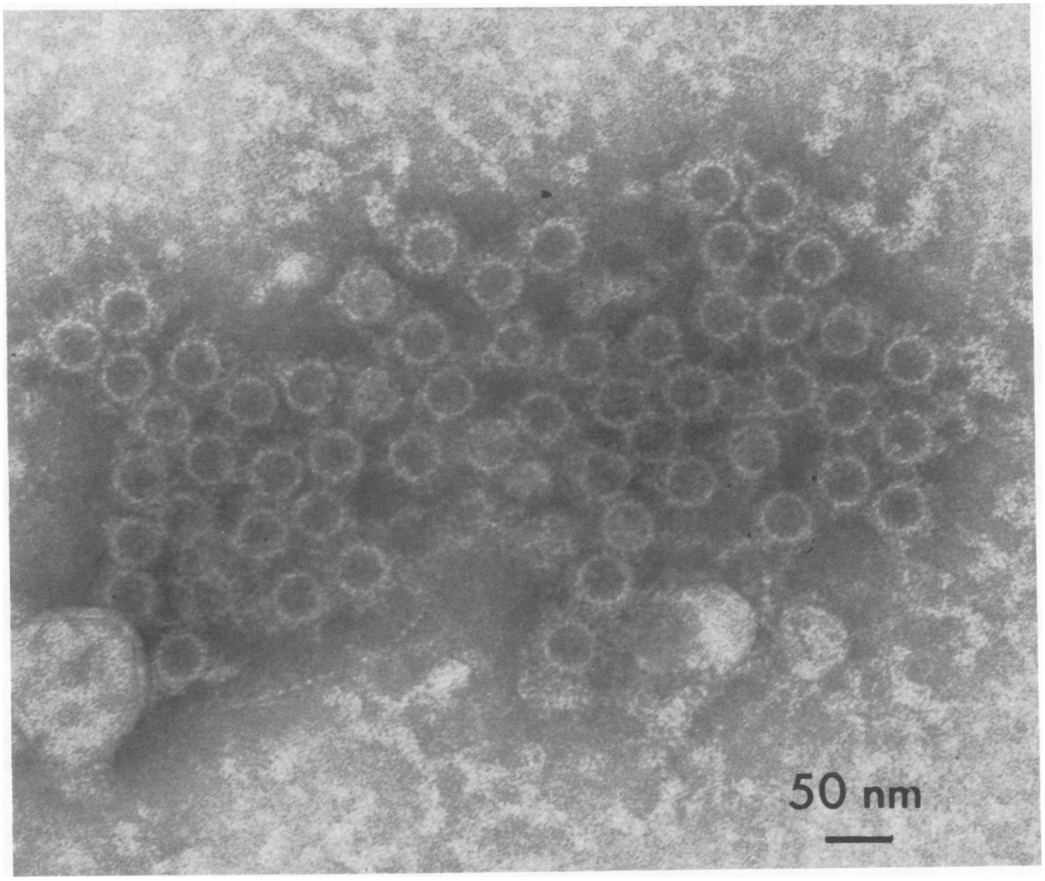

FIG. 1. *Virus particles in antibody-treated urine sample of patient #73-239, prepared for EM by the negative staining method. Magnification, ×170,000.*

satisfactorily tested for their ability to induce BKV-specific IF, three were positive. Those specimens which were positive for IF induction also contained virus particles, as visualized by EM.

The details of tests on specimens from the three virus-excreting patients are summarized in Table 3. Several of the urines which were tested by EM could not be examined for IF induction or virus isolation because of the toxicity of the concentrated urine for cells. BKV was isolated from three urines obtained from two patients (Table 3). Virus-specific cytopathic effect in inoculated Vero cells was detected as early as 18 days after inoculation of one specimen. Virus was reisolated from each of the three positive urines. Although EM examination was the most sensitive of the three methods for virus detection, BKV was identified in at least one urine specimen from each of the three patients by IF induction or virus isolation.

TABLE 2. *Frequency of papovaviruses in urine by EM and by induction of BKV-specific immunofluorescence*

Urine donors	No. positive/no. tested			
	EM		IF induction	
	Individuals	Specimens	Individuals	Specimens
Patients				
Adults	2/9	5/21	2/7	2/13
Children	1/12	1/22	1/8	1/14
Controls				
Adults	0/9	0/9		
Children	0/12	0/12		

Patient #73-77 was a 12-year-old female with acute myelomonocytic leukemia who had been in chronic relapse for the two years since the diagnosis for her illness. She was followed as an outpatient and was receiving prednisone (15 mg/day) and vincristine (1.5 mg each week). Each urine specimen examined was pooled from three separate spot urine samples. The first pooled specimen was positive and the second negative for virus by each of the three detection methods.

Patient #73-191 was a 24-year-old male with acute myelogenous leukemia who had undergone a bone marrow transplant. Prior to the collection of specimens, he had received high doses of cyclophosphamide (200 mg/kg), which had been tapered so that at the same time he provided the urine specimens he was on cyclophosphamide (5 mg/kg). Virus particles were detected by EM examination in the second and third urine specimens, which were collected over a 2-week interval. BKV was also demonstrated in the third specimen by IF induction.

Patient #73-239 was a 53-year-old male with a seminoma who was treated with cyclophos-

TABLE 3. *Summary of tests on specimens from virus-excreting patients*[a]

Patient no.	Patient identification	Age (yr)/sex[b]	Days of collection[c]	Concentrated urine					Serum	
				Virus			Antibody			
				EM	IF induction WI38	Isolation Vero cells[d]	Reciprocal HI titer	IFN	Reciprocal HI titer	IFN
1	73-77	12/F	0-28	Pos[e]	Pos	Pos (18)	Neg			
			42-97	Neg[e]	Neg	Neg	8	Pos		
2	73-191	24/M	0	Neg			16		Neg	Pos
			12	Pos			Neg			
			25	Pos	Pos		Neg	Pos		
3	73-239	53/M	0	Pos	Neg	Pos (66)	32	Pos	160	Pos
			15	Pos	Neg	Neg	32	Pos		
			24	Pos	Pos	Pos (30)	8	Pos		

[a] Diagnoses: patient 1, acute myelomonocytic leukemia in relapse; patient 2, acute myelogenous leukemia, after bone marrow transplant; patient 3, seminoma, under intensive chemotherapy.

[b] F, Female; M, Male.

[c] Day of first collection designated day 0. Each specimen of patient 1 was a pool of three consecutive spot urines.

[d] Number in parenthesis after positive specimens indicates number of days post-inoculation when cytopathic effect was first observed.

[e] Pos, Positive; Neg, negative.

phamide (50 mg/kg) and cytosine arabinoside (50 mg/kg) prior to and during the study period. Virus particles were observed by EM in all three specimens and represented virus excretion over a 24-day period. BKV was detected in one of the three specimens by IF induction and in two of three specimens by isolation.

Antibodies in urine. The frequency of virus-specific antibodies in the urines of the four groups is summarized in Table 4. The highest frequency was found in the urines of the adult patients; a majority of the urines had BK virus antibodies detectable by HI and IFN tests and four of 21 had BKV-specific IgG antibodies demonstrable by indirect FA tests. The antibody prevalence was lower in the urines of pediatric patients. About one-fifth of the specimens had BKV antibodies detectable by HI and IFN tests and only one had IgG antibodies which stained BKV antigen. Antibodies were found infrequently in normal urines with the exception that 5 of 23 urines from normal children had antibodies demonstrable by IFN tests. BKV-specific IgA antibodies were not detected in indirect FA tests of urines from either patients or controls. None of the urines contained SV40-specific antibodies as determined in IFN and indirect FA tests.

The correlation between results of HI and IFN tests is given in Table 5. All but one of nine urine specimens with an HI titer of 1:16 or above were positive in IFN tests. Of the 58 urines with HI titers of 1:4 or less, only nine (16%) were positive in IFN tests. None of the five urines with IgG antibodies had an HI titer above 1:8; three of these five were positive in IFN tests. The urine antibody data for the three virus-excreting patients (Table 3) suggest a correlation between virus excretion and presence of antibodies in urine. Antibodies were detected in all three samples of patient #73-239, two of three samples of patient #73-191 and in one of two samples of patient #73-77. All of the urines of the virus-excreting patients were negative for BKV-specific IgG antibodies reactive in indirect FA tests.

The BKV-specific antibodies in urine, although unequivocally present in some specimens, were at low levels even in these concentrated urines.

Antibodies in serum. In the 10 sera from patients, HI, IFN, IgG, and IgA antibodies to BKV were detected, respectively, in six, eight, nine, and three specimens. Two of these sera were from virus-excreting patients; their antibody data are given in Table 3. The HI antibody titers of the positive sera ranged from 1:20 to 1:320. In tests of concentrated urines corresponding to these sera, HI, IFN, IgG, and IgA antibodies were detected, in 6, 5, 0, and 0 specimens, respectively. In five cases where both urine and serum from a patient had BKV HI antibodies, the titers in (unconcentrated) urines were 20- to 300-fold lower.

DISCUSSION

Papovavirus particles were detected in the urines of three patients treated with drugs having immunosuppressive side effects. One of these patients had received a bone marrow transplant, and the other two had received no organ transplants. The observation suggests that papovavirus activation is related to immunological deficiency rather than to the transplantation itself. Although the virus particles in urine were not directly identified by immune EM, it is very likely that they were BKV virions; BKV was isolated from three particle-positive urines and BKV antigen was induced by two of these three urines and by one additional particle-positive specimen which could not be tested for virus isolation. The IF induction test, although it was not as sensitive as examination by EM, was positive for at least

TABLE 4. *Frequency of virus-specific antibodies in urine of patients and controls*

Virus	Antibody test	No. of specimens positive[b]/No. tested			
		Patients		Controls	
		Children	Adults	Children	Adults
BKV	HI test[a]	7/31 (23)	13/21 (62)	1/23 (4)	1/17 (6)1
	IFN	5/27 (19)	11/16 (70)	5/23 (22)	1/16 (7)
	indirect FA, IgG	1/31 (3)	4/21 (19)	0/23	0/17
	indirect FA, IgA	0/31	0/21	0/23	0/17
SV40	IFN	0/11	0/7	0/7	0/3
	indirect FA, IgG	0/31	0/21	0/23	0/17

[a] Urines with titer of 1:8 or greater considered positive.
[b] Number in parentheses is the percentage positive specimens.

TABLE 5. *Correlation between results of tests of concentrated urines for BK virus hemagglutination-inhibiting and IF neutralizing antibodies*

HI test		No. of specimens positive in IFN/no. tested			
		Patients		Controls	
Titer	No. of urines	Children	Adults	Children	Adults
1:8	58	3/20	1/1	5/22	0/15
1:8	9	2/6	1/2	0/1	
1:16	3		2/2		1/1
1:32	5		5/5		
1:64					
1:128	1		1/1		

one of the urine samples of each of the three patients. The frequency of occurrence of virus particles in urine was related to the degree of immunosuppression; particles were seen in 24% of the urines of heavily immunosuppressed, hospitalized adults, in 5% of urines of children receiving lower maintenance doses of drugs as outpatients, and in none of the normal urines examined.

Normal human urine contains small amounts of immunoglobulins of the IgG, IgA (7*S*), sIgA (11*S*), and IgE classes (3), but little is known about the quantities, immunoglobulin class, site of synthesis or function of virus-specific antibodies in human urine. Poliovirus-specific antibodies of the IgG and IgA classes have been detected in human urine (2, 11). In this study, antibodies to BKV were found in urines from all four groups and were most frequently present in the urines of the heavily immunosuppressed adult patients. The correlation between the results of BKV IFN, and HI tests (Table 5) of the urines and the absence of SV40 IF neutralizing antibodies in these urines indicate that the tests were specific for BKV antibodies. The antibody titers were low and virus-specific antibodies of the IgA class were not detected. Previously, Gardner et al. (7) described antibody-like material coating papovavirus particles in negatively stained urine sediment examined by EM, and Shah et al. (14) reported BKV antibodies in the urine of five renal transplant recipients. The presence of antibodies in urine could be a result of glomerular leakage of serum antibodies or of local antibody synthesis. The demonstration of virus-specific antibodies of the sIgA class in urine would have strengthened the case for local BKV antibody synthesis. However, IgG is the predominant immunoglobulin class present in urine, and in some experimental systems the bulk of specific antibodies shown to be produced in the urinary tract is of the IgG class. Specific antibodies in urine have been detected in two other viral infections of the urinary tract: to SV40 in urines of experimentally infected rhesus (15) and African green monkeys (1) and to cytomegalovirus in urines of normal and immunosuppressed humans (L. Asher and K. Shah, unpublished data). It is probable that the antibodies in urine in such viral infections are a result of local antibody synthesis in the urinary tract in response to viral multiplication at the same site. The high prevalence of BKV-specific antibodies in the urine of immunosuppressed individuals may well result from activation and multiplication of BKV in the urinary tract of these patients.

ACKNOWLEDGMENTS

We are grateful to Herbert Kaizer and Donald Trump for their assistance in collection of the specimens and to Rose Warszawski, Janet Valis, and Lucille Jeffries for technical help.

This study was supported by Public Health Service grant CA-13478 and by research contract NO1-CP-43266 from the National Cancer Institute.

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