Lack of Serologic Evidence for Prevalent Simian Virus 40 Infection in Humans

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Background: Propagation of poliovirus in monkey kidney cells led to the inadvertent contamination of poliovirus vaccines with simian virus 40 (SV40) between 1955 and 1963. Recent studies using polymerase chain reaction–based strategies have detected SV40 DNA in a large number of tumor types. The finding of SV40 DNA in tumors from individuals who are too young to have been exposed to SV40-contaminated vaccines has led to the suggestion that SV40 has become a prevalent transmissible human pathogen. To test this hypothesis, we screened human sera for antibodies to SV40 using direct and competitive enzyme-linked immunosorbent assays (ELISAs). Methods: An ELISA was developed using recombinant SV40 virus-like particles (VLPs) and was validated using sera from naturally infected macaques. VLPs of SV40 and the related ubiquitous human polyomaviruses, JCV and BKV, were used to screen human sera to determine the prevalence of SV40, JCV, and BKV antibodies among a normal population of control subjects (n = 487) and among case patients with either osteosarcoma (n = 122) or prostate cancer (n = 90). A competitive ELISA in which sera were pre-adsorbed with each type of VLP was used to identify cross-reactive antibodies. Correlations of reactivity among the three polyomavirus types were calculated using the Spearman correlation coefficient. All statistical tests were two-sided. Results: BKV and JCV antibodies were prevalent in all case patients and control subjects examined. In contrast, only 6.6% (46/699) of serum samples were positive for SV40 antibodies by ELISA; however, none of these samples could be confirmed as having authentic SV40 antibodies following pre-adsorption with JCV or BKV VLPS. Conclusion: These data indicate that some individuals have BKV and/or JCV antibodies that cross-react with SV40, but they do not provide support for SV40 being a prevalent human pathogen. [J Natl Cancer Inst 2003;95:1522–30]
the tumors of these common types were found to harbor SV40 DNA sequences (12).

Given the variety and the substantial number of tumors that have been reported to harbor SV40 DNA sequences, especially in individuals who were not directly exposed to SV40-contaminated vaccines, many investigators have hypothesized that SV40 has become adapted to humans and is circulating widely in the human population. Several research groups have begun to explore this hypothesis. Searches for SV40 DNA in urine and in sewage have been negative, whereas searches for BKV and JCV DNA, two ubiquitous human polyomaviruses, have been positive (15). Serologic evidence of SV40 has also been sought, mostly by using plaque-reduction assays that measure the ability of human serum to neutralize SV40 infection in vitro. SV40 seropositivity in individuals who had not received poliovirus vaccines was reported as early as 1971 (16). More recently, Butel et al. (17) screened 337 unselected (i.e., randomly chosen) sera from hospitalized children and found that 5.9% of children were seropositive for SV40. Using the same serologic assay, SV40 seropositivity was found in 16.4% and 12.1% of HIV-positive and HIV-negative men born before 1962, respectively; among men born after 1962, SV40 seropositivity was found in 11.8% and 9.7% in HIV-positive and HIV-negative men, respectively (18).

One difficulty in interpreting serologic data is the potential for cross-reactivity between antibodies to SV40 and antibodies to JCV and BKV. The major capsid proteins of SV40, JCV, and BKV have been shown by western blot to exhibit a cross-reactivity between antibodies to SV40 and antibodies for cross-reactivity between antibodies to SV40 and antibodies to JCV or BKV. The major capsid proteins of SV40, JCV, and BKV have been shown by western blot to exhibit a cross-reacting epitope (19). Brown et al. (20) examined a population of North American Indians who had not been exposed to SV40 and found that about one-third of individuals who had high titers of anti-BKV antibodies also had anti-SV40 antibodies, leading the authors to conclude that BKV and SV40 antibodies cross-react. It is interesting that, in a survey of both South American Indian and Japanese populations that had also presumably not been exposed to SV40, Major et al. (21,22) reached the opposite conclusion—that is, there was no cross-reactivity between JCV and BKV antibodies and no cross-reactivity of SV40 antibodies to JCV or BKV.

Because it has not been possible to use the same assay to determine antibody reactivity for all three types of polyomaviruses, it has been difficult to determine whether antibodies were virus-specific or cross-reactive. For example, SV40 antibodies have been detected using plaque-reduction assays; however, because BKV forms plaques poorly and JCV does not form plaques at all, plaque-reduction assays have not been useful in detecting BKV and JCV antibodies. Conversely, BKV and JCV antibodies have been assayed by the ability of these viruses to agglutinate erythrocytes; however, because SV40 does not agglutinate erythrocytes, this assay has not been useful in detecting SV40 antibodies. Thus, antibodies to SV40, BKV, and JCV have not been compared in the same assay.

There are many similarities between the polyomaviruses and the human papillomaviruses (HPVs), and the lessons learned from HPV serology have been informative in terms of our approach to detecting SV40 antibodies. Both virus groups have T = 7 icosahedral capsids that are composed of a major capsid protein; these major capsid proteins self-assemble to form virus-like particles (VLPs) (23–25). Robust (i.e., sensitive and type-specific) serologic assays have been developed using HPV VLPs in enzyme-linked immunosorbent assays (ELISAs) to detect type-specific HPV antibodies [reviewed in (26)]. Both virus groups cause widespread infection in their natural hosts, establish latent infections, and result in humoral immune responses that persist for decades (26,27). In the case of HPVs, antibodies are directed against conformational epitopes on the major capsid protein, L1. Although there is a common epitope on the denatured L1 protein, it is not seen during natural infection, resulting in type-specific HPV antibody responses (28,29). Antibody titers to HPV are generally low (≈ 1/125 geometric mean titer), presumably because HPVs remain solely in the epithelium. In contrast, JCV and BKV infections result in high antibody titers, presumably because of the systemic nature of their infections. Because the major capsid proteins (i.e., VP1s) of SV40, BKV, and JCV are more distantly related to each other than are many of the HPV L1 proteins to each other, we hypothesized that polyomavirus VLPs would provide a useful reagent in detecting type-specific antibodies for SV40, BKV, and JCV. We used these VLPs in an ELISA to screen human sera for the prevalence of SV40, JCV, and BKV antibodies in control subjects and case patients with osteosarcoma or prostate cancer.

**MATERIALS AND METHODS**

**Macaque Serum**

A breeding colony of cynomolgus macaques was set up by the National Institute for Biological Standards and Control, United Kingdom. Care of the animals was closely monitored and approved by the National Institute for Biological Standards and Control in accordance with Her Majesty’s Home Office regulations. Cohort 3, which consisted of 16 monkeys, was weaned on May 2, 2000. Sera (multiple samples from each animal) were collected for routine health screening purposes on May 9, 2000, June 13, 2000, and July 18, 2000, for all but one animal. A fourth sera collection was taken at a time interval ranging from 1 month to 1 year after the third collection. During that time interval, 5 of 14 monkeys became naturally infected with SV40 (i.e., seroconverted), and none of those monkeys had declining SV40 antibody titers within that year. Sera were tested for neutralization of SV40 antibodies (by P. A. Pipkin and P. D. Minor), and a subset of the sera (n = 216) was shipped to the laboratory of D. A. Galloway, where it was tested in a blinded fashion (by J. J. Carter and G. C. Wipf) using the VLP ELISA described below.

**Human Serum**

Population-based control subjects were identified using random-digit telephone dialing as part of an ongoing case-control study of anogenital cancer (30). Briefly, we obtained serum samples from 415 individuals (205 males and 210 females) who were between 19 and 78 years of age and of whom 91.4% were white, 2.1% were black, 2.1% were Native American, 1.9% were Asian, and 2.4% were other. All control subjects lived in the three-county area of western Washington State, which includes Seattle, at the time of their in-person interview and blood draw (1987–1999). Sera were also obtained from a population-based study on prostate cancer that has been previously described (31). Briefly, we obtained serum samples from 162 men (90 case patients and 72 control subjects) who were between 40 and 64 years of age; 97.2% of the case patients and 100% of the control subjects were white. Serum samples were also obtained for 122 osteosarcoma case patients (provided to R. L. Garcea) from the
Cooperative Human Tissue Network and the Children’s Oncology Group Solid Tumor Tissue Bank. Demographic data were available for 114 of the 122 osteosarcoma case patients (60 males and 54 females), who were between 4 and 21 years of age; information on race was not available. The Fred Hutchinson Cancer Research Center institutional review board approved all studies, and all case patients and control subjects provided written informed consent for antibody testing.

VLP Preparation

Recombinant baculoviruses expressing either the BKV or JCV VP1 proteins were provided by P. N. Jensen and E. O. Major (National Institutes of Health, Bethesda, MD). To clone the SV40 VP1 open reading frame into a baculovirus expression system, a cDNA clone was amplified by initially using the primers 5'-AAAGCAGGCTGCCACCATGGCTCGGAAAGCTTAAGGG-3’ and 5’-AGAAAGCTTGTTCACTGCAATCTTA GGTGTGTTGTCC-3’. After 10 cycles of amplification by PCR, 10 µL of the reaction was removed and a second round of amplification was performed using the attB1 and attB2 adapter primers (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Deep VentR DNA polymerase (New England Biolabs, Beverly, MA) was used for all PCR amplifications. The SV40 VP1 amplifier was cloned into pDest8 after initial cloning in pDONR201 using Gateway technology (Invitrogen). The pDest8-VP1 clone was sequenced in both directions to verify fidelity. Sequencing was conducted using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions on a model 377 DNA Sequencer (Applied Biosystems). Bacmids containing the SV40 VP1 were generated in DH10Bac cells (Invitrogen) and verified by PCR according to the manufacturer’s instructions with the following exception. A 10-mL bacmid-containing culture was grown in Luria Broth at 37 °C for 24 hours, and bacmid DNA was purified using a QIAGEN-tip 20 column (Qiagen, Valencia, CA). Insect cells (sf9) were transfected with 1 µg of bacmid DNA using Cellfectin (Invitrogen) according to the manufacturer’s instructions. The expression of SV40 VP1 in sf9 cells was verified after 3 days of culture at room temperature (without additional CO2) by immunoblots using rabbit anti-VP1 antibodies (1:1000 dilution; provided by J. Butel, Baylor College of Medicine, Houston, TX), and high-titer baculovirus stocks were generated by serial passage.

sf9 cells were split from 100-mm plates onto 150-mm plates (Corning, Corning, NY) and allowed to adhere at room temperature without additional CO2. Plates (20 × 150 mm plates) were infected with high-titer baculovirus at 100 µL per plate. After 3 days of incubation, the cells (approximately 65% confluent) were pelleted by centrifugation at 3000g for 20 minutes at 4 °C, and the supernatant was discarded. The cells were then resuspended in 10 mL of phosphate-buffered saline (PBS) with protease inhibitors (Complete; Roche, Indianapolis, IN) and Dounce-homogenized for 50 strokes on ice. The nuclei were then collected by centrifugation at 16 000g for 20 minutes at 4 °C. The pellet was resuspended in 3 mL of 1 M NaCl and 0.02 M Tris–HCl (pH 7.6) and sonicated on ice (Branson Sonifier 250; Branson Ultrasonics, Danbury, CT) using a microtip at a setting of 6 (3 × 45 seconds with 1 minute between pulses). The sample was centrifuged again at 16 000g for 20 minutes at 4 ºC, and the resultant supernatant was layered onto a cushion contain-
second set was polypropylene plates (Nalgene Nunc International, Nutting Lake, MA) used to pre-incubate the human (or rabbit) sera with VLPs in solution. Briefly, VLPs were serially diluted (1:3) down the plate in 30 μL of blocking solution starting at a concentration of 8.3 μg/mL for the rabbit serum experiments and 18 μg/mL for the human sera experiments. Human (or rabbit) serum that had been diluted (1:50 human, 1:1000 rabbit) in blocking solution were then added to each well (30 μL per well). Both sets of plates were then incubated overnight at 4 °C. The VLP-coated plates were washed and blocked as described above. After 2 hours, the blocking solution was removed and human (or rabbit) sera were transferred from their polypropylene plates to the VLP-coated plates (50 μL per well). All subsequent steps were identical to the ELISA procedure described above.

**SV40 Neutralization Assay**

The ability of macaque sera to block infectivity of BSC-1 cells by SV40 in microtiter wells was used as the neutralization assay. The cell line BSC-1 was grown in minimal essential medium supplemented with 10% fetal bovine serum and buffered with 15 mM HEPES and 0.088% sodium bicarbonate. The SV40 strain used in this assay was a laboratory strain from the 1960s (32), and the viral dose required to cause cytopathic effects in BSC-1 cells was first established by titration. Briefly, SV40 was diluted in growth medium containing minimal essential medium, antibiotics, 10% fetal bovine serum, 1.5% HEPES, and 2.5% sodium bicarbonate buffer in half log steps from 10^{-1.0} to 10^{-6.5}. An aliquot of diluted SV40 (50 μL) was transferred to the wells of a Falcon Microtest 96-well plate, and 200 μL of the BSC-1 cell suspension (2.5 × 10^5 cells/mL) was added to each well. The plate was incubated at 37 °C and read after 10 days at 405 nm on an Elx808 plate reader (Bio-Tek Instruments) for microscopic evidence of an SV40 cytopathic effect.

Antibody screening was carried out by placing 70 μL of growth medium in each well of a Falcon 3072 microtiter plate and adding 10 μL of heat-inactivated sera, making a 1:8 dilution that could be used for screening purposes. Diluted sera (25 μL) was then added to each well of a microtiter plate as a control, and SV40 (25 μL; 1000 tissue culture infectious dose [TCID] per 50 μL) was added to the duplicate plate. Both plates were sealed and incubated at 35 °C for 1 hour. BSC-1 cells were trypsinized (in 1 mL of 0.5% trypsin and 5.3 mM EDTA), and a cell suspension containing 2.5 × 10^5 cells/mL in growth medium was prepared. A 200-μL aliquot of the cell suspension was added to each well. The plates were again sealed and incubated at 35 °C and read after 10 days for microscopic evidence of an SV40 cytopathic effect. Those sera found to be positive for SV40 antibodies were subsequently titrated. A hyperimmune rabbit antiserum (provided by J. Butel) was used as a control in each assay. The titer of this antiserum was 1 in 40,000, which is comparable to that found by Jafar et al. (18), confirming the sensitivity of the screening assay for detecting SV40 antibodies.

**Statistical Analyses**

All statistical analyses were performed using Prism software (GraphPad, San Diego, CA). Correlations of reactivity among the three types of polyomavirus (i.e., SV40, BKV, and JCV) were calculated using the Spearman correlation coefficient. All statistical tests and results are reported as two-tailed P values.

**RESULTS**

VLPs were generated by using baculovirus recombinants expressing VP1 from SV40, JCV, and BKV. The recombinants expressed appropriately sized capsid proteins, and the morphology of the VLPs was confirmed by electron microscopy (data not shown). Sera were tested by using the VLPs as antigen targets in an ELISA using methods that have been previously described for HPV VLPs (28). Repeated testing of the same sera indicated that the lowest correlation between replicates was for JCV (r = .401; Spearman coefficient), and the highest correlation between replicates was for SV40 (r = .85; Spearman coefficient). The correlation for repeated testing was r = .500. All of the correlations were statistically significant, indicating that the ELISAs for the three VLP types were reproducible (P<.001; n = 65).

To verify that the SV40 VLP ELISA was capable of detecting SV40 antibodies, sera from a cohort of 16 cynomolgus macaques were screened. The cohort was followed for a few months after weaning, during which time some of the monkeys became naturally infected with SV40. The sera were assayed independently by both a neutralization assay for SV40 antibodies and the VLP ELISAs. ELISA data and neutralization titers were obtained for 214 and 211 of the 216 available sera, respectively. Fig. 1A, shows that the majority of the monkey sera had reactivities that were close to zero; however, 59 sera (28.0%) from a subset of monkeys had reactivity to SV40 that was greater than 1.0, whereas only four sera (0.2%) had reactivity to JCV or BKV that was greater than 1.0. Comparison of the SV40 ELISA data with the neutralization titers (Fig. 1B) indicated that, for samples with high neutralization titers (i.e., >512) or undetectable titers (i.e., ≤16), the assays provided very similar results. However, for the small number of samples with intermediate neutralization titers (i.e., 32–256), the ELISA was not a reliable indicator of titer.

An ELISA cut point for the SV40 VLP assay of more than 0.1 (i.e., sample would be considered positive) was selected on the basis of the distribution of two normal populations (28). There was an increase from 0% (0/82) of seropositive sera that had neutralization titers of less than 8 to 100% (31/31) of seropositive sera that had neutralization titers of more than 8192 (chi-square test for trend χ² = 140, P<.001), indicating that the two assays can both discriminate between negative and positive sera. By considering a neutralization titer of more than 8 as positive (cut point was chosen by the distribution presented in Fig. 1B), the ELISAs and antibody neutralization assays produced the same classification for 186 sera (82 negative and 104 positive) and different classifications for 25 sera (all of which were positive on neutralization assay and negative on ELISA). Because infection with BKV and JCV has been thought to be restricted to humans, it was noteworthy that 46 and 27 sera had a reactivity to BKV and JCV, respectively. It should be noted that the cut points for the BKV and JCV ELISAs with macaque sera were set to be the same as the SV40 cut point because there were not two normal distributions. There was a statistically significant correlation between the optical density values for SV40 and BKV (r = .703, Spearman coefficient; P<.001) and to a lesser extent between SV40 and JCV (r = .500, Spearman coefficient; P<.001).

In an initial screening of human sera for SV40 antibodies, we evaluated a population-based sample from western Washington State consisting of 415 individuals who were contacted by ran-
The mean ELISA values (i.e., log difference values) were 0.090 (95% confidence interval [CI]) for vaccine-like particles (VLPs). The results from all serum samples are shown. The mean ELISA values (i.e., log difference values) were 0.090 (95% CI = 0.001 to 0.045) for the JCV test, and 0.584 (95% CI = 0.491 to 0.703) for the SV40 test; each mean is presented at the top of the scatter plot. A) Comparison of SV40 antibody neutralization titer with ELISA values. Sera were categorized by their neutralization titer for SV40 on the x-axis, and the result of the SV40 ELISA for each serum is indicated on the y-axis. The mean ELISA values for sera in each category are indicated on the top of the scatter plot. The mean ELISA values (95% CIs) at each titer are as follows: for 64: 0.084 (95% CI = 0.066 to 0.102); for 256: 0.084 (95% CI = 0.074 to 0.102); for 1024: 0.084 (95% CI = 0.074 to 0.102); for 2048: 0.084 (95% CI = 0.074 to 0.102). B) Comparison of SV40 antibody neutralization titer with ELISA values. Sera were categorized by their neutralization titer for SV40 on the x-axis, and the result of the SV40 ELISA for each serum is indicated on the y-axis. The mean ELISA values for sera in each category are indicated on the top of the scatter plot. The mean ELISA values (95% CIs) at each titer are as follows: for <8: 0.007 (95% CI = 0.000 to 0.002); for 64: 0.012 (95% CI = 0.006 to 0.018); for 256: 0.012 (95% CI = 0.006 to 0.018); for 1024: 0.012 (95% CI = 0.006 to 0.018); for 2048: 0.012 (95% CI = 0.006 to 0.018).
coefficient; \( P < .001 \) for both). In addition, of the 46 sera samples that were seropositive for SV40 (i.e., all SV40-seropositive samples, 46/699 = 6.6%), all were also found to be seropositive for BKV, for JCV, or for both.

Because we observed that all 46 human serum samples that were seropositive for SV40 were also seropositive for JCV and BKV, it was critical to determine whether the overlap between SV40 and BKV/JCV seropositivity observed in human and macaque sera was due to cross-reactivity or to infection with both viruses. To address this issue, immunoreactive sera to SV40 were pre-adsorbed with VLP types for each of the three polyomaviruses. Elimination of seroreactivity to a particular VLP only with a homologous VLP would be evidence of specific antibodies, whereas elimination of seroreactivity to a particular VLP by a heterologous VLP would be evidence of either cross-reactive antibodies or reactivity to a shared protein present in both VLP preparations. Fig. 3, A, shows data from a competitive

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Fig. 2. A) Comparison of the reactivity of human sera to three polyomavirus types. Human sera from random-digit dialing control subjects (n = 415) were tested for reactivity to BKV, JCV, and simian virus 40 (SV40). The results from all serum samples are indicated on the y-axis. The mean enzyme-linked immunosorbent assay (ELISA) values (i.e., log difference values) were 1.121 (95% confidence interval [CI] = 1.039 to 1.203) for the BKV test, 0.724 (95% CI = 0.652 to 0.797) for the JCV test, and 0.181 (95% CI = 0.149 to 0.21) for the SV40 test; each mean is presented at the top of the scatter plot. B) The percentage of seropositive male (solid bars) and female (open bars) control subject sera for BKV (top panel), JCV (middle panel), and SV40 (bottom panel) are shown after stratification by age. C) For ease of reading, the relationship of SV40 seropositivity with age and sex is shown again with an adjusted scale. Again, the percentages of positive sera from males are shown as solid bars and the percentages of positive sera from females are shown as open bars.

Fig. 3. Virus-like particle (VLP) competition assay to assess antibody specificity. A) An immune rabbit serum was pre-adsorbed overnight with increasing concentrations of simian virus 40 (SV40) (circles), BKV (squares), or JCV (triangles) VLPs. The pretreated samples were then tested using an enzyme-linked immunosorbent assay (ELISA) against BKV VLPs (upper panel) or SV40 VLPs (lower panel). B–D) Human sera (1–3) were pre-adsorbed overnight with increasing concentrations of SV40 (circles), BKV (squares), or JCV (triangles) VLPs before use in ELISAs against reactivity to BKV (B and D), JCV (C), and SV40 (B–D). Representative experiments are shown in the figures; each assay was repeated two or three times.
inhibition assay that uses an experimental rabbit serum known to neutralize SV40 infection. This serum reacted to both BKV and SV40 without VLP pre-adsorption (data not shown). Reactivity to BKV was effectively blocked by competition with either BKV or SV40 VLPs; in contrast, only SV40 VLPs had the ability to block SV40 reactivity, and this reactivity required a much higher concentration of VLPs than was necessary to inhibit BKV binding. The rabbit serum did not show reactivity to JCV (data not shown), and JCV VLPs were not effective in competing with either JCV or BKV reactivity.

Similar competitive inhibition assays (Fig. 3, B–D) were performed on all human sera that had reactivity to SV40 VLPs of greater than 0.7 on ELISA. On no occasion was a human serum found to be SV40 type-specific. Human serum 1 is an example of a serum that reacted to both BKV and SV40. Reactivity to BKV could be eliminated only with pre-adsorption with BKV VLPs, whereas reactivity to SV40 was eliminated by pre-adsorption with both SV40 and BKV VLPs (Fig. 3, B). Human serum 2 was reactive to JCV and SV40. Reactivity to SV40 was effectively inhibited by JCV VLPs; however, SV40 VLPs could not inhibit JCV reactivity (Fig. 3, C). There were two sera for which specificity could not be defined. For these two sera, BKV and SV40 VLPs inhibited reactivity to both BKV and SV40 (human serum 3, Fig. 3, D). Macaque sera reactive to BKV or JCV were examined by competitive inhibition assays and were found to contain no BKV- or JCV-specific responses (data not shown).

**DISCUSSION**

Because SV40 DNA sequences have been found in numerous tumor types, it was important to determine the prevalence of SV40 in both normal and cancer populations by using a serologic assay that could distinguish antibodies to SV40 from those elicited by the related human polyomaviruses BKV and JCV. Our data could not confirm the presence of SV40-specific antibodies in the general population or in individuals with prostate cancer or osteosarcoma. We did find, however, that approximately 6.6% (46/699) of human sera reacted weakly to SV40 VLPs, but that pre-adsorption with either BKV or JCV VLPs could eliminate this reactivity. This finding may explain why previous studies (17,18) have reported SV40 antibodies in human sera. In contrast to the reactivity to SV40, reactivity to BKV and JCV was prevalent in all populations examined, and reactivity could not be eliminated by pre-adsorption with heterologous viruses. The combined use of a VLP ELISA to detect all three polyomaviruses, coupled with pre-adsorption of sera with homologous and heterologous VLPs, has provided a relatively specific and sensitive strategy for detecting specific polyomavirus antibodies. The SV40 VLP ELISA was shown to be effective for detecting antibodies to SV40 in naturally infected monkeys. The fact that 25 of the 211 evaluable monkey sera were positive for SV40 by neutralization assay but not by ELISA was probably not due to the misclassification of low-titer sera (data not shown), but rather it was probably due to adverse shipping conditions of the monkey sera. Unfortunately the shipment of sera was held by U.S. customs for 2 weeks, and the conditions under which the sera were held may not have been optimal. Hence this delay in shipping could have resulted in degradation of antibodies. Thus, the true sensitivity of the neutralization assay is unknown, but it is at least 88.2% (186/211 concordant sera).

A potential weakness in the analysis of data presented in this study is the use of cut points that were defined for one polyomavirus type being used for the analysis of other polyomavirus types. Using a cut point on the basis of the mathematical distributions of two normal populations assumes that there will be an infected population and an uninfected population. For example, when monkey sera were screened, it was only possible to determine a cut point for SV40 but not for BKV and JCV using that statistical approach. Similarly, when human sera were screened, it was possible to select cut points for BKV and JCV but not for SV40. It should be emphasized that a central conclusion of this paper—that antibody responses to SV40 VLPs in individuals are due to cross-reactive responses to other polyomavirus types—is not cut point–dependent. The evidence to support this conclusion is, first, the demonstration of a correlation between SV40 ELISA values and BKV ELISA values (and to a lesser degree JCV ELISA values) and second, the inhibition of SV40 reactivity in human sera using BKV and JCV VLPs. The results that were cut point–dependent were the estimation of seropositivity for the three polyomaviruses among random-digit dialing control subjects, prostate cancer case patients and control subjects, and osteosarcoma case patients and the association of seropositivity with age and sex. We performed additional analyses using quartiles in the place of cut points for these data; however, these analyses did not alter the conclusions of this study (data not shown).

When serum samples were seropositive for both BKV and JCV, it appeared that there were two antibody specificities rather than cross-reacting epitopes; however, BKV/JCV cross-reactivity was tested only in a subset of samples that had both reactivities. It is reasonable to assume that there has been evolutionary pressure for the immunogenic epitopes of JCV and BKV to diverge so that both viruses can establish infections in their natural host, which happens to be human. Moreover, perhaps the greater degree of cross-reactivity seen between SV40 and the other human polyomaviruses may be reflective of SV40 remaining in macaques without the need to establish an immunologically distinct niche from BKV and JCV. The different patterns of seroreactivity between BKV and JCV suggest that there might be differences between the natural histories of infection in these two viruses. It is possible that BKV is acquired early in life and that antibodies to BKV wane over time, whereas JCV infection continues into adulthood.

In this study, it was not possible to follow the natural history of SV40 infection in macaques. In the cohort of cynomolgus monkeys we studied, 5 of 14 monkeys seroconverted within the first year, and none of those monkeys had declining SV40 antibody titers within that year; however, follow-up was too short to study persistence of SV40 antibodies. If SV40 infection in humans occurred during the years when SV40-contaminated poliovirus vaccines were used, it is possible that SV40 antibodies could have waned in the intervening 40 years (i.e., the approximate time from vaccination to collection of sera), particularly if there was no active infection. In addition, if osteosarcoma were associated with prior SV40 infection, we would have anticipated that SV40 antibodies would have been prevalent in that young population. However, only 2.46% of the case patients with osteosarcoma had reactive antibodies to SV40, whereas the highest levels of cross-reactive antibodies (8.4%) were seen in the oldest individuals tested, i.e., the population-based controls born before 1962. Hence, these findings indicate that the SV40 antibodies
may develop as cross-reactive responses to BKV or JCV over time.

It is difficult to reconcile the lack of circulating SV40 antibodies in the cohorts we studied with the widespread detection of SV40 DNA in other studies. Low sensitivity of the ELISA in detecting SV40 antibodies must be considered as a possible explanation for the observed discord between the SV40 antibody and DNA results; however, this type of assay can detect low-titer antibodies to HPV (25,26). We would not anticipate SV40 antibodies to be low titer in humans if the virus is capable of infecting many tissues. Indeed, in monkeys, SV40 elicits high-titer antibodies. In addition, other viruses that have jumped species, for example, HIV and Ebola, are immunogenic in their new hosts (33,34). Therefore, it would be unusual for SV40 infection levels to be so low that they fail to generate a detectable immune response, yet produce enough virus to be widely transmitted. Another possibility is that the strains of SV40 that circulate in humans have variant capsid proteins, eliciting antibodies not detected by the ELISA. Although most sequencing of tumor-associated SV40 DNA has focused on the regulatory regions or T-antigen coding sequences, two studies (9,35) found no differences in VP1 sequences between laboratory- and tumor-derived strains. A formal possibility is that the SV40 genome is pseudotyped by another capsid; however, this phenomenon has never been demonstrated. The issue of artifactual detection of SV40 DNA due to contamination of PCRs has been explored extensively but inconclusively (7,8).

This study also has several limitations. With the exception of the osteosarcoma case patients, all of the sera were drawn from populations in western Washington State, and no information was available regarding the patients’ poliovirus vaccination histories. However, Washington State was considered to have a high level of exposure to SV40-contaminated poliovirus vaccines (36), so it is likely that many of the individuals (born before 1962) tested would have been exposed to SV40. We have not yet examined sera from population-based case–control studies of other cancers that have been associated with SV40. However, sera from a large case–control study of lymphomas from Spain (37) were tested for SV40 using an SV40 VLP-based ELISA. Similar to the results reported here, seropositivity was infrequent, with 9.5% of control subjects and 5.9% of case patients testing seropositive for SV40; this proportion of individuals testing seropositive for SV40 was reduced by BKV VLPs. Because no SV40 DNA was found in lymphomas from Spain (13), it remains possible that seropositivity for SV40 could be associated with lymphomas in other geographic areas. Our results have demonstrated an accurate and sensitive assay that distinguishes seropositivity to three polyomaviruses, but do not provide serologic evidence to support the hypothesis that SV40 is a prevalent infection in humans.

References

(21) Major EO, Neel JV. The JC and BK human polyoma viruses appear to be recent introductions to some South American Indian tribes: there is no serological evidence of cross-reactivity with the simian polyoma virus SV40. Proc Natl Acad Sci U S A 1998;95:15525–30.
NOTES

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