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Prevalence and stability of human serum antibodies to simian virus 40 VP1 virus-like particles

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Possible human infection with simian virus 40 (SV40) has been of great concern ever since SV40 was discovered in polio vaccines. Human populations are SV40-seropositive, but because of serological cross-reactivity between SV40 and the human polyomaviruses BK virus (BKV) and JC virus (JCV), it is debatable whether these antibodies are specific. An SV40-specific serological assay was established, based on purified virus-like particles (VLPs), where the SV40 VLPs were blocked with hyperimmune sera to BKV and JCV. Competition with SV40 hyperimmune sera was used as a confirmatory test. Among 288 Swedish children of between 1 and 13 years of age, 7–6% had SV40-specific antibodies. SV40 seroprevalence reached a peak of 14% at 7–9 years of age. Among 100 control patients with benign tumours, 9% were SV40-seropositive. However, SV40 DNA was not detectable in corresponding buffy-coat samples. In serial samples taken up to 5 years apart from 141 Finnish women participating in the population-based serological screening for congenital infections, only two of 141 women were SV40-seropositive in both samples. Six women seroconverted and eight women had a loss of antibodies over time. None of the SV40-seropositive samples contained detectable SV40 DNA. In conclusion, there is a low prevalence of SV40-specific antibodies in the Nordic population. The SV40 antibodies appear to have a low stability over time and their origin is not clear.

INTRODUCTION

Simian virus 40 (SV40) of monkey origin was discovered in 1960 as a contaminant of human polio vaccines produced in monkey cells during the 1950s (Sweet & Hilleman, 1960). Both the oral and the inactivated polio vaccines were found to contain high titres of SV40 virus (Hilleman, 1998). In Sweden, a polio vaccine of US origin was administered in 1957 to children born between 1945 and 1953. The nationwide population coverage was about 80%, resulting in exposure of about 700 000 Swedish children to the potentially SV40-contaminated vaccine (Olin & Giesecke, 1998; Vilchez et al., 2003).

Many case-control studies of poliovirus vaccination and cancer have been performed, but results have been inconsistent (Strickler & Goedert, 1998). An increased incidence of cancer in vaccine-treated individuals was reported in five studies, but five other studies found no link (Strickler & Goedert, 1998). A cohort study of exposed and unexposed birth cohorts found no increased risk (Vilchez et al., 2003). Several studies have reported the detection of SV40 nucleotide sequences in human tumours, mainly mesothelioma, osteosarcoma, ependymomas and chorid plexus tumours (Bergsagel et al., 1992; Butel & Lednicky, 1999; Minor et al., 2003; Shah, 2000). SV40 oncogenesis is mediated by the large tumour antigen (T-Ag), which is capable of transforming several different types of cells in the absence of other viral genes. SV40 T-Ag promotes the onset of S phase in host cells and induces DNA synthesis through binding and functional inactivation of the cellular tumour-suppressor proteins p53 and pRb (Vilchez et al., 2003).

Studies of neutralizing antibodies to SV40 in human sera from the UK, Poland and Africa found an overall seroprevalence of between 3 and 5% (Minor et al., 2003). An English study reported a 1.3–5% SV40 antibody prevalence, with mostly low titres (Knowles et al., 2003). Occasionally, humans with serum neutralizing-antibody titres of very high magnitude (similar to those found in experimentally infected monkeys) are found (Minor et al., 2003). In the few
and limited surveys that have been performed, there has been no correlation of SV40 seroprevalences with history of poliovirus vaccination. This has been interpreted as suggesting that SV40 is now circulating in human populations (Butel & Lednicky, 1999).

Serological cross-reactivity between antibodies to SV40 and the human polyomaviruses BK virus (BKV) and JC virus (JCV) is strong. Both the prevalence and the level of antibodies correlate strongly between the three viruses (Knowles et al., 2003; Minor et al., 2003; Stolt et al., 2003; Viscidi et al., 2003). BKV and JCV have extensive sequence similarity to SV40 (Knowles et al., 2003).

The objective of the present study was to establish an enzyme immunoassay (EIA) for SV40-specific antibodies, devoid of cross-reactivity with BKV and JCV, and subsequently to investigate the age-specific SV40 seroprevalences in the Nordic countries. We also wished to investigate whether SV40 seropositivity correlated with detectability of SV40 DNA and whether SV40-specific antibodies are stable over time.

**METHODS**

**Study population.** Serum samples from a consecutive series of 1031 samples from children between 0 and 13 years of age were originally obtained from the Department of Clinical Virology, Karolinska Hospital, Sweden, and have been characterized in a previous study of the seroepidemiology of papillomavirus infection (af Geijersstam et al., 1999). The children were stratified in 2-year age groups and a stratified, random subsample of 290 sera was selected. In this study, 50 serum samples each were from children between 1-1 and 3 years, 3-1 and 5 years, 5-1 and 7 years, 7-1 and 9 years, 9-1 and 11 years of age and 40 serum samples were from children between 11-1 and 13 years of age.

The Finnish Maternity Cohort of the National Public Health Institute in Finland contains samples from the population-based serological screening programme for congenital infections in the first trimester of pregnancy (af Geijersstam et al., 1998). In this study, an age-stratified, random subsample of 300 serum samples taken from 150 women during their first and second pregnancies were collected. The women were stratified by age at their first pregnancy, and having had a second pregnancy during a 5-year follow-up period was an eligibility requirement. Fifty women each were between 14 and 19 years, 20 and 25 years and 26 and 31 years of age at their first pregnancy (Stolt et al., 2003).

Two childhood serum samples could not be analysed because of reactivity with blank (uncoated) ELISA plates. Serial samples from nine women were excluded due to inadequate amounts of serum. The final numbers of samples from children and from pregnant women were 288 and 282, respectively.

One hundred Swedish patients with benign skin tumours (mostly common warts and seborrhoeic keratosis) had been enrolled as controls for a case-control study of skin cancer. Buffy-coat samples of these patients were analysed for both SV40 DNA and BKV DNA by using real-time PCR and the corresponding serum samples were tested for SV40 antibodies.

**Polyoma virus-like particles (VLPs).** Polyoma VLPs from SV40, BKV and JCV were produced in *Sacccharomyces cerevisiae* yeast cells as described previously (Gedvilaité et al., 2000). VLPs are empty capsids consisting of the major capsid protein, VP1. The VP1 gene was inserted into the yeast expression vector pFX7. The pFX7-derived expression plasmids carrying the VP1 genes were transformed into *S. cerevisiae* for cultivation and vector replication. Expression of VP1 proteins results in spontaneous assembly into VLPs that retain sialic acid-binding and antigenic properties of native virions (Sasnauskas et al., 1999).

**Sero logical analysis.** The optimal concentration of polyomavirus VLPs and the serum dilutions to be used were determined by titration, using positive and negative controls. Purified VLPs were, for all the viruses, added to the wells at a concentration of 1-25 ng per well in ice-cold PBS (pH 7.2). Half-area Costar 3690 EIA plates were incubated overnight at 4 °C. After washing with 0-1 % PBS/Tween, a blocking buffer consisting of 10 % horse serum in PBS (HS-PBS) was added and incubated for 1 h at 37 °C.

Rabbit hyperimmune sera against BKV (AS strain) and JCV (55 μl per well), each diluted 1/100 in HS-PBS, were added and incubated for 2 h at 37 °C. The plates were washed five times with 150 μl PBS/Tween per well. Serum samples (50 μl), diluted 1/60 in HS-PBS, were added per well and incubated for 1 h at room temperature. The plates were washed five times with 150 μl PBS/Tween, and anti-human IgG (mouse monoclonal; Eurodiagnostica), diluted 1/800, was added and incubated for 90 min at 37 °C. The plates were washed five times with 150 μl PBS/Tween, and goat anti-mouse IgG–peroxidase conjugate (Southern Biotechnology), diluted 1/2000 in HS-PBS containing 2 % normal rabbit serum, was added and reacted at 37 °C for 60 min. Following another washing step, the peroxidase substrate ABTS was added and incubated for 30 min at room temperature, whereafter A415 was measured.

Sera testing positive for antibodies to SV40 in the EIA were confirmed by an anti-SV40 inhibition test. The same EIA as described above was performed, except that the plate wells were also blocked with a rabbit hyperimmune serum to SV40.

Generation of hyperimmune sera was carried out as described previously (Christensen et al., 1996; Dillner et al., 1991). Briefly, 50 μg VLPs from BKV, JCV and SV40 was injected subcutaneously in the neck, firstly in Freund’s complete adjuvant, whereas all subsequent injections were in Freund’s incomplete adjuvant. The optimal dilution of hyperimmune serum was determined by titration test. Boosters were given 3 and 6 weeks after the third injection.

One of the children’s samples that had > 50 % inhibition in the SV40 test was used as a positive control for SV40. One negative serum sample from a child aged 1 year 9 months was used as negative control.

Human reference sera from three renal-transplant recipients who tested positive for BKV DNA in urine by PCR and were strongly positive for BKV antibodies in EIA were also used as negative controls for SV40 (Stolt et al., 2003). These sera were used at a dilution twofold lower than the end-point titre (1/10 240, 1/640 and 1/40 960, respectively).

For definition of cut-off values, the mean value and SD were calculated from the log-transformed values in the group of children between 1-1 and 3 years of age and the cut-off values were defined as the mean value + 2SD of the log A415 values among the 1-1-3-year-old children. For the confirmation test, a cut-off point of significant inhibition was set arbitrarily at 50 % blocking. Correlation between seropositivities was evaluated by using Pearson’s correlation coefficient.

A 50 μl aliquot of each of the 40 sera that were confirmed as positive for SV40 antibodies and the 100 Buffy-coat samples was extracted for PCR by using a QiAamp MinElute Virus Spin kit (Qiagen). Two sera were excluded due to low volume of serum.
SV40 quantitative PCR. A real-time (TaqMan) PCR method for SV40 was established by using a primer pair and an oligonucleotide probe with the reporter fluorescein dye FAM attached to the 5’ end and a rhodamine dye (TAMRA) quencher linked to the 3’ end. A threshold cycle value (Ct) was calculated for each sample by determining the point at which the fluorescence exceeds the threshold limit chosen for the specific plate (Heid et al., 1996; Tedeschi et al., 2001).

SV40-specific primers and a probe detecting the VP2 region of the SV40 genome were designed by using Primer Express software version 2.0 (PE Applied Biosystems). The real-time PCR assay used the forward primer 5'-CACAGGCTATGCTGTGATATCTG-3' (nucleotide position 752–775), the reverse primer 5'-AAAATCTA-TACCCCCAGTGGACAA-3' (nucleotide position 863–839) and the fluorescent Taqman probe 5'-CACCTTTACTGCAAATCCTGAACGGTTGAG-3' (nucleotide position 803–833) (DNA Technology A/S) to amplify and detect a 112 bp amplicon within the VP2 region of the SV40 genome.

To each well of a 96-well plate, we added 5 μl sample and 20 μl PCR mixture, consisting of 10× buffer (1:10), dNTPs (each 1-25 mM), MgCl2 (25 mM), AmpliTaq Gold (0.625 U) and H2O (PE Applied Biosystems). The optimum concentrations were determined by titration using the positive standard and negative water sample controls. Forward primer, reverse primer and probe, at concentrations of 300, 500 and 200 nM, respectively, were added. Each sample was run in duplicate. Cycling parameters were 50°C for 2 min, 95°C for 10 min and 50 cycles of 95°C for 15 s and 60°C for 1 min.

Rolling-circle amplification was used on all extracted template sera before retesting by real-time PCR (TempliPhi Amplification kit; Amersham Biosciences) (Rector et al., 2004). Amplification was performed according to the instructions from the manufacturer. Briefly, 1 μl extracted template serum was diluted in sample buffer and incubated at 95°C for 3 min. Reaction buffer (5 μl), 0.2 μM enzyme mix and 0.5 μl 20-7 mM dNTPs were added to each sample before incubation at 30°C for 20 h. The enzyme was inactivated by incubation at 65°C for 10 min.

Negative-control water samples, negative serum, positive controls (SV40 standard solutions) and spiked negative sample were included on each plate and produced a standard curve from which the number of genomes in the samples could be calculated. The SV40 quantities in the standards used were 100,000, 10,000, 1000, 100 and 10 copies in 5 μl, i.e. per well.

The PCR sensitivity was detection of 10 copies. All serum samples and controls were run by using a GeneAmp 5700 sequence detection system (Applied Biosystems). The standard curve was created by the GeneAmp 5700 SDS software by plotting the Ct values against each known concentration of the SV40 standards.

The standard stock solution, plasmid pBRSV (ATCC 45019), containing SV40 genome was cultivated in terrific broth medium and the culture was purified according to the manufacturer’s instructions (QIAprep Spin Miniprep kit; Qiagen). The A260 of the plasmid solution was measured and DNA copy number was calculated.

A similarly designed real-time (TaqMan) PCR method was used for detection and quantification of BKV DNA (Stolt et al., 2005). Negative-control water samples and positive controls (diluted from a standard BKV stock solution) were included on each plate and produced a standard curve from which the number of genomes in the samples could be calculated. The BKV quantities in the standards used were 4, 40, 400 and 4000 copies in 5 μl, i.e. per well.

RESULTS

Assay development

Testing of 288 children's sera in ordinary EIAs based on purified VLPs of BKV, JCV and SV40 showed that antibody titres to SV40 VLPs were related strongly to the BKV titres, in particular to the titres against the BKV AS strain. There were also significant correlations between SV40 antibody levels and BKV SB strain antibody levels (data not shown), as well as with SV40 and JCV antibody levels.

By addition of hyperimmune sera to BKV AS and JCV to the SV40 VLPs before addition of human sera, the antibody reactivity to SV40 was reduced strongly, but not eliminated. In the blocked assay, the correlation between presence of SV40 antibodies and presence of antibodies to BKV AS was much weaker (r=0.30) than that in the unblocked assay (r=0.51). The correlation between the presence of SV40 and JCV antibodies was also reduced in the blocked assay (r=0.22, compared with r=0.31 in the unblocked assay).

As a further test of specificity, all initially SV40-positive sera were also tested in an inhibition assay with a hyperimmune serum to SV40. There were many examples of serum samples that were completely inhibited, not at all inhibited and partially inhibited. There were 91 serum samples that were reactive with SV40 in the BKV and JCV-blocked ELISA, but only 40 of these sera could be inhibited to >50% with the hyperimmune sera to SV40. The presence of confirmed SV40 antibody reactivities had an even lower correlation with the presence of BKV AS (r=0.24) and the correlation with presence of JCV antibodies was almost eliminated (r=0.09).

For comparison, we performed similar inhibition of BKV reactivity with anti-BKV hyperimmune sera. Three high-titrated sera from patients with haemorrhagic cystitis (BKV PCR-positive) were blocked almost entirely, whereas only two of four sera from BKV-seropositive healthy children were blocked to >50% (not shown).

Confirmed SV40 antibody reactivities among children

In the following, only serological reactivities that were confirmed by competitive inhibition with the anti-SV40 antibody are considered. The children between 1 and 13 years of age had an overall SV40 seroprevalence of 7.6%. SV40 seropositivity increased with increasing age of the children, reaching 14% seroprevalence at 7–9 years of age, followed by a decrease (Table 1; Fig. 1).

Stability over time of confirmed SV40 antibody reactivities in serial samples from pregnant women

The SV40 seropositivity among a population-based sample of 141 mothers at their first pregnancy was 7.1%. The SV40 seroprevalence of the same mothers at their second
pregnancy up to 5 years later was 5.7%. Only two of 141 women were SV40-seropositive in both samples. Six women had a SV40 seroconversion and eight women had a loss of SV40 antibodies between the two pregnancies (Table 2).

Thirty-eight serum samples testing positive for SV40 antibodies were also tested for SV40 DNA by using a real-time PCR (with a sensitivity for SV40 detection of 10 viral-genome copies) but were all negative. Non-specific amplification of the viral DNA by using rolling-circle amplification before the specific PCR, a method known to increase sensitivity (Rector et al., 2004), was also performed on all 38 serum samples, but even after rolling-circle amplification, all samples were negative for SV40 DNA in the real-time PCR.

**Comparison of confirmed SV40 antibody reactivities and presence of viral genomes in peripheral blood mononuclear cells in a hospital-based control population**

Nine of 100 serum samples from control patients with benign tumours had confirmed SV40 antibody reactivities. By contrast, none of these sera and none of the corresponding buffy-coat samples from the same subjects contained detectable SV40 DNA or BKV DNA in real-time PCR.

**DISCUSSION**

The present study has established an SV40 seroassay based on blocking of cross-reactive epitopes with hyperimmune sera to human polyomaviruses, followed by competitive inhibition with an SV40 hyperimmune serum. The assay has significantly curtailed the strong and well-known cross-reactivity between SV40 and the human polyomaviruses. There still remains an SV40-specific antibody response in human sera, but the origin of these antibodies is not clear. The age-specific SV40 seroprevalence among children was low (compared with previously reported seroprevalences of the human polyomaviruses BKV AS, BKV SB and JCV; Stolt et al., 2003). The age-specific seroprevalence curves were somewhat similar between SV40 and BKV, but clearly different from the JCV age-specific seroprevalence curve. The low stability over time of SV40 antibodies that we observed in serial samples is probably mostly attributed to the fact that the antibody levels were low in most of the positive sera. However, lasting infections expressing specific antigen would have been expected to result in stable seropositivity over time. Indeed, both BKV and JCV seropositivities are stable over time, in line with the fact that these viruses are persistently present in most humans.

Because of the theoretical possibility that immunization of rabbits with VLPs could lead to hyperimmune sera containing antibodies against epitopes different from those that the human response recognizes, we added the inhibition test with rabbit antibodies against SV40 as part of the assay. In order for a human serum to be classified as positive in our test, the human antibodies are not blocked by rabbit antibodies against BKV or JCV, but are blocked by rabbit antibodies against SV40.

Differential recognition by humans and rabbits as an explanation for the observed reactivities seems unlikely, because it would require that the cross-reactive BKV or JCV epitope would be immunogenic to rabbits in the context of SV40, but not in the context of BKV or JCV themselves. An alternative explanation is that the SV40-reactive antibodies are induced by some human virus other than BKV or JCV, perhaps an as-yet-unknown human polyomavirus. The latter possibility can unfortunately not be addressed.

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**Table 1. Seroprevalence of SV40 infections in children between 1 and 13 years**

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. sera</th>
<th>No. SV40-positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–3 years</td>
<td>50</td>
<td>0 (0)</td>
</tr>
<tr>
<td>3–5 years</td>
<td>49</td>
<td>3 (6–1)</td>
</tr>
<tr>
<td>5–7 years</td>
<td>50</td>
<td>6 (12–0)</td>
</tr>
<tr>
<td>7–9 years</td>
<td>50</td>
<td>7 (14–0)</td>
</tr>
<tr>
<td>9–11 years</td>
<td>49</td>
<td>3 (6–1)</td>
</tr>
<tr>
<td>11–13 years</td>
<td>40</td>
<td>1 (2–5)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>288</strong></td>
<td><strong>22 (7–6)</strong></td>
</tr>
</tbody>
</table>

**Table 2. SV40 seroprevalence in first and second pregnancies**

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. sera</th>
<th>SV40 serostatus in first/second pregnancy [no. (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+/+</td>
</tr>
<tr>
<td>14–19 years</td>
<td>46</td>
<td>1 (2–2)</td>
</tr>
<tr>
<td>20–25 years</td>
<td>46</td>
<td>1 (2–2)</td>
</tr>
<tr>
<td>26–31 years</td>
<td>49</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>141</strong></td>
<td><strong>2 (1–4)</strong></td>
</tr>
</tbody>
</table>

**Fig. 1. Prevalence of antibodies to the polyomaviruses BKV SB (●), BKV AS (●), JCV (▲) and SV40 (■) among children between 1 and 13 years of age.**
Other studies have used a competitive-inhibition method, where sera were absorbed with VP1 VLPs from BKV and JCV before being added to the SV40 VLP EIA plates (Carter et al., 2003; Engels et al., 2004). In the study by Engels et al. (2004), SV40 reactivity was defined as SV40-specific if it was also inhibited to at least 50% by SV40 VLPs. Whilst Carter et al. (2003) could not find any SV40-specific sera among 699 tested samples, Engels et al. (2004) found that 1–1.6% of human serum samples contained confirmed SV40 reactivity. Both the strategies with competitive inhibition with the antigen and those with inhibition with antibodies against the antigen are intended to control for possible differences in epitope exposure of the antigen absorbed to solid phase as compared with the native antigen in solution. Both we and Engels et al. (2004) found that reactivities that cannot be confirmed are indeed common and a confirmatory step is therefore likely to be important in these serological assays. The fact that we find a higher prevalence of confirmed reactivities than Engels et al. (2004) appears to reflect a higher sensitivity of our assay in general.

As we were not able to demonstrate SV40 genomes in the sera or buffy coats of seropositive individuals, we do not know whether the SV40-reactive antibodies have indeed been induced by SV40 infection. However, detection of viraemia in serum or viral DNA in buffy coat is not a regular phenomenon, even for the near-ubiquitous human polyomaviruses, and urine samples (which are commonly SV40 DNA-positive in SV40-infected monkeys) were unfortunately not available. Indeed, we also did not detect any BKV DNA in these samples. Therefore, our inability to detect SV40 DNA does not exclude the possibility that SV40 infection may have been present.

Our reported prevalences of SV40 antibodies in populations of Swedish children and Finnish women are similar to those reported in previous studies of other human populations (Minor et al., 2003; Olin & Giesecke, 1998; Vilchez et al., 2003). However, the origin of the SV40-specific antibodies found in the present population remains to be established.

In conclusion, the present study has highlighted several important problems in SV40 seroepidemiology, such as a need for assessment of cross-reactivity with human polyomaviruses and limited stability over time of antibody responses, making inferences of serological data difficult. Further studies are needed, particularly regarding demonstration of SV40 genomes and comparison with presence of SV40 antibodies, before more informative seroepidemiological studies can be performed.

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