

Some Oral Poliovirus Vaccines Were Contaminated with Infectious SV40 after 1961

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Abstract

Some polio vaccines prepared from 1954 to 1961 were contaminated with infectious SV40. It has been assumed that all polio vaccines were SV40 free in the United States after 1961 and in other countries after 1962. Following a WHO requirement that was prompted by the detection of SV40 in some human tumors, we conducted a multilaboratory study to test for SV40 polio vaccines prepared after 1961. Vaccine samples from 13 countries and the WHO seed were initially tested by PCR. The possible presence of intact and/or infectious SV40 DNA in PCR-positive samples was tested by transfection and infection of permissive CV-1 cells. All results were verified by immunohistochemistry, cloning, and sequencing. All the vaccines were SV40 free, except for vaccines from a major eastern European manufacturer that contained infectious SV40. We determined that the procedure used by this manufacturer to inactivate SV40 in oral poliovirus vaccine seed stocks based on heat inactivation in the presence of MgCl₂ did not completely inactivate SV40. These SV40-contaminated vaccines were produced from early 1960s to about 1978 and were used throughout the world. Our findings underscore the potential risks of using primary monkey cells for preparing poliovirus vaccines, because of the possible contamination with SV40 or other monkey viruses, and emphasize the importance of using well-characterized cell substrates that are free from adventitious agents. Moreover, our results indicate possible geographic differences in SV40 exposure and offer a possible explanation for the different percentage of SV40-positive tumors detected in some laboratories. (Cancer Res 2005; 65(22): 10273-9)

Introduction

Inactivated poliovirus vaccine (IPV) and live oral poliovirus vaccines (OPV) were prepared in primary cell cultures derived from rhesus monkey kidneys. Studies of these vaccines led to the discovery of a new virus called SV40 in 1959. This DNA virus caused vacuolization of green monkey cell cultures and was found to be highly oncogenic in hamsters (reviewed in refs. 1, 2). It was found that SV40 was endemic in rhesus monkeys. For this reason, the rhesus kidney cell cultures used to manufacture poliovirus vaccines as well as some seed stocks of poliovirus contained infectious SV40

(2–5). Therefore, the early batches of OPV contained infectious SV40 (3–5). Because formaldehyde treatment used to prepare IPV failed to completely inactivate SV40, some batches of IPV contained infectious SV40 (2, 4). As a result, it has been estimated that >100 million people in the United States and many more worldwide received potentially contaminated vaccines prepared during the years 1954 to 1961 (2).

Regulations adopted in 1961 required new batches of poliovirus vaccines prepared in the United States to be free of SV40 and it has been assumed that they were based on quality-control testing done during vaccine manufacture (documents concerning the contamination of polio vaccines with SV40 and the history of polio vaccination can be found in the text and appendices to ref. 4). In the United States, OPV was licensed after SV40 was discovered; therefore, these vaccines should have been free from SV40. In addition to the United States, other countries followed the WHO recommendations issued in November 1960 and attempted to produce poliovirus vaccines free of SV40 (3–5). To eliminate SV40 from polio vaccines, the manufacturers had to address two main problems. The first problem was that ~50% of rhesus monkeys were endemically infected with SV40 and that infection readily spread to uninfected caged monkeys (4, 5). Moreover, because harvest from kidneys from different monkeys was often pooled together during production, even one SV40-infected monkey could contaminate the entire vaccine batch (2, 4). To address this issue between 1961 and 1963, manufacturers switched to the use of African green monkeys because they are SV40 free (although they can occasionally be infected with SV40, various monkey viruses, and filoviruses, including the Marburg virus that caused a deadly outbreak of hemorrhagic fever in Yugoslavia and Germany in 1967; ref. 4). It was therefore assumed (incorrectly; see Discussion) that rhesus monkeys were not used for polio vaccine production since the early 1960s, thus removing a very important cause of vaccine contamination by SV40 (2–9). The second problem was that the Sabin virus seed stocks that were used to prepare polio vaccines were contaminated with SV40, as stated by Sabin and Boulger (3) and independently confirmed by others (5). To purify the Sabin seeds from SV40, different manufacturers used different techniques (3–5). The United Kingdom, the United States, and the WHO used an anti-SV40 antiserum; the USSR used a methodology based on the addition of MgCl₂ (see below; refs. 3–5); we do not know what methodology was used by other countries.

Because of these actions (replacing rhesus with green monkeys for production and the purification of seed stocks from SV40), it is widely assumed that after the early 1960s all polio vaccines were free from infectious SV40.

The hypothesis that some vaccines prepared after the early 1960s were contaminated with SV40 has been discussed numerous times (4, 6–8). We (9, 10) and others (11) did not find evidence of SV40

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contamination in any tested United States and UK vaccines prepared after 1961 (IPV and OPV), whereas SV40 was detected in the IPV prepared in United States in 1954 (9). However, only a limited number of samples were available for testing and they were not representative of all poliovirus vaccine produced after 1961 (9–11). Moreover, a recent study conducted by PCR reported that current vaccines produced in the USSR are also free of SV40 (12).

Therefore, attempts to identify and determine the magnitude of potential SV40 contamination remains an important priority. This was confirmed by the WHO recommendation issued in 2000 to test seed stocks used for polio vaccine manufacture for the presence of SV40. The present multilaboratory study was conducted in response to this recommendation and involved testing current vaccines from 13 countries as well as the WHO seed stocks and some earlier vaccine samples that had been deposited at the National Institute for Biological Standards and Control (NIBSC; Herts, United Kingdom). The samples were initially tested at the NIBSC using the PCR test for SV40. All samples tested negative, except for the vaccine samples from a major eastern European vaccine manufacturer (EEVM). These SV40-positive samples and the UK samples (control) underwent a second round of detailed independent scrutiny using multiple technical approaches in the laboratory of Dr. Carbone. Some of the results, infection, viral cloning, and $MgCl_2$ inactivation, were further validated independently in the laboratory of Dr. Lednický as described below.

Materials and Methods

Vaccines. All vaccines were provided by the NIBSC. The samples of oral polio vaccines from different manufacturers were sent to NIBSC in the mid-1970s as a part of the WHO Collaborative Study to standardize the monkey neurovirulence test and to compare the neurovirulence of vaccine strains grown in monkey cells and in WI38 cells. EEVM vaccines and control UK vaccines were tested with numerous techniques and in three separate laboratories following initial PCR testing that revealed the presence of SV40 in EEVM samples (Table 1). Each vaccine vial produced by the EEVM was obtained from the USSR by the late Dr. David Magrath (NIBSC) and stored at -70°C . The numbers of vaccine batches and the dates of their manufacture indicated on the vials stored at the NIBSC were consistent with the production records that are kept by this manufacturer. Sample EEVM lot 492 (see Table 1) was prepared from a type 3 seed batch that was in use from 1965 to 1978. Sample 39 was prepared from type 1 seed lot 360 that was produced in 1966 and remained in use until 1978 when a new seed was produced. Only one seed was used at each time for production of each of the three variants of poliovirus vaccines. In other words, the type 1 and 3 poliovirus in all of the OPV produced by the EEVM from 1965/1966 to 1978 originated from the same type 1 and 3 seed stocks. Therefore, if these seeds

were contaminated, it could be expected that vaccines produced from these seeds might also be contaminated.

A third EEVM batch stored at NIBSC along with the above samples was not clearly labeled (NIBSC no. 40), and it was assumed that it was from roughly the same time period based on the NIBSC archive number. In the 1980s, EEVM switched to the use of WHO seeds that we found to be SV40 free (see below).

The UK oral vaccines tested were prepared in 1982 (lot 314) and 1997 (lot 335; two separate samples; see Table 1 and text for additional information).

Precautions to prevent and/or detect PCR contamination. Eight mock extractions (both for RNA and DNA studies) were done in parallel with six polio vaccine samples (e.g., three from EEVM and three from the United Kingdom; Table 1) and processed thereafter as polio vaccine samples (negative controls). Extractions were done in a sterile hood that we use only for the purpose of extracting DNA or RNA for PCR (there are two hoods: one is used for DNA extraction and one for RNA extraction). PCR assembly was done in a separate room during which one additional negative control was added. The template DNA (except for the positive control) was added in a third separate room and one more negative control was prepared. (As an additional precaution, these hoods are treated with UV light when they are not in use.) PCR reactions were amplified in a fourth room (where the positive control was added) and PCR-amplified samples were opened in a fifth room. Each room and hood has its own separate set of pipettes and there is no sharing of equipment among these stations. The fourth and fifth rooms are located in a different building than rooms 1 to 3, and no reagents or equipment that entered rooms 4 and 5, including laboratory coats, go back to rooms 1 to 3. We use disposable laboratory coats, shoe covers, microcentrifuge rack holders, etc., to work in rooms 1 to 3 and to transport the assembled PCR reactions to rooms 4 and 5. These steps are used in Dr. Carbone's laboratory to diminish the risk of PCR contamination and to facilitate the identification of the source of contamination if such event occurred. Moreover, to test for possible laboratory contamination by plasmids, we routinely run PCR reactions using the primers 5'-GCTCAGCTGTAGGTATCTC-3' and 5'-TCTAGTGTAGCCGTAGTTAG-3' that amplify a 241 portion of the pUC origin of replication present in pBR313 and in virtually all plasmids that are propagated in *Escherichia coli*. Similarly, extensive precautions were used at the NIBSC and in Dr. Lednický's laboratory to prevent or eventually detect PCR contamination.

Positive control. pSV21-N (13, 14) is a SV40 strain 776-based plasmid, which contains engineered *SalI* and *XhoI* restriction sites in the nonarchetypal regulatory region to allow the differentiation of this control DNA from other SV40 DNAs (a further precaution to detect eventual PCR/plasmid contamination).

PCR. Reactions were run on agarose gel, blotted, and hybridized with specific SV40 probes. Primer sequences R2/R1, R2/R5, R6/R7, R8/R9, R10/R11, R12/R13, TA2/TA1, T5/T6, P_{yy}.rev/P_{yy}.for, P_{yy}.rev.nes/P_{yy}.for.nes, SV_{rev}/SV3, SV2/SV_{rev}, and CPC-MEN primer pairs, PCR conditions, probes, and Southern hybridization were described (9). All positive results were verified by DNA sequencing of the PCR amplicons. PCR has limitations because positive results can be questioned for the possibility of contamination with plasmids containing SV40 sequences, and negative results can be caused by the presence of PCR inhibitors. Because all samples were handled in parallel, the absence of any positive results in the negative controls, in the UK samples, etc., indicated that the positive results obtained in the EEVM samples could not originate from PCR contamination. Moreover, CPC-MEN and VA-45-54-1 (the two SV40 strains identified in the EEVM samples, see below) contain only one 72-bp enhancer element in their archetypal regulatory region and contain differences in the COOH terminus that make these strains easy to distinguish from our positive control pSV21-N and from SV40 strain 776 (13, 14). In our laboratory (Carbone's; where these viruses were identified), we do not have SV40 VA-45-54-1 and CPC-MEN, we have never grown these viruses, and we have never detected these strains in our previous work. The issue of PCR inhibitors producing false-negative results is addressed in Results, and we did not detect any inhibitors in the UK samples that could cause false-negative results.

DNA extraction. Sterile $5\times$ lysis buffer [10 μL ; 50 mmol/L Tris-HCl (pH 8.0), 2.5% Tween 20] with 0.4 mg/mL proteinase K were added to 40 μL

Table 1. Source of the vaccine and date of production

NIBSC ID	Type	Description	Date	Lot no.
UK 10	3	Vaccine bulk	05/14/97	335
UK 12	3	Vaccine bulk	1982	314
UK 16	3	Vaccine bulk	05/14/97	335
EEVM 39*	1	Seed	10/16/66	360
EEVM 40*	1	Vaccine bulk	—	—
EEVM 44*	3	OPV	05/13/69	492

*SV40-contaminated EEVM vaccines. Lot 492 was from a seed that was in use from 1965 to 1978. See also Materials and Methods.

vaccine. Samples were incubated for 1 hour at 55°C followed by enzyme inactivation at 95°C for 10 minutes and standard phenol/chloroform purification and ethanol precipitation. G25 spin columns (Amersham, Arlington, IL), when noted, were used for DNA purification. DNA was extracted from 40 µL of each vaccine in five separate extractions, each independently tested by PCR; the results were reproducible.

RNA extraction. RNA from each OPV/seed sample (UK and EEVM) and Poliovirus 1 (attenuated) Strain Chat (WCH Wy 4B-5) and Poliovirus 3 (attenuated) Strain Fox (Wy 3), purchased from American Type Culture Collection (Manassas, VA), was extracted using the QIAmp Viral RNA Mini Spin kit (Qiagen, Valencia, CA) following the manufacturer's suggested protocol. RNA extraction was from 0.4 µL, except for EEVM sample 40 for which only 0.2 mL was available.

Real-time PCR. cDNA was synthesized using the First-Strand cDNA Synthesis kit (Fermentas, Hanover, MD) following the manufacturer's protocol. cDNA was amplified using the type 1 and 3 poliovirus-specific primers and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) using Perkin-Elmer ABI 7900HT thermal cycler. cDNA from type 1 and 3 poliovirus (American Type Culture Collection) served as the standard for determining calibration curves. cDNA standards and primers were provided by our coauthor (K.C.). PCR products for the regulatory region and for the COOH terminus (EEVM samples 39 and 40) were cloned into the pGEM-TA cloning vector (Promega, Madison, WI). About 20 inserts positive of each clones were picked and sequenced.

Lipofection. DNA was extracted from aliquots of vaccine using a standard proteinase K digestion method, phenol/chloroform extraction, precipitated in 70% ethanol, and solubilized in water and then treated with 0.01 mg (total amount) RNase A for 10 minutes to inactivate polioviruses, mixed with an equal volume of Lipofectin reagent (Invitrogen, Carlsbad, CA) for 30 minutes, and added to CV-1 cells as described (13, 14). We did not standardize the amount of DNA used in these transfections because we would not be able to distinguish among viral and cellular mitochondrial DNA and the latter may vary among different samples. Instead, we transfected whatever amount of DNA we could extract from 40 µL vaccine.

Cloning and sequencing. Low molecular weight DNA was extracted and cloned into pUC19 (13, 14) and 20 full genomic clones from each transfection or infection were picked and sequenced for the COOH terminus, the regulatory regions, and the capsid proteins. Representative clones were fully sequenced and SV40 strains CPC-MEN and VA-45-54-1 were identified.

Immunostains. These were conducted according to standard procedures (2) and cells were fixed in cold acetone for 10 minutes. The anti-Tag used was pAb-419 (Calbiochem, San Diego, CA). The anti-VP-1 was AB-597 mouse monoclonal antibody kindly provided by Dr. F.J. O'Neill (University of Utah, Salt Lake City, UT). Reactions were developed using ABC Vectastain Elite (Vector Laboratories, Burlingame, CA).

MgCl₂ treatment. Following the procedures outlined in ref. 16, multiple aliquots (1 mL each) of 3×10^7 , 3×10^5 , and 3×10^3 plaque-forming units (pfu)/mL SV40 strain 776 were thoroughly mixed with 1 mL sterile 2 mol/L MgCl₂ in sterile polypropylene tubes and heated in a 50°C water bath for 1 hour. A final concentration of 1 mol/L MgCl₂ was chosen, as this was the concentration of MgCl₂ used by the EEVM to inactivate SV40 in OPV. Equivalent aliquots of SV40 were mixed with PBS (instead of MgCl₂) and were heat treated in parallel. After heat treatment, virus survival was determined by adding either 1 mL or lower amounts (0.1 or 0.01 mL) of the treated virus preparations to CV-1 cells in 75-cm² flasks containing 25 mL growth medium. The infected cells were maintained until the development of characteristic SV40 cytopathic effects; the presence of SV40 was confirmed by PCR, sequencing of the PCR product, and immunofluorescence assays for the SV40 proteins. No SV40 cytopathic effects were formed in negative control CV-1 cells held in parallel. To estimate the extent of virus inactivation, serial dilutions were done on MgCl₂/heat-treated samples, and aliquots were inoculated into CV-1 cells grown in sterile 12-chamber slides. After 24 hours, the cells were fixed in ice-cold acetone and examined by immunofluorescence for Tag. Fields showing well-separated infected cells were chosen and the number of infected cells in MgCl₂/heat-treated samples was compared with those in equivalent heat-treated samples alone.

Results

Testing strategy: PCR analyses. First, using a PCR approach and methods described previously (10), the current or recent seed lots of all three poliovirus serotypes from Belgium, Canada, France, Germany, Indonesia, Iran, Japan, Mexico, United Kingdom, United States, EEVM, Vietnam, and the former Yugoslavia as well as WHO seed viruses supplied to manufacturers were tested at the NIBSC. This is not a comprehensive list of producers. Moreover, except for the EEVM, vaccines that were in use during the years 1965/1966 to 1978, and the UK vaccines prepared in 1982 and 1997, only polio vaccines and WHO seeds in use in 1998 to present were tested in the current studies. Testing of additional samples was reported previously (10). The samples tested consistently negative, except that the EEVM vaccine produced positive PCR signals, and signals were occasionally detected and attributed to possible PCR contamination (although the presence of PCR inhibitors or of very low SV40 amounts could not be completely ruled out) on testing of UK lot 335 (data not shown). These results prompted a detailed multilaboratory investigation of two separate samples from UK lot 335 (one stored at the NIBSC and a new one provided by its manufacturer), of UK lot 314 (control), and of three separate EEVM OPV samples stored at the NIBSC (Table 1; see Materials and Methods).

A new extensive set of PCR analyses was done in Dr. Carbone's laboratory on each of the samples described in Table 1 to search for SV40 sequences. PCR primers were used that amplify the SV40 regulatory region, the SV40 late region that encodes for the capsid proteins VP1, VP2, and VP3, and the SV40 DNA regions that encode the NH₂ and COOH termini of the SV40 Tag (the primers were described in ref. 9). The NH₂ terminus of Tag is conserved among many SV40 strains, whereas the COOH terminus is not and variations in its sequence are important for the identification of SV40 strains and to identify PCR or plasmid contamination (13, 14). Moreover, three different regulatory regions have been described: protoarchetypal, archetypal, and nonarchetypal (13, 14). Non-archetypal regulatory regions are present in commonly studied laboratory strains of SV40 (these strains were formerly called wild-type SV40) and in most plasmids containing SV40 DNA (13, 14).

All three UK samples and all negative controls repeatedly tested negative with all primers (Fig. 1A and B). EEVM OPV sample 39 repeatedly tested positive with all primers. EEVM sample 44 repeatedly tested positive in nested PCR reactions but only sporadically in direct PCR reactions. To test for the presence of possible PCR inhibitors, we added pSV21-N to each PCR preparation and positive controls (13, 14). This is a SV40 strain 776-based plasmid, which contains engineered *SalI* and *XhoI* restriction sites in the nonarchetypal regulatory region to allow the differentiation of this control DNA from other SV40 DNAs (a further precaution to detect possible PCR contamination). Using this approach, the presence of a PCR inhibitor was detected in EEVM OPV sample 40 (Fig. 1C). After purification through G25 spin column, SV40 DNA was detected in this sample (Fig. 1D). Direct DNA sequencing of these PCR products suggested the presence of SV40 strain VA-45-54-1 in EEVM sample 39 and SV40 strain CPC-MEN in EEVM sample 40. However, the presence of overlapping peaks in the DNA sequence from sample 39 suggested the possibility that CPC-MEN was also present in this sample. This was tested using specific primers for the COOH terminus of Tag of CPC-MEN that allowed the identification of a sequence like that of CPC-MEN in EEVM sample 39. In summary, PCR analyses indicated that the UK samples were SV40 free and that three EEVM samples

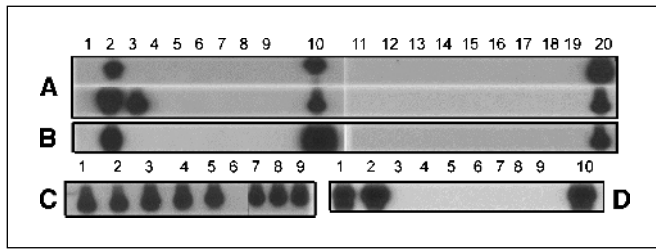


Figure 1. Representative Southern blot hybridization of PCR products obtained after amplification of DNA extracted from UK and EEVM poliovirus vaccines (for technical details, see ref. 9 and Materials and Methods). *A*, hybridization of PCR products obtained using primers specific for the Tag NH₂-terminal portion (P_{YV} primers). *Top*, regular PCR; *bottom*, nested PCR. *B*, hybridization of PCR products obtained using primers specific for the Tag COOH-terminal portion (TA primers). *Lane 1*, EEVM sample 40; *lane 2*, EEVM sample 39; *lane 3*, EEVM sample 44; *lanes 4 to 9*, negative controls; *lane 10*, SV40 strain 776 positive control; *lane 11*, UK 10; *lane 12*, UK 12; *lane 13*, UK 16; *lanes 14 to 19*, negative controls; *lane 20*, SV40 strain 776 positive control. *C*, Southern blot hybridization of PCR products obtained after amplification of DNA extracted from UK and EEVM polio vaccine samples spiked with SV40 before DNA extraction to test for possible PCR inhibitors. PCR products were obtained using the same set of primers used in (*B*). *Lane 1*, UK 10; *lane 2*, UK 12; *lane 3*, UK 16; *lanes 4 and 5*, SV40 strain 776 positive control; *lane 6*, EEVM sample 40; *lane 7*, EEVM sample 39; *lane 8*, EEVM sample 44; *lane 9*, SV40 strain 776 positive control. Note that sample 40 fails to produce PCR amplification even when SV40 was spiked into the vaccine before extraction, indicating the possible presence of a PCR inhibitor in sample 40 vaccine that was not removed using the standard DNA extraction protocol. *D*, Southern blot hybridization of PCR products obtained after amplification of DNA extracted from UK and EEVM poliovirus vaccines after an additional gel filtration purification step. PCR products were obtained using the same set of primers used in (*B* and *C*). *Lane 1*, sample 40; *lane 2*, sample 39; *lane 3*, sample 44; *lanes 4 to 9*, negative controls; *lane 10*, SV40 positive control. Note the positive result obtained after gel filtration of sample 40. Sample 39 is positive, and sample 44 contains low amounts of SV40 that become detectable only in nested reactions.

contained SV40 DNA. EEVM sample 40 initially produced false-negative results because of the presence of a PCR inhibitor, and sample 44 repeatedly tested positive only in nested reactions, suggesting that a low amount of SV40 DNA was present.

Poliovirus analyses to test the integrity of the samples. We quantified the amount of poliovirus present in each vaccine as an indirect measure of the integrity of viral nucleic acids in the samples. This was done using real-time PCR according to a previously published protocol (15). The highest amounts of poliovirus RNA were detected in the UK samples and in EEVM sample 39 (Fig. 2). The lowest amounts of poliovirus RNA were found in EEVM sample 44, the one that tested reproducibly SV40 positive only in nested PCR reactions. Northern blot analyses (data not shown) showed a correlation with the real-time PCR analyses and indicated that the RNA in EEVM sample 44 was mostly degraded and that the best quality RNA was present in the two separate samples of UK lot 335 and in EEVM sample 39. These data indicate that the UK samples were adequate for testing and did not contain SV40. In contrast, the EEVM samples contained poliovirus RNA that was partially to mostly degraded: the efficiency of SV40 detection in these samples correlated with the quality of the RNA.

Transfection analyses to test for the presence of intact SV40 DNA. We transfected DNA isolated from vaccine samples into CV-1 cells (derived from African green monkey kidneys). These cells are permissive for SV40 replication, and the virus produces characteristic vacuoles. Vacuolization was detected in CV-1 cells 2 to 5 days after transfection with DNA from each of the three EEVM vaccines and after 24 hours in cells transfected with the positive control SV40 strain 776 DNA. Low molecular weight DNA was extracted

and whole viral genomes were cloned into pUC19 and representative clones were fully sequenced. These analyses confirmed 100% homology with SV40 VA-45-54-1 and CPC-MEN in EEVM sample 39 and CPC-MEN in EEVM samples 40 and 44.

Infection studies: poliovirus and SV40. Next, we tested for the presence of infectious poliovirus in the UK and EEVM samples. This test was done by adding 1 μ L of each vaccine to 75-cm² tissue culture flasks containing CV-1 cells. All cells developed characteristic poliovirus cytopathic effects (swelling of the cells and cell detachment) and all cells were lysed within 3 days. Sample 44 did not produce any cytopathic effect. The test was repeated by adding 14 μ L of sample 44 and still no cytopathic effects could be detected after 60 days, indicating that this sample did not contain infectious poliovirus. These findings were consistent with RNA degradation in this sample. These results suggested that the vaccine samples (except for EEVM sample 44) should have been adequate for testing whether infectious SV40 was present.

To test for the presence of infectious SV40, we added 14 μ L of each polio vaccine to CV-1 cells in the presence of rabbit anti-poliovirus serum type 1 or 3 depending on the vaccine being tested. Characteristic SV40 vacuolization was detected 14 to 17 days after inoculation in EEVM samples 39 and 40 (Fig. 3); thus, these vaccines seemed to contain infectious SV40. Vacuolization was not observed in EEVM sample 44 and in any of the UK samples even when the experiment was repeated by inoculating 74 μ L of the vaccine and subsequent incubation for 60 days. SV40 Tag and VP-1 immunostainings confirmed that vacuolization was caused by SV40 (Fig. 3). DNA was extracted from the vacuolated cells and full viral genomes were cloned in pUC19 and representative clones were fully sequenced. The results confirmed the presence of infectious SV40 strains VA-45-54-1 and CPC-MEN in EEVM sample 39 and CPC-MEN in EEVM sample 40.

In summary, three different technical approaches (PCR, transfection, and direct infection of susceptible cell cultures) showed the presence of two separate SV40 strains in EEVM.

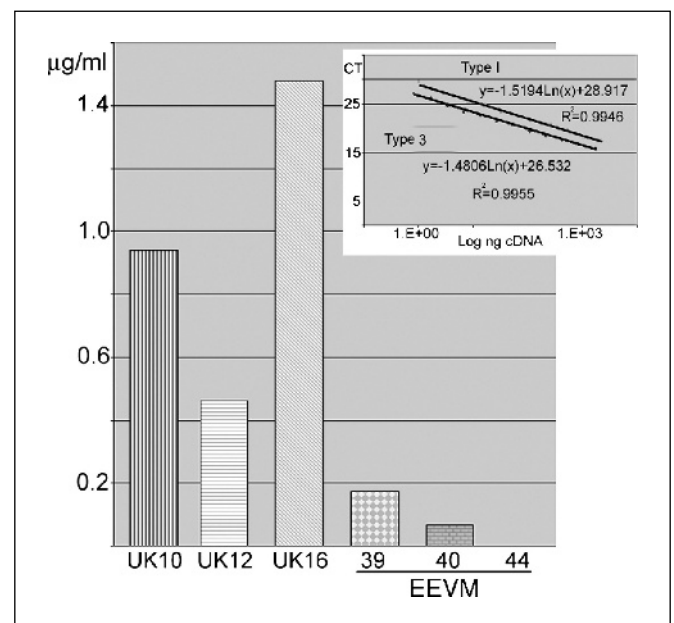
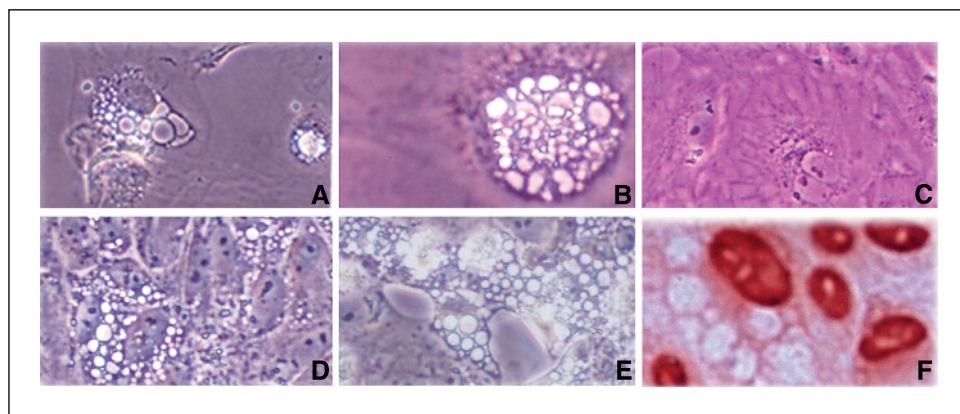


Figure 2. Concentration of type 1 and 3 poliovirus in the UK and EEVM polio vaccines/seed using quantitative SYBR Green real-time PCR. *Inset*, calibration curves determined by using type 1 and 3 poliovirus cDNA.

Figure 3. Characteristic SV40 cytopathic effects and immunohistochemical detection of SV40 Tag. CV-1 cells lipofected with (A) SV40 strain 776 positive control, (B) EEVM sample 44, and (C) negative control (no DNA). CV-1 cells infected with (D) EEVM sample 39 and (E) EEVM sample 40. Vacuolated cells characteristic of SV40 in CV-1 cells are evident in (A, B, D and E). Photographs ($\times 200$) were taken 3 days (A-C), 14 days (D), and 17 days (E) after infection or lipofection. F, CV-1 cells infected with EEVM 39 and immunostained for SV40 Tag. Photograph ($\times 400$) taken 2 days after infection shows vacuolated cells with Tag-positive nuclei.



Tests to identify the cause of the residual SV40 contamination in eastern European vaccine manufacturer oral poliovirus vaccines. We asked why SV40 was still present in the EEVM vaccines but had been successfully removed from the UK vaccines. The EEVM received its seed stocks from Dr. Sabin in the late 1950s. These stocks were later found to be contaminated with 10^5 pfu/mL SV40 (5). Moreover, $\sim 47\%$ of the rhesus monkeys used to prepare polio vaccines were infected with SV40 (5). In 1962, the EEVM switched to primary African green monkey cells that were supposedly SV40 free, and rigorous quality-control measures to test for the presence of SV40 in these monkeys were implemented (5). In addition, Sabin poliovirus stocks were treated with a procedure proposed in 1961 (16) to remove live SV40 (5). The procedure involved thermal inactivation of SV40 under conditions where poliovirus was selectively protected by the addition of 1 mol/L $MgCl_2$. Heating at $50^\circ C$ for 1 hour in the presence of 1 mol/L $MgCl_2$ was followed by growth in cell culture and was shown to “decrease or eliminate SV40 infectivity” (16). Because no other validation of this SV40 elimination procedure was published, and because testing procedures available for SV40 detection in the early 1960s may have not been as sensitive as current techniques, we attempted to evaluate the efficacy of this procedure. Three different quantities of SV40 (3×10^7 , 3×10^5 , and 3×10^3 pfu) were mixed with an equal volume of 2 mol/L $MgCl_2$ (1 mol/L final concentration) and heated at $50^\circ C$ for 1 hour (16). CV-1 cells were then infected with serial dilutions of the treated SV40 samples. This treatment caused $\sim 80\%$ reduction of Tag-positive staining cells infected with 3×10^7 pfu SV40, $\sim 87\%$ reduction of 3×10^5 pfu SV40, and up to 92% reduction of 3×10^3 pfu SV40, respectively (see Table 2, which shows the average of three independent experiments). Thus, $MgCl_2$ /heat treatment of the samples resulted in incomplete reduction of virus viability. In 1962, after $MgCl_2$ /heat inactivation of the Sabin seed viruses, the EEVM did plaque purification of seed stocks in African green monkey cells (5). This step should have further reduced the risk of infectious SV40 present in subsequent vaccines. However, some SV40 may have

contaminated these plaques. Alternatively, SV40 was reintroduced into the EEVM vaccines by cultivating the vaccine in African green monkey cells. Green monkeys are usually free of SV40 but are susceptible to SV40 infection. Of note, SV40 strain VA-45-54 (one of the strains we detected in this study) was derived from primary African green monkey cells (17). The other strain that we detected, CPC-MEN, was originally isolated in 1984 from a human brain tumor in a German patient (18). The same virus was independently isolated from a brain tumor of a U.S. patient in 1995 (14).

Discussion

Our results indicate that heat inactivation in the presence of $MgCl_2$ failed to completely inactivate SV40 in poliovirus seed stocks. In contrast, inactivation procedures based on antibody treatment (19) used by most other manufacturers seem to have been successful as evidenced by the absence of SV40 from OPV made by any other manufacturer. As a note of precaution, we cannot completely rule out the hypothesis that in some vaccine preparations SV40 titers dropped and became undetectable during the years. Additional information about the procedures used to remove SV40 from polio vaccines can be found in refs. 3–5; ref. 20 discusses possible problems associated with some of these procedures. In addition to the seed stocks prepared in the 1950s, the choice of the cell substrate used to prepare vaccines is critical to prevent possible additional sources of infection for humans by SV40 or by other monkey viruses. In this regard, the hypothesis that rhesus monkeys have not been used for vaccine production since the early 1960s when it was discovered that they were often infected with SV40 (e.g., see refs. 3–6, 19) is incorrect. Presently, in China, $\sim 50\%$ of oral attenuated poliovirus vaccines are still prepared in primary rhesus monkey kidney cells. The manufacturer that prepares OPV in these primary rhesus cells relies on screening rhesus and derived cell cultures for SV40 to prevent human infection. We did not receive these vaccines for testing.

Our findings underscore that there is a risk in using primary monkey kidney cells for preparing vaccines, because monkey cells can be infected with SV40 (and with other monkey viruses) and it may be difficult to completely eliminate or detect (9) this contamination. Instead, our results support the use of well-characterized continuous cell lines for vaccine production as originally proposed by Hayflick et al. (ref. 21; see also ref. 4, chapter 10).

When vaccines are prepared in monkey cells, the most sensitive testing should be used to try to detect possible viral contaminants. These tests have usually been done in green monkey cells exposed

Table 2. Decreased SV40 infectivity after $MgCl_2$ and heat inactivation

Input SV40 (pfu)	3.0×10^7	3.0×10^5	3.0×10^3
Residual SV40 (pfu)	$6.0 \times 10^6 \pm 1.05 \times 10^6$	$3.9 \times 10^4 \pm 0.84 \times 10^4$	$2.4 \times 10^2 \pm 0.91 \times 10^2$

in vitro to vaccine aliquots and observing these cells for 2-week cycles for degeneration and vacuolization (4, 19, 20). These tests may not be always sufficiently sensitive to detect occasional slow-growing SV40 strains (9). The possible addition of PCR to the quality-control arsenal improves sensitivity of detection. However, careful attention to the details of the protocol must be paid because, as shown here, low SV40 levels and/or the presence of PCR inhibitors in some vaccines can result in false-negative findings. Therefore, demonstration of the absence of PCR inhibitors must be a part of quality-control testing (e.g., by spiking the samples with SV40). We found that transfection of the DNAs extracted from vaccines into green monkey cells that are permissive for viral replication was the most sensitive way to detect SV40 contamination. We hope that this technical approach will be useful to those who may test future vaccines prepared in monkeys for possible viral contaminants.

The EEVM samples that we tested were produced in 1966 and 1969; therefore, we show that SV40 contaminated some polio vaccines at least until that period. The EEVM vaccine samples we tested (Table 1) were produced with the same seed virus that was used until 1978, apparently without any further purification to remove SV40. Therefore, it is possible that the EEVM vaccines may have remained SV40 contaminated until 1978 when a new seed was produced. We have no information about this seed virus or vaccines produced from it. Therefore, based on our data, we could not determine the exact time when EEVM vaccines became SV40 free but can suggest that it may have happened in the 1980s when EEVM switched to seed virus stocks provided by the WHO that we found to be free of SV40.

The finding of live SV40 in EEVM vaccines suggests that epidemiologic studies done to explore possible links between the exposure of human populations to SV40 and the risk of developing cancer should consider that large blocks of population may have been vaccinated with vaccines containing live SV40. Vaccines produced by the EEVM were widely used in many countries, including the USSR, the countries of eastern Europe, Asia, and Africa. Extensive migration of people from former eastern European countries into the West may have influenced the results of epidemiologic and molecular studies in which cohorts born before and after 1961 were compared for SV40 infection and cancer incidence. In fact, previous epidemiologic studies to test for possible negative health outcomes associated with the administration of SV40-contaminated poliovirus vaccines (reviewed in refs. 8, 22) were based on the assumption that all poliovirus vaccines produced after 1961/1962 were free of SV40, a hypothesis that is not supported by our results. Our findings also suggest that geographic differences in exposure to SV40-contaminated vaccines may exist and that these differences extended well beyond 1962. Therefore, our results provide support to the hypothesis that geographic differences may account for some of the differences in the percentage of SV40-positive samples that were detected in different cohorts (23–26). Animal experiments showed that SV40 was highly oncogenic on injection but not on oral administration (2). Therefore, it should not be automatically inferred that SV40-positive tumors in the eastern world were linked to the administration of oral polio vaccines. At the same time, it should be emphasized that there are major differences among humans and hamsters that make the former susceptible to oral infection and the latter resistant (2). Hamsters and rodents are nonpermissive to SV40 replication; humans, like monkeys, are. Therefore, in hamsters and rodents (the species in which SV40 oncogenicity was tested), the viral load corresponds to the amount that is given and infection can only occur on direct

injection of the virus into the body. Instead, because human cells are permissive to SV40 infection, millions of viral particles are produced on infection of relatively few cells: accordingly, humans, but not rodents, can be infected through airway or gastrointestinal exposure to SV40 (27, 28). This evidence suggests that the potential risk of oral SV40 administration should not be underestimated.

A recent seroprevalence study of SV40 infection in Kazakhstan, which is in the geographic area of distribution of the contaminated EEVM vaccines, reported that 60% of the subjects seropositive for SV40 were born from 1960 to 1980s (29). The authors of this report noted that this was “a time period in which the vaccines should have been expected to be free of SV40 (29).” Our results provide a possible explanation for these findings because it is possible that the vaccines distributed in Kazakhstan from 1960 to 1980 were not SV40 free.

In addition, our results confirmed that, when careful precautions are taken (see Materials and Methods), PCR studies to detect SV40 DNA or other tumor viruses are reliable. We did not find evidence of PCR/plasmid contamination; however, we found that low SV40 DNA amounts or the presence of PCR inhibitors, which can be present in DNA extracted following standard procedures, as observed in EEVM sample 40, could cause false-negative results.

SV40 is a DNA tumor virus (1). Early studies showed that SV40 induced chromosomal aberrations and caused malignant transformation of human cells in culture (30). SV40-transformed human cells grew as s.c. tumor nodules when injected into human volunteers, suggesting that SV40 could promote tumor growth in humans (31). Some human cell types, such as mesothelial cells, were shown to be more susceptible than others to SV40 infection and transformation (32, 33). Recent studies have associated SV40 with some human mesotheliomas, brain tumors, lymphomas, and osteosarcomas and have elucidated possible molecular mechanisms of carcinogenesis (reviewed in refs. 1, 34). For additional information, see ref. 1 and the conclusions of three different panels that have reviewed the evidence in favor and against a link between SV40 and human cancers and/or among SV40-contaminated vaccines and human cancers (6–8). The detection of SV40 in human tumors and the biological significance of this finding have been controversial, because although >50 different laboratories reproduced these findings some did not and either failed to detect SV40 at all or, more frequently, detected SV40 only in a small percentage of samples (reviewed in refs. 1, 2, 6–8, 34). These discrepancies have been attributed to geographic differences (23–26), technical deficiencies (1, 2, 34), and plasmid contamination (35). Moreover, in the latter study, the amount of “true” SV40 (i.e., not due to contamination) that was detected in ~6% of the mesothelioma biopsies tested was considered too low to be etiologically relevant (35). An alternative hypothesis is that SV40 alone may be insufficient to cause cancer in humans but that it may be a cocarcinogen (e.g., with asbestos in causing mesothelioma). This hypothesis was based on the observed cocarcinogenesis between SV40 and asbestos in causing transformation of human mesothelial cells in tissue culture (32). Very recently, this hypothesis received independent *in vitro* and molecular-epidemiologic support (36–38).

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