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Advances in Brief

Unique Strains of SV40 in Commercial Poliovaccines from 1955 Not Readily Identifiable with Current Testing for SV40 Infection[1]

Paola Rizzo, Ilaria Di Resta, Amy Powers, Herbert Ratner,[2] and Michele Carbone[3]

Loyola University Medical Center, Cardinal Bernardin Cancer Center, Cancer Immunology Program, Department of Pathology, Maywood, Illinois 60153

Abstract

SV40 was first identified as a contaminant of poliovaccines used from 1955 until 1963. Recently, SV40 has been detected in several human tumors. The virus detected in human tumors often contained only one 72-bp enhancer in the regulatory region, in contrast to the SV40 originally isolated from poliovaccines, which contained two 72-bp enhancers. The origin of viruses with one 72-bp enhancer in humans was unknown, because it was thought that these viruses were not present in poliovaccines. It was also thought that all poliovaccine vials produced from 1955 until 1963 had been discarded, thus the possibility that one 72-bp virions contaminated those vials could not be tested. We unexpectedly obtained what appear to be the last available vials of poliovaccine produced in 1955. In these vials, we detected and sequenced SV40 containing only one 72-bp enhancer in the regulatory region. The tissue culture cytopathic test currently used in the United States to screen oral poliovaccines was designed to detect rapidly proliferating SV40 virions containing two 72-bp enhancers. We found that this test is not sensitive enough to detect low amounts of the slow-replicating SV40 virions containing one 72-bp enhancer. This virus was easily detected in the same cells by immunostaining and PCR. Twelve current vials of poliovaccines tested uniformly negative for SV40, suggesting that the precaution of preparing poliovaccines from kidneys obtained from monkeys bred in isolated colonies prevented SV40 contamination. Our data demonstrate that humans were exposed to SV40 viruses with both one 72-bp enhancer and two 72-bp enhancers SV40 through contaminated vaccines. Our data also suggest that instead of cytopathic tests, immunohistochemical and/or molecular studies should be used to screen poliovaccines for SV40 to completely eliminate the risk of occasional contamination.

Introduction

The development and distribution of poliovaccines that began in the early 1950s have virtually eliminated paralytic poliomyelitis and have been of unquestionable benefit to the entire human race. However, questions concerning the safety of the vaccine were raised in 1959 when Bernice Eddy found that hamsters injected with poliovaccine preparations developed sarcomas and suggested that a virus was contaminating poliovaccines. In 1960, Sweet and Hilleman reported that a monkey virus called SV40 contaminated both Salk and Sabin poliovaccines and caused the tumors observed by Eddy in hamsters (reviewed in Ref. 1). Poliovaccines were produced in kidney cell cultures derived from rhesus, green, and patas monkeys (reviewed in Ref. 2). Rhesus monkeys are natural hosts of SV40, and, in captivity, related species caged with infected animals, including the cynomolgus macaque and African green monkey, are also easily infected (2). SV40 infection appears harmless in immunocompetent hosts, indicating that monkeys carrying the virus showed no obvious signs of illness and thus were not excluded for use in vaccine production. Thus, it has been estimated that millions of people worldwide were inadvertently exposed to SV40 through contaminated vaccines administered from 1955 to 1963 (reviewed in Refs. 1–3). Similar to poliovaccines, adenovaccines 3 and 7 distributed to a limited extent among military and civilian personnel between 1961 and 1965 were shown to contain SV40 sequences (4).

Soon after its discovery, SV40 was shown to be an oncogenic papovavirus that is capable of inducing tumors in hamsters, mastomys, and some strains of mice and of transforming human cells in tissue culture. Recently, SV40 DNA sequences have been detected in several human tumor types, including mesotheliomas, ependymomas, and osteosarcomas (reviewed in Refs. 2 and 3).

The SV40 genome is composed of a closed circular double-stranded DNA molecule (5.2 kb) that codes for six genes, including the large and small t antigens, the primary oncoproteins of the virus (reviewed in Ref. 2). The nontranslated regulatory region of the virus spans approximately 400 bp and is comprised of four regions: (*a*) the origin of replication (ori); (*b*) the G + C-rich domain, also called the 21-bp repeat region; (*c*) the enhancer region, which includes a 72-bp element that is sometimes partially or completely duplicated; and (*d*) the region containing the late-promoter/initiator (reviewed in Ref. 2). Studies have revealed that although there is only a single SV40 serotype, different strains of SV40 exist that are characterized primarily by differences in the COOH-terminal Tag[4] sequence (5). Furthermore, three different general arrangements of the regulatory region are encountered in SV40, which, according to Butel and Lednicky (2), are: (*a*) a protoarchetypal arrangement that lacks a duplicated sequence within the G + C-rich domain and contains a single 72-bp enhancer element (6, 7); (*b*) an archetypal arrangement, which contains duplications within the G + C domain and a single 72-bp enhancer element (6, 8); and (*c*) a nonarchetypal arrangement, which contains partial or complete duplications of the 72-bp enhancer element as well as duplications within the G + C-rich domain and is also known as SV40 wild type (6, 7). Butel and Lednicky have proposed calling SV40 strains with one 72-bp enhancer sequence "archetypal" and calling those with two 72-bp enhancer sequences "nonarchetypal" (2).

Primary isolates of SV40 from healthy and immunocompromised monkeys usually contain archetypal regulatory regions (6–9), although nonarchetypal strains have also been isolated from SIV-infected monkeys (6). In contrast, nonarchetypal (wild-type) SV40 variants SV40-776, SV40-VA45-54-2, and SV40-Baylor-2 were isolated from poliovaccines or adenovaccines or from uninoculated cell cultures derived from monkeys in the early 1960s (2). These isolates had a nonarchetypal regulatory region; thus, initially it was not appreciated that there were different variants of SV40. Most of the

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[2] H. R. is deceased.

[3] To whom requests for reprints should be addressed, at Loyola University Medical Center, Cancer Center, Building 112, Room 205, 2160 S. First Avenue, Maywood, IL 60153.

[4] The abbreviations used are: Tag, SV40 large T antigen; pfu, plaque-forming unit(s); MOI, multiplicity of infection; nt, nucleotide; RB, retinoblastoma; SIV, simian immunodeficiency virus; Ab, antibody.

experiments during the last 40 years have been conducted with SV40 strain 776, which was commonly called wild-type SV40.

Whereas the original source(s) of SV40 in the human population is unknown, it has been assumed that widespread human exposure occurred via contaminated poliovaccines administered in the period from 1955–1963. It has been traditionally thought that all batches of contaminated vaccines contained nonarchetypal SV40, because only nonarchetypal SV40 variants (SV40-776 and SV40-Baylor-2) were isolated from these vaccines. Secondly, SV40 strains with nonarchetypal regulatory regions were shown to outgrow archetypal versions in culture (10). This observation suggested that although predominantly archetypal strains were isolated from the monkeys used to make these vaccines, it was likely that only nonarchetypal SV40 strains were present or predominated in the final vaccine preparations, due to their growth advantage in culture. Thus, if contaminated poliovaccines were a major source of SV40 infection in humans, it was thought that the SV40 present in human tumors would be nonarchetypal in nature. Surprisingly, archetypal SV40 has been detected in several human tumors (2), suggesting the possibility that human exposure to SV40 with archetypal regulatory regions occurred via a separate unknown cross-species transfer event. It should be noted that archetypal SV40 has been detected primarily in ependymomas that developed in children born after 1963, who were therefore not exposed to SV40-contaminated vaccines. Nonarchetypal SV40 has been often detected in mesotheliomas, which develop in an older population potentially exposed to contaminated vaccines.

To determine whether SV40 with archetypal regulatory regions might also have been present in poliovaccines distributed between 1955 and 1963, examination of lots prepared during this period would be necessary. However, this type of analysis seemed impossible, because vaccines from the aforementioned period were reportedly no longer in existence. In our own search, we attempted to obtain these vaccines from the manufacturers and agencies responsible for their distribution. All of the sources that we contacted, however, no longer had any of the lots in stock. We were unexpectedly able to acquire what appear to be the last remaining vials of poliovaccine produced from 1955–1963, allowing us to investigate the SV40 strains present in these early vaccines. Furthermore, because of the results of our analyses, we investigated whether the cytopathic test currently used in the United States to screen poliovaccines for SV40 is sufficiently sensitive to detect slow-growing archetypal SV40 virions.

Materials and Methods

Poliovaccine Vials. Two sealed 9-ml vials of poliovaccine manufactured by Parke Davis were analyzed in this study: (*a*) lot #028863B, (expiration date, October 15, 1955); and (*b*) lot #028846B, (expiration date, October 12, 1955). These vials, along with five additional unopened vials, were kindly given to us by Dr. Herbert Ratner, who received them from the National Foundation of Infantile Paralysis when he served as the Director of Public Health in Oak Park, Illinois. He stored these seven vials together in his refrigerator for future analysis until 1997, when he gave them to our laboratory for testing. Dr. Ratner died in December 1997. Five of these vials were labeled lot #028846B, one vial was labeled lot #028863B, and the remaining vial was unlabeled. Oral poliovaccines, prepared in primary monkey cells (Lederle, Pearl River, NY) and parenteral poliovaccines prepared in cultures of Vero cells (Pasteur Merieux, Lyon, France) from 1996 were purchased from the Pharmacy of the University of Chicago Hospitals and analyzed in parallel with the 1955 vials.

SV40 Detection and Sequencing. A total of 40 μl of vaccine were added to 10 μl of 5× lysis buffer containing 50 mM Tris-HCl (pH 8.0), 2.5% Tween 20, and proteinase K (0.4 mg/ml), and incubated at 55°C for 1 h, followed by inactivation for 10 min at 95°C. The sample was transferred onto ice and analyzed immediately by PCR. The initial screening was performed using the sets of primers routinely used in our laboratory to screen samples for the presence of SV40: (*a*) SV2/SV.rev to amplify the RB-binding pocket of the large T antigen; (*b*) 2902/2573 to amplify the large T antigen COOH terminus; (*c*) R1/R2 to amplify part of the origin of replication and early region promoter; and (*d*) L1/L2 to amplify the VP1 capsid protein. Sequences of the primers and PCR cycling conditions were as described previously (11). The identity of the PCR products was determined by Southern blot using an internal radioactive oligoprobe specific for SV40 and by direct sequencing. Thirteen new sets of primers were then synthesized to sequence the entire SV40 genome from the vaccine. The primers were: (*a*) agnoprotein LP-1 primers R1.rev/R5 (262 bp; R1.rev, 245–266; R5, 482–506); (*b*) VP2 minor structural protein primers R6/R7 (338 bp; R6, 461–484; R7 (772–798); (*c*) VP2/VP3 minor structural protein primers R8/R9 (345 bp; R8, 796–820; R9, 1118–1140); (*d*) VP2/VP3 minor structural protein primers R10/R11 (309 bp; R10, 1138–1161; R11, 1423–1446); (*e*) VP1/VP2/VP3 minor structural protein primers R12/R13 (310 bp; R12, 1441–1463; R13, 1727–1750); (*f*) VP1 minor structural protein primers R14/R15 (297 bp; R14, 1748–1771; R15, 2021–2044) and R16/LA1REV (235 bp; R16, 2040–2062; LA1REV, 2251–2274); (*g*) VP1/large T primer LA3/LA4 (344 bp; LA3, 2540–2560; LA4, 2866–2883); and (*h*) large T primers V7/V8 (372 bp; V7, 3233–3254; V8, 2883–2909), V5/V6 (397 bp; V5, 3615–3644; V6, 3248–3269), V3/V4 (387 bp; V3, 3996–4019; V4, 3633–3656), V1/V2 (372 bp; V1, 4359–4381; V2, 4011–4034), and ILA1/ILA2 (363 bp; ILA1, 4873–4896; ILA2, 5212–5235). PCR conditions were as described previously (11). The average size of the fragment was 330 bp, and annealing was at 58°C. The fragments obtained by PCR were gel-purified and sequenced.

Cytopathic Test to Detect SV40. SV40-776 with one 72-bp enhancer sequence and SV40-776 containing two 72-bp enhancer sequences were kindly provided by John Lednicky (Baylor College of Medicine, Houston, TX). The cytopathic test was performed according to the protocol currently used in the United States to screen poliovaccines for SV40 (12). Viral titers were determined in TC-7 (Green monkey kidney cells) according to the procedure described in Ref. 13. TC-7 cells were seeded in 6-well plates containing 5% FCS-DMEM and infected in triplicate with different MOIs of 1, 10^{-2}, 10^{-3}, and 10^{-4}/pfu cell. Three wells were left uninfected as negative controls. The plates were observed daily and fed every 4 days. Thirteen days after the infection, cells from one well for each MOI were scraped, reseeded on a tissue culture coverslip, and analyzed by immunohistochemistry the following day for T antigen expression using the monoclonal Ab 419 from Oncogene Science as described previously (14). On day 14 after the infection, the supernatant from the infected cells was removed, spun, and added to new wells containing 70% confluent TC-7 cells. These wells were observed daily for 2 weeks.

Results

Virus Detection and Sequencing

A sample from a 1955 poliovaccine (lot #028846B) obtained from Dr. Ratner was first analyzed for the presence of the COOH terminus (Fig. 1*A*) and the RB binding pocket of Tag. Six 1996 samples of oral poliovaccine and six samples of parenteral poliovaccine were simultaneously analyzed and served as negative controls (Fig. 1*B*). All of the 1996 vaccines tested negative for these SV40 sequences, whereas the 1955 vaccine tested positive with both sets of primers. We then tested a second sealed vial from the same lot (lot #028846B) for these sequences. Using the same primers described above, we amplified identical viral sequences corresponding to the COOH terminus and RB binding pocket of Tag. We then analyzed the vaccine with the R1/R2 set of primers that amplify the origin of replication and the early region promoter. The vaccine tested positive with this set of primers; however, the band observed from the vaccine was smaller than the band obtained from the positive control SV40 strain 776. Sequencing analysis of this band showed that the SV40 early region promoter present in vaccine lot #028846B contained only one 72-bp enhancer sequence as opposed to SV40-776, which contained two 72-bp enhancer sequences (Fig. 2, *A* and *B*).

To sequence the entire SV40 genome from lot #028846B, we synthesized 13 sets of primers for the amplifications of fragments of SV40 DNA with an average size of 330 bp. Using these primers, we

sequenced the entire SV40 genome from the poliovaccine vial. To read the sequences in the region where the primers annealed, several combinations of primers were used to amplify overlapping fragments of DNA. Therefore, as shown in Fig. 1*C*, each region of the SV40 genome was sequenced with at least one and usually three different set of primers.

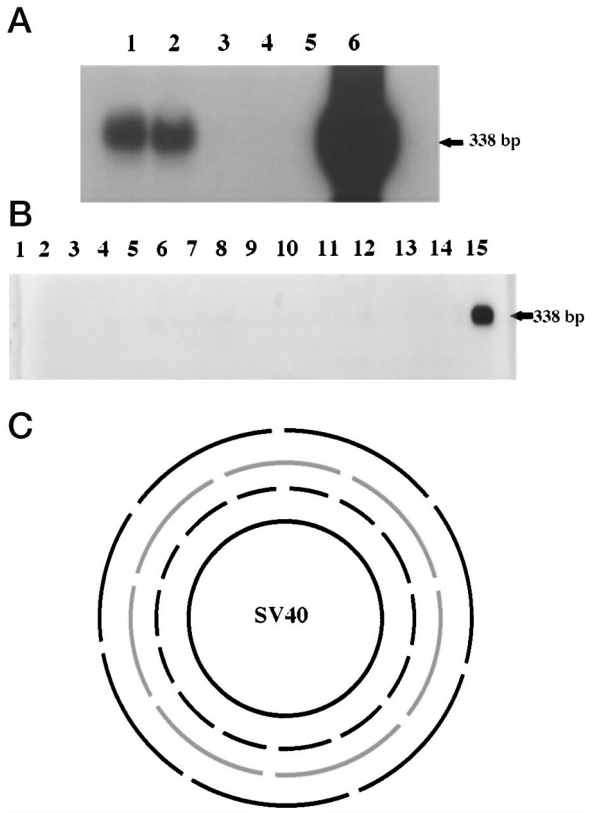

Fig. 1. *A*, Southern blot hybridization of PCR products obtained from the 1955 poliovaccine with the primer set 2902/2573. *Lane 1*, 10 μl of poliovaccine; *Lane 2*, 2 μl of poliovaccine; *Lanes 3* and *5*, empty; *Lane 4*, mock DNA extraction; *Lane 6*, SV40 DNA positive control. *B*, Southern blot hybridization of PCR products obtained from 1996 poliovaccines with the primer set 2902/2573. *Lanes 1–6*, 10 μl of oral poliovaccine; *Lanes 7–12*, 10 μl of parenteral poliovaccine; *Lane 13*, mock DNA extraction; *Lane 14*, water; *Lane 15*, SV40 DNA positive control. *C*, scheme showing the PCR strategy used to sequence the SV40 genome from the poliovaccine. The *inner circle* represents the first set of PCRs amplifying fragments of an average size of 330 bp. Only 14 of the 17 amplified fragments are shown due to space limitations. The *empty space* between fragments represents the region where the primers anneal. The *middle* and *outer circles* represent different combinations of the above cited primers to read the SV40 sequence in the region where the primers anneal and to confirm the polymorphisms detected.

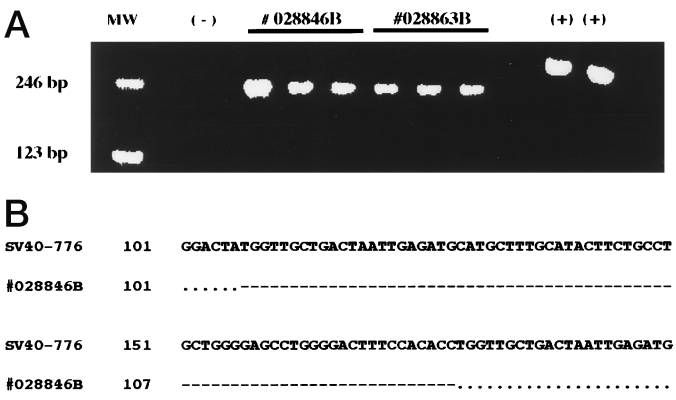

Fig. 2. *A*, products of PCR amplification with the R1/R2 set of primers from two different lots of 1955 poliovaccines (ethidium bromide staining of 2% agarose gel). Each lot was tested in triplicate: −, H_2O negative control, +, SV40 DNA positive control. *B*, the alignment of the sequence of SV40-776 origin of replication and early region promoter with the sequence obtained from the poliovaccine 028846B shows a deletion of one of the two 72-bp enhancers in the poliovaccine. •, homology; −, deletion.

Table 1 *Polymorphisms found in PV028846B and their location in the SV40 genome*

nt no.	SV40-776	Vaccine isolate
36	G	A
107–178		del. 72 bp
732	T	A
747	C	A
1756	A	C
1939	T	A
2239	T	G
2716–2721	TGGGAG	del. 6 bp
2751	A	G
2757	G	A
2766–2771	ATTATG	del. 6 bp
2795	—	TGAGGGCTG
2813	C	T
2851	T	A
2908–2913	TCATCA	del. 6 bp
2950	A	G
3117	T	C
3727	C	G
3755	A	G
3873	C	T
4834	G	A
4839	C	T
4879	C	T

Sequencing of the SV40 contained in lot #028846B revealed a number of nucleotide differences compared to the reference strain SV40-776. These differences are presented in Table 1. Among them, we found a 9-bp insertion in position 2795 that was previously described in two human osteosarcomas, one choroid plexus tumor, in SVCPC/SVMEN, SV40-B2, and VA45-54 (6). The nt differences observed at nt 2950, nt 2751, and nt 2757 have also been found in human tumors (5, 6) and in monkey isolates (8). Finally, the same nucleotide sequences were detected in a sample of the same vaccine lot that we received from Dr. W. John Martin as an aliquot of a sample sent to him by Dr. Ratner.

Because the majority of nucleotide substitutions that we detected were in the early region promoter and in the COOH end of the Tag gene, we tested only these regions in the second lot of vaccine lot #028863B. Similar to lot #028846B, PCR analysis revealed that lot #028863B contained SV40 that was characterized by an early region promoter containing only one 72-bp enhancer. Furthermore, two slightly different sequences of the COOH end of Tag were detected, both of which were dissimilar to the sequence amplified from vaccine lot #028846B. It has been suggested that the sequence encoding the COOH terminus of Tag characterizes different SV40 strains (10). Thus, lot #028863B contained two different strains of SV40, both with one 72-bp enhancer in the regulatory region (These sequences have been deposited in the GenBank with accession numbers AF180737, AF180738, and AF180739). The COOH terminus of Tag and the early region promoter of each of these three SV40 viruses showed more polymorphisms than other regions (Table 1). In each virus, these regions were sequenced with three different set of primers (Fig. 1*C*), and the results of each of these set primers was confirmed in two separate experiments. Therefore, we are confident about the reliability of the polymorphisms presented in Table 1.

Detection of SV40 Viruses with One 72-bp Infection of TC-7 Cells

The finding of slow-growing archetypal strains in the 1955 poliovaccines raised concerns, because the test used to detect possible SV40 contamination in today's poliovaccines has been designed to visualize rapidly growing nonarchetypal SV40 strains (12). Therefore, we tested whether the screening currently used to investigate possible

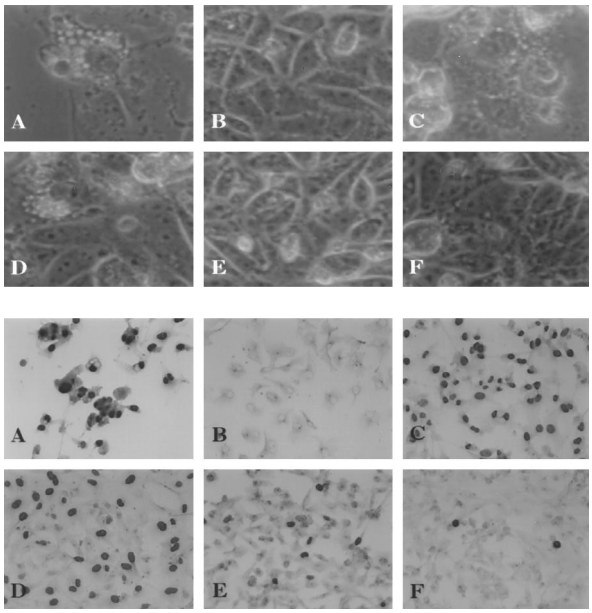

Fig. 3. *Top*, infection of TC-7 cells with nonarchetypal (2 72bP) SV40 at a MOI of 10^{-2} (*A*) and a MOI of 10^{-4} (*C*). Infection of TC-7 cells with archetypal (1 72bP) SV40 at MOIs of 10^{-2} (*D*), 10^{-3} (*E*), and 10^{-4} (*F*). Uninfected TC-7 cells (*B*). The pictures were taken 14 days after infection. (×400). *Bottom*, Tag immunostaining of TC-7 cells infected with nonarchetypal (2 72bP) SV40 at MOIs of 10^{-2} (*A*) and 10^{-4} (*C*). TC-7 cells infected with archetypal (1 72bp) SV40 at MOIs of 10^{-2} (*D*), 10^{-3} (*E*), and 10^{-4} (*F*). The staining was done on day 14 after the infection. Uninfected TC-7 cells (*B*). (×200).

poliovaccine contamination by SV40 would be sensitive enough to detect SV40 virions with one 72-bp fragment. TC-7 cells were infected with either archetypal or nonarchetypal SV40. Infections were performed at four different MOIs (1 pfu/cell, 10^{-2} pfu/cell, 10^{-3} pfu/cell, and 10^{-4} pfu/cell). At the lowest MOI, about 30 pfu were added to each well.

Cells Infected with Nonarchetypal SV40. In cells infected with a MOI of 1 pfu of SV40/cell, cytopathic and cytolytic effects were seen 4 days after infection as evidenced by intracellular vacuoles, followed by cell lysis and disruption of the cell monolayer. At the lowest concentration of infection, cytopathic and cytolytic effects were seen 11 days after infection. Cells infected with intermediate viral dilutions showed vacuoles and lysis between day 4 and 10 (Fig 3).

Cells Infected with Archetypal SV40. At the MOI of 1 pfu/cell, SV40 induced vacuolization of some cells on day 10. By day 14 occasional cells infected at 10^{-2} pfu/cell showed vacuolization. However, cell lysis was not readily detectable, and the cell monolayer appeared to remain intact until day 19 after infection, when cell lysis was seen in some cells. Cells infected with 10^{-3} and 10^{-4} pfu/cell did not show vacuolization until day 19 (Fig. 3).

Following the protocol currently used to screen poliovaccines for SV40, we then infected fresh TC-7 cells with the supernatants obtained from the infection experiments described above. On day 3, all plates containing the supernatant from cells infected with nonarchetypal SV40, including supernatants from the lowest dilutions, showed clear cytopathic and cytolytic effects. TC-7 cells cultured with supernatants from cells infected with 1 pfu of archetypal SV40/cell showed some vacuoles at the end of the 14-day observation period. However, they lacked clear-cut cytopathic effects. Occasional vacuolated cells were also seen in cells cultured with supernatants from the 10^{-2} pfu/cell dilution. One single cell showed small vacuoles suggestive of a possible cytopathic effect in only one of the dishes containing supernatant from cells infected with 10^{-3} pfu/cell. Cells exposed to supernatants derived from cells infected with 10^{-4} pfu of archetypal SV40/cell showed no vacuoles or lysis and were indistinguishable from uninfected controls at day 14. According to the current protocol used to detect SV40 in poliovaccines, cells are screened for cytopathic effects in 14-day cycles (12). Our data suggest that this test might have failed to detect low amounts of SV40 virions containing only one 72-bp repeat in the regulatory-enhancer region. However, our data confirmed that this test is more than adequate to detect SV40 with two 72-bp repeats.

Tag staining of these same cells 14 days after infection revealed that all of the nonarchetypal SV40-infected cells (those that had not been lysed yet) were Tag positive, including those infected at the concentration of 10^{-4} pfu/cell (Fig. 3). The number of Tag-positive cells in infections with archetypal SV40 was proportional to the amount of initial infection, and occasional Tag-positive cells were even seen at the 10^{-4} dilution (Fig. 3). Therefore, Tag staining is more sensitive than cytopathic tests in detecting SV40 infection with virions containing only one 72-bp repeat.

Discussion

SV40 DNA has been found in several human tumor types, including mesotheliomas (14, 15), ependymomas (16), and osteosarcomas (17). In many cases, these DNA sequences have corresponded to viruses with archetypal regulatory regions (reviewed in Ref. 2). Although the original source(s) of SV40 in humans remains unclear, SV40-contaminated poliovaccines and adenovaccines administered in the 1950s and 1960s may have infected millions of individuals with the virus. However, only nonarchetypal SV40 strains had been previously isolated from vaccines developed during this period (reviewed in Ref. 2). Thus, questions arose regarding the source(s) of SV40 archetypal strains in the human population. We examined what appear to be the last remaining poliovaccines from this time period in existence today. Using PCR analysis and DNA sequencing, we analyzed samples from lots #028863B and #028846B distributed by Parke Davis in 1955 for SV40 DNA. Both contained SV40. Sequencing analysis of the SV40 present in lot #028846B demonstrated the presence of numerous mutations (polymorphisms) compared to the reference strain SV40 776 (see Table 1). Most of these nucleotide differences were localized in the COOH terminus of Tag, similar to SV40 strains found in human tumors and monkeys. In addition, the regulatory region contained only one 72-bp enhancer characteristic of archetypal SV40. Because the majority of the nucleotide differences in the aforementioned lot were localized to the regulatory region and the COOH terminus of Tag, we subsequently analyzed lot #028863B only with primers R1/R2 and 2902/2573. Similar to lot #028846, strains amplified in lot #028863B were archetypal in nature. PCR analysis of the Tag COOH-terminal sequence produced two bands. Sequencing of these bands showed that two strains of SV40 were present in this lot, and both had mutations that were dissimilar to those found in lot #028846B. The presence of polymorphisms and/or deletions was confirmed in two or more separate experiments using different aliquots from the vaccine. Each aliquot was tested with multiple set of primers that amplified overlapping fragments of DNA (see Fig. 1*C*). Several of these polymorphisms were unique in that they were never detected in other SV40 isolates. Together, these findings confirm the reliability of the results and rule out laboratory artifacts including PCR contamination.

Similar to strains found in human tumors and monkey isolates, SV40 strains with one 72-bp sequence in the enhancer of their regulatory regions were found in both lots of the 1955 poliovaccines. To our knowledge, this is the first time that archetypal SV40 has been isolated from a commercial vial of poliovaccines, and these findings indicate that SV40-contaminated poliovaccines are one possible

source of the archetypal strains that have recently been discovered in some human tumors.

Our finding of archetypal SV40 in these lots raised questions regarding the safety of current poliovaccines. Before this study, it was thought that only nonarchetypal SV40 had contaminated poliovaccines, primarily because only this type of virus has been isolated from distributed lots. Furthermore, a 1995 study by Lednicky *et al.* (10) demonstrated that duplication of the 72-bp sequence gives SV40 a growth advantage in culture, because SV40 strains in culture containing two 72-bp sequences in the enhancer region outgrew strains with only one 72-bp sequence. In early cultures of the vaccines, monkey kidney cells would have most likely contained some mixture of archetypal and nonarchetypal SV40 strains, as observed in wild monkeys. However, after several passages in cell culture, it was thought that the faster-growing nonarchetypal strains would have been selected. Thus, in the final preparation of the vaccine, only nonarchetypal SV40 strains would have remained. However, we found that archetypal stains of the virus were still present in the final vaccine product. Currently, some poliovaccines are still made in cultures of monkey cells. The monkeys used as a source of kidney tissue are presumably SV40 free because they are purposely bred in isolated breeding colonies and tested for viral antibodies. As a second measure of protection from SV40 contamination, monkey kidney cell cultures are monitored for cytopathic effects of the virus. We were surprised to learn that neither molecular nor immunohistochemical techniques are used to screen poliovaccines for SV40 (12). Thus, we were concerned that archetypal SV40 may still contaminate some poliovaccines. To test this possibility, we screened six parenteral and six oral poliovaccines produced in 1996 by Pasteur Merieux and Lederle, respectively, for the presence of SV40 DNA. We found that no SV40 sequences corresponding to archetypal or nonarchetypal strains were present in any of the vaccines. To determine whether the current SV40 detection methods used by some poliovaccine manufacturers were adequate to detect archetypal SV40 strains, we then infected TC-7 monkey cells with SV40-776 strains containing either one 72-bp or two 72-bp enhancer sequences and compared how long each took to produce a cytopathic effect in these permissive cells. Whereas SV40-776 with two 72-bp repeats produced a cytopathic effect within 14 days at all MOIs tested, SV40-776 with one 72-bp repeat only demonstrated vacuolization of occasional cells at days 10 and 14 with MOIs 1 and 10^{-2}, respectively. MOIs of 10^{-3} and 10^{-4} did not demonstrate a cytopathic effect until day 19. Subsequent exposure of uninfected TC-7 cells from supernatants derived from these infections confirmed that the cytopathic and cytolytic effects were readily observed only in cells exposed to supernatants from two 72-bp-repeat SV40 infections. Thus, low levels of archetypal SV40 may be difficult or impossible to detect in the 14-day screening cycles used in the manufacturing of poliovaccines. It should be noted, however, that different monkey kidney cells may have a different susceptibility to SV40 infection. Thus, it cannot be excluded that those used by the manufacturers of the oral poliovaccines might be sufficiently sensitive to allow the detection of low amounts of one 72-bp SV40 virions. However, it is also possible, that because of the obvious cytopathic and cytolytic effects induced by SV40 with two 72-bp virions, the subtle cytopathic effects induced in rare cells by one 72-bp-repeat virions would easily be missed.

Whereas no archetypal strains of SV40 were found in recently distributed poliovaccines, the implications of their presence in early lots of the vaccine are significant. SV40 archetypal sequences have been found and expressed in a variety of human tumors (2). In hamsters, injection of SV40 causes the development of several tumor types. Interestingly, SV40 has been detected in the same types of human tumors that it has been proven to cause in hamsters (3). It is also known that Tag is mutagenic and induces cellular DNA replication. Furthermore, Tag has been shown to bind and inactivate p53 and the RB binding family of tumor suppressors in human mesotheliomas (18, 19). SV40 small t antigen also contributes to SV40 oncogenesis (20). Finally, SV40 transforms human cells in tissue culture, and SV40-transformed human cells injected into human volunteers are capable of producing s.c. tumors (Ref. 21; reviewed in Refs. 1–3). Very recently, David Schrump and colleagues at the National Cancer Institute (Bethesda, MD) demonstrated that the expression of Tag is necessary for the maintenance of the transformed phenotype of human mesothelioma cell lines established from SV40 positive mesotheliomas (22).

The presence of archetypal strains in poliovaccines may be of further significance to development of human tumors because some studies suggest that archetypal SV40 may have greater transforming capabilities than nonarchetypal strains. In culture, archetypal SV40 typically forms small plaques in permissive cells, whereas strains of the virus with two 72-bp sequences form larger plaques (10). Small plaque-forming SV40 has been shown to be more efficient at transforming cells in tissue culture than the larger plaque-forming laboratory strains (23). A study performed by Sack (24) confirmed these observations, stating that small plaque-forming SV40 was approximately 200-fold more effective in producing transformed foci than large plaque-forming strains. Together, these data suggest that one 72-bp SV40 may be pathogenic for humans. Therefore, great care should be taken to insure that this virus does not contaminate poliovaccines. We found no evidence of SV40 in six vials of current oral poliovaccines we tested. However, our data suggest that the absence of SV40 was most likely related to the fact that these vaccines are prepared from SV40-free monkeys, rather than due to the sensitivity of the cytopathic test used to screen for SV40. We found that this cytopathic test has limited sensitivity to detect low amounts of SV40 virions containing only one 72-bp repeat. Therefore, it may be important to study a larger number of commercial poliovaccines produced after 1963 to rule out occasional contamination with archetypal SV40. Occasional contamination of vaccines with archetypal SV40 could help to explain the presence of these SV40 variants in pediatric tumors of children born after 1963. Therefore, we suggest that more modern and sensitive techniques, such as immunostaining or molecular techniques, be used in place of or in addition to the cytopathic test to screen poliovaccines. Such measures will insure that any accidental one 72-bp SV40 contamination is promptly detected and will prevent further human exposure to this potentially pathogenic virus.

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Paola Rizzo, Ilaria Di Resta, Amy Powers, et al.

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