RAPID COMMUNICATION

No Evidence of HIV and SIV Sequences in Two Separate Lots of Polio Vaccines Used in the First U.S. Polio Vaccine Campaign

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We obtained sealed vials of two different polio vaccine lots, expiration date 1955, which were used in the first U.S. polio vaccine campaign. These early lots were pulled from the market because they contained live infectious poliovirus which caused polio in some of the vaccines. Theoretically, these vaccines could have contained other infectious retroviruses, including HIV. No viral sequences were detected using RT-PCR analyses with primers capable of amplifying chimpanzee SIV and HIV-1-related viruses nor with primers for macaque SIV, sooty mangabey SIV, and HIV-2-related viruses. Poliovirus sequences were readily amplified by RT-PCR, suggesting that the technique used would have detected SIV or HIV sequences, if present.   © 2001 Academic Press

Introduction. The polio vaccine–HIV hypothesis stating that polio vaccine lots in the 1950s may have been contaminated with HIV or SIV, and thus caused the AIDS epidemic, has recently gained major attention in both the scientific and the lay press (*7, 8*). The lack of documented evidence that only rhesus and green monkeys were used for the preparation of the early polio vaccine lots, together with the apparent complete destruction of these early lots, which made testing for SIV and HIV impossible, has fueled a controversy over the polio vaccine–HIV hypothesis. No evidence of HIV or SIV gene sequences was detected in 15 pools of oral polio vaccine produced between 1975 and 1984 (*6*). Until recently, no vaccines produced prior to 1975 have been available for analysis. Edward Hooper, a proponent of the oral polio vaccine–HIV hypothesis, called for the remaining samples of CHAT 13, a vaccine lot he suspected of causing the AIDS epidemic, to be analyzed for the presence of HIV/SIV (*3, 7*). The results of this multilaboratory "blinded" study were presented at a 2-day conference sponsored by Britain's Royal Society (*2*). All laboratories that tested the vaccine samples could not detect the presence of HIV/SIV. All HIV-1 strains are closely related phylogenetically to SIVcpz (chimpanzee) strains infecting *Pan troglodytes troglodytes,* indicating that this subtype of chimpanzee was the source of the AIDS epidemic (*5*). Questions concerning the exact species of monkeys used in the production of early polio vaccines have recently arisen

(*7*), but the analysis of the CHAT 13 lot indicates that the vaccines had been grown in macaque kidney cells (*2*).

Injectable polio vaccines could have represented a much more dangerous source for HIV infection than oral polio vaccines, if contaminated with live HIV. However, injectable polio vaccines were inactivated with formaldehyde, which killed the poliovirus and presumably any other contaminating retrovirus. Thus, injectable polio vaccines are unlikely contributors to the spread of the AIDS epidemic, except for the very early polio vaccine lots. The inactivating process was inefficient in the early injectable polio vaccine lots and live, infectious poliovirus, and presumably any possible contaminating retrovirus, was present in some of those lots, which resulted in a few polio epidemics. For these reasons, some of these lots had to be pulled from the market. We acquired sealed injectable polio vaccine vials from two such lots (028846B and 028863B; Parke Davis) prepared some time in 1954 (*9*). Letters from the FDA and polio vaccine manufacturers stated that they had no additional vials from before 1963, thus it appears that our vials are the last existing vials of the very early polio vaccine. The two lots we obtained were used in the first polio vaccine campaign in the United States. They were prepared in "monkey kidney cells," but the type of monkey was not specified. This is not unexpected, since there were no major concerns at that time associated with the use of monkey cells, which were often shipped to laboratories without further labeling. In these lots, we demonstrated the presence of archetypal SV40 (*9*). In the

present study, using RT-PCR analysis, we analyzed both lots of the vaccine for HIV and related viruses.

Results. Both lots were tested for the presence of HIV and SIV. Three different RNA extraction procedures were used to verify the accuracy and reproducibility of the results. No viral sequences were detected using two different sets of primers specific for HIV-1 group M (Fig. 1a). We next used the HPOL set of primers, which amplifies HIV-1 sequences of very diverse geographical and genetic origin. This set of primers amplifies HIV group M, group N, and group O and SIVcpzGAB-1, SIVcpzGAB-2, and SIVcpzANT. Polio vaccines tested repeatedly negative for HIV or SIV sequences (Fig. 1b). Furthermore, we tested two additional sets of primers specific for two different strains of SIVcpz. Polio vaccines tested negative with SIVcpzANT-specific primers, whereas controls were easily amplified (Fig. 1c). Primers specific for SIVcpzUS were designed by us (HPOL-US.for, 5′ CCA TAC AAT CCA CAA AGT CAA AGT CAA GG; HPOL-US.rev, 5′ TAC TGC CCC TTC ACC TTT CCA; HPOL-US.for.nested, 5′ AAA GAC AGC TGT ACA GAT GGC AG; HPOL-US.rev.nested, 5′ CTC TGC TGT CCC TGT AAT A). Polio vaccines tested negative with SIVcpzUS-specific primers. We could not obtain any SIVcpzUS positive control; however, these primers also amplified the closely related HIV-1 and SIVcpzANT, which were included as controls in these experiments (Fig. 1d). An ELISA for the detection of p24, the HIV core protein, in the polio vaccines was also negative.

Subsequently we analyzed the vaccines for the second group of immunodeficiency viruses, which includes several types of SIV and HIV-2. Rhesus monkey kidney cells were commonly used to prepare polio vaccines. It is unknown if the rhesus monkeys used in 1954–1955 were infected with SIVmac, which occurred when rhesus monkeys were housed together with macaques. We designed primers (SIVgag) for a nested PCR in the gag region of SIVmac. No sequences were detected in the polio vaccine (Fig. 1e). We repeated the screening for SIVmac using a new set of primers (SIV5′gag/SIV3′gag) that amplifies a different sequence in the gag gene, which is conserved among different strains of SIV, including SIVmac, SIVsm (sooty mangabey), and all HIV-2-related SIV isolates. No SIV or HIV-2-related sequences were detected in the polio vaccine RNA (Fig. 1f). No viral sequences were detected using RT-PCR analyses with primers capable of amplifying SIV chimpanzee and HIV-1-related viruses nor with primers for macaque SIV, sooty mangabey SIV, and HIV-2-related viruses. In every experiment we were able to amplify poliovirus RNA, which suggests that the technical procedure was adequate.

Discussion. If AIDS had been spread through contaminated polio vaccines, it could have been spread through the oral (Sabin and Koprowski) or the injectable (Salk) polio vaccine. The injectable polio vaccines would have been a far more dangerous source of infection if live
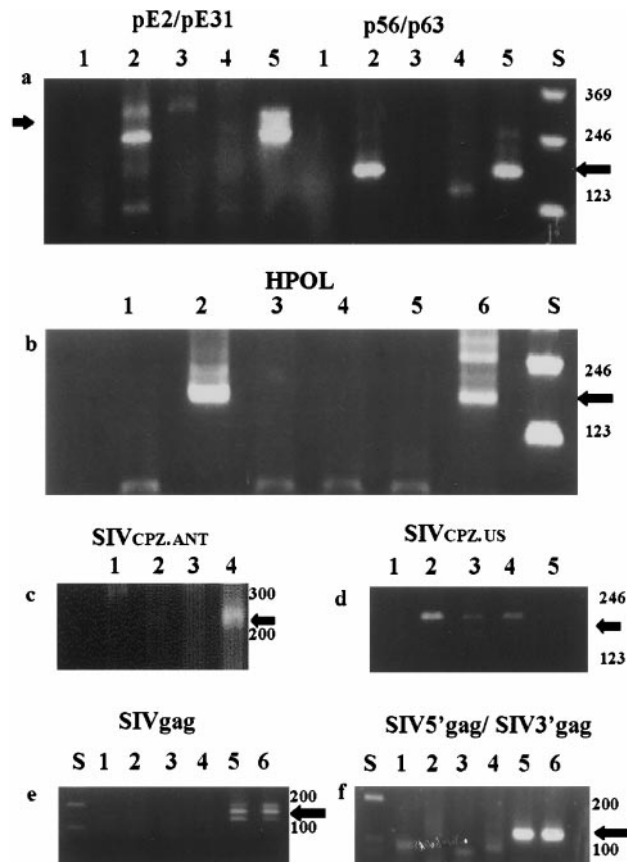
FIG. 1. (a) Ethidium bromide staining of the seminested PCR products obtained with P56/P63 primers, which amplify a fragment of the HIV-1 pol region, and with PE2/P31 primers, which amplify a fragment of the gp 120–gp 41 junction region. The arrow points to the target sequence amplified in the controls. Lane 1, polio vaccine; lane 2, serum of HIV-1-positive patient, positive control; lane 3, water, negative control; lane 4, human DNA, negative control; lane 5, HIV-1 DNA, positive control; S, DNA ladder. (b) Ethidium bromide staining of the nested PCR products obtained with the primer set HPOL, specific for HIV-1 pol. The arrows point to the target sequence amplified in the controls. Lane 1, polio vaccine; lane 2, serum of HIV-1-positive patient, positive control; lane 3, water, negative control; lane 4, human DNA, negative control; lane 5, water, negative control; lane 6, HIV-1 DNA, positive control; S, DNA ladder. (c) Ethidium bromide staining of the nested PCR products obtained with the primers specific for SIVcpzANT. The arrow points to the target sequence amplified in the controls. Lane 1, polio vaccine; lane 2, polio vaccine; lane 3, polio vaccine; lane 4, SIV cpzANT DNA (500 ng). Samples 1–3 represent three different RNA extractions (see Appendix). (d) Ethidium bromide staining of the nested PCR products obtained with the primer set HPOL, specific for SIVcpzUS. The arrow points to the target sequence amplified in the controls. Lane 1, polio vaccine; lane 2, HIV-1-positive human serum; lane 3, SIV cpzANT DNA (500 ng); lane 4, SIVcpzANT DNA (50 ng); lane 5, PCR negative control. (e) Ethidium bromide staining of the nested PCR products obtained using the SIVgag set of primers. The arrow points to the target sequence amplified in the controls. Lane 1, polio vaccine; lane 2, SIV-negative monkey serum; lane 3, water, negative control; lane 4, SIV-negative monkey DNA from peripheral white blood cells; lane 5, SIV-positive monkey DNA from peripheral white blood cells; lane 6, SIV-positive monkey serum. S, DNA ladder. (f) Ethidium bromide staining of the seminested PCR products obtained with the SIV5′gag/SIV3′ gag primers. The arrow points to the target sequence amplified in the controls. Lane 1, polio vaccine; lane 2, SIV-negative monkey serum; lane 3, SIV-positive monkey serum (no RT); lane 4, water, negative control; lane 5, SIV-positive monkey DNA from peripheral blood cells; lane 6, SIV-positive monkey serum; S, DNA ladder.

virus was present. Although these vaccines were inactivated with formalin, the inactivation process was initially not very efficient. Infectious poliovirus (which is also an RNA virus) was sometimes present, resulting in the development of occasional polio epidemics. For this reason, some of the early batches of polio vaccines were pulled from the market and replaced with safer vaccines (*7*). HIV and SIV could potentially have spread through this route if present in these early lots of polio vaccines. Because there are no records regarding the types of monkeys used in the preparation of the first lots of injectable polio vaccines, there was some reason for concern. In the years after 1955, the inactivation process was improved and the types of monkeys used were recorded, making it unlikely that later inactivated polio vaccines contained infectious HIV or related viruses. We found no evidence of SIV- or HIV-related sequences in two separate lots of polio vaccine used in the first U.S. campaign against polio. In addition, samples of lot CHAT 13 prepared by Dr. Koprowski have also tested negative through the multilaboratory study for HIV and related viruses, presented at the Royal Society Conference (*2, 3*). Together, these findings with our data make it reasonable to state that it is extremely unlikely that polio vaccines caused the AIDS epidemic.

Materials and Methods. Two unopened 9-ml vials of polio vaccine manufactured by Parke Davis were analyzed in this study: the first was from Lot 028863B—expiration date October 15, 1955; the second was from Lot 028846B—expiration date October 12, 1955. These vials were kindly provided 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. Dr. Ratner stored these vials for future analysis until 1997, when he gave them to our lab for testing. SIV-positive and SIV-negative rhesus monkey sera and DNA from peripheral blood cells of SIV-positive and SIV-negative rhesus monkeys were kindly provided by Ann Rosenthal at the California Regional Primate Research Center, University of California at Davis. HIV-1-positive and HIV-1-negative human sera were obtained from the Department of Pathology at Loyola. Sooty mangabey SIV-positive and SIV-negative serum was kindly provided by Dr. H. McClure at the Yerkes Regional Primate Research Center, Emory University, Atlanta, Georgia. SIVcpzANT DNA and PCR primers specific for SIV cpzANT DNA were provided by Dr. Heeney at the Biomedical Research Primate Center, Rijswijk, The Netherlands.

RNA extraction. Extraction from the human and monkey sera was performed using the Qiagen viral RNA kit (Valencia, CA). To reduce the possibility of false negative results, three different technical approaches were used to extract RNA from Lot 028846B, of which several vials were available. Only one approach was used, however, for Lot 028863B because only one vial was available.

First approach: To pellet eventual RNA viruses present in the polio vaccine, we spun 2 ml of polio vaccine (1 ml of polio vaccine Lot 028863B and 1 ml of Lot 028846B, combined) overnight at 30,000 rpm on a TLA100.2 rotor in a Beckman ultracentrifuge, resuspended the pellet in 140 μl of PBS, and extracted the RNA using the Qiagen viral RNA kit according to the protocol of T. Zhu *et al.* (*10*). Second approach: 1 ml of vaccine (Lot 028846B) was incubated with 4 ml of lysis buffer from the Qiagen viral RNA kit. The RNA was then precipitated and resuspended in 50 μl of RNase-free water. Third approach: The RNA-STAT-50 solution (Tel-Test, Friendswood, TX) was used to extract RNA from 500 μl of polio vaccine Lot 028846B, according to the manufacturer's protocol. The RNA pellet was resuspended in 100 μl of RNase-free water. All three approaches gave RNA suitable for RT-PCR amplification, as demonstrated by the successful amplification of poliovirus sequences (see below). The second and third approach gave a higher yield of RNA.

RT-PCR for HIV-1 and Poliovirus. Twenty-two microliters of RNA was incubated with one of the following: an oligonucleotide (POL1, see below) specific for the poliovirus to verify that the RNA was suitable for RT-PCR analysis or a mixture of both an oligonucleotide (P58) specific for the HIV-1 pol gene and an oligonucleotide (PE2) specific for the gp120–gp41 junction (*10*) or with the HPOL 4538 oligonucleotide (*4*). The primer concentration was 1.6 ng/μl in a final volume of 24 μl. The mixture was heated at 70°C for 10 min and then equilibrated at room temperature. The RNA was reverse transcribed using a Promega kit. Briefly, total RNA was incubated for 1 h at 42°C in a reaction containing 0.3 mM dNTP, 2.5 mM MgCl$_2$, reverse transcriptase buffer 1$\times$, 0.87 μl of RNase inhibitor, and 40 units of AMV reverse transcriptase. The enzyme was then inactivated by incubation at 70°C for 15 min. Ten microliters of the reaction was analyzed in by PCR using AmpliTaq polymerase (Perkin–Elmer). The following primers were used at the final concentration of 0.5 μM: POL1/POL2 (control, human poliovirus, strain Sabin 1 POL2 nt 203–226 and POL1 nt 61–83), P63/P58, and PE11/PE2 (*10*) and HPOL 4538/HPOL 4235 (*4*). Cycling conditions for all of the primers were 3 min at 95°C (1 cycle), 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C for 45 cycles followed by a 10-min extension, except for the HPOL set of primers, for which the annealing temperature was lowered to 50°C. Ten microliters of the PCR product obtained with the HIV-1-specific primers was reamplified for 45 cycles in a seminested PCR using primers P56/P63, for the product obtained with P63/P58, and P31/PE2 for the product obtained with PE11/PE2. In a nested PCR, the primers HPOL 4327/HPOL 4481 were used to reamplify the products obtained with HPOL 4538/HPOL 4235.

Sensitivity of HIV-1 Primers. To test the sensitivity of the primers HPOL 4538/HPOL 4235 (outer primers) and HPOL 4327/HPOL 4481 (inner primers) and primers P58/
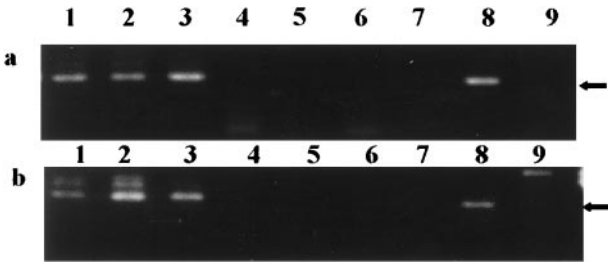
FIG. 2. Sensitivity of HIV-1 primers. (a) Ethidium bromide staining of the seminested PCR products obtained with the primers P63/P56 specific for HIV-1 pol. The arrow points to the target sequence amplified in the positive controls. Lane 1, undiluted HIV-1-positive serum (16,380 copies); lane 2, HIV-1-positive serum diluted in polio vaccine (1638 copies); lane 3, HIV-1-positive serum diluted in polio vaccine (148 copies); lane 4, HIV-1-positive serum diluted in polio vaccine (13 copies); lane 5, HIV-1-positive serum diluted in poliovaccine (1 copy); lane 6, polio vaccine; lane 7, HIV-positive serum (no RT); lane 8, PCR-positive control; lane 9, water negative control. (b) Ethidium bromide staining of the nested PCR products obtained with the primers HPOL specific for HIV-1 pol. The arrow points to the target sequence amplified in the positive controls. Lane 1, undiluted HIV-1-positive serum (16,380 copies); lane 2, HIV-1-positive serum diluted in polio vaccine (1638 copies); lane 3, HIV-1-positive serum diluted in polio vaccine (148 copies); lane 4, HIV-1-positive serum diluted in polio vaccine (13 copies); lane 5, HIV-1-positive serum diluted in polio vaccine (1 copy); lane 6, polio vaccine; lane 7, HIV-positive serum (no RT); lane 8, PCR-positive control; lane 9, PCR-negative control.

P63 and p56/p63 we used sera of two HIV-positive patients. The titers of these two sera were measured using a commercially available kit (Amplicor HIV1 Monitor test from Roche Diagnostic, Indianapolis, IN). HIV-1-positive sera were serially diluted down to 1 copy/140 μl of polio vaccine and the RNA was extracted using a Qiagen kit and tested by RT-PCR, as described above. In these experiments the limit of detection was between 50 (for the HIV-1-positive serum 1) and 148 (for the HIV-1-positive serum 2) copies of HIV-1 in the initial 140 μl of the polio vaccine mixture (Fig. 2).

RT-PCR for SIV. Fifteen microliters of RNA was incubated with either an oligonucleotide specific for the poliovirus (POL1) or an oligonucleotide specific for the SIVmac gag gene (SIVgag.rev.outer, 5′ CAC CAG ATG ACG CAG ACA GT). cDNA synthesis was performed as described above for HIV-1. Ten microliters of the reaction was analyzed in a 100-μl PCR using AmpliTaq polymerase (Perkin–Elmer). The following primers were used at a final concentration of 0.5 μM: POL1 and POL2 (human poliovirus, strain Sabin 1) and SIVgag.rev.outer and SIVgag.for.outer (5′ CGG CGG AAA GAA AAA GTA CA). Cycling conditions were 3 min at 95°C (1 cycle), 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C for 45 cycles followed by a 10-min extension. Ten microliters of the PCR products obtained with the SIV-specific primers was reamplified for 45 cycles in a nested PCR (SIVgag.for.inner, 5′ GCA TGT AGT ATG GGC AGC AA, and SIVgag.rev.inner, 5′ TTC TGA GCC TGT TGG CAC TA). The same experiment was then repeated using a different set of
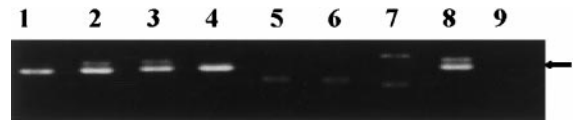
FIG. 3. SIVsm serum diluted in polio vaccine. Ethidium bromide staining of the seminested PCR products obtained using the primers SIV5′gag/SIVgag.rev.outer. The arrow points to the target sequence amplified in the positive controls. Lane 1, undiluted SIV-positive Sooty Mangabey serum; lane 2, SIV-positive Sooty Mangabey serum diluted in polio vaccine (1 to 10); lane 3, SIV-positive Sooty Mangabey serum diluted in polio vaccine (1 to 10^2); lane 4, SIV-positive Sooty Mangabey serum diluted in polio vaccine (1 to 10^3); lane 5, SIV-positive Sooty Mangabey serum diluted in polio vaccine (1 to 10^4); lane 6, polio vaccine; lane 7, SIV-positive serum (no RT); lane 8, PCR-positive control; lane 9, PCR-negative control.

primers (SIV5′gag/SIV3′gag) which also targets the gag gene of all HIV-2-related SIV strains and amplifies "all the HIV-2 related SIV isolates" as described by S. I. Staprans *et al.* in "Quantitative methods to monitor viral load in simian immunodeficiency virus infections" (*1*). Ten microliters of PCR product obtained with the SIV-specific primers was reamplified for 45 cycles in a seminested PCR using primers SIV5′gag/SIVgag.rev.outer for the product obtained with SIV5′gag/SIV3′gag. In the experiments described above we used SIVmac-positive sera. Concerning other SIV strains, these should have been detected if present, at least by the primers designed by Staprans *et al.* (see above). However, inspection of the sequences of these primers with the Los Alamos sequences database revealed that the alignment contained some mismatches with SIVsm. We therefore tested the ability and sensitivity of the primers SIV5′gag/SIV3′gag to amplify SIVsm.

Testing the Sensitivity of the SIV5′gag/SIV3′gag and Seminested Primers for SIVsm Sequence Amplification. Sooty Mangabey SIV (SIVsm) RNA was amplified using the set of primers SIV5′gag/SIV3′gag and seminested SIV5′gag-SIVgag.rev.outer (see above). To test the sensitivity of these primers, SIVsm-positive serum was serially diluted in polio vaccine; the RNA was extracted and analyzed by RT-PCR. This serum was kindly provided by Dr. McClure, Yerkes Regional Primate Center (Atlanta, GA) and the SIVsm titer was unknown. However, we were able to detect SIVsm sequences in serum diluted up to 1 to 10^4 in polio vaccine (Fig. 3). These experiments confirmed that the primers we used amplify SIVsm sequences.
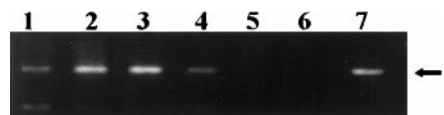
FIG. 4. Ethidium bromide staining of the nested PCR products obtained with the HPOL primers. The arrow points to the target sequence amplified in the controls. Lane 1, 500 ng SIVcpzANT DNA; lane 2, 250 ng SIVcpzANT DNA; lane 3, 125 ng SIVcpzANT DNA; lane 4, 61 ng SIVcpzANT; lane 5, 5 ng SIVcpzANT DNA; lane 6, PCR-negative control; lane 7, PCR-positive control.

Test to Verify the Ability of the HPOL Primers to Amplify SIVcpzANT. DNA from SIVcpz ANT was serially diluted in water and tested by PCR using the HPOL set of primers. These experiments revealed that the limit of detection was 61 ng (Fig. 4). Polio vaccines tested negative for these primers (see Fig. 1).

ACKNOWLEDGMENTS

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