Correlation between amount of virus with altered nucleotide sequence and the monkey test for acceptability of oral poliovirus vaccine

(attenuation/type 3 poliovirus/polymerase chain reaction/restriction enzyme analysis)

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ABSTRACT Production of live attenuated oral poliomyelitis vaccine (OPV) requires rigorous neurovirulence safety testing of each vaccine lot, currently carried out in monkeys. It has been reported that a change from 472-U to 472-C in the type 3 OPV RNA is associated with an increased histologic lesion score produced upon intraspinal inoculation of the mutant virus in monkeys. We have developed a method, based on polymerase chain reaction, for measuring the relative abundance of these mutant sequences directly in vaccine preparations and used this method to evaluate the proportion of 472-C in 40 different lots of type 3 OPV. Six vaccine lots that had failed the intraspinal monkey neurovirulence test contained a higher proportion of 472-C than all other lots that had passed this test. OPV type 3 virus containing 472-C was rapidly selected during serial passages in African green monkey kidney cells that are used for manufacturing of the vaccine. We have also found that the wild-type poliovirus type 3 strain Leon/37, from which the vaccine strain was originally derived, contained a mixture of 472-U and 472-C sequences. No other mutations in OPV type 3 RNA have been detected by similar assays at position 2034, also associated with attenuation, or at several other positions reported to be altered in some vaccine preparations. Our results suggest that molecular diagnostics may provide a supplement or a potential alternative to animal testing of live attenuated vaccines.

The production of the Sabin live attenuated oral poliomyelitis vaccine (OPV) (1–4) during the past 30 years has been rigorously controlled by neurovirulence tests in large numbers of monkeys (5). Over the last 7 years the complete nucleotide sequences of genomes of all three antigenically distinct strains used in the production of the vaccine have been determined (6, 7), making it possible to investigate the basis of OPV attenuation at the molecular level. The RNA of the type 3 vaccine strain (Leon 12a,b) has been found to have 7434 nucleotides and only 10 of them were different from the wild-type progenitor strain Leon/37 (8). It has been shown that, following multiplication in the intestinal tract of vaccinees, there can be a quantitative difference in some of the properties of the ingested virus—e.g., reproductive capacity at higher temperature (rct) with or without change in neurovirulence as measured by intraspinal and intrathalamic tests in monkeys but not in chimpanzees (9). Studies on type 3 viruses excreted by healthy children after ingestion of the trivalent OPV regularly showed a change in the nucleotide at position 472 from uridine (U), found in the genome of the type 3 vaccine strain, to cytosine (C), found in wild-type strains (8, 10, 11). This mutation was shown to result in a quantitative

increase in histologic lesion scores produced in monkeys after intraspinal inoculation of the virus (10, 11). We have therefore asked whether this virus containing C at position 472 (472-C) can be detected in the preparations of vaccine and whether its amount can be correlated with the results of a strict test for acceptability as measured by the World Health Organization (WHO) intraspinal monkey neurovirulence test (5).

Sequence changes in poliovirus RNA have been previously analyzed by direct sequencing of viral RNA (10, 12), a method that is not particularly sensitive for detection of sequence variants present at low abundance. The advent of the polymerase chain reaction (PCR) technology (13, 14) provided a tool with which we could readily test type 3 vaccine preparations for changes at position 472. To accomplish this, we have developed a highly sensitive molecular assay for these mutants and used it to test 40 lots of type 3 OPV, produced by different manufacturers. We have also tested these vaccine preparations for mutations at several other positions associated with attenuation (position 2034) (10) or reported to be altered in some lots that failed the monkey neurovirulence test (positions 445, 448, and 449) (12). The results of our assays showed correlation between the nucleotide substitution at position 472, but not at any other tested position, and the results of the intraspinal monkey neurovirulence test. 472-C mutants were rapidly selected during serial passages in African green monkey kidney (AGMK) cells that are used for manufacturing the vaccine. Our results suggest a potential use for molecular diagnostics in the testing of live attenuated vaccines.

MATERIALS AND METHODS

Virus Growth, RNA Isolation, and cDNA Preparation. Viral RNA was isolated directly from 0.4 ml of vaccine stocks or cell culture fluid by phenol extraction with 1% SDS at room temperature. AGMK cells were obtained from Quality Biologics (Gaithersburg, MD) and infected either with 0.1 tissue culture dose (TCD₅₀) per cell ("low multiplicity") or 10 TCD₅₀ per cell ("high multiplicity") of the appropriate virus stock. cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (SuperScript; Bethesda Research Laboratories) and random hexadeoxynucleotide primers (15).

Analysis of 472-T → 472-C Mutants. PCR amplification of a cDNA segment between positions 440 and 532 in poliovirus type 3 RNA (7) was carried out using Taq DNA polymerase (*Thermus aquaticus* DNA polymerase; AmpliTaq; Perkin-Elmer/Cetus) for 30 two-step cycles (each cycle included

melting for 1 min at 94°C and annealing/elongation for 5 min at 65°C). The sense strand PCR primer (pS) was 5'-TGAGAGTCCT CCGCCCCCTG AATGCGGCTG AT-3', corresponding to positions 440–471 of poliovirus type 3 RNA except for an A → G change at position 469. This change creates a *HinfI* restriction site in PCR products amplified from vaccine-specific sequences and an *Mbo* I restriction site in PCR products amplified from revertant-specific sequences. The antisense strand PCR primer (pA) was 5'-ACGGACTTGC GCGTTACGAC AGGCTGGCTGC-3', complementary to positions 502–532 of poliovirus type 3 RNA. The pA primer was 5' end-labeled with [γ -³²P]ATP (Amersham) prior to PCR. Each reaction contained 0.5 μ g of the pS primer and 0.05 μ g of the ³²P-labeled pA primer. Amplified 440–532 DNA fragments were digested with *Mbo* I restriction endonuclease and separated by electrophoresis in 15% or 10% polyacrylamide gels (30 × 40 cm). The undigested 93-base-pair (bp) PCR product and the 61-bp *Mbo* I cleavage product were excised from the gel and their associated radioactivity was determined by liquid scintillation spectroscopy. The identity of the 93-bp PCR product was confirmed by digestion with *HinfI*, specific for 472-T, and with *Pvu* II, which cuts poliovirus type 3 RNA at position 491 (data not shown).

Two DNA fragments were synthesized for the construction of a standard set. A 93-bp fragment containing 100% 472-T was prepared by reverse transcription and PCR using a vaccine-derived RNA template and a modified pS primer (pS/T) containing an additional T residue at the 3' end, corresponding to 472-T. A 93-bp fragment containing 100% 472-C was made using RNA from the DM Pg35aP-396 strain of poliovirus type 3 vaccine (a gift of P. D. Minor, NIBSC, U.K.) containing 472-C and a modified pS primer (pS/C) containing an additional C residue at the 3' end, corresponding to 472-C. These standard PCR amplified fragments containing 100% 472-T and 100% 472-C were mixed in different proportions and used as templates in PCR reactions. The amount of 472-C measured in triplicate samples for every lot of vaccine was corrected using the calibration line obtained in the same experiment using the above described standard mixtures. Three to 10 independent determinations were performed for each vaccine lot. In tests with some of the older vaccine preparations, we observed a high background due to "smearing" of radioactive material, which made the determination of 472-C abundance irreproducible. The reason for this smearing is unknown. The data with such samples were not included in this paper.

Analysis of 2034-T → 2034-C Mutants. A DNA fragment corresponding to the genome region 1970–2070 of poliovirus type 3 genome was amplified using ³²P-labeled sense primer 5'-GTTACTCTGA GCGACAGTGC CGATCTATCG CAACC-3' and antisense primer 5'-ACTTCCCCAA GCATGGTGTG TGACAAGCGC GGATTA-3'. The vaccine-specific 2034-T containing sequence was detected by digestion with *Mse* I restriction enzyme, and wild-type-specific 2034-C containing sequence was detected by digestion with *Sfa*NI restriction enzyme.

Analysis of Mutations at Positions 445, 448, and 449. A 124-bp DNA fragment corresponding to the genome region 409–532 of poliovirus type 3 genome was amplified using ³²P-labeled antisense primer 5'-ACGGACTTGC GCGTTACGAC AGGCTGGCTGAT-3' and sense primer 5'-GAA-CAGGGTG TGATGAGGCTT ATTGTGCTAC TG-3'. The latter corresponds to positions 409–441 of the type 3 poliovirus genome except for changes of A → T at position 422 (to remove a *Mbo* II restriction site) and A → T at position 433 (to remove an *Alu* I restriction site). 445-A → 445-C mutants were detected by digestion of the 124-bp PCR-amplified fragment with *HinfI* and *Hga* I restriction enzymes, which recognize the vaccine- and mutant-specific sequences, re-

spectively. 448-C → 448-G mutants were detected by digestion with *Mnl* I (or *HinfI*) and *Bsp*1286 (restriction enzymes recognizing the vaccine- and wild-type specific sequences, respectively). 449-C → 449-A mutants were detected by digestion of the 124-mer with *Mnl* I and *Fok* I restriction endonucleases, cutting the vaccine- and mutant-specific sequences, respectively.

RESULTS

We have hypothesized that the failure of some batches of type 3 poliovirus vaccine in the WHO monkey neurovirulence test may be due to the presence in the vaccine preparations of 472-C mutants, previously shown to produce increased lesion scores in this test (10). To measure the relative abundance of 472-C mutants in mixed populations of the virus, we have developed an assay illustrated in Fig. 1a. This assay is based on reverse transcription of viral RNA, followed by PCR amplification of a cDNA segment that includes position 472 and subsequent detection of sequences with the 472-T → 472-C substitution by digestion with a restriction enzyme. Since this substitution does not alter any known restriction sites in the cDNA, we have mutated the sequence of the PCR product by using an imperfectly matched primer for second-strand cDNA synthesis and PCR. This modification leads to the appearance of an *Mbo* I restriction site that is characteristic for 472-C. The proportion of 472-C revertants is estimated after *Mbo* I digestion of single-end-labeled PCR products by determining the proportion of radioactivity associated with *Mbo* I-digested fragments after separation in a polyacrylamide gel. The tested samples are analyzed in parallel with a series of standard cDNA mixtures, containing known proportions of 472-C (Fig. 1b). Less than 0.2% of 472-C would be detected in these mixtures. The proportion of 472-C in the original cDNA template could be readily measured by this assay using a calibration curve (Fig. 1c).

Forty lots of type 3 poliovirus vaccine produced from six different seed virus preparations were tested by the above assay. Almost all batches contained measurable levels of 472-C mutants, indicating that such mutants were indeed present in the vaccine preparations. There was, however, a major difference in the frequency of 472-C mutants between the vaccines that passed versus those that failed the monkey neurovirulence test. Six vaccine lots that failed this test had 472-C contents above 1.17%, whereas all batches that passed the test contained <0.79% 472-C revertants (Table 1). There were differences in frequency of 472-C mutants among acceptable vaccines manufactured from different batches of both SO and RSO seed virus strains (Table 1).

To identify the factors that may influence the frequency of 472-C mutants in vaccine preparations, we have asked if the proportion of these mutants is altered by the conditions of growth and the initial source of the virus. We have analyzed two vaccine preparations, one derived from SO and the other from RSO stock, after serial passage at 34.6°C or 37°C. Fig. 2 demonstrates that at both temperatures 472-C mutants steadily accumulate in the course of passaging. This accumulation is much more rapid at 37°C than at 34.6°C and is more rapid for SO-derived virus than for RSO-derived virus. The rate of mutant accumulation was also influenced by the type of cells used for propagating the virus and by the multiplicity of infection during passages (to be published elsewhere). These results indicate that selection for 472-C mutants occurs in the cells used for vaccine manufacturing and suggest that the conditions for propagation of the virus and the starting virus stock can determine the proportion of 472-C mutants in vaccine preparations.

The observed correlation between the results of the monkey neurovirulence test and the proportion of 472-C mutants

FIG. 1. (a) Scheme for detection of 472-U $\rightarrow$ 472-C mutation in poliovaccine type 3 RNA. The nucleotide at position 472 is boxed, and the residue altered by the use of mismatched primer is underlined. An asterisk indicates the primer and DNA fragments that are ^{32}P labeled at the 5' end. (b) Autoradiography of a polyacrylamide gel containing *Mbo* I-digested PCR products. Lanes 1–9, analysis of mixtures of standard 472-T and 472-C DNAs containing 100, 5, 2.5, 2, 1.5, 1, 0.5, 0.2, and 0% of 472-C, respectively. Lanes 10–15, analysis of RNA from different batches of poliovaccine type 3, including three batches that failed the monkey neurovirulence test (lanes 10–12) and three batches that passed this test (lanes 13–15). Bands corresponding to the undigested 93-bp (a) and *Mbo* I-digested 61-bp (b) PCR products are indicated with arrowheads. The presence of a small amount of the 61-bp fragment in lane 9, containing 100% 472-T, visible in the original autoradiogram but poorly reproduced in the photograph, apparently indicates low-level misincorporation of a C residue at position 472 during PCR. (c) Calibration curve for quantitation of the proportion of 472-C revertants. The points correspond to the above-described standard cDNA mixtures. Mean values and standard deviations from 15 experiments are presented.

confirms the significance of this position as an important marker of the original type 3 attenuated poliovirus strain. We were interested to determine whether the vaccine-specific 472-U sequence arose during the isolation of the vaccine strain or preexisted in the original wild-type poliovirus. We have therefore analyzed the RNA from the wild Leon/37 strain that served as the progenitor for the Sabin live vaccine

strain Leon 12a₁b. We found that two batches of the Leon/37 virus obtained from the American Tissue Culture Collection (ATCC) contained a mixture of 472-U and 472-C (Fig. 3). The proportions of 472-C and 472-U were determined by digestion of the PCR product with *Mbo* I and *Hinf*I restriction enzymes, respectively (the latter enzyme recognizes the 472-U sequence). Leon/37 virus (ATCC VR-1004 lot V-003-001-019

Table 1. Percent of 472-C in type 3 poliovirus vaccines made from different seed viruses by different manufacturers

Group	No. of vaccine lots in group	% 472-C	
		Mean	Range
Failed monkey test			
SO group I	2	1.39 ± 0.36	1.37 ± 0.19–1.40 ± 0.46
SO group II	3	1.98 ± 0.39	1.83 ± 0.40–2.21 ± 0.44
SO group III	1	1.17 ± 0.21	
Total	6	1.67 ± 0.52	1.17 ± 0.21–2.21 ± 0.44
Passed monkey test			
SO group I	9	0.58 ± 0.15	0.39 ± 0.09–0.79 ± 0.05
SO group II	1	0.28 ± 0.19	
RSO group I	14	0.12 ± 0.09	0–0.30 ± 0.03
RSO group II	10	0.32 ± 0.15	0.17 ± 0.07–0.49 ± 0.17
Total	34	0.29 ± 0.23	0–0.79 ± 0.05

SO groups I–III are vaccines that were prepared from passages of Sabin Original seed virus; RSO groups I and II vaccines were prepared from RNA-plaque-purified seed. Standard deviations of values obtained in 3–10 independent determinations are presented.

prepared for ATCC at the National Institute of Allergy and Infectious Diseases by one passage in baboon kidney cells) showed 78.43% 472-U and 21.64% 472-C. In contrast, Leon/37 virus contained in the ATCC VR-62 culture (that was prepared at ATCC by passaging three times in AGMK and once in LLC-MK₂ rhesus monkey kidney continuous cell line) showed 9.16% 472-U and 90.14% 472-C (Fig. 3, lanes 4–6). After one additional passage of the above ATCC VR-62 Leon/37 culture in the HEP-2 human epithelial carcinoma cell line at low multiplicity of infection, this strain possessed almost 100% 472-C (Fig. 3, lanes 7–9). The sum of 472-U and 472-C amounts is very close to 100%, suggesting that no 472-A or 472-G could be detected in these virus preparations. This result demonstrates that a wild poliovirus isolate comprises a mixed population of virus particles and that the viruses containing 472-C in their RNA are selectively enriched under certain conditions.

We were interested to determine whether mutations in other regions of the viral genome can also contribute to an increased lesion score in the WHO monkey test. In addition to position 472, another position, 2034, has been suggested to play a functional role in the attenuation of poliovirus type 3 (11). To test for reversion to the wild-type sequence at this position, we have amplified a DNA fragment spanning the

region 1970–2070 of the virus genome and digested it with *Mse* I restriction enzyme, which is expected to cut the 2034-T-containing vaccine sequence, and with *Sfa* NI, which should cut the 2034-C-containing wild-type sequence (Fig. 4). We found that whereas the latter enzyme cleaves wild-type Leon/37 sequence completely, we were unable to find any detectable amount of 2034-C either in lots of type 3 OPV that failed or passed the monkey test or in the vaccine virus that was passaged in cell culture under the conditions that favored the selection of 472-C revertants.

Weeks-Levy *et al.* (12) reported that by using direct sequencing they were able to detect mutations at positions 445, 448, and 449 in several type 3 poliovirus vaccine lots that failed the monkey neurovirulence test. To test for the presence of such mutations, we have amplified the DNA fragment corresponding to the region 409–532 of the virus genome and digested it with restriction enzymes that should discriminate between the vaccine and the mutated sequences (see *Materials and Methods*). We have found no mutations at any of these positions in 18 tested individual lots of vaccine that included those that failed and those that passed the intraspinal monkey test (data not shown). Instead, the DNA fragments obtained for all vaccine lots were cleaved completely with restriction enzymes recognizing unaltered sequence.

FIG. 2. Accumulation of 472-C in the course of passaging of two poliovaccine type 3 strains in AGMK cells. Bars represent the proportions of 472-C in the population of SO-derived and RSO-derived batches of vaccine during serial passages in AGMK cells either at 34.6°C or at 37°C. Cells were infected at a low multiplicity (≈ 0.1 infectious unit per cell). (Inset) Percent accumulation for passages 0 and 1 (expanded).

FIG. 3. Sequence heterogeneity at position 472 in the wild-type 3 poliovirus Leon/37 strain. PCR-amplified DNA fragments corresponding to positions 440–532 (lanes 1, 4, and 7) were cut with *Hinf*I (lanes 2, 5, and 8) or *Mbo* I (lanes 3, 6, and 9) restriction enzymes. Lanes 1–3, Leon/37 batch ATCC VR-1004; lanes 4–6, Leon/37 batch ATCC VR-62; lanes 7–9, Leon/37 batch VR-62 grown in HEP-2 cells (one passage). Arrowheads indicate the positions of the uncut 93-bp fragment and the 61-bp restriction fragment.

FIG. 4. Gel electrophoresis of PCR-amplified DNA fragment corresponding to positions 1970–2070 of type 3 OPV genome (WHO-III reference strain, corresponding to the second passage of the Sabin Original Leon 12a₁b strain in AGMK cells) digested with restriction enzymes that recognize 2034-T (*Mse* I, lane 2) or 2034-C (*Sfa*NI, lane 3). Lane 1, uncut fragment. Arrowheads indicate the position of uncut 101-bp fragment and 65-bp restriction fragment.

DISCUSSION

Neurovirulence of poliovirus has been shown to cover an extensive quantitative spectrum when tested by different routes (intrathalamic, intraspinal, oral, parenteral) in monkeys and chimpanzees (1–4). The WHO monkey intraspinal neurovirulence test (5) is based on production of the histological lesions (without paralysis) assessed as the lesion score after the injection of virus directly into the anterior horns. This stringent test is purposefully designed to detect the variation from the seed virus used to produce the vaccine lots for general use. Quantitative changes along the neurovirulence spectrum and changes of other properties have been demonstrated in the earliest work with the attenuated poliovirus used for vaccine production (1–4), and recently molecular changes have also been reported (10, 12).

The monkey test for acceptability of vaccine lots has been satisfactorily used for >30 years. However, the test requires many monkeys and is expensive. Accordingly, the demonstration in the present communication of a remarkable difference in the abundance of 472-C sequences between lots of type 3 OPV that pass the monkey test and those that do not provides a molecular procedure for assessment of acceptability of vaccine lots, supplementing the monkey test.

We have shown that serial cultivation of the virus *in vitro* results in a rapid accumulation of 472-C, and the conditions of virus growth greatly influence the rate of mutant selection. Significantly faster selection of 472-C at 37°C as compared to 34.6°C indicates that this mutation is implicated in the *rct* marker of vaccine virus. It is noteworthy that two strains of type 3 OPV seed, one derived directly from the original stock of the seed virus and the other obtained by its RNA plaque purification, differ in their rates of selection of 472-C despite the approximately equal proportion of mutants in the initial preparations. The selection seems to be specific for position 472, since analysis of another position, 2034, that was implicated in the increased neurovirulence (11), or of positions 445, 448, and 449, reported to be altered in some vaccine lots (12), showed no changes either in acceptable or unacceptable vaccine lots or after four experimental passages in AGMK cells *in vitro*. Certainly we cannot rule out that changes in positions other than 472 could be found upon the further studies.

The presence of C at position 472 apparently increases the translation efficiency of viral RNA (16); this increased efficiency is likely to provide the basis for the selective advantage of 472-C mutants during growth in AGMK cells as well as in human gut (10) and in a neuroblastoma cell line (17). In this respect it is interesting to find out if 472-U also provides some selective advantage to the virus under some conditions and to understand the mechanism of attenuation of poliovirus

in the Sabin vaccine. Our finding that the extremely virulent wild-type Leon/37 strain contained 472-U and 472-C indicates that an equilibrium between these forms may exist in the native virus population. From these data it is evident that the presence of 472-U in the vaccine strain cannot be regarded as a mutation, but rather it was present in the wild-type parental strain. Similarly, change of 472-U for 472-C taking place in vaccinees (10) cannot be regarded as a true “reversion.” Another question raised by these findings is whether some other mutation(s) in the wild-type strain may modify the effect of 472-U mutation.

Several specific mutations have also been implicated as the determinants of attenuation in type 1 and type 2 polioviruses (18–20). The approach used in the present study can be applied to determine which mutations may be predictive for increased neurovirulence in vaccines of these two types. Our results suggest that molecular assay of the OPV may provide a supplement or an alternative to the current monkey test of the final product and may also be used for the in-process monitoring of poliovirus vaccine production. Furthermore, analysis of the mutant frequency at key positions associated with attenuation may also serve as a general principle for molecular evaluation of other attenuated viral or bacterial vaccines.

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