RNA Sequence Variants in Live Poliovirus Vaccine and Their Relation to Neurovirulence

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Mutant analysis by polymerase chain reaction and restriction enzyme cleavage (MAPREC) was used to study sequence heterogeneity and stability in attenuated poliovirus type 3 at positions in which the vaccine virus differs from its wild-type progenitor. Of seven genomic positions tested, only two (positions 472 and 2493) show nucleotide heterogeneity. Propagation of the vaccine virus in cell cultures leads to rapid selection of virus with reversions at these two positions of the genome. The relative abundance of reversions at position 472 correlates with the results of monkey neurovirulence tests, while the mutation at position 2493 is not directly associated with neurovirulence of the virus in monkeys. Instead, the abundance of mutations at the latter position correlates with the source of the seed virus and its passage level. These results further indicate that MAPREC at position 472 can be used to assess the quality of poliovirus type 3 vaccine.

Sabin oral poliovirus vaccine (OPV) consists of live attenuated viruses of three serotypes. These viruses replicate in the intestinal tracts of vaccinees, inducing long-lasting immunity but unlike wild-type strains, they cause no disease. According to the World Health Organization (WHO) requirements for OPV (9), the neurovirulence safety of the vaccine is tested by intraspinal inoculation of monkeys with each manufactured vaccine lot along with the homotypic reference lot, followed by 17 to 22 days of observation for neurological signs and necropsy. Histologic assessment of specific lesions in the monkeys central nervous systems allows a comparison of the degree of neurovirulence of a vaccine lot with that of the reference lot. For type 3 virus, the test requires at least 20 monkeys for each vaccine and reference lot.

Using a new sensitive method for quantitation of base substitutions in viral RNA, we recently found that live poliovirus type 3 vaccines contain varying proportions of the virus with altered nucleotide sequences. A high abundance of reversions at position 472 of the 5' noncoding region correlates with increased neurovirulence in monkeys (1). Therefore, we proposed that the molecular procedure for quantitation of these revertants could be used as a supplement to the monkey test.

We also showed that these revertants rapidly accumulate in the vaccine in the course of cell culture passage (1). In addition to position 472, several other positions were shown to differ in wild-type 3 Leon/37 virus and its attenuated derivative Leon 12a,b strain (4–6). We asked whether these other mutations contribute to the instability of the poliovirus genome and the increased neurovirulence of some vaccine lots. To answer this question, we designed quantitative assays for mutations at positions of the genome at which the attenuated poliovirus type 3 strain differs from the wild-type progenitor. In this communication, we present the data showing that, with the exception of positions 472 and 2493, all positions remain stable. We also present evidence that of these two variable sites in the virus genome, only position 472 is related to neurovirulence in monkeys, while position 2493 has no apparent influence on the attenuated phenotype of the virus.

MATERIALS AND METHODS

Viruses used. Sabin Original (SO) and RNA-plaque-purified SO (RSO) derived vaccines were studied by analyzing RNA extracted directly from vaccine lots. Wild-type Leon/37 preparations were obtained from the American Type Culture Collection (ATCC) (VR-62, VR-63, and VR-1004) and from O. Kew (Centers for Disease Control, Atlanta, Ga.). Wild-type Fox Wy-3 and attenuated Fox WM-3 strains were obtained from ATCC (VR-193 and VR-300), and a wild-type Saukett strain was given to us by P. Albrecht (Food and Drug Administration—Center for Biologies Evaluation and Research, Bethesda, Md.). The revertant type 3 strain DM Pg35aP-396 was a gift from P. Minor (National Institute for Biological Standards and Control, London, United Kingdom). A recombinant plasmid, pT7/- Leon, containing the complete copy of Sabin poliovirus type 3 was kindly provided by J. Almond (University of Reading, Reading, United Kingdom).

Determination of the abundance of specific mutations. Determination of the abundance of specific mutations at different positions of the poliovirus genome was performed as previously described (1). In brief, this mutant analysis by polymerase chain reaction (PCR) and restriction enzyme cleavage method includes (i) synthesis of viral cDNA with reverse transcriptase and random primer, (ii) PCR amplification of a specific region with synthetic primers modified to create restriction enzyme recognition sites, and (iii) cutting of PCR product with restriction enzymes and quantitative analysis of cleavage products by gel electrophoresis. The method allows one to quantitate sequence heterogeneity at specific positions of the genome at a level above 0.1%. The sequences of primers used to analyze several positions in the poliovirus type 3 genome and respective restriction enzymes are listed in Table 1. In some cases, a single PCR fragment could be cleaved with several different restriction enzymes recognizing both wild-type and vaccine sequences (positions

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TABLE 1. PCR primers and restriction enzymes used to detect mutations

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (position)</th>
<th>Restriction enzyme and specificity</th>
</tr>
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<tbody>
<tr>
<td>P220S</td>
<td>(194) ATAGACTTGT CCCCAGGTTG AAAGAG (219)</td>
<td>HaeIII, 220-G (wild type)</td>
</tr>
<tr>
<td>P220A</td>
<td>(290) GAGCCGCAAGC CTGCGAGAT ATTCGAG (266)</td>
<td>Hinfl, 220-U (vaccine)</td>
</tr>
<tr>
<td>P472S</td>
<td>(440) TGAGGCTTCC CGCGGCCCCTG ATTCGCGGGCT AT (471)</td>
<td>MboI, 472-C (wild type)</td>
</tr>
<tr>
<td>P472A</td>
<td>(623) CTCGCTTATA GATAAATCT TATGA (599)</td>
<td>Hinfl, 472-U (vaccine)</td>
</tr>
<tr>
<td>P2034A</td>
<td>(2070) ACCTCCCAAG CCGGTGTTG TGAAAGGCGG GATTA (2035)</td>
<td>Msel, 2034-U (vaccine)</td>
</tr>
<tr>
<td>P2493S</td>
<td>(2463) CTGCGCTTCC ACAGGTATG GAAGATCTGA (2492)</td>
<td>ClaI, 2493-U (wild type)</td>
</tr>
<tr>
<td>P2493S C</td>
<td>(2463) CTGCGCTTCC ACAGGTATG GAAGATCTGA (2492)</td>
<td>HinclI, 2943-C (vaccine)</td>
</tr>
<tr>
<td>P2493S G</td>
<td>(2463) CTGCGCTTCC ACAGGTATG GAAGATCTGA (2492)</td>
<td>DdeI, 2493-G*</td>
</tr>
<tr>
<td>P2493G</td>
<td>(2558) CTTTTGAGAT GTGGCGGGCA CTGGAGATT (2559)</td>
<td>EcoRI, 2493-A*</td>
</tr>
<tr>
<td>P3333S</td>
<td>(3301) GGUACCUAUA UAAGGACAG GGGUGGACUC U (3332)</td>
<td>DdeI, 3333-A (wild type)</td>
</tr>
<tr>
<td>P3333A</td>
<td>(3590) TGAGGCCAAG ACAGCATATG GCTCAAACT TTTTC (3566)</td>
<td>Mael, 3333-G (vaccine)</td>
</tr>
<tr>
<td>P3464S A</td>
<td>(3434) CAUCUGG CGCAUAGGAGA UUUACAA AGU (3463)</td>
<td>ScaI, 3464-A (wild type)*</td>
</tr>
<tr>
<td>P3464S G</td>
<td>(3434) CAUCUGG CGCAUAGGAGA UUUACAA AGU (3463)</td>
<td>NsiI, 3464-G (vaccine)*</td>
</tr>
<tr>
<td>P3464A</td>
<td>(3548) TGCAATGGTGC CTTGCTTTG AAGTGGCTG AGATT (3519)</td>
<td></td>
</tr>
<tr>
<td>P6061S</td>
<td>(6967) CUCAGAAGT AAAUGGAAAC GAGG (6992)</td>
<td>CfoI, 6061-C</td>
</tr>
<tr>
<td>P6061A</td>
<td>(6086) CTCCTTCAAA CACATAGTGG AGACC (6062)</td>
<td>HgiAl, 6061-U</td>
</tr>
</tbody>
</table>

a PCR product made with P2493S-P2493A primers.
b PCR product made with P2493S-P2493A primers.
c PCR product made with P2493S-P2493A primers.
d PCR product made with P2493S-P2493A primers.
e PCR product made with P3464S-P3464A primers.
f PCR product made with P3464S-P3464A primers.

220, 472, 2034, 3333, and 6061). In other cases, separate sense primers were synthesized and used to make separate PCR fragments which could be cleaved by enzymes specific to wild-type and vaccine sequences (positions 2493 and 3464).

**Monkey neurovirulence test.** The monkey neurovirulence test was performed according to the WHO procedure (9) by intraspinal inoculation of 12 or 24 rhesus monkeys with subsequent assessment of histologic lesions in the central nervous system.

**RESULTS**

Comparison of nucleotide sequences of wild-type and attenuated strains of Leon poliovirus type 3 previously performed by Stanway et al. (4) revealed 10 point substitutions, at positions 220, 472, 871, 2034, 3333, 3464, 4064, 6127, 7165, and 7432. In addition, the sequence of attenuated Leon 12a,b reported by Toyoda et al. (6) contained U instead of C at position 6061, and recently Tatem et al. (5) have found that type 3 vaccines contain C and not U at position 2493. Three of these substitutions are located in untranslated regions, and of nine substitutions in the coding region, only four cause amino acid changes in viral proteins, namely, position 2034 in VP3 and positions 2493, 3333, and 3464 in VP1. We decided to study the stability of these four positions along with the stability of two positions in the 5′ noncoding region and position 6061, at which a discrepancy between two published sequences occurred (4, 6). By using the highly sensitive quantitative mutant analysis by PCR and restriction enzyme cleavage method (1), we determined the sequence heterogeneity at these positions in several vaccine lots made from SO or RSO seeds. Higher-level passages of these strains, as well as some wild-type strains and a cDNA-derived clone of poliovirus type 3, were also tested to study the stability of these positions.

Analysis of position 6061 showed that all vaccines, as well as experimental high-level passage preparations and all wild-type strains, including Leon/37, Fox, and Saukett, contain U, which corresponds to the sequence published by Toyoda et al. (6). The only sample that showed C at position 6061 (6061-C) was the recombinant plasmid pT7 Leon containing the complete cDNA of poliovirus type 3 (results not shown). No sequence heterogeneity was detected at this position, and there is no discrepancy in published sequences that may be the result of a cDNA cloning procedure described in reference 4. Anyway, the mutation at position 6061 is silent and does not change the amino acid sequences of viral proteins.

No sequence heterogeneity was observed at positions 220, 2034, and 3464 in either vaccine lots, high-level passages, or wild-type strains (data not shown). The homogeneity of these positions in higher-level passages of the vaccine virus indicates that they remain stable and thus do not contribute to the increased neurovirulence of some vaccine lots. Some heterogeneity was detected at position 3333 in two Fox strains and in the Saukett strain of poliovirus type 3 but not in the wild-type or attenuated Leon strains (Fig. 1). In addition to 3333-A, preparations of Fox Wy-3 (ATCC VR-193), Fox WM-3 (ATCC VR-300), and the Saukett strain contained approximately 2% 3333-G, which is characteristic of the Sabin strain.

Position 2493 showed the highest heterogeneity. With preparations of the Fox strain, we could demonstrate the presence of all four nucleotides at this position (Fig. 2A). In addition to the predominantly U, there was approximately 1 to 2% (each) A, G, and C. All wild-type Leon preparations showed U at position 2493, while preparations of the Sabin strain contained variable proportions of C and U (Fig. 2B). Vaccine lots prepared from the RSO seed contained predominantly C with roughly 1% U at this position, while vaccines made of SO seed at the SO+2 passage level contained 60 to 85% U and vaccines made of SO+3 level contained about 99% U at this position. Thus, the abundance of U at position
2493 allows determination of the type (SO versus RSO) and the passage level of the seed virus used for vaccine production. This finding suggests that C at this position may be unstable during passage in cell culture. To test this, we passaged the vaccine virus (RSO+2) in different cell cultures at 34 and 37°C with high (1 to 3 IU per cell) and low (0.01 to 0.1 IU per cell) multiplicities of infection. Figure 3 shows that passaging of the vaccine virus in Vero cells results in rapid outgrowth by the revertant similar to the outgrowth we described earlier by the reversion at position 472 (1). Selection of 2493-U-containing mutants was observed with all the cells tested so far, which included Vero, primary African green monkey kidney, WI-38, and HEp-2 cells (data not shown).

We analyzed four different preparations of wild-type Leon strains. One (Leon/37 KP7) was obtained from Olen Kew (Centers for Disease Control), and three (VR-62, VR-63, and VR-1004) were obtained from ATCC. Analysis of positions 220, 2034, 3333, and 3464 showed that the RNA sequence of the last virus, VR-1004, is not consistent with the sequences of other Leon strains but is similar to the sequence of the Sabin strain. At position 2493, VR-1004 has U, which is characteristic of the wild-type strains and of the Sabin strain at a passage level higher than SO+3. At position 472, VR-1004 has ca. 20% C, which is also characteristic of higher-level passages of the Sabin strain. Thus, although we did not sequence the entire genome of this strain, the data for positions that distinguish the Sabin strain from its progenitor suggest that ATCC VR-1004 represents a higher-level passage of the Sabin vaccine strain.

Mutation at position 472 has long been considered a major determinant of attenuation (2). This conclusion was based on the isolation and analysis of neurovirulence of revertant strains, which were presumed to contain only a single base substitution at position 472. However, analysis shows that these revertants contain two base substitutions, at positions 472 and 2493. Thus, it is important to reevaluate the influence of each of these mutations on the neurovirulence of poliovirus type 3. We analyzed several virus preparations containing various amounts of both 472-C and 2493-U. The preparations included commercial vaccine lots, various passages of the Sabin strain cultured under conditions favoring selection of revertants, and clones obtained by plaque purification with subsequent sequence characterization. All preparations were tested for the abundance of reversions at positions 472 and 2493 and inoculated intraspinally in rhesus monkeys according to the WHO procedure.

Figure 4A shows the results of the monkey neurovirulence test presented as a plot of the mean histologic lesion scores versus the content of 472-C in the preparations. A clear correlation showing that the abundance of 472-C in excess of 1% agrees with the failure of the vaccine lots in the preparations. A clear correlation showing that the abundance of 472-C in excess of 1% agrees with the failure of the vaccine lots in the monkey test can be observed. In contrast, the plot of neurovirulence scores versus the content of 2493-U (Fig. 4B) does not reveal such a correlation. It demonstrates the following results: (i) A group of samples containing 100% 2493-U produced a wide range of mean lesion scores, from 0.13 to 2.13. (ii) Vaccine samples made of SO seed at the SO+2 passage level which have approximately the same high content of 2493-U either passed or failed the monkey test. (iii) Commercial vaccine lots which produced the same range of low mean lesion scores and which accordingly passed the monkey test have a 2493-U content ranging from less than 1 to 100%.
lack of the apparent influence of 2493-U abundance on the neurovirulence of the virus in monkeys is also demonstrated by the results of analysis of clone L111, which was obtained by plaque purification from the ATCC VR-1004 virus (Fig. 4). At all genome positions differentiating wild-type and attenuated strains, this clone was found to contain sequences characteristic of an attenuated Sabin strain. Quantitative sequence characterization showed that this clone contained no detectable reversions at position 472 and 100% reversions at position 2493 and showed the lowest histologic lesion score.

**DISCUSSION**

Because of a high mutation rate, RNA viruses cannot be considered a homogenous population. Rather, they are quasispecies consisting of a population of viral particles containing RNA with slightly different nucleotide sequences. Some of these sequence variants are not viable and contribute to the high ratio of physical to infectious particles, while others are selectively neutral and persist in the population. Under certain growth conditions, they may provide some benefit to the virus and be selected. To what extent can this sequence heterogeneity influence important biological properties of the virus such as neurovirulence?

Recently we demonstrated that the presence of 472-C revertants at a level as low as 1% predicts the failure of the vaccine in the monkey neurovirulence test (1). Thus, the presence of a small fraction of mutants in the virus population should be studied before any conclusions about the relationship between the genomic sequence of the virus and its biological properties are drawn.

In this communication, we present the results of a study of the genetic stability of live poliovirus vaccine and the sequence variability of selected wild-type strains of poliovirus type 3. We studied positions in the virus genome at which the attenuated Sabin strain differs essentially from its wild-type Leon/37 progenitor. Initially, the sequences of both attenuated and wild-type poliovirus were determined with cDNA clones, which allowed identification of 10 base substitutions between these strains. Recently, direct RNA sequencing (5), as well as the data reported in the present communication led to the refinement of the RNA sequence of Sabin poliovirus type 3 and identification of two positions at which the sequence of poliovirus type 3 RNA differs from the sequence of cloned cDNA (positions 2493 and 6061). The high level of sequence heterogeneity at some positions of the virus genome may be due either to a higher mutation rate at these positions or to higher selective pressure toward the selection of mutants or both. Of all positions studied, one (position 3333) revealed minor heterogeneity in wild-type strains and two (positions 472 and 2493) showed major heterogeneity in commercially produced vaccines and experimental preparations of the Sabin virus. At both of these positions, attenuated virus is unstable and rapidly reverts during cultivation in cell cultures. The major question, therefore, is which of these two mutations determines the reversion to neurovirulence in monkeys, which occurs upon passaging of the attenuated virus in vitro.

The results obtained in this study show a direct correlation between the abundance of mutations at position 472 and
neurovirulence in monkeys, providing additional proof of the important role of this position. The plot of histologic lesion scores versus the incidence of mutations at position 2493 indicates that this position of the genome has no direct influence on neurovirulence. Some apparent correlation between the two parameters in this plot can be attributed to the selection of both 472-C and 2493-U during cultivation in cell culture and may not reflect a cause-effect relationship. Samples with a high content of 2493-U also tend to have higher contents of neurovirulent 472-C reversions. Our unpublished data show that selection of these two mutations is independent, enabling preparations enriched in either one of these mutations to be obtained. By plaque purification, we were able to isolate a clone that contained complete reversion at position 2493 and no reversion at position 472, and this clone showed extremely low neurovirulence. Although we did not determine the entire RNA sequence of this clone and cannot exclude the presence of some other mutations which may modulate the expression of the mutation at position 2493, the data for other OPV samples also show that high 2493-U content is not necessarily accompanied by increased neurovirulence. The current WHO-I reference lot, which is the vaccine lot produced from SO seed at an SO+2 passage level, contains 85% 2493-U, and vaccines made by some manufacturers contain up to 100% 2493-U (but contain very few 472-C [unpublished data]) and still produce small lesions in monkeys and have a very good safety record (3). These data provide the evidence for the apparent inability of the mutation at position 2493 alone to increase neurovirulence in monkeys. Whether the mutation at position 472 alone is sufficient for this remains to be established in direct experiments, but the excellent correlation between the abundance of 472-C and the results of the monkey test implies the cause-effect relationship. While the increased content of 472-C predicts the failure of a vaccine lot in the monkey test, the content of U at position 2493 appears to reflect only the source and passage level of the virus and does not predict the results of the monkey neurovirulence test. Still, the mutation at position 2493 is not neutral, a conclusion which is proved by the rapid selection of 2493-containing revertants in passages. It would be important to find the biological significance of the threonine-to-isoleucine substitution at position 6 of capsid protein VP1 caused by this mutation and its relation to virus attenuation in a broader sense.

Recently, Westrop et al., by studying recombinant strains, showed that base substitution at position 2034 increases the neurovirulence of the virus (7). Our findings indicate that this position in the vaccine poliovirus type 3 genome appears to be stable during cultivation in cell culture and shows no heterogeneity in vaccine lots. This suggests that reversions at position 2034 have no selective advantage over the attenuated virus and therefore are not selected. In this study, we tested only those positions that are different in wild-type and attenuated strains of poliovirus type 3 and thus cannot rule out the possibility that some other mutations, different from the direct reversions that we studied, can occur during vaccine manufacturing. More extensive study of the virus genome stability should be performed to explore this possibility.

Finally, it should be emphasized that the results describing the roles of positions 472 and 2493 presented in this communication are relevant only to neurovirulence in monkeys. Although it is an important aspect of attenuation of the poliovirus, it may not cover the whole phenomenon. The excellent correlation between the results of the monkey test and the assessment of 472-C content suggests that this simple molecular procedure may be used for routine evaluation of acceptability of OPV type 3, at least as a supplement to the monkey test. At the same time, although determination of the abundance of reversions at position 2493 does not correlate with the results of the monkey test, it may be used as an additional means of monitoring the consistency of the vaccine manufacturing process.

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REFERENCES


