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Changes in Population Dynamics during Long-Term Evolution of Sabin Type 1 Poliovirus in an Immunodeficient Patient[▽]

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The evolution of the Sabin strain of type 1 poliovirus in a hypogammaglobulinemia patient for a period of 649 days is described. Twelve poliovirus isolates from sequential stool samples encompassing days 21 to 649 after vaccination with Sabin 1 were characterized in terms of their antigenic properties, virulence in transgenic mice, sensitivity for growth at high temperatures, and differences in nucleotide sequence from the Sabin 1 strain. Poliovirus isolates from the immunodeficient patient evolved gradually toward non-temperature-sensitive and neurovirulent phenotypes, accumulating mutations at key nucleotide positions that correlated with the observed reversion to biological properties typical of wild polioviruses. Analysis of plaque-purified viruses from stool samples revealed complex genetic and evolutionary relationships between the poliovirus strains. The generation of various coevolving genetic lineages incorporating different mutations was observed at early stages of virus excretion. The main driving force for genetic diversity appeared to be the selection of mutations at attenuation sites, particularly in the 5′ noncoding region and the VP1 BC loop. Recombination between virus strains from the two main lineages was observed between days 63 and 88. Genetic heterogeneity among plaque-purified viruses at each time point seemed to decrease with time, and only viruses belonging to a unique genotypic lineage were seen from day 105 after vaccination. The relevance of vaccine-derived poliovirus strains for disease surveillance and future polio immunization policies is discussed in the context of the Global Polio Eradication Initiative.

During their replication in humans, polioviruses accumulate mutations very rapidly due to the high error rate of their RNA-dependent RNA polymerase. This has been estimated to be between 10^{-4} and 10^{-5} substitutions per base per replication cycle (50). Mutations accumulate sequentially during human infection at a nearly uniform rate of 1 to 2% nucleotide changes per year overall, or 2.5 to 3% for synonymous positions, acting as a molecular clock and making it possible to establish epidemiological and temporal links between polio cases (18, 27, 43). After immunization with live attenuated oral polio vaccine (OPV), key mutations are selected for very rapidly in viruses replicating in the gut, which often leads to an increase in the virulence of poliovirus isolates excreted by vaccinees (50). However, the rate of vaccine-associated paralytic poliomyelitis (VAPP) among healthy vaccinees is very low, and the vaccine is considered very safe (1). The situation is different in persons with defects in their immune systems. Immunodeficient individuals, particularly those with antibody deficiencies, have been shown to have a much higher risk of VAPP (an estimated 3,000-fold excess) (63). While immunecompetent individuals excrete poliovirus for short periods after vaccination with OPV, which rarely exceeds several weeks, immunodeficient patients can excrete poliovirus for several months and even several years (43). To date, less than 40 such cases have been reported around the world (21, 43, 67). Among them, an individual in the United Kingdom is known to have been excreting Sabin 2-derived poliovirus for an estimated 20 years and is still excreting despite several attempts to interrupt the poliovirus excretion (41). Sabin viruses are also known to be transmitted from person to person and to circulate for prolonged periods in areas with low polio herd immunity (30). In order to devise strategies to deal with these poliovirus strains, derivatives of the Sabin live attenuated vaccine strains present in OPV have been classified into two broad categories for programmatic use. OPV-like viruses represent the vast majority of vaccine-related isolates and have a close sequence relationship (>99% VP1 sequence identity) to the original OPV strains. In contrast, vaccine-derived polioviruses (VDPVs) are those strains showing ≤99% VP1 sequence identity to the corresponding parental Sabin strain and are much rarer. The sequence drift shown in VDPVs is indicative of prolonged replication of the vaccine strain either in one individual (immunodeficient VDPVs [iVDPVs]) or in the community (circulating VDPVs [cVDPVs]). The prevalence of long-term polio excretion among individuals with immunodeficiencies is not well known, although recent studies involving 347 individuals with B-cell immune deficiencies in four different countries (United States, United Kingdom, Brazil, and Mexico) found no long-term excretors among them, which suggested that this could be a relatively rare phenomenon (21). However, these patients are considered a potential risk for the reintroduction of poliomyelitis epidemics in a prospective world without circulating wild poliovirus. cVDPVs are certainly of public health concern and have been associated with several polio epidemics (28). Examples of such events have recently been reported in Hispaniola (26), the Philippines (60), Egypt (69), Madagascar (59), China (35), Cambodia (67), Myanmar (66), Indonesia (67), and Nigeria (66). Other examples of possible VDPV circulation have been described in Belarus

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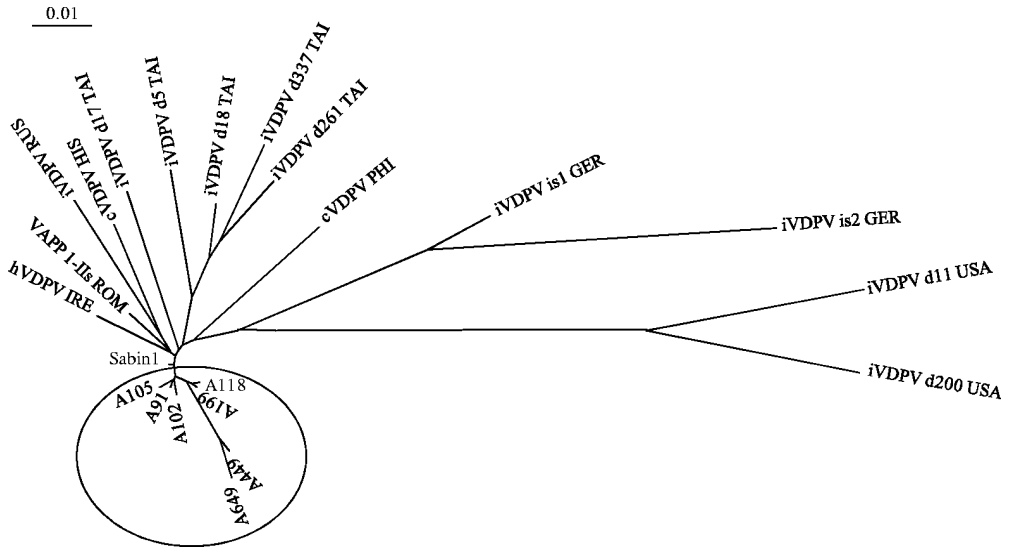


FIG. 1. Neighbor-joining rooted tree showing phylogenetic sequence relationships in the VP1 coding region between isolates from the immunodeficient patient (circled) and other type 1 vaccine-derived poliovirus strains described previously. The analysis included isolates from immunodeficient patients (iVDPVs), a healthy child (hVDPV), circulating epidemic strains (cVDPVs), and a VAPP case. The accession numbers and literature references are as follows: iVDPV USA, AF083931 and AF083937 (29); iVDPV GER, AJ132960 and AJ132960 (2); iVDPV RUS, AF462418 (8); iVDPV TAI, AF538840, AF538841, AF538843, AY928383, and AY928387 (68); hVDPV IRE (45); cVDPV HIS, AF405634 (30); cVDPV PHI, AB180070 (60); and VAPP ROM (19).

(33), Romania (11), and the Russian Federation (8). A single highly evolved type 3 VDPV strain has been recently isolated from sewage in Estonia (5), and several type 2 VDPV strains have also been found in environmental samples from Israel (61) and Slovakia (58). The increasing awareness of the implications of VDPVs has changed the perspective of the WHO-led Global Polio Eradication Initiative (GPEI), aimed at interrupting wild poliovirus circulation, in terms of disease surveillance strategies and present and future polio immunization policies (28). However, several questions about the biology of these poliovirus strains, which could determine how they are best controlled and managed in the context of the GPEI, remain unanswered.

A number of reports have described the evolution and properties of type 1 iVDPV strains from different patients, showing that viruses from various genetic lineages often coreplicate in the gut of the same individual and that all iVDPV isolates analyzed to date had lost the attenuation properties characteristic of the Sabin 1 vaccine strain (2, 8, 19, 29, 45, 68). Notably, a recent study by Yang et al. (68) describes the evolution of type 1 VDPVs over a 337-day period from day 10 after the onset of paralysis, estimated at 567 days after the last OPV dose. As many as five different genetic lineages were identified among isolates from this patient, and the presence of mosaic genome structures, indicative of recombination between lineages, was shown. However, all studies to date correspond to paralytic cases, involve virus isolates highly drifted from Sabin 1 after several months/years of replication, and often correspond to a single plaque-purified virus from a given stool sample. Patients with known immunological anomalies are generally not vaccinated with OPV, so poliovirus isolates from immunodeficient long-term excretors are available only after the diagnosis of VAPP, which can be several years after immunization, in patients who are diagnosed with immunodeficiency after adolescence/maturity when they have already received a full or partial course of routine OPV. Therefore, events that occur during the early and middle stages of poliovirus replication in these individuals remain largely unknown.

Here, we describe the evolution of the Sabin strain of type 1 poliovirus during a 646-day excretion period in an immunodeficient individual. The study analyzes isolates from day 21 to day 646 after immunization and includes samples taken at short intervals during the first ~100 days of poliovirus excretion. The patient was part of a study in the United Kingdom in 1963, during which a number of immunodeficient individuals were given monovalent OPV while undergoing immunoglobulin therapy (40). This article describes the properties of poliovirus strains isolated from 12 sequential samples from this patient. The evolution of viral properties, such as temperature sensitivity, antigenic structure, and neurovirulence, as well as phylogenetic relationships and changes in population dynamics throughout the period of virus excretion, were examined in detail. The results are discussed in the context of the GPEI strategy.

MATERIALS AND METHODS

Case history. The patient was a 3-year-old boy who had been on immunoglobulin replacement therapy for 2 years when he was first fed type 1 virus monovalent vaccine (40). The boy had shown very low immunoglobulin levels from early childhood and suffered from recurrent infections. On admission to the study, he showed immunoglobulin G (IgG) levels of 1.6 g/liter. At this stage, no detectable antibodies to poliovirus were found. At the time when he was given the vaccine, his IgG was 5.6 g/liter, his IgA was 1% that of the normal reference serum, and his type 1 poliovirus antibody titer was 1:64. Type 1 poliovirus was detected in stools collected during the next 32 months. At that point, the boy developed Sonne dysentery and was treated with colomycin. The next stool culture, 7 days after the onset of the dysentery, was negative for poliovirus, as were all subsequent specimens. The titer of excreted virus detected in tissue cultures was stable throughout the excretion period at about 10^4 cell culture

infective doses ($CCID_{50}$) per gram of feces. In an attempt to interfere with the excretion of type 1 virus, type 3 poliovirus was administered to the boy 5 months after excretion of type 1 poliovirus started, but no type 3 virus was detected in the stools. Type 3 was given again 3 months later, and a small amount was detected in the stool collected on the seventh day, but this had no effect on the amount of type 1 virus present. Monovalent type 2 polio vaccine was given 4 weeks later but was not recovered in stool cultures. No illness attributed to the poliovirus vaccine occurred at any time, but the boy died on 21 November 1966, more than 2 years after the last virus excretion. The polioviruses were originally isolated using monkey kidney cell cultures.

Poliovirus isolates. Fecal specimens were processed according to standard protocols for virus isolation and characterization (42). Virus isolations, plaque purifications, and preparation of virus stocks were performed in cell culture under the most permissive conditions so that less advantageous viruses would be able to replicate and cell line-specific selection of mutants would be minimized. Primary monkey kidney cells were used for virus isolation at 35°C. Plaque-purified viruses were obtained after 3 days of incubation of the original virus isolates on HEp-2c cell monolayers in a semisolid medium containing 1% Bacto agar as described previously. The plaque-purified viruses were then amplified by growth in HEp-2c cells for further characterization. Poliovirus isolates from days 21, 49, 63, 84, 88, 91, 102, 105, 118, 199, 449, and 649 after vaccination were available for characterization. The isolates were named with the letter A, the initial of the patient's name, followed by a figure corresponding to the day of excretion. Plaque-purified viruses were described by adding a number from 1 to 10 to the name of the virus isolate. Virus lineages were indicated by the letters a to d. Poliovirus type 1 Sabin vaccine and wild-type Mahoney strains were used as references in our experiments.

Reverse transcription, PCR, and nucleotide sequencing of poliovirus genomes. Poliovirus RNA was purified from cell culture supernatants and used for reverse transcription and PCRs using standard procedures. The purified viral reverse transcription-PCR DNA products were directly sequenced using the Big-Dye Terminator Cycle Sequencing Ready Reaction kit on the ABI Prism DNA 377 sequencer as specified by the manufacturer. Standard primers with Sabin sequences were used. Sequence data were stored as standard chromatogram format (*.scf) files, analyzed, and edited using the Wisconsin Package version 10.0-UNIX (GCG) and AlignIRV11 (Li-Cor) software. Sequencing was performed in both directions, and every nucleotide position was sequenced at least once on each strand.

Sequence analysis. Phylogenetic relationships between strains were established by comparing the sequences determined and aligning them using the alignment program CLUSTAL X (65). The degrees of nucleotide sequence identity and of protein similarity between strains were determined using the default scoring matrices. Phylogenetic relationships between sequences were inferred by the maximum likelihood method, with DNADIST/NEIGHBOR of PHYLIP (Phylogeny Inference Package) version 3.6, and a distance matrix was calculated using the F84 model of nucleotide substitution with a transition/transversion ratio of 10.0 (14). The robustness of phylogenies was estimated by bootstrap analyses with 1,000 pseudoreplicate data sets generated with the SEQBOOT program of PHYLIP. Phylogenetic trees were constructed using neighbor joining of PHYLIP and drawn using TREEVIEW (56) or NJ Plot (57) software.

Temperature sensitivity. Temperature sensitivity was assayed by comparison of plaque formation on HEp-2C cells at 35.0°C, 39.5°C, and 40.0°C as described previously (49).

Neurovirulence in transgenic mice. Tg21-Bx transgenic mice expressing the human poliovirus receptor were used for these experiments. Tg21-Bx mice are the product of crossing TgPVR21 mice (32) with BALB/c mice, followed by repeated backcrossing of offspring with BALB/c mice, interbreeding, and selection by PCR screening of tail DNA. The mice are homozygous for PVR and class II IA b genes ($H2^d$). The mice were inoculated intramuscularly (left hind limb) with 50 μl of a virus solution containing 10^6 50% $CCID_{50}$, and daily clinical scores were monitored for 14 days.

Antigenic characterization. The antigenic properties of the virus isolates were studied by a microneutralization assays using Sabin-specific monoclonal antibodies corresponding to antigenic sites 1 to 4 as described previously (52). One hundred copies of the challenge virus were used in the test. Monoclonal antibodies in ascytic fluid were used at a 1:100 dilution.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this article are available from the GenBank nucleotide sequence database under accession numbers EU794953 to EU794964.

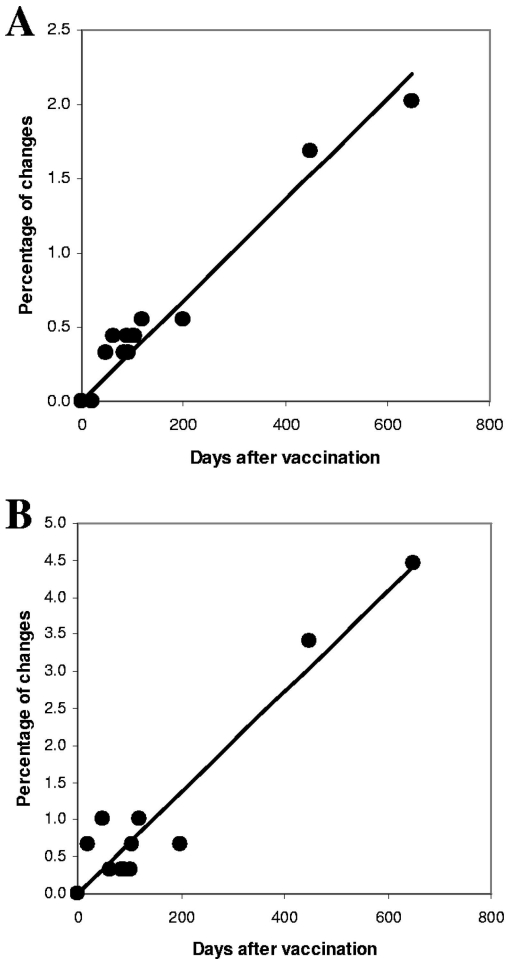


FIG. 2. Accumulation of nucleotide changes in the VP1 region of type 1 isolates from the immunodeficient patient during a 649-day period after vaccination with Sabin 1. (A) Percentages of synonymous plus nonsynonymous mutations with respect to Sabin type 1. (B) Percentages of synonymous substitutions with respect to Sabin type 1.

RESULTS

Phylogenetic analysis of poliovirus isolates. Sequence comparisons in the genomic region coding for the VP1 capsid protein are commonly used for the genotypic analysis of poliovirus isolates and to measure sequence evolution within genotypic lineages (25). Analysis of VP1 sequences confirmed the sequential nature of poliovirus isolates from the immunodeficient patient, which followed evolutionary pathways separate from those shown by other type 1 VDPV isolates from different origins described elsewhere (Fig. 1). This was due to the incorporation of mutations at different nucleotide positions, particularly at those involving synonymous sites. Figure 2 shows the accumulation of changes in the VP1 genomic region of sequential type 1 poliovirus isolates from the case under study. The number of nucleotide mutations is expressed as the percentage of changes with respect to the Sabin type 1 genome. The data were adjusted to linear functions for the accumulation of synonymous substitutions ($y = 0.0068x$; $R^2 = 0.92$) or synonymous plus nonsynonymous changes ($y = 0.0034x$; $R^2 = 0.95$). The rates of sequence evolution were estimated at 2.48%

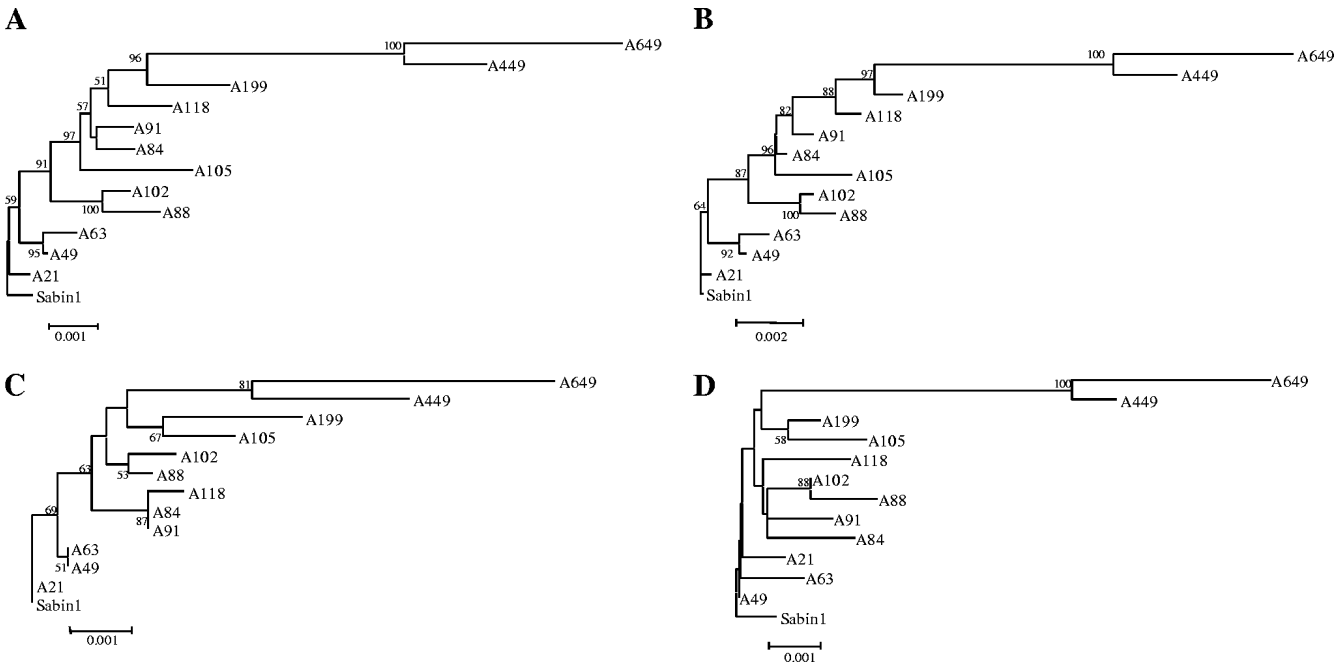


FIG. 3. Neighbor-joining trees representing phylogenetic relationships between the 12 strains and the Sabin type 1 strain across different genomic regions. (A) Complete genome. (B) P1 coding region. (C) P2 coding region. (D) P3 coding region. The numbers at the nodes indicate the percentages of 1,000 bootstrap pseudoreplicates supporting the cluster.

and 1.24% nucleotide substitutions per year, respectively, similar to those reported before for comparable series of VDPV isolates (2, 8, 19, 29, 45, 68). The genomic sequences of all isolates were completed from nucleotide 52 to the 3′ end and compared to the Sabin 1 nucleotide sequence. The analysis of phylogenetic trees based on nucleotide sequences from different genomic segments showed that there was close genetic relationship among isolates in all genomic regions analyzed (Fig. 3). None of the 12 isolates showed evidence of intertypic recombination with other polio or nonpolio enteroviruses. The phylogenetic layout of the isolates from the immunodeficient patient was statistically more robust, as judged by the higher bootstrap values of tree branches, when it was based on nucleotide sequence comparisons through the P1 (capsid) region (Fig. 3).

Genetic heterogeneity among viral isolates. The nucleotide sequence analyses described above revealed sequence heterogeneity at certain nucleotide positions in some isolates (observed as mixed peaks in the sequencing chromatograms), suggesting the possible presence of mixed viral populations (data not shown). To test this hypothesis, 10 plaque-purified viruses were selected and grown from each of the original virus samples and then sequenced through the VP1 coding region. The results confirmed the presence of mixed viral populations in virus samples from the immunodeficient patient, particularly A49, A63, and A118. The genetic heterogeneity at each time point was estimated using two parameters. First, the average number of mutations between the 10 plaque-purified viruses from each stool sample based on pairwise comparisons was calculated, and second, the average number of mutations between unique virus variants identified at each time point was estimated. These two parameters are not necessarily identical and can vary to different extents depending on the number of mutations, the number of virus variants, and the proportion of these among the 10 plaque-purified viruses obtained from each virus sample. As shown in Fig. 4A, plaque-purified viruses containing few mutations with respect to the original sequence were identified in several of the virus samples, but most showed less than two nucleotide changes between them, the vast majority being synonymous substitutions. However, samples A49, A63, and A118 contained four, three, and two virus variants, with averages of 6.8, 9.0, and 9.0 mutations between them, respectively (Fig. 4A). Virus variants are defined here as those showing more than two VP1 nucleotide differences from the consensus sequence and any other variant at that time point. The virus variants were named with the subindexes a to d; related variants at different time points were assigned the same letter code for convenience. Indeed, the different virus variants found in samples A49 and A63 were genetically related and suggested the presence of at least two main groups in terms of the VP1 sequence, one represented by variant b and the other by variants a, c, and d (Fig. 4B). The virus variants showed differences at key nucleotide positions, some resulting in amino acid changes, including the deletion of 6 nucleotides that led to the deletion of 2 amino acids in antigenic site 1, found in variants A49c and A63c. Virus sample A118 consisted of two virus variants, a major variant (8 of the 10 plaque-purified viruses) and a minor variant (2 of the 10 plaque-purified viruses). The minor variant contained nine mutations with respect to the A118 consensus sequence (represented by the majority variant). In this case, none of the nucleotide changes except one (at nucleotide 2782, corresponding to amino acid VP1-101) resulted in an amino acid difference. Further nucleotide sequence analysis of the plaque-purified

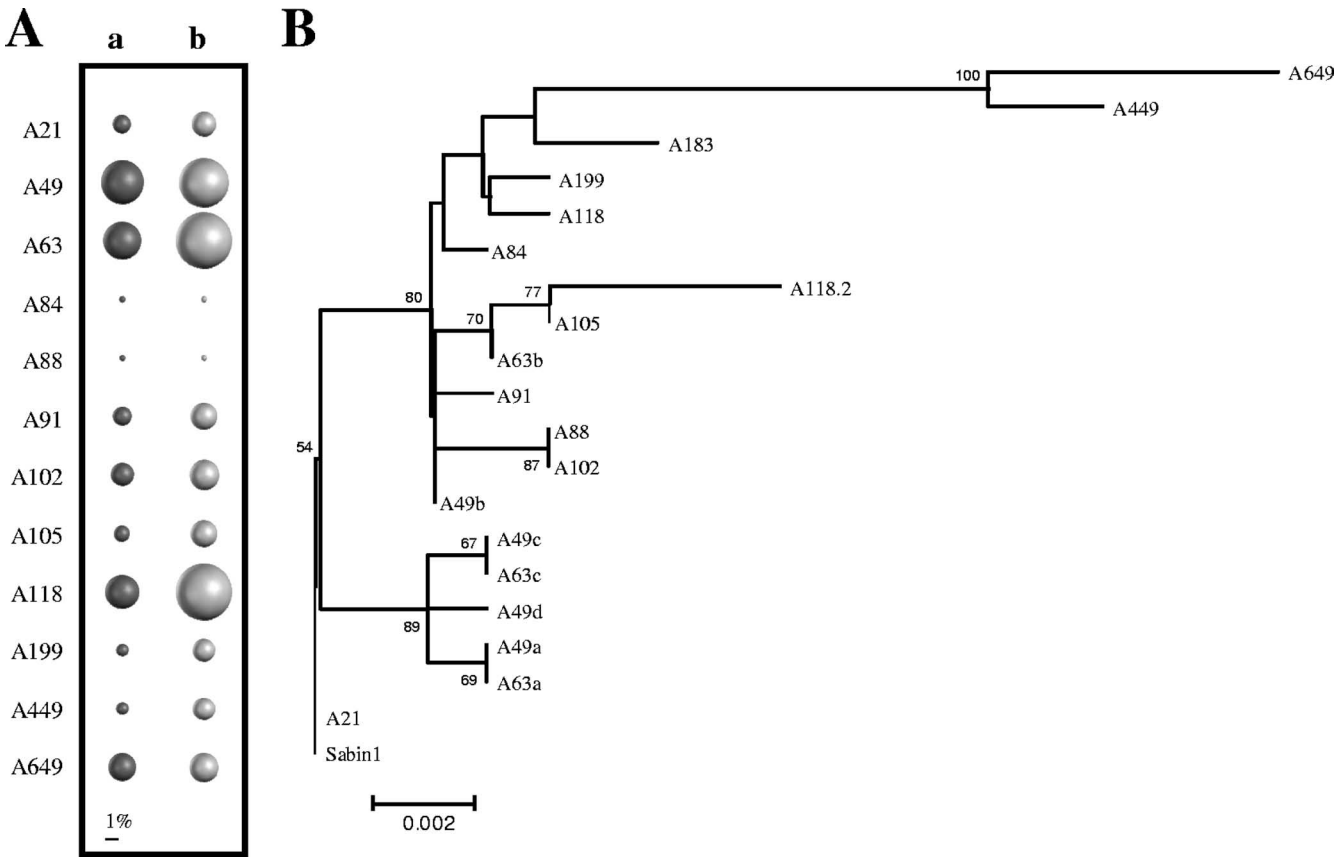


FIG. 4. Genetic heterogeneity among 10 plaque-purified poliovirus strains at each excretion time point. (A) The average number of mutations between 10-plaque purified viruses (a) or between unique virus variants (b) at each time point was estimated. The values are represented by bubble diagrams with various diameters. (B) Neighbor-joining tree showing phylogenetic relationships between plaque-purified viruses and Sabin 1. A comparison of unique virus variants identified at each time point is shown. The numbers at the nodes indicate the percentages of 1,000 bootstrap pseudoreplicates supporting the cluster. The lowercase letters represent genetic variants. The minor variant found on day 118 is shown as A118.2.

viruses to include fragments of the 5′ NCR (nucleotides 49 to 570) and the 3C/3D coding region (nucleotides 5760 to 6536) confirmed the above findings, although very few mutations were found between variants in the 3C/3D region to establish relevant conclusions (data not shown). Remarkably, important sequence differences were found between some of the virus variants from days 49 and 63 in the 5′ NCR, particularly at locations in domain V of the internal ribosomal entry site (IRES) known to have a role in the attenuation of Sabin vaccine strains (39) (Fig. 5). From day 84 onward, all 10 plaque-purified viruses from stool samples taken at a particular time point showed identical or very close nucleotide sequences, with the exception of the sample from day 118, which, as mentioned above, contained two different virus variants with nine VP1 nucleotide differences between them.

In order to determine the number and proportion of each virus variant in sample A49 more precisely, 100 plaque-purified viruses were selected and their VP1 nucleotide sequences were analyzed. Similarly, 100 plaque-purified viruses were selected and characterized from virus sample A649. This helped to determine if viruses from all four variants, such as those containing deleted VP1 amino acids, had survived. The same four variants a to d described above were found in sample A49 at proportions of 37%, 33%, 21%, and 9%, respectively. All 100 plaque-purified viruses from isolate A649 were closely related to each other, showing very few mutations between them (data not shown), as seen among the 10 A649 plaque-purified viruses initially analyzed. Mutations were found at only four VP1 amino acid positions among the 100 A649 plaque-purified viruses, and only one of them was present in more than one plaque-purified virus (the mutation at VP1-67 was found in three plaque-purified viruses).

Genetic relationships between iVDPV strains. The nucleotide sequence analyses discussed above revealed that complex genetic relationships existed among isolates from the immunodeficient patient, particularly during the first 9 weeks of poliovirus excretion. The nucleotide sequences of plaque-purified viruses representative of each of the four A49 variants and three A63 variants were completed in the 5′ NCR and capsid region (from nucleotides 52 to 3386), where mixed sequences had been detected. Figure 6A shows nucleotide sequence alignments at polymorphic sites between the isolates from the immunodeficient patient and Sabin 1 in this genomic interval. The results confirmed the close genetic relationship between the 12 iVDPV isolates as mutations accumulated with time. However, a number of nucleotide mutations appeared in isolates that were not carried forward to subsequent isolates, suggesting the existence of several coevolving and related lin-

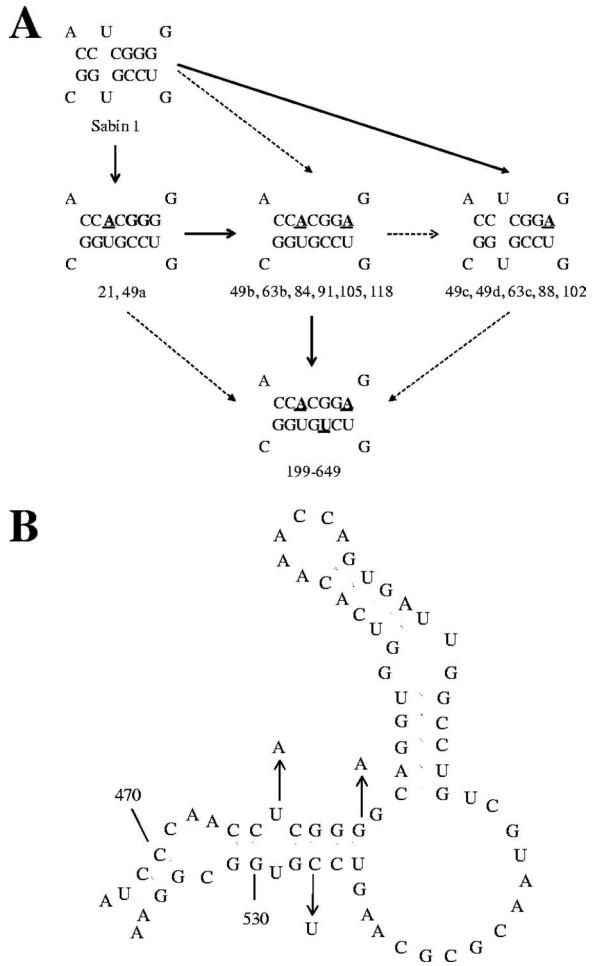


FIG. 5. Mutations found in domain V of the 5′ NCR in isolates from the immunodeficient patient. (A) Accumulation of nucleotide substitutions in the longer stem of domain V during the period of virus excretion. Mutations with respect to Sabin type 1 strain are underlined. The dates (number of days after vaccination) at which different genetic structures were found are shown. The lowercase letters indicate genetic variants. A continuous line indicates the most likely evolutionary pathway between two genetic structures based on sequencing data in other regions of the genome. The dashed lines indicate alternative sequence evolutionary routes. (B) Predicted secondary structure of domain V of the 5′ NCR of the Sabin 1 type 1 strain (64). The locations of mutations found at nucleotides 476, 480, and 525 are shown.

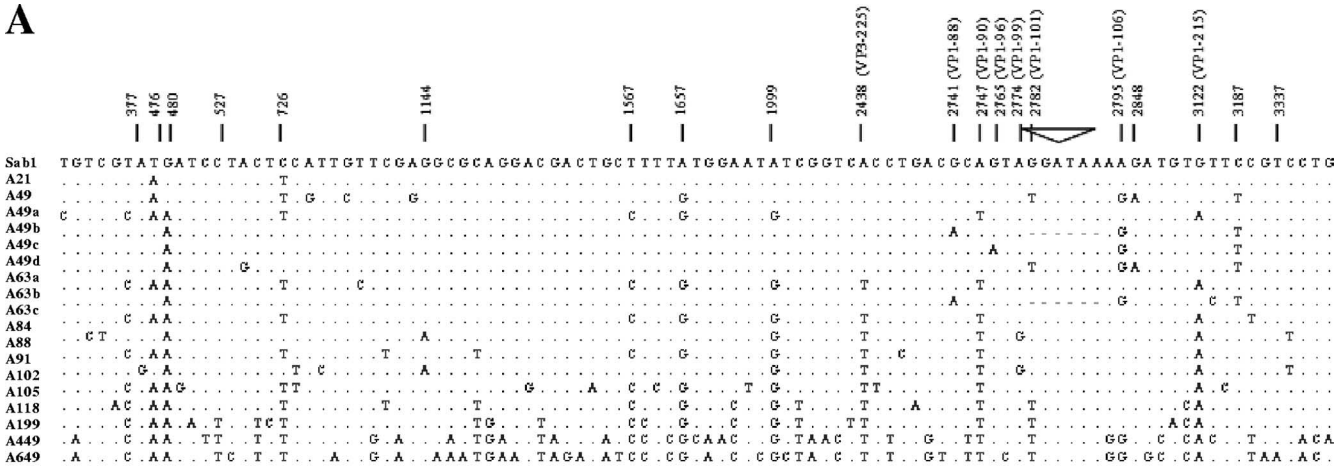
eages. There were also mutations linking two or more isolates that did not follow a strict sequential time course. Isolates A88 and A102, for example, shared seven unique nucleotide mutations, six of them at synonymous positions, that mapped throughout the genome and were not found in any other isolate.

Interestingly, virus isolates A88 and A102 showed a structure in domain V of the 5′ NCR similar to that of variant c (Fig. 5) but VP1 sequences related to that of variant b (Fig. 4B). As shown in Fig. 6A, identical sequences were identified at six nucleotide positions in the 5′-end section of the genome (between nucleotides 377 and 1657) that related viruses A84, A105, A118, A199, A449, and A649 but were different in isolates A88 and A102. All of these mutations were already present in virus variants A49b and A63b, found on days 49 and 63, respectively. None of the mutations found downstream of nucleotide 1657 followed the same pattern, and they accumulated in a more truly sequential manner, with viruses A88 and A102 showing mutations common to viruses A49b, A63b, and all those from day 84 in that region (Fig. 6A). The results indicate that genetic relationships between isolates were different in separate intervals of the genome, suggesting the possible occurrence of recombination events during the evolution of Sabin 1 in this patient. Isolate A88 is the most likely to have its origin in a recombination event. As illustrated in Fig. 6B, recombination between virus variants A63c and A63b to give a precursor for A88 would mean that the recombinant virus would have incorporated, in a single replication cycle, eight nucleotide mutations between nucleotides 377 and 1657 or eight mutations between nucleotides 1999 and 3187 with respect to either of the parental viruses. This is highly unlikely to have occurred by sequential acquisition of single mutations in such a short time and precisely at those nucleotide positions. Variant A63a also showed a number of nucleotide differences upstream of nucleotide 1657 with respect to its relative A49a, even though sharing a common VP1 sequence (Fig. 6A), which indicates that virus A63a may have also been the result of intratypic recombination.

The identification of virus variants at different time points suggested that viruses from two main genetic lineages coevolved between days 21 and 102. It is particularly interesting that virus isolates from days 84, 88, 91, 102, and 105 alternated the presence of viruses from either of the two genotypic lineages and that no mixed genotypes were identified in any of the stool samples from those dates.

Locations and biological significance of nucleotide and amino acid changes. Mutations were found at 17 positions in the 5′ NCR in isolates from the immunodeficient patient, 12 of them within the IRES and 5 in the hypervariable region at nucleotides 673, 713, 719, 726, and 730. Seven of the IRES mutations in isolates from the immunodeficient patient were found in single isolates at nucleotides 318 (in isolate A88), 331 (A88), 345 (A118), 442 (A102), 495 (A105), 503(A199), and 519 (in isolate A449). A mutation at nucleotide 203 was found only in the late isolates A449 and A649. Mutations at the other four IRES positions were present in several isolates, at nucleotides 377 in domain IV and at nucleotides 476, 480, and 527 in domain V, where mutations are known to play a very important role in the attenuation phenotype of all three Sabin strains (39). Isolates from the immunodeficient patient appeared to have followed two separate evolutionary pathways, incorporating different mutations in the main stem region of domain V (Fig. 5). Isolates found on days 49, 63, 88, and 102 had acquired a mutation at nucleotide 480 with respect to Sabin 1, which is a straight reversion to the sequence present in Mahoney virus and is commonly found in isolates from VAPP patients and healthy vaccinees (19, 34, 51). This mutation restores an AU base pair, strengthening the stem structure in domain V (62). Viruses that followed the second pathway first incorporated, by day 21, a mutation at nucleotide 476 that converted a UU mismatch to an AU base pair, increasing the stability of domain V (62). This mutation had also been seen before in type 1 isolates from vaccinees (12, 51). Domain V was further stabilized by the introduction between days 21 and 49 of the reversion at nucleotide 480 mentioned above. A third

A



B

Virus	Nucleotide position																					
	318	331	377	476	480	703	726	865	1144	1567	1657	1999	2438	2747	2774	2782	2795	2848	3122	3187	3337
A88	C	U	U	U	A	A	C	U	A	U	A	G	U	U	G	G	A	G	A	C	U
A63a	U	C	-	-	G	G	-	-	G	-	-	A	A	A	A	U	G	A	G	U	C
A63b	U	C	C	A	-	-	U	C	G	C	G	-	-	-	A	-	-	-	-	-	C
A63c	U	C	-	-	-	-	-	-	G	-	-	A	A	A	A	*	G	-	G	U	C

FIG. 6. Accumulation of nucleotide substitutions in isolates from the immunodeficient patient. (A) Alignment of nucleotide sequences at polymorphic sites in the 5′ NCR-P1 genomic region (nucleotides 49 to 3386) between isolates from the immunodeficient patient and the Sabin type 1 vaccine strain. Separate virus variants identified on days 49 and 63 were included. The locations of key nucleotide positions (discussed in the text) are shown. The location of nucleotides 2782 to 2787, deleted in variants A49c and A63c, is indicated by an inverted triangle. (B) Nucleotide differences between virus A88 and virus variants A63b and A63c. The dashes indicate that the sequence is the same as that of A88. *, virus A63c had a deletion of nucleotides 2782 to 2787.

mutation in the same stem region in domain V at nucleotide 527 was selected between days 118 and 199, which changed a GC to a GU base pair, slightly weakening the structure of domain V (Fig. 5A). This structure containing mutations at nucleotides 476, 480, and 527 was seen in all isolates thereafter.

Mutations were found at 25 capsid amino acid positions in isolates from the immunodeficient patient (Table 1). Most mutations were located on the external surface of the virus particle. Mutations in amino acids VP1-88, VP1-90, VP1-96, VP1-99, VP1-101, and VP1-102 were located at the BC loop that constitutes antigenic site 1 (23). A mutation at VP1-99 (from Lys to Asn or Glu) eliminated a trypsin cleavage site characteristic of Sabin type 1 (17). Amino acid substitutions were also found at antigenic site 2b (residues VP2-165 and -168 and VP2-173), antigenic site 3a (VP1-295 and VP1-298), and antigenic site 3b (VP3-60) (48). Amino acids VP2-140, VP2-141, and VP2-142 were located in the south rim of the canyon, a surface depression that encircles the fivefold axis, in a region that contacts the cell receptor and close to antigenic site 2b (3, 22). Mutations at VP1-106 and VP1-168 were found in the north rim of the canyon close to the BC loop. VP1-196 was located next to the conserved hydrophobic pocket, which is normally occupied by an endogenous lipid and where drugs that prevent virus uncoating are inserted (15, 53). A mutation at VP4-47 was located on the inner surface of the capsid shell at the interface between protomers. This region undergoes dramatic structural rearrangements after virus attachment to susceptible cells (15, 16). Mutations at VP1-215, VP2-222, VP3-4, and VP3-225 occurred in domains internalized in the native virion. Four of the capsid mutations, at VP1-88 (Ala to Thr), VP1-106 (Thr to Ala), VP2-165 (Asp to Asn), and VP3-225 (Met to Leu), were direct reversions to the parental Mahoney sequence.

Fewer mutations were found in nonstructural polypeptides. Mutations were identified at positions previously seen in other type 1 iVDPV isolates in the protease 2A at positions 2A-36 and 2A-134, in protein 2B at residue 2B-88, and in the polymerase 3D at amino acid 3D-73 (2, 8, 19, 29). The mutation at 3D-73 from His to Tyr is a straight reversion to the sequence in the Mahoney wild-type strain and has been associated with the temperature sensitivity of the Sabin 1 strain and, to a limited extent, to its attenuation phenotype (6, 47, 64). Amino acid mutations were mostly incorporated sequentially in isolates from the immunodeficient patient. However, as detailed in Table 1, mutations at some capsid and nonstructural amino acid residues were seen in only one or a few isolates, and some of them were not carried forward to subsequent isolates.

Temperature sensitivity and neurovirulence. The results for the temperature sensitivity and neurovirulence of isolates from the immunodeficient patient are shown in Table 2, together

TABLE 1. Amino acid changes between isolates from the immunodeficient patient and Sabin 1 and Mahoney strains

Protein	Position	Amino acid in strain[a]:													
		Sabin 1	A21	A49	A63	A84	A88	A91	A102	A105	A118	199	A449	A649	Mahoney
VP4	47	Ser	-	-	-	-	-	-	-	-	-	-	Ala	Ala	Ser
VP2	140	Thr	-	-	-	-	-	-	-	-	-	-	Asn	Asn	-
	141	Met	-	-	-	-	-	-	-	-	-	-	-	Ile	-
	142	His	-	-	-	-	-	Tyr	-	-	Tyr	Thr	Tyr	Tyr	-
	153	Lys	-	-	-	-	-	-	-	-	-	Arg	Arg	Arg	-
	159	Gly	-	-	-	-	-	-	-	-	-	-	Ser	Asp	-
	160	Thr	-	-	-	-	-	-	-	Ala	-	-	-	-	-
	165	Asp	-	-	-	-	-	-	-	-	-	-	Asn	Asn	Asn
	168	Thr	-	-	-	-	-	-	-	-	-	-	-	Glu	-
	173	Arg	-	-	-	-	-	-	-	-	-	-	Lys	Lys	-
	222	Val	-	-	-	-	-	-	-	-	-	Ala	Ala	Ala	-
VP3	4	Val	-	-	-	-	-	-	-	-	-	-	Ile	-	-
	60	Lys	-	-	-	-	-	-	-	Asn	Thr	Thr	Thr	Thr	Thr
	225	Met	-	-	Leu[b]	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
VP1	88	Ala	-	Thr[b]	Thr[b]	-	-	-	-	-	-	-	-	-	-
	90	Ile	-	Leu[b]	Leu[b]	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Met
	96	Ala	-	Thr[b]	-	-	-	-	-	-	-	-	-	-	-
	99	Lys	-	-	-	-	Glu	-	Glu	-	-	-	-	-	Thr
	101	Lys	-	Asn[b]	Asn[b]	-	-	-	-	-	Asn	Asn	Asn	Asn	-
	102	Asp	-	Del[b]	Del[b]	-	-	-	-	-	-	-	-	-	-
	106	Thr	-	Ala[b]	Ala[b]	-	-	-	-	-	-	-	Ala	Ala	Ala
	168	Glu	-	-	-	-	-	-	-	-	-	-	-	Gly	-
	196	Val	-	-	-	-	-	-	-	-	-	Ile	-	-	-
	215	Val	-	-	-	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	-
	295	Ser	-	-	-	-	-	-	-	-	-	-	Pro	Pro	-
	298	Asp	-	-	-	-	-	-	-	-	-	-	Asn	-	-
2A	36	Asn	-	-	-	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Ser
	86	Asn	-	-	-	-	-	-	-	-	-	-	Asp	Asp	-
	116	His	-	-	-	-	-	-	-	-	-	Asn	-	Asn	-
	134	Thr	-	Ser	Ser	Ala	Ser	Ala	Ser	Ser	Ala	Ser	Ala	Ser	Ser
2B	88	Val	-	-	-	-	-	-	-	-	Ile	-	Ile	Ile	-
	95	Thr	-	-	-	-	Ile	-	-	Ile	-	Ile	Ile	Ile	Ile
2C	51	Arg	-	-	-	-	-	-	-	-	-	-	Lys	Lys	-
3A	17	Leu	-	-	-	-	-	-	-	-	Pro	-	-	-	-
	35	Asp	-	-	-	-	-	Asn	-	-	-	-	-	-	-
3C	21	Ser	-	-	-	-	-	-	-	Asn	-	-	-	-	-
	53	Glu	-	-	-	-	-	-	-	Lys	-	-	-	-	-
	107	Ser	Asn	-	-	-	-	-	-	Asn	-	-	Asn	Asn	Asn
	180	Thr	-	-	-	-	Asn	-	-	-	-	-	-	-	-
3D	53	Asn	-	-	-	Ser	-	-	-	-	-	-	Ser	Ser	-
	73	His	-	-	-	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
	75	Lys	-	-	-	-	-	-	-	-	-	-	Arg	-	-
	95	Met	-	-	-	-	Val	-	-	-	-	-	-	-	-
	362	Ile	-	-	-	-	-	-	-	-	-	-	-	Thr	Thr

[a] -, same as in Sabin 1; Del, deleted.
[b] Mutation present in only some of the virus variants found on this date.

with a list of the mutations that could be responsible for the observed phenotypes as determined in studies reported elsewhere (6, 9, 24, 31, 47, 55, 64) (virus strains showing similar phenotypes are grouped together). These included reversions to sequences in the Sabin 1 wild parental virus, the Mahoney strain. The isolates evolved toward non-temperature-sensitive and neurovirulent phenotypes, as shown in studies carried out in tissue culture and Tg21-Bx transgenic mice that expressed the human poliovirus receptor. The results appear to confirm that mutations in the 5′ NCR, capsid amino acids, and polymerase residue 3D-73 contributed to the observed phenotypes (Table 2). A mutation at nucleotide 476 in domain V of the 5′ NCR seemed to only slightly affect both phenotypes, resulting in minor virus growth at 39.5°C and one paralyzed mouse (out of eight challenged) (Table 2). Changes at nucleotide 480, also in domain V of the 5′ NCR, and at various capsid amino acids seemed to have a noticeable effect on neurovirulence but led to only partial reversion of temperature sensitivity in Hep-2c cells. The effect on temperature sensitivity appeared to be increased by mutations at internal capsid residue VP3-225 and polymerase amino acid 3D-73 without a further increase in neurovirulence in transgenic mice. The results also indicate that a third mutation in domain V of the 5′ NCR at nucleotide 527 has a strong effect on both neurovirulence and temperature sensitivity, leading to virtually full reversion and properties comparable to those of the wild Mahoney strain. Other possibly relevant mutations were located at protease 2A, where changes have been shown to compensate for mutations in domain V of the 5′ NCR (38). A mutation at 2A-36 seen from day 84 may have an effect similar or additional to those of mutations at VP3-225 and 3D-73 (Table 2).

TABLE 2. Temperature sensitivities and neurovirulence of isolates from the immunodeficient patient

Virus	Nucleotide or amino acid position in strain[a]:													Temp sensitivity[b]		Neurovirulence[c] (no. of paralyzed mice/ total)
	5′ NCR			Capsid region						2A		3D Pol	3′ NCR			
	476	480	527	VP3-1	VP1-88	VP1-90	VP1-99	VP1-101	VP1-106	2A-36	2A-134	3D-73	7441	39.5°C	40°C	
Sabin 1	U	G	C	Met	Ala	Ile	Lys	Lys	Thr	Asn	Thr	His	G	>6.0	>6.0	0/8
A21	A	-	-	-	-	-	-	-	-	-	-	-	-	4.3	>6.0	1/8
A49a	A	-	-	-	-	-	-	Asn	*Ala*	-	*Ser*	-	*A*	1.9	4.7	5/8
A49b	A	*A*	-	-	-	*Val*	-	-	-	-	*Ser*	-	*A*	2.3	6.1	5/8
A49c	-	*A*	-	-	*Thr*	-	-	Del	*Ala*	-	*Ser*	-	*A*	2.1	4.6	6/8
A49d	-	*A*	-	-	-	-	-	-	*Ala*	-	*Ser*	-	*A*	2.6	5.2	4/8
A63	-	*A*	-	-	-	-	-	Asn	*Ala*	-	*Ser*	-	*A*	3.3	5.0	5/8
A84	A	*A*	-	*Leu*	-	*Val*	-	-	-	Asp	Ala	*Tyr*	*A*	0.9	2.6	5/8
A88	-	*A*	-	*Leu*	-	*Val*	Glu	-	-	Asp	*Ser*	*Tyr*	*A*	1.0	3.6	5/8
A91	A	*A*	-	*Leu*	-	*Val*	-	-	-	Asp	Ala	*Tyr*	*A*	0.6	3.5	2/8
A102	A	*A*	-	*Leu*	-	*Val*	Glu	-	-	Asp	*Ser*	*Tyr*	*A*	1.7	3.4	5/8
A105	A	*A*	-	*Leu*	-	*Val*	-	-	-	Asp	*Ser*	*Tyr*	*A*	0.4	2.8	6/8
A118	A	*A*	-	*Leu*	-	*Val*	-	Asn	-	Asp	Ala	*Tyr*	*A*	0.6	3.6	5/8
A199	A	*A*	U	*Leu*	-	*Val*	-	Asn	-	Asp	*Ser*	*Tyr*	*A*	0.0	0.4	7/8
A449	A	*A*	U	*Leu*	-	*Val*	-	Asn	*Ala*	Asp	Ala	*Tyr*	*A*	0.0	0.3	8/8
A649	A	*A*	U	*Leu*	-	*Val*	-	Asn	*Ala*	Asp	*Ser*	*Tyr*	*A*	0.1	0.6	8/8
Mahoney	U	A	C	Leu	Thr	Val	Lys	Lys	Ala	Ser	Ser	Tyr	A	0.0	0.1	6/8

[a] Mutations that reverted to Mahoney sequences are shown in italics, the same nucleotide or amino acid as in Sabin 1; Del, deletion.
[b] Virus titer reduction in HEp-2c cells at the given temperature with respect to virus titer at 35°C expressed as log $CCID_{50}$.
[c] Number of mice paralyzed with a dose of virus equivalent to 6.0 log $CCID_{50}$ (±0.25) by the intramuscular route.

Antigenic properties. The antigenic properties of the iVDPV isolates were determined by studying their reactivities with a panel of monoclonal antibodies of known specificity in tissue culture neutralization assays. The results are shown in Table 3. At some point during replication in the immunodeficient patient, the viruses lost reactivity with antibodies specific for antigenic sites 1, 2, and 3, but none showed an altered antigenic site 4. Antigenic site 1 was the first to be modified, and viruses from day 49 onward did not react with monoclonal antibody 955, specific for antigenic site 1. Mutations were later introduced in antigenic site 3 on day 105 and in antigenic site 2 on days 91, 118, 449, and 649. Resistance to neutralization by particular antibodies could be easily attributed to mutations in amino acids at or close to known antigenic sites in the three-dimensional viral structure (Table 3) (23). The only exception was isolate A84, which failed to react with one of the eight monoclonal antibodies specific for antigenic site 2. No obvious mutation(s) was found that could explain this phenotype. The observed antigenic modifications did not result in drastic structural changes, since all isolates were readily neutralized with sera from immunized individuals, showing neutralization titers comparable to those against the Sabin 1 vaccine strain (data not shown).

TABLE 3. Reactivities of isolates from the immunodeficient patient with Sabin 1-specific monoclonal antibodies

Virus	No. of neutralizing antibodies/total tested at site:			
	1	2	3	4
Sabin 1	2/2	4/4	2/2	2/2
A21	1/1	8/8	1/1	2/2
A49a	0/1	8/8	1/1	2/2
A49b	0/1	8/8	1/1	2/2
A49c	0/1	8/8	1/1	2/2
A49d	0/1	8/8	1/1	2/2
A63	0/1	8/8	1/1	2/2
A84	0/1	7/8	1/1	2/2
A88	0/1	8/8	1/1	2/2
A91	0/1	7/8	1/1	2/2
A102	0/1	8/8	1/1	2/2
A105	0/1	8/8	0/1	2/2
A118	0/1	7/8	0/1	2/2
A199	0/1	7/8	0/1	2/2
A449	0/1	4/8	0/1	2/2
A649	0/1	2/8	0/1	2/2

DISCUSSION

A thorough nucleotide analysis of poliovirus isolates found in sequential stool samples from an immunodeficient patient who had been fed Sabin 1 vaccine revealed complex genetic and evolutionary relationships between the poliovirus strains. The likely evolutionary pathways, based on sequencing data and analysis of plaque-purified viruses from each stool sample, are shown in Fig. 7. The first isolate, taken 21 days after vaccination, contained only six nucleotide mutations with respect to Sabin 1; two were in the 5′ NCR at nucleotide 476 in domain V of the IRES and at nucleotide 726 in the hypervariable region, and the other four changes were found in the nonstructural coding region, all resulting in synonymous changes except one mutation at amino acid 3C-107. This virus was temperature sensitive but showed only slightly increased

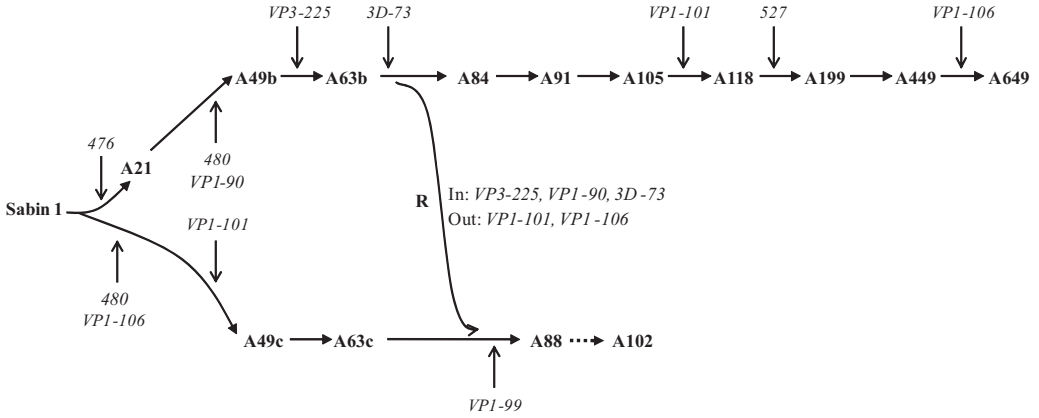


FIG. 7. Likely evolutionary pathways that link the virus isolates from the immunodeficient patient. The two main genetic lineages are represented. Incorporation of mutations at key nucleotide positions in the 5′ NCR (numbers) and relevant amino acid residues in the coding region (letters followed by amino acid numbers) is shown in italics. Isolates are shown in boldface. R indicates recombination across lineages. The lowercase letters represent genetic variants. The dashed arrows show possible links between two isolates in only part of the sequence.

neurovirulence in transgenic mice with respect to the Sabin 1 vaccine strain.

Four different variants designated a, b, c, and d were identified in the stool sample from day 49, possessing characteristic identifying mutations; subsequently, there was a narrowing of the lineages found. Viruses related to groups a, b, and c, but not d, were detected on day 63, and only viruses related to groups b and c were found until day 102. From day 105, only viruses related to group b were detected, including all viruses isolated as 100 separate plaques from the last positive sample taken on day 649. The virus seems to have evolved along several lineages, one of which eventually dominated, consistent with previous studies (44). Viruses A88 and A102 were of particular interest; the sequence suggested that virus A88 originated by recombination between a virus of group b and another of group c, as shown by the change in characteristic mutations and the absence of the 6-base deletion found in group c downstream of nucleotide 1999 (Fig. 5B).Virus A102 resembles A88 downstream from base 1999 but is different from all other viruses in the upstream region and therefore seems to have evolved independently of any of the other common viruses detected, but its history also involved recombination. It is remarkable that the recombination site involved the capsid region, which is not thought to be common, although it has been previously described (4, 37, 46, 68).

The common occurrence of recombinants during replication of poliovirus Sabin vaccine strains following immunization with trivalent OPV or during circulation of wild and cVDPV strains in epidemics (7, 20, 26, 36, 59, 60, 68, 69) means that a single cell must be doubly infected at some stage. The rapid drift in the virus, which must be a consequence of population bottlenecks, is also consistent with the existence of relatively few infectible cells at any one time. This could also explain why no type 2 and 3 viruses were detected in any of the stools, despite the fact that Sabin 2 (once) and Sabin 3 (twice) monovalent vaccines were given to the patient on separate occasions. The results suggest that replication of type 2 and 3 viruses, if it occurred, did not have an effect on type 1 virus evolution, but this cannot be completely ruled out.

The development of multiple virus lineages as commonly detected among sequential VDPV isolates from a single immunodeficient patient (2, 29, 45) could be a result of viral invasion of new areas of the gastrointestinal tract. As might be expected, the number of individual variants and the phenotypic significance of the mutations are greater in the early stages of infection, when the virus must adapt rapidly. Indeed, during the early period of the example shown here, the main driving force for diversity appears to have been the selection of mutations at known attenuation sites, particularly in the 5′ NCR and the VP1 BC loop.

Domain V of the 5′ NCR plays an important role in the attenuation of all of the live attenuated Sabin vaccine strains (39). Some isolates from the patient had acquired a mutation at base 480 that reverted directly to the wild-type sequence, generating an AU base pair from a GU, thus strengthening the stem structure in domain V (62). Others incorporated a mutation at base 476 that also strengthens the structure, while at a later date, isolates were obtained that had altered base 527 to weaken it, as shown in Fig. 5A. At the same time, the virus phenotype changed in ways that were predictable (Table 2). These observations emphasize the biological significance of maintaining the particular structure and the fine adjustments that lead to an optimal configuration, which is clearly not necessarily the most thermodynamically stable. The involvement of base 476 has implications for the use of molecular methods for the assessment of OPVs, such as MAPREC (Mutational Analysis by PCR and Restriction Enzyme Cleavage) (10), which currently focus only on bases 480 and 525 (13).

The majority of the capsid amino acid changes were in surface loop structures making up antigenic sites. Three of the four known antigenic sites varied over the course of the virus excretion period, including a deletion of 2 amino acids (residues 101 and 102 in VP1) in antigenic site 1 in one lineage. A similar virus, in which residues 102 and 103 were deleted, was isolated from a suspected polio case in Turkey in 1997 (54). Changes were observed throughout the period of infection up to the last isolate. This is consistent with a response to weak immune pressure originating from deficient immune responses or the humoral immunoglobulin given to the patient. However as the sites are surface loops, they would be expected to be

more accommodating to random mutations in any event, and the drift may have been purely stochastic. Other mutations were also identified, including changes in the canyon, drug-lipid binding pocket, internal sequences, and monomeric interfaces that might affect receptor binding or virus uncoating. Other changes of unknown significance were also seen. Changes in some of the capsid amino acids (VP1-88, -90, and -106 and VP3-225) and in 3D-73 were reversions to the wild-type Mahoney sequence and probably contributed to the phenotypic changes observed (50). Some of the mutations occurred in several lineages, suggesting a role in adaptation to the gut.

It is not known how any of the changes affect the transmissibility of the virus to others, although this has obvious implications for vaccination, surveillance, and outbreak control strategies. The VDPVs described here and elsewhere are essentially wild-type viruses in their measurable properties, and the phenomenon will condition future vaccination policies both for routine immunization and outbreak control and the eventual cessation of vaccination. The use of inactivated nonreplicating vaccine instead of the live attenuated strains would prevent the occurrence of long-term excreters of the type described here in future. However, there are uncertainties associated with inactivated nonreplicating vaccine with respect to its ability to control established outbreaks and the technical considerations associated with achieving the high coverage required. Development of efficient treatments to eliminate poliovirus from immunodeficient patients and effective methods for identifying long-term excreters of virus would be desirable. Understanding the pathogenesis and ecology of poliovirus infections may help us to find suitable intervention strategies for the polio endgame and after.

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