Evolution of the Sabin Type 1 Poliovirus in Humans: Characterization of Strains Isolated from Patients with Vaccine-Associated Paralytic Poliomyelitis

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Attenuated strains of the Sabin oral poliovirus vaccine replicate in the human gut and in rare cases cause vaccine-associated paralytic poliomyelitis (VAPP). Reversion of vaccine strains toward a pathogenic phenotype is probably one of the main causes of VAPP, a disease most frequently associated with type 3 and type 2 strains and more rarely with the type 1 (Sabin 1) strain. To identify the determinants and mechanisms of safety versus pathogenicity of the Sabin 1 strain, we characterized the genetic and phenotypic changes in six Sabin 1-derived viruses isolated from immunocompetent patients with VAPP. The genomes of these strains carried either few or numerous mutations from the original Sabin 1 genome. As assessed in transgenic mice carrying the human poliovirus receptor (PVR-Tg mice), all but one strain had lost the attenuated phenotype. Four strains presented only a moderate neurovirulent phenotype, probably due at least in part to reversions to the wild-type genotype, which were detected in the 5’ noncoding region of the genome. The reversions found in most strains at nucleotide position 480, are known to be associated with an increase in neurovirulence. The construction and characterization of Sabin 1 mutants implicated a reversion at position 189, found in one strain, in the phenotypic change. The presence of 71 mutations in one neurovirulent strain suggests that a vaccine-derived strain can survive for a long time in humans. Surprisingly, none of the strains analyzed were as neurovirulent to PVR-Tg mice as was the wild-type parent of Sabin 1 (Mahoney) or a previously identified neurovirulent Sabin 1 mutant selected at a high temperature in cultured cells. Thus, in the human gut, the Sabin 1 strain does not necessarily evolve toward the genetic characteristics and high neuropathogenicity of its wild-type parent.

Poliomyelitis is an acute paralytic disease caused by poliovirus, an enterovirus of the Picornaviridae family which is classified into three distinct and stable serotypes. Since the 1960s, poliomyelitis has been effectively controlled by the use of inactivated or live attenuated vaccines. The Sabin live oral poliovirus vaccine (OPV) is constituted of attenuated strains of each of the three serotypes which have been selected by numerous passages of wild-type strains in monkey tissues in vivo and in vitro (60). The OPV strains (Sabin 1, 2, and 3) replicate in the human gut and induce a strong and long-lasting immune response, including a local intestine immunity (59). Therefore, OPV has been widely used; because it acts against the feco-oral transmission of poliovirus strains in humans, it has been the tool of choice for the eradication of poliomyelitis. However, in rare cases (1 case per 0.2 to 2.5 million doses), OPV strains have been implicated in vaccine-associated paralytic poliomyelitis (VAPP) (62, 63).

Like other RNA viruses, poliovirus is highly mutable (14). Phenotypic changes due to the genetic variability of Sabin strains are probably one of the main causes of VAPP. Indeed, neurovirulent vaccine-derived strains are found in the gut of healthy vaccinees and in the central nervous system (CNS) of patients with VAPP (24, 43). Sabin 3 and Sabin 2 are more frequently isolated from VAPP cases than is Sabin 1 (about 12% of cases) (62, 63).

The poliovirion is a nonenveloped virus composed of a 7.5-kb positive-sense single-stranded polyadenylated RNA genome surrounded by an icosahedral capsid. The capsid is made of 60 promoters, each containing a single copy of the four structural capsid proteins (VP1 to VP4). The structure of the capsid proteins in virions has been established by X-ray crystallography (27). The genetic organization of the poliovirus genome has been reviewed by Wimmer et al. (67). The viral RNA contains a long open reading frame flanked by 5’ and 3’ noncoding (5’NC and 3’NC, respectively) regions involved in viral replication and translation. The predicted secondary structure of the 5’NC region defines six domains (numbered from I to VI [67]), corresponding to highly conserved structures formed by stems and loops (55, 61). Domains II, IV, and V and part of domain VI contribute to the internal ribosomal entry site which allows cap-independent initiation of translation of the viral genome (53). Structural and nonstructural viral polypeptides originate from a single polypeptide precursor which is cleaved by viral proteases.

The molecular determinants of attenuation have been intensively studied (reviewed in reference 43) to elucidate the mechanisms involved and to improve the safety of Sabin vaccine strains. The genome of Sabin 3 differs from that of the virulent wild-type progenitor at 12 nucleotide positions, three of which determine the attenuated phenotype. One substitution in the 5’NC region of the genome and another that changes an amino acid residue in a capsid protein are the major determinants of the attenuation of Sabin 3. Similarly, Sabin 2 has two major
attenuation determinants. The genome of Sabin 1 differs from that of its parental virulent Mahoney virus by 54 point mutations (49). As with other OPV strains, Sabin 1 is temperature sensitive (ts), and the ts phenotype of Sabin 1 correlates rather well with the attenuated phenotype (7, 51). Molecular determinants of the attenuation and temperature sensitivity Sabin 1 have been found in the 5’NC and 3’NC regions of the genome, as well as in the regions encoding VP1, VP3, and VP4 capsid proteins and the RNA polymerase (3D) (6, 7, 23, 28, 29, 51, 66). The presence of numerous attenuation determinants is believed to slow down the reversion of Sabin 1 toward pathogenicity in vaccinees.

Some aspects of the genetic and phenotypic changes undergone by the Sabin 1 strain after passages in cultured cells, monkeys, and healthy vaccinees have previously been described (11, 17, 36, 57). Preliminary molecular studies of a few Sabin 1-derived strains associated with paralytic disease have also previously been reported (20–22, 25, 43, 52). To study further the determinants and mechanisms which contribute to the safety versus pathogenicity of the Sabin 1 strain, we investigated the genetic and phenotypic changes this strain undergoes when it is involved in VAPP and thus thought to have acquired some particular pathogenic characters. We report detailed genomic and phenotypic analyses of six Sabin 1 vaccine-derived strains isolated from patients with VAPP in Romania from 1980 to 1992. The genomes of these strains diverged by various degrees from the original Sabin 1 vaccine strain. This study reveals a new determinant in the 5’NC region of the genome that is involved in reversion toward neurovirulence. The neurovirulence of each VAPP strain was evaluated in transgenic mice carrying the human poliovirus receptor (PVR-Tg mice). Most had lost the attenuated phenotype but appeared to be only poorly or moderately neuroviral, and none were as neuroviral as was the neuroviral parent of Sabin 1. A comparison of these VAPP strains with a previously identified highly neuroviral Sabin 1 mutant selected at a high temperature in cultured cells suggested that in the human gut, the Sabin 1 strain does not encounter factors (temperature and/or cell types) that favor rapid reversion toward high neurovirulence.

MATERIALS AND METHODS

Cells and viruses. HEP-2c cells were grown in monolayers in Dulbecco modified Eagle’s medium (DMEM) supplemented with 5% newborn calf serum. The attenuated poliovirus vaccine Sabin 1 strain (S1/lab) was obtained from the World Health Organization master seeds for OPV preparation [Behringwerke (S0 + 1)]. The second passage at 34°C of the original seed was used to prepare the viral stock (S0 + 1 + 2). The neurovirulent strain PV1/Mahoney (Mah/lab) was maintained in our laboratory and grown at 37°C in HEP-2c cells.

The type 1 field strains analyzed in this study were isolated from stools and, in one case, cerebrospinal fluid of patients with VAPP. The viruses were isolated from primary monkey kidney cells and passaged once or twice on HEP-2c cells. The virus stocks resulted from a further passage on HEP-2c cells at a multiplicity of infection of 5 to 10 PFU per cell. For all passages, vaccine-derived viruses were grown at 34°C. Viruses were counted. Plaque purification of viruses from stocks was performed as described above for plaque titration, except that cells were stained with neutral red and then individual plaques were picked.

Nucleotide sequencing. Viral RNA was extracted as previously described (40). Briefly, HEP-2c cells were infected with the appropriate virus stock and were lysed (5 h after infection) in the presence of 0.5% Nonidet P-40. Cytoplasmic RNAs were recovered by phenol-chloroform extraction in the presence of 1% sodium dodecyl sulfate and sequenced with avian myeloblastosis virus reverse transcriptase (18).

Viral RNA was sequenced with Sabin 1-specific primers (lengths of about 20 nucleotides) spread every 150 to 250 nucleotides over the entire genome. This allowed us to determine, when necessary, the nucleotide sequence of the whole genome, except for the 25 terminal nucleotides in the 3’NC region just upstream from the poly(A) tract. This fragment was sequenced after reverse transcription of the genomic RNA and PCR by using the primers and method of Rezapkin et al. (57). The presence of the Sabin-like nucleotide at position 7441 of the 3’NC region of the RNA genome was confirmed by comparative sequence analysis with a cDNA primer, (T20)C (66).

The DNA sequences of plasmid DNA and PCR products were determined with a Sequenase kit (United States Biochemical) or by dye-labelled terminator cycle sequencing (Perkin-Elmer) with an automated DNA sequencer (model 377; Applied Biosystems).

Construction of infectious cDNAs and mutant viruses. Escherichia coli DH5α (Bethesda Research Laboratories) was used for propagation of plasmids. DNAs were isolated with restrictions on plasmid sequences under conditions recommended by the manufacturer (New England BioLabs).

pKK17 contains the full-length Mahoney cDNA downstream from the simian virus 40 (SV40) late promoter, with the origin of replication, enhancer sequences, and T antigen gene from SV40 (50). pVN1(3’C-07T) contains similar SV40 regulatory sequences, including the T antigen gene, and the entire genomic cDNA of the L-Sc, 2ab Sabin strain of PV1 (33).

pS1/189C was obtained by replacing the PmlI-AgeI fragment (nucleotides 38 to 339) of the Sabin 1 cDNA in pVS1(1JC-0T) with the homologous fragment isolated from the Mahoney cDNA in pk17. The reciprocal exchange was also done to construct pMH189U. The corresponding mutant strains, S1/189C and Mah/189U, differed from their parental viruses at nucleotide position 189 only. To construct pS1/480A, the AgeI–SnaBI fragment (nucleotides 339 to 2545) of plasmid S1F/480A (41) was isolated and inserted in place of the corresponding fragment in pVS1(1JC-0T). The corresponding mutant virus, S1/480A, differed from Sabin 1 only by carrying the wild-type nucleotide at position 480.

Prior to transfection, selected sequences of the infectious cDNAs were determined to verify the presence of the introduced mutations and the integrity of each restriction site used for plasmid construction. For pS1/189C and pMH/189U, the fragmented inserts were sequenced entirely.

Transfection of cDNAs. Plasmids containing full-length PV infectious cDNAs and regulatory sequences of SV40 were used to transfect simian Vero cells (1.5 µg of DNA per 10⁶ cells) by the calcium phosphate technique previously described (9). Transfected cells were incubated at 34°C until the cytopathic effect was complete (70 to 2 h for all plasmids). Two independently obtained cDNAs (a and b) were transfected for pS1/189C and pMH/189U, and one cDNA each was transfected for pS1/189C, pS1/480A, and pMH/189U. Viral RNA of each virus stock was sequenced to ensure the presence of the introduced mutations.

The specific infectivity of each plasmid was measured. Cells were transfected with various dilutions of plasmid DNA, maintained under a 0.9% agar overlay, and stained (23). The number of viral plaques per amount of plasmid DNA was determined. The differences between the specific infectivities of parental and mutant cDNAs (from 3 to 4 PFU/pmol of pk17 and pMH/189U DNA/10⁶ cells) and from 15 to 50 PFU/pmol of pVS1(1JC-0T), pS1/189C, and pS1/480A DNA/10⁶ cells were not large.

Ret marker. Reproductive capacities at different temperatures (Ret marker) were evaluated by an Ret test. Ret is defined as the difference between the log⁴₀ virus titer of a viral stock measured at the optimal temperature (37°C) and that at each of the suboptimal temperatures (35.5, 39.5, 40.1, and 40.5°C ± 0.1°C). Ret titers were determined on HEP-2c cells by an endpoint microtiter method (47) after 6 days of incubation at the appropriate temperatures and were expressed in % protection culture infective dose units (TCID₅₀) per ml Viruses with Ret values at 40°C (between 37 and 40°C) of 2 were considered to be ts.

Assay of neurovirulence in PVR-Tg mice. Viruses were tested for neurovirulence in homozygous PVR-Tg21 mice, which are susceptible to poliovirus infection. Groups of six 4- to 5-week-old mice (three males and three females) were inoculated intracerebrally (i.c.) with 30 µl of virus. The lengths of virus stock or concentrated virus were made in DMEM containing 0.1% fetal calf serum. Mice were inoculated to cover the viral titer range, causing disease in 100 to 0% of mice (i.e., PD₅₀ [see below] test). In rare cases, although the viral suspensions with the highest titer available was inoculated, the 100% paralyzed gene could not be reached. To confirm the inoculated dose, viral suspen-
were titrated before and after inoculation. The viral dose that induced paralysis or death in 50% of mice (PD₅₀) after 21 days was calculated by the method of Reed and Muench (56). To check the identity of the mutant virus which caused the disease, the spinal cord was recovered from at least one diseased mouse, the virus was isolated, and its genome was partially sequenced to verify the presence of the introduced mutation.

**RESULTS**

Type 1 VAPP cases. Six patients with VAPP were selected for this study because type 1 vaccine-derived poliovirus strains were isolated from stools and, in one case, cerebrospinal fluid (CNS isolate). The patients were infants between 4 and 13 months of age and had clinically confirmed paralytic poliomyelitis (65). These VAPP cases were classified according to the epidemiologic and laboratory classification system for paralytic poliomyelitis cases (64). Epidemiological data are presented in Table 1. Cases 1-III, 1-IV, 1-V, and 3-IV were classified as recipient cases because the patients received the OPV 4 to 30 days prior to the onset of the disease. Although the patients received OPV, two cases (1-I and 1-II) were classified as community cases, a category used for patients neither recently vaccinated nor known to live in contact with individuals having received OPV (contact cases). In case 1-I, a vaccine-derived poliovirus was isolated from stool samples and a nasopharyngeal swab, but the long delay (9 months) between the vaccination of the patient and disease onset precluded its classification in the recipient category. Conversely, for case 1-II, febrile illness started 3 days prior to OPV administration (not shown) followed by paralysis onset, and a highly mutated vaccine-derived virus was isolated from stool samples (see below). Case 1-II was therefore classified as a VAPP community case.

The viruses were isolated on primary monkey kidney cells and passed two or three times on HEp-2c cells at 34°C. The viruses isolated from samples were identified by neutralization with serotype-specific sera and characterized as vaccine-derived by intratypic differentiation with monoclonal antibodies and by restriction fragment length polymorphism analysis (Table 1). In three VAPP cases (1-I, 1-II, and 1-III), only Sabin 1-derived polioviruses were isolated from the clinical samples. In case 1-IV, the type 1 vaccine-derived poliovirus was detected in two stool samples in association with coxsackie A5 (CA5) virus. Although the prototype strain of CA5 virus was isolated from a stool sample of a patient with poliomyelitis (42), CA5 virus is known to be associated in humans with vesicular enteroviral stomatitis (4), hand, foot, and mouth disease (42), and herpeticangina (68). Moreover, the anti-CA5 neutralizing antibody titers (<10 and 10 [for two blood samples]) in this patient’s blood samples were negligible. Thus, it is likely that the paralytic disease of case 1-IV was due to a Sabin 1-derived poliovirus. For case 1-V, the presence of two poliovirus serotypes in the single stool sample available made the identification of the true etiologic agent of the disease impossible. In case 3-IV, as noted previously (24), a type 1 vaccine-derived strain was isolated from the cerebrospinal fluid. However, in two stool samples collected on consecutive days, only mixtures of type 3 vaccine-derived polioviruses (type 3 nonrecombinant and type 3-type 2 intertypic recombinant) were detected, suggesting that the ancestor of the strain identified in the CNS was not excreted on the days of sample collection (24).

The presence of an antipolio virus antibody response indicated that each patient was immunocompetent (Table 1). The antibody titer for PV1 increased from the first to the second blood samples in most cases, suggesting active infection by this virus. In three cases (1-II, 1-III, and 1-IV), only anti-PV1 antibodies were detected. In case 1-I, the presence of anti-PV2 antibodies, in addition to anti-PV1 antibodies, may have resulted from immunization of the patient 9 months before the onset of disease. In this case, the presence of a single PV1 strain in stool and nasopharynx samples strongly suggested that a Sabin 1-derived poliovirus was the etiologic agent. In case 3-IV, serological data did not further implicate a PV1 strain in the disease, as titers of antibodies against all three serotypes increased between days 8 and 17 of the disease, indicating that all vaccine strains actively replicated in the vaccine.

**Genomic analysis of the type 1 strains isolated from VAPP cases.** To evaluate the genetic shift undergone by the Sabin 1 strain associated with VAPP, the genomes of the strains isolated from stool samples of cases 1-I to 1-V (strains 1-Is to 1-Vs) and that of the strain from cerebrospinal fluid of case 3-IV (strain 3-IVc) were analyzed by RNA sequencing. Strains isolated from the first stool samples were studied, except for case 1-IV, for which the second stool sample was analyzed. The genomic regions implicated in attenuation or temperature sensitivity, i.e., the 5'NC and 3'NC regions and the capsid and polymerase 3D coding regions of the viral genome, were in-

### Table 1. Epidemiological data for VAPP cases with type 1 vaccine-derived poliovirus isolates

<table>
<thead>
<tr>
<th>Case</th>
<th>Yr of isolation</th>
<th>OPV history</th>
<th>Time (days)</th>
<th>VAPP case classification</th>
<th>Virus(es)</th>
<th>Delay (days)</th>
<th>Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-I</td>
<td>1981</td>
<td>2</td>
<td>270</td>
<td>Community</td>
<td>PV1 (2 s, 1 n)</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>1-II</td>
<td>1980</td>
<td>1</td>
<td>5</td>
<td>Community</td>
<td>PV1 (3 s)</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>1-III</td>
<td>1980</td>
<td>1</td>
<td>14</td>
<td>Recipient</td>
<td>PV1 (1 s)</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>1-IV</td>
<td>1981</td>
<td>1</td>
<td>13</td>
<td>Recipient</td>
<td>PV1, CA5 (2 s)</td>
<td>14</td>
<td>128</td>
</tr>
<tr>
<td>1-V</td>
<td>1984</td>
<td>1</td>
<td>28</td>
<td>Recipient</td>
<td>PV1, PV3 (1 s)</td>
<td>14</td>
<td>180</td>
</tr>
<tr>
<td>3-IV</td>
<td>1992</td>
<td>1</td>
<td>20</td>
<td>Recipient</td>
<td>PV1 (1 c), PV3 (2 s)</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

**a** Number of doses of trivalent OPV.

**b** Delay between vaccination and paralysis onset.

**c** The numbers of stool(s), nasopharynx (n), and/or CNS (c) samples from which the indicated virus(es) were isolated are noted parenthetically.

**d** Delay between paralysis onset and blood sampling. The delay between paralysis onset and the first stool sampling was approximately the same as that for the first blood sampling (±1 day).

"NK," not known.

**e** Data are reciprocal serum dilutions that neutralized 100 TCD₅₀ of homotypic reference type 1 (PV1), type 2 (MEF1-PV2), and type 3 (Saukett-PV3) polioviruses.

"f" Delay between paralysis onset and blood sampling.
vestigated first. Because critical Sabin 1-specific nucleotides are likely to be selected against in vaccinees, all nucleotides and amino acid codons which differentiate the Sabin 1 and Mahoney strains in these genomic regions were checked. As the effect of attenuating Sabin 1 nucleotide 480 (5’9NC region) can be suppressed by a second-site mutation at nucleotide 525 (7), this nucleotide was also checked. In all strains, at least 25% of the genome was sequenced. Moreover, for a more accurate view of the mutation patterns that characterize strains implicated in VAPP, the sequences of the entire genomes of strains 1-IIs and 1-IIIs (except positions 7414 through 7440 in strain 1-IIs) were determined, as they appeared to be highly and poorly mutated, respectively. These two strains were plaque purified before sequencing. Sequencing results are presented in Fig. 1 and 2.

There were few differences between most of the strains analyzed (1-Is, 1-IIs, 1-IVs, and 3-IVc) and Sabin 1. Six mutations were found in the entire 1-IIs genome, and only one or two mutations were found in strains 1-Is, 1-IVs, and 3-IVc. In these three last strains, 25% of the genome was sequenced, suggesting an average of four to eight mutations per total genome. (The genomic fragments sequenced and the corresponding nucleotide positions are indicated in Fig. 2). In contrast, 71 mutations were detected in 1-IIs; we estimate that there were about 24 mutations in strain 1-Vs.

All VAPP strains, except strain 3-IVc, displayed nucleotide substitutions in the 5’9NC region, at nucleotide positions 480 (strains 1-Is, 1-IIs, 1-IVs, and 1-Vs) and 189 (strain 1-IIIs), two positions which differentiate Sabin 1 and Mahoney. These substitutions were reversions to the wild-type Mahoney genotype. Reversion at nucleotide position 480 is associated with increased neurovirulence (29, 40, 41), probably by restoring base pairing in a stem of domain V in the 5’9NC region (61). A reversion at position 189 was found in the plaque-purified and the original nonpurified 1-IIIs viral stocks. The contribution of this reversion to deattenuation was analyzed (see below).

Other mutations were discovered in the 5’9NC region of strains 1-IIs, 1-IVs, and 1-Vs, mostly outside the structural domains of the 5’NC region (nucleotides 105, 443, 576, 701, and 721). Two others, located in stems of domain VI, would not be expected to affect base pairing (nucleotides 609 and 618) (55, 61).

Missense mutations were found in the capsid coding regions of only two strains, strains 1-Vs and 1-IIs. In both strains, reversion at codon 106 of VP1 (VP1-106) was detected. This
reversion in vaccinees has previously been described (31). Three missense mutations, including two reversions, were found in strain 1-IIs at codons VP4-65, VP3-60, and VP1-90, which differentiate Sabin 1 and Mahoney. Reversions at codons VP4-65 and VP1-106 are believed to play a significant role in neurovirulence (6, 7). Some of the other missense mutations, at codons VP1-222 and VP3-59-60, may affect the antigenic structures of the viruses, as the corresponding amino acids map in neutralization antigenic sites 2 and 3a, respectively (44).

In the polymerase 3D coding region of the genome, a missense mutation in codon 3D-52 and a reversion in codon 3D-73 of strain 1-IIs were found. This last change contributes to loss of temperature sensitivity and may be moderately involved in reversion toward neurovirulence (7, 41, 66). A reversion at position 7441, also shown to contribute (although poorly) to loss of temperature sensitivity (23) was found in the 3'NC region of the 1-IIs genome.

Missense mutations were also identified in the 1-IIs genome in regions encoding nonstructural proteins. Surprisingly, two of them were located in protease 2A codons (2A-36 and 2A-134) which differentiate Sabin 1 and Mahoney, suggesting the presence of a selection pressure against these Sabin 1 codons. The role of these two mutations is unknown. Nevertheless, mutations in protease 2A have been associated with the adaptation and neurovirulence of poliovirus in normal mice (35).

The only mutation detected in strain 3-IVc was at position 1885. This mutation is silent and in a VP3 codon. Nucleotide 1885 differentiates the Sabin 1 and Mahoney genomes. The same mutation was found in strain 1-IIs, but not in the other four VAPP strains analyzed. Among the 64 mutations located in the 1-IIs coding region, 51 were silent. These silent mutations were evenly distributed throughout the 1-IIs genome. In addition to the silent mutation at position 1885, one appeared at another locus that differentiates Sabin 1 and Mahoney, position 3919. Reversion at position 3919 has also been found in one Sabin 1 virus isolated from an inoculated monkey (36). Thus, the effect of most of the 26 silent mutations in Sabin 1 (compared with Mahoney) on viral multiplication may be neutral since they appeared to be stable during replication in humans. However, a few, including those at positions 1885 and 3919, may be selected against. A silent mutation at nucleotide 3955 was found in 1-IIs and a Sabin 1 mutant isolated from persistently infected cells (5).

Temperature sensitivity of strains. To evaluate the residual of Sabin 1 strains, after replication in patients with VAPP, the reproductive capacity of each strain (Rct marker) was determined (Table 2). Most of the VAPP strains were clearly ts with Rct values at 40°C of >4.0 (loss of titer of >4.0 log_{10} TCID_{50}/ml between 37 and 40°C). The highly mutated VAPP strain 1-IIs was slightly ts with an Rct value at 40°C of 2.7. Only two of the VAPP strains, 1-Vs and particularly 1-IIs, showed no signs of temperature sensitivity at 39.5°C, with all other VAPP strains displaying Rct values at 39.5°C of >3.0. The reduction in temperature sensitivity correlated well with the number of mutations in the genomes of VAPP strains (compared with Sabin 1); however, none of these strains showed full reversion to the non-ts phenotype of the Mahoney strain.
Neurovirulence of strains in PVR-Tg mice. To test the neurovirulence of VAPP strains, PVR-Tg mice were i.c. inoculated with various dilutions of viral stocks (i.e., PD_{50} tests). The PD_{50}s of VAPP strains were calculated and compared to those of the reference laboratory strains Sabin 1 and Mahoney.

Although PVR-Tg mice have been shown to be suitable for investigating poliovirus neurovirulence (28), we first checked the validity of this PVR-Tg mouse test for evaluating the neurovirulence of VAPP strains, PVR-Tg mice were i.c. inoculated with various dilutions of viral stocks (i.c.-PD_{50} tests). The PD_{50}s of VAPP strains were calculated and compared to those of the reference laboratory strains Sabin 1 and Mahoney.

All strains but 3-IVc had lost the attenuated phenotype and had PD_{50}s between those of the Mahoney and Sabin 1 strains (Table 2). Strain 3-IVc displayed a PD_{50} similar to that of the Sabin 1 strain.

Strains 1-Is, 1-IVs, and 1-Vs had roughly similar moderate levels of neurovirulence. Reversion to the wild-type genotype at position 480 of the 5′NC region thus correlates with a drop in PD_{50} of about 2 log_{10} PFU (compared with that of Sabin 1), although other mutations may jointly contribute to this phenotype. Apparently, reversion at codon VP1-106, previously believed to be involved in reversion to neurovirulence (6, 7), did not correlate with increased neurovirulence in strain 1-Vs. Nevertheless, other missense mutations, like those detected in the capsid coding region of this strain, may exert an antagonist effect on the neurovirulent phenotype.

Strain 1-IIIs lost the attenuation phenotype of the Sabin 1 strain, although to a lesser extent than did the other VAPP strains. Among the six mutations found, reversion to the wild-type genotype at position 189 of the 5′NC region seems to be the best candidate for the mutation responsible for this phenotypic change (see below).

The most neurovirulent VAPP strain was 1-Is, which contained the largest number of nucleotide differences compared to the Sabin 1 strain. However, its neurovirulence was lower than that of the Mahoney strain, suggesting that in contrast to strain S1-39°C_{CA}, strain 1-Is did not contain a set of molecular determinants to allow full neurovirulence.

**Comparison of the genomic characters of strains 1-IIIs and S1-39°C_{CA}** Sabin 1 mutant strain S1-39°C_{CA} was more neurovirulent than was strain 1-Is (7). The patterns of mutations in these two strains were compared in detail. S1-39°C_{CA} carries mutations in the 5′NC and 3′NC regions (nucleotide positions 525 and 7441, respectively), the 3D coding region (3D-73), and the capsid coding region (VP3-225, VP1-88, and VP1-106) of the genome (7). The sequences of previously unsequenced parts of the S1-39°C_{CA} genome were determined (except for the first nucleotide) (Fig. 1). Beside three silent mutations, missense mutations were found in the capsid coding region at codons VP1-4 and VP1-168 and another one was found in the region coding for the nonstructural protein 2C at codon 2C-253. Although Sabin 1 nucleotide 480 and codons VP4-65 and VP1-134 are believed to play a significant role in attenuation
The incidence of VAPP cases due to type 1 vaccine-derived poliovirus is significantly lower than that for type 3 or type 2 virus, suggesting that the type 1 vaccine strain has the most stable attenuated phenotype (63). The rarity of type 1 VAPP cases makes it difficult to study the evolution in humans of the type 1 vaccine strains associated with this disease. Here, we present a detailed analysis of the genomes and phenotypes of six vaccine-derived PV1 strains isolated from patients with VAPP. Surprisingly, although the genomes of some of the VAPP strains carried many mutations compared to the original Sabin 1 strain, none were as neurovirulent as highly neurovirulent PV1 strains.

The Sabin 1-derived field strains were recovered, with one exception (case 3-IVc), from stool samples. As previously shown, strains detected in stools do not necessarily correspond to the true etiologic agent which multiplies in the CNS and causes paralysis (24). This is obviously relevant to recipient cases which present mixtures of strains in stool samples. For example, for case 1-V, it was impossible to determine among the type 1 and type 3 strains isolated from stools which strain induced the disease. Nevertheless, for cases 1-I, 1-II, 1-III, and 1-IV, the absence of other paralytogenic strains in stools and in most cases the serology of the patients suggest that the recov-
tered Sabin 1-derived strains were the etiologic agents of the disease. The difficulty of isolating poliovirus from cerebrospinal fluids (42) is such that only one Sabin 1 strain (strain 3-IVc) was available from this source. In this case, only type 3 polioviruses were found in stool samples; the 3-IVc ancestor may have been absent from stools when the samples were collected (24). Surprisingly, most of the sequences of genomic fragments of 3-IVc and its neurovirulence in PVR-Tg mice were similar to those of Sabin 1. However, one silent mutation which differentiated the genome of 3-IVc and that of Sabin 1 was found, suggesting that 3-IVc replicated in the patient and indicating that laboratory contamination was unlikely. Moreover, this mutation was not found in five vaccine and reference Sabin 1 strains at the laboratories where 3-IVc was isolated or analyzed.

The fact that poorly or moderately neurovirulent strains, like those investigated in this study, may be implicated in VAPP appears to be surprising and leads to questions about the validity of the PVR-Tg mouse model for evaluating the pathogenic potentials of Sabin 1-derived poliovirus mutant strains for humans. To date, poliovirus neurovirulence in PVR-Tg mice correlates with low pathogenicity in monkeys (28) and low neurovirulence in monkeys correlates with low pathogenicity in humans (59). The PVR-Tg mouse line used in this study (homozygous PVR-Tg21 mice) has been proposed as an animal model for safety testing of OPV preparations (1). Moreover, the levels of neurovirulence of Sabin 1 mutants S1-38°C5 and S1-39°C6 and those of the reference strains Sabin 1 and Mahoney and Sabin 1 strain viral RNAs. Nucleotide positions in the genomic RNAs are indicated.

FIG. 3. Predicted stem-loop structure of domain III in the 5' NC region of the Mahoney and Sabin 1 strain viral RNAs. Nucleotide positions in the genomic RNAs are indicated.

that restores base pairing with Sabin 1 nucleotide 480 (5, 7, 11, 17, 21, 34, 36, 45, 57). One of our strains (1-III) which was no longer attenuated carried a true reversion to the wild-type genotype at position 189 in the 5' NC region. It was previously suggested that this could affect the attenuated phenotype of the Sabin 1 strain (28). By comparing the neurovirulence of site-directed mutant viruses containing the wild-type nucleotide at position 189 or 480 in a Sabin 1 background for PVR-Tg mice, we found that each of these mutations contributed to the reversion toward neurovirulence of the Sabin 1 strain, with the mutation at position 480 having a more pronounced effect on the clinical outcomes of affected mice. However, Sabin 1 nucleotide 189 in a Mahoney context did not lead to any detectable attenuation effect, indicating that the role of nucleotide 189 may depend on other Sabin 1 determinants. Nucleotide 189 is located in the lower stem of domain III of the 5'NC region (55, 61). Unlike domain V, domain III of poliovirus type 3 is not required for ribosomal entry (54). However, deletion of domain III from a type 2 poliovirus 5'NC region reduced internal translation by 50% (48) and a PV1 variant lacking domain III displayed delayed growth (15). A cellular 50-kDa protein binds to this domain (46), and this protein-RNA interaction is conserved among other picornaviruses (16). The interaction is dependent upon maintenance of the secondary structure of the lower stem of domain III (16). Thus, the U → C mutation at position 189 found in VAPP strain 1-III may modify some viral function and restore some level of neurovirulence by favoring base pairing in the lower stem of domain III (Fig. 3).

Among the type 1 field strains analyzed, the most neurovirulent strain (1-III) was highly divergent from Sabin 1, with 71 nucleotide differences, including 13 missense mutations. The localization of these mutations clearly indicated that this strain was not a Mahoney-like wild-type poliovirus but a strain derived from Sabin 1 poliovirus, since 44 nucleotides that differentiate Sabin 1 from Mahoney were maintained. Only 11 mutations, including 7 missense mutations, appeared at nucleotides or codons which differentiate the Sabin 1 and Mahoney strains. Strain 1-III had thus considerably diverged not only from Sabin 1 but even more from Mahoney (107 nucleotide differences). However, the difference between the polypeptide sequences of Mahoney and 1-III (22 amino acids)
was comparable to that between Mahoney and Sabin 1 (21 amino acids). Possibly, structural and/or biological constraints restrict the genetic shift of poliovirus polypeptides. We cannot exclude the possibility that some of the mutations in strain 1-IIs were selected during isolation and passages in cultured cells. However, the use of primary monkey kidney or HEp-2c cells and a temperature of 34°C minimize the accumulation of mutations in Sabin 1 during passing (8, 57). Since all the viruses described here were treated in a similar manner and some of them displayed very few nucleotide differences with Sabin 1 (strains 1-Is and 3-IVc), it is probable that the genetic shift of the analyzed strains in cultured cells was very limited. Thus, the large divergence between strain 1-IIs and the original Sabin 1 strain mainly reflects in vivo genetic variation and suggests that this vaccine strain can survive for a long time in the field. Indeed, case 1-II was a community case; therefore, the patient was infected by contact, indicating that strain 1-IIs had replicated in at least two humans before isolation. During wild-type PV1 epidemics, nucleotide substitutions are fixed in the genome with a frequency of one to two substitutions per week (50) and most of them in the coding region are silent (58). Presumably, the 51 silent substitutions in 1-IIs (compared with Sabin 1) were selected with a similar frequency. To our knowledge, this is the most mutated and thus oldest vaccine-derived poliovirus isolate described. This could have important implications for the poliomyelitis eradication program.

In contrast to strain 1-IIs, the highly neurovirulent Sabin 1 mutant 1-IIs (7), selected at a high temperature in human HEp-2c cells, was genomically less divergent. Twelve nucleotides, including seven missense mutations in the coding region, differentiated Sabin 1 and 1. (Suppl.) 39°c (7). The high neurovirulence and loss of temperature sensitivity of strain S1.39°c correlated with the presence of mutations at nucleotide 525 (probably restoring the 480-525 base pairing), codons VP3-225, VP1-88, VP1-106, and 3D-73, and nucleotide 7441. All these positions have been implicated in temperature sensitivity and/or attenuation of Sabin 1 (2, 7, 23, 66). Surprisingly, two other Sabin 1 residues, VP4-65 and VP1-134, which contribute to attenuation (6) have been maintained. However, the mutations at codons VP1-4 and VP1-168 may suppress the effect of VP4-65 and VP1-134. VP1-4 and VP4-65 are located in two distinct but neighboring regions on the inner face of the poliovirus capsid (27) which simultaneously control the release of viral RNA in the early step of infection and morphogenesis of new infectious particles in the last step (12, 32). Residue VP1-168 is located in the EF loop and is exposed at the surface of the virion on the north rim of the canyon near neutralization antigenic site 1; this region, like the hydrophobic pocket area which contains VP1-134, controls the early events in poliovirus infection (10, 19, 26). The eventual role in neurovirulence of the missense mutation in codon 253 of the nonstructural membrane-associated protein 2C is not known. Thus, in strain S1.39°c, at least 8 of the 12 mutations (compared with Sabin 1) may contribute to increased neurovirulence and loss of temperature sensitivity. In contrast, in the moderately neurovirulent VAPP strain 1-IIs, the maintenance of Sabin 1 residues VP3-225 and VP1-134, which have been implicated in attenuation (6, 7), may at least in part restrict the neurovirulence of this strain.

None of the type 1 VAPP strains analyzed were as neurovirulent as the Mahoney strain, the parental wild-type strain of Sabin 1. This contrasts with the presence in stools of patients with VAPP of neurovirulent Sabin 3-derived isolates inducing high lesion scores in monkeys, with many of them being as neurovirulent as the parental wild-type progenitor or even more so (2, 37). Type 2 VAPP isolates had intermediate to high lesion scores (39). Similar results were obtained when the neurovirulence of type 3 and type 2 stool isolates from patients with VAPP was evaluated in intraperitoneally inoculated PVR-Tg mice (24). Thus, from this work and another work (34), there is a clear difference between type 1 and at least type 3 isolates found in the gut of patients with VAPP. Sabin 3 strains passaged in infants achieved neurovirulence levels comparable to that of the parental wild-type strain within a few days of a single OPV dose, whereas passaged Sabin 1 did not (11). It is probable that the large number of genetic differences between Sabin 1 and Mahoney, including numerous attenuation determinants, inhibits the shift of the Sabin 1 strain toward Mahoney-like characteristics, particularly neurovirulence. Nevertheless, a small number of passages and the cloning of Sabin 1 at high temperature on HEp-2c cells were sufficient to introduce all the mutations (no more than 12 mutations) necessary for S1.39°c to become as neurovirulent as Mahoney (7). This may indicate that factors encountered at high temperatures in cultured cells, but not those encountered in the human gut, favor rapid and direct reversion toward a full neurovirulent phenotype. Presumably, in the human gut, the Sabin 1 strain is not subject to strong selection pressures (temperature and/or cell types) that favor the rapid appearance of the Mahoney-like neurovirulent character. As strain 1-IIs diverges from Sabin 1 mainly by silent mutations, the Sabin 1 strain may be well adapted to grow in the human gut. Alternatively, the high genetic divergence between Mahoney and Sabin 1 may also give Sabin 1 characteristics which differ in some way from those of a simple Mahoney mutant. This may favor the evolution of the Sabin 1 strain in the human gut toward other new genotypic and phenotypic characteristics which differ from those of Mahoney and do not necessarily involve neurovirulence, thereby explaining the safety of Sabin 1 in vaccines.

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