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High Diversity of Poliovirus Strains Isolated from the Central Nervous System from Patients with Vaccine-Associated Paralytic Poliomyelitis

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To establish the etiology of vaccine-associated paralytic poliomyelitis (VAPP), isolates from the central nervous system (CNS) from eight patients with VAPP were compared with stool isolates from the same patients. The vaccine (Sabin) origin was checked for all of the available isolates. Unique and similar strains were recovered from paired stool and CNS samples for five of the eight VAPP cases and the three wild-type cases included in the study. In the remaining three VAPP cases, the stool samples and, in one case, the CNS samples contained mixtures of strains. In two of these cases an equivalent of the CNS isolate was found among the strains separated by plaque purification from stool mixtures, and in one case different strains were isolated from CNS and stool. This shows that the stool isolate in VAPP might not be always representative of the etiologic agent of the neurological disease. A wide variety of poliovirus vaccine genomic structures appeared to be implicated in the etiology of VAPP. Of nine CNS vaccine-derived strains, four were nonrecombinant and five were recombinant (vaccine/vaccine or even vaccine/nonvaccine). The neuropathogenic potential of the isolates was evaluated in transgenic mice sensitive to poliovirus. All of the CNS-isolated strains lost the attenuated phenotype of the Sabin strains. However, for half of them, the neurovirulence was lower than expected, suggesting that the degree of neurovirulence for transgenic mice is not necessarily correlated with the neuropathogenicity in humans.

Poliomyelitis is an acute paralytic disease caused by three distinct serotypes of poliovirus. Over the past 30 years, the disease has been controlled by the use of two vaccines: the inactivated poliovirus vaccine and the oral poliovirus vaccine (OPV). The OPV, the most widely used vaccine, has the advantage of conferring solid intestinal immunity and thus efficiently interrupting the transmission of wild-type poliovirus strains. The OPV consists of live, attenuated Sabin poliovirus strains (35) and has a good general safety record. However, in a small number of cases, estimated as 1 in 500,000 to 1 in 1,200,000 first-vaccine-dose recipients, vaccine-associated paralytic poliomyelitis (VAPP) may appear as a consequence of the vaccination (27). VAPP is defined as an acute paralytic illness in which vaccine-like poliovirus is isolated from stool and is believed to be the cause of the disease (1). Such disease is most frequently associated with the type 2 and type 3 vaccine strains and rarely with the type 1 vaccine strain (2, 30).

When the trivalent OPV is administered, the opportunity is given for strains of the three different serotypes to multiply in the receiver's gut. The isolation of a strain from stool in a case of paralysis temporally associated with OPV vaccination does not necessarily mean that this is the actual strain which induced the neurological disease. The fact that the stool-isolated strain switched to a neurovirulent phenotype is evidence for its neuropathogenic potential, but this still does not constitute a formal proof that it caused the disease, since similarly modified strains are recovered systematically from healthy vaccinees (6, 17). Moreover, the existence of other enteroviruses, for instance, enterovirus 71, which is implicated in polio-like paralytic syndromes, throws another shadow on this causal association (10, 11, 19). As a consequence, the term vaccine-associated poliomyelitis has been used, instead of vaccine-caused poliomyelitis, to express the uncertainty regarding the actual etiologic agent of paralysis cases (34).

Poliovirus strains isolated from the central nervous system (CNS) from patients with VAPP are the most probable etiologic agents of the neurological disease. Assuming that the strains isolated from the CNS of such patients are the result of multiplication of the neuropathogenic virus in the CNS, we focused our analysis on such strains. We found that all the CNS isolates studied lost the attenuated phenotype of the original Sabin strains, as determined from tests on transgenic mice sensitive to poliovirus infection (12, 16). The poliovirus replicating in the CNS is thought to be derived from the virus population initially multiplying in the patient's gut (20). Thus, it was necessary to compare the strain multiplying in the gut and excreted in the stool (stool isolate) with the strain isolated simultaneously from the CNS of the same patient. This also gave us the opportunity to determine whether the virological diagnosis is reliable. The stool-isolated strain usually serves as the basis for the diagnosis. However, this strain neither is specific for the diseased condition nor necessarily coincides with the strain that multiplies in the CNS.

MATERIALS AND METHODS

Cells and viruses. HEp-2c and simian Vero cells were grown in monolayers in Dulbecco modified Eagle's medium supplemented with 5% fetal calf serum.

One attenuated strain and one neurovirulent reference laboratory poliovirus strain were used for each serotype. The attenuated viruses, Sabin 1, 2, and 3, were obtained from the World Health Organization "master seeds" for the OPV preparation (Behringwerke [S0+1]). The second passage of

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TABLE 1. PCR primers and restriction enzymes used for RFLP assay

Primer	Sequence (nucleotide position)[a]	Assay	Restriction enzymes[b]
UG13	(3617) CCCACCTTCCAGTACATGGA (3636)	RFLP-2AB	*Hae*III, *Dde*I
UC22	(4152) TCAGTAAATTTCTTCAACCA (4133)		
UG23	(4169) AAGGGATTGGAGTGGGTGTC (4188)	RFLP-2C	*Hae*III, *Dde*I, *Hin*fI, *Rsa*I
UC15	(4965) CATCTCTTGAAGTTTGCTGG (4946)		
UG15	(4936) CTGTCACCAACCAGCAAACTT (4956)	RFLP-3AC	*Hae*III, *Hin*fI, *Rsa*I
UC16	(5940) TGTGAACCGTTCCCACCAAC (5921)		
UG16	(5921) GTTGGTGGGAACGGTTCACA (5940)	Nested PCR	
UC12	(6516) TCAATTAGTCTGGATTTTCCCTG (6494)		
481G	(440) GAGTCCTCCGGCCCCTGAATGCGGCTAATCC (470)	SSR-481[c]	*Afl*III (A, vaccine)
481C	(521) GCGCGTTACGACAAGCCAGTCACTGGTTCGCGACCACGTG (482)		*Bsp* 1286 (G, wild type)
2908G	(2870) GACATGGAATTCACCTTTGTGG (2891)	SSR-2908	*Ase*I (A, vaccine)
2908C	(2946) TGATAAACTTGGTTCAATGCATGTCCGTTATTTGCATTAA (2907)		*Mae*II (G, wild type)

[a] Numbers refer to the nucleotide position of the primer on the Sabin type 1 strain genome (41).
[b] The detected nucleotide and its specificity are indicated in parentheses.
[c] SSR, site-specific RFLP.

the original seed at 34°C in HEp-2c cells was used to prepare viral stocks. The virulent strains PV1/Mahoney, PV2/MEF1, and PV3/Leon/37 were the reference strains maintained in our laboratory and grown at 37°C in HEp-2c cells.

The field strains analyzed in this study were isolated from the stools and CNS (cerebrospinal fluid [CSF], spinal cord, medulla, or cerebral cortex) of patients with VAPP or epidemic paralytic poliomyelitis. The viruses were isolated on primary monkey kidney cells and passaged once or twice on HEp-2c cells. The virus stock resulted from a further passage at a multiplicity of infection of about 5 to 10 PFU per cell. For all passages, vaccine-derived virus was grown at 34°C and wild-type virus was grown at 37°C.

For the neurovirulence test, virus from the stock was concentrated by centrifugation for 2 h at 30,000 rpm in a Kontron 50.38 rotor at 4°C and resuspended in 1 ml of phosphate-buffered saline plus Ca^{2+} and Mg^{2+}.

Titers of the stocks were determined by plaque assay in HEp-2c-seeded six-well plates under 3 ml of 0.9% agarose in Dulbecco modified Eagle's medium supplemented with 2% fetal calf serum and 50 mM $MgCl_2$. After 72 h of incubation at 34 or 37°C and removal of the agarose overlay, cell monolayers were stained with crystal violet and plaques were counted. In the case of mixtures of strains with the same serotype but with different genotypes, virus cloning was performed as for plaque titration, except that the cells were stained with neutral red and then individual plaques were picked.

Virus identification. Serotype identification was carried out with a standard microplate neutralizing index assay, for which Vero cells and type-specific neutralizing sera were used. When necessary, mixtures of viruses with different serotypes were separated by recovering the virus from the last-dilution wells, passaging once, and retesting the serotype.

The intratypic differentiation of wild-type or vaccine-derived strains was performed by using two assays previously described, the neutralization index test with strain-specific monoclonal antibodies (7) and the restriction fragment length polymorphism (RFLP) assay (3). The neutralization index test measures the difference between the $\log_{10}$ titer with a monoclonal antibody specific for a wild-type or vaccine strain and that without the antibody in a standard microtitration assay on Vero cells. The monoclonal antibodies used were 1_0, I_0 for the type 1 polioviruses, 2_a, II_a for the type 2 viruses, and 3_0, III_0 for the type 3 viruses (7).

Genomic analysis. Recombinant strains were detected by an approach previously described by Furione et al. (9). Briefly, PCR amplification followed by enzyme digestion (RFLP) was performed on two distant regions of the viral genome, the first corresponding to nucleotides 2402 to 2881 in the VP1 capsid region (RFLP-1) and the second corresponding to nucleotides 6086 to 6376 in the virus polymerase-encoding region (RFLP-3D). Restriction profiles obtained with three different restriction enzymes were compared with profiles of the homotypic Sabin strain and of some of the known wild-type strains.

For the reverse transcription of the viral RNA, we used hexanucleotide random primers instead of a single downstream primer. This was done to improve the efficiency of the synthesis of the cDNA and to use as substrate the same cDNA products for the PCR amplification of different regions of the viral genome. Fourteen microliters of infected cell supernatant was incubated with 1.5 μl of a 0.74-μg/μl hexamer solution (Pharmacia) for 5 min at 90°C (denaturation) and then for 5 min at 37°C (primer annealing). The concentration of the random hexanucleotides was chosen so as to yield cDNA fragments of between 0.5 and 3 kb in length. To the annealed template-primer solution were added 12 U of avian myeloblastosis virus reverse transcriptase in 10 μl of transcription buffer (Promega), 5 μl of deoxynucleoside triphosphates (10 mM each), 20 U of RNasin, and distilled water to a final volume of 50 μl; this mixture was incubated for 1 h at 42°C.

PCR amplification was carried out as previously described (3) with 5 μl of the transcription product. When necessary, a seminested or nested PCR was performed by using external primers for the first PCR and standard RLFP primers for the second one. The external primers used were UC11 (33) and UG16 and UC12 (Table 1). One microliter of the first PCR product was used for the second reaction. A negative control was included in each series of reactions.

When heterotypic recombinant genomes were found by using RFLP-1 and RFLP-3D, new RFLP assays were performed for the connecting genomic regions in order to localize the recombination junction. Three assays, each permitting differentiation of the three Sabin strain genomes, were used

(see the three pairs of primers and the respective restriction enzymes listed in Table 1).

Specific point mutations associated with reversion to neurovirulence of vaccine-derived strains were detected by a site-specific RFLP assay. When the nucleotide of interest was included in an already existing restriction site (positions 480, 2741, 6203, and 7441 of the type 1 Sabin strains), the fragment encompassing this site was amplified and the suitable restriction endonuclease was used (30). When the nucleotide did not belong to an existing restriction site, a new site was introduced by PCR amplification with modified synthetic primers. The modified primers and the restriction enzymes used for positions 472 and 2034 have been previously described (5). New primers were designed for positions 481 and 2908 of the type 2 poliovirus. They are listed in the Table 1, together with the respective site-specific restriction enzymes. PCR and restriction analysis were performed as described previously (3).

Viral RNA extraction and nucleotide sequencing. Viral RNA was extracted as previously described (18). Briefly, HEp-2c cells were infected with the appropriate virus stock and 6 h later were lysed by a 10-min incubation at 4°C in TEN (10 mM Tris [pH 7.4], 1 mM EDTA, 140 mM NaCl) containing 0.5% Nonidet P-40. Cytoplasmic RNAs were extracted in the presence of 1% sodium dodecyl sulfate with phenol-chloroform followed by chloroform. After ethanol precipitation, total cytoplasmic RNA was used for sequencing with avian myeloblastosis virus reverse transcriptase, as previously described (8).

Phenotypic markers. We determined the plaque diameter on virus-infected HEp-2c cell monolayers stained after 3 days of incubation at 34°C under a 0.9% agar overlay.

The reproductive capacity at different temperatures (RCT marker) was evaluated by an RCT test. The RCT value is defined as the difference, after 7 days of incubation, between the $\log_{10}$ virus titer (50% tissue culture infective dose [$TCID_{50}$] per milliliter) at the optimal temperature (37°C) and that at a supraoptimal temperature (39.5 or 40.1°C) as determined by an end point micromethod (26). Viral titers were determined by using HEp-2c cells. Viruses were considered thermosensitive (*ts*) if the RCT value (between 37 and 40.1°C) was >4.00, thermoresistant (non *ts*) when the RCT value was <2.00, and partially thermosensitive for intermediate cases.

Neurovirulence assay with PVR-Tg mice. In order to test the neurovirulence of the field isolates, an assay (IP-MHT test) was developed by using transgenic mice with the human poliovirus receptor (PVR-Tg mice). The homozygous Tg21 PVR-Tg mice, kindly provided by A. Nomoto and T. Nomura, were used throughout this study. Five or six mice per virus strain were inoculated intraperitoneally with a fixed virus dose of 10^8 PFU per mouse in 0.5 ml. The mice were observed for 14 days for the onset of clinical signs (paresis, paralysis, and prominent instability during walking were considered clinical signs suggestive of a neurological disease). The disease incidence and the time to disease onset were registered, and a neurovirulence score was computed for each strain from these two variables: the mean healthy time (MHT) was expressed as the mean of the disease-free survival times for all mice inoculated with the same virus. The field isolates were classified as highly neurovirulent (MHT, <8 days), intermediary (MHT, between 8 and 14 days), or attenuated (MHT, 14 days) by comparing the data with the values obtained for the reference attenuated (Sabin) and neurovirulent (PV1/Mahoney, PV2/MEF1, and PV3/Leon/37) strains.

RESULTS

Case selection. In order to compare stool and CNS isolates from VAPP cases, we selected eight cases, for which we isolated at least one poliovirus strain from the CNS and one from the stool. Three wild-type poliomyelitis cases were also included in the study to compare our results with an etiologically well-defined clinical poliomyelitis reference. All cases studied were recorded in Romania over a 20-year period (1973 to 1992).

Clinical and epidemiological data (Table 2) were also used to select the cases. The patients were infants between 4 and 25 months of age. All VAPP cases in this study were clinically confirmed paralytic poliomyelitis cases, according to the U.S. confirmatory case definition (39). Briefly, to be defined as poliomyelitis, a patient must have had paralysis clinically and epidemiologically indicative of poliomyelitis and at 60 days after the onset of symptoms must have had a residual neurological deficit or have died. All cases were virologically confirmed, and all the viruses isolated from these patients were characterized as vaccine derived by intratypic differentiation. All the patients had seroconverted (Table 2); hence, they were considered immunocompetent and classified as sporadic, recipients, or contacts, according to the U.S. classification system (38). In the U.S. system, the categories for classification of VAPP are defined as follows: for a recipient case the patient must have received OPV 4 to 30 days prior to illness onset, and for a contact case the patient must have had onset of illness 4 to 75 days after OPV was fed to a recipient in contact with the patient; however, receipt of OPV more than 30 days or contact more than 75 days prior to onset does not preclude classification in these categories. The assignment of the case V2-II (recorded during a time period free of reported wild-type disease; clinically and virologically confirmed VAPP with disease-specific seroconversion) to the recipient category appeared to be unwise, considering the long delay between vaccination and paralysis onset (80 days). Therefore, it was classified as a VAPP contact case.

The wild-type poliomyelitis cases were also clinically and virologically confirmed and were classified (U.S. system) as epidemic for the cases due to wild-type 1 and 2 viruses and sporadic for the case due to the wild-type 3 virus.

Four type 2 and four type 3 VAPP cases were studied in detail (Table 2). For the CNS isolates, the virus was recovered from the CSF in five of eight cases and from necroptic specimens (spinal cord, medulla, or cerebral cortex) in the three lethal cases. Two or three stool isolates were recovered for the five nonlethal cases. Only one stool isolate was recovered for each of the lethal cases because death ensued quickly after hospitalization. Other isolates from blood clot or nasopharyngeal swabs were recovered whenever possible.

For the epidemic cases associated with wild-type 1 and 2 viruses (w1 and w2 in Table 2), three isolates were recovered from stool, one or two from the CNS, and one from a nasopharyngeal swab. For the sporadic wild-type 3 case (w3 in Table 2), virus could not be isolated from stool but could be isolated from CSF, nasopharyngeal swab, and blood clot. For this particular case, the CNS isolate (CSF) was compared with the nasopharynx isolate.

The nomenclature of the strains includes the following: the origin, v for vaccine-derived strain and w for wild-type strain; the serotype, 1, 2, or 3, to which the case had been assigned by common diagnostic procedures (only the serotype of the stool-isolated strain was taken into account for the nomenclature); the VAPP case number, I to IV for each type 2 and 3

TABLE 2. Clinical and epidemiological data for the VAPP and wild-type cases studied

Case	Age (mos)	OPV history[a]	Time[b] (days)	Illness outcome	Case classification	Yr of isolation	Sample[c] (day[d])	Serology[e]			
								Day[d]	PV1	PV2	PV3
v2-I	25	Nonvaccinated		Death	VAPP contact	1980	s (1) ct (2)	1	190	512	<4
v2-II	7	2 T	80	Paralytic residue	VAPP contact	1985	s (2), s (3), s (4) cc (2)	2 14	350 512	128 180	<4 <4
v2-III	11	1 T	17	Paralytic residue	VAPP recipient	1980	s (3) cc (2) b (2)	2 12	128 180	64 90	16 32
v2-IV	7	1 T	20	Paralytic residue	VAPP recipient	1987	s (3), s (4), s (5) cc (3) n (3)	3 14	<4 <4	22 128	22 22
v3-I	6	Nonvaccinated		Death	VAPP contact	1977	s (1) ct (3), n (1)	1	<16	<16	4,096
v3-II	9	1 T	40	Death	VAPP recipient	1980	s (4) ct (4)	4	<4	<4	128
v3-III	8	2 T	10	Paralytic residue	VAPP recipient	1973	s (4), s (5), s (6) cc (7)	4 25	256 128	128 128	128 512
v3-IV	4	1 T	20	Paralytic residue	VAPP recipient	1992	s (8), s (9) cc (8)	8 17	4 22	90 256	22 128
w2	13	1 M1	120	Death	Wild-type virus, epidemic	1980	s (1), s (2), s (3) ct (5), n (3)	3	10	6	<4
w3	16	1 M1	353	Paralytic residue	Wild-type virus, sporadic	1980	cc (12) b (12), n (12)	ND[f]			
w1	7	Nonvaccinated		Paralytic residue	Wild-type virus, epidemic	1982	s (1), s (2), s (3) cc (2) n (2)	2 21	40 1,280	<4 <4	<4 <4

[a] Number of doses of trivalent (T) or type 1 monovalent (M1) OPV.
[b] Delay between vaccination and paralysis onset.
[c] Sample origin: s, stool; n, nasopharynx; b, blood clot; cc and ct, CNS (CSF [cc] and necroptic tissue [ct]).
[d] Delay between paralysis onset and sampling.
[e] Reciprocal of the serum dilution neutralizing 100 $TCID_{50}$ of homotypic reference type 1 (PV1), type 2 (PV2), or type 3 (PV3) poliovirus.
[f] ND, not determined.

case; and the site of isolation, s for stool, c for CNS, b for blood clot, and n for nasopharynx.

Strain identification. The strains were identified by serotyping, intratypic differentiation, and molecular characterization (RFLP analysis and sequencing). Recombinant strains, which are frequently found in VAPP cases, were detected by RFLP analysis (9). In some cases, the viral genome was partially sequenced to check for the recombination junction and/or to confirm the identity between viral genomes isolated from the same case.

The recombination junction of heterotypic recombinant genomes was localized by RFLP screening followed by partial genome sequencing and was used as an identity marker for CNS and stool strains derived from the same VAPP case. The strategy employed to localize the recombination junction of virus genomes consisted of comparing the RFLP profiles of different subgenomic fragments with the corresponding RFLP profiles of the Sabin strains (Fig. 1). For example, the recombination junction of strain 2-IIc, an S2 × S1 recombinant, was localized by RFLP to a 360-nucleotide segment of the 3AC region, between nucleotides 5337 (*Rsa*I restriction site present, as in the Sabin 2 strain) and 5696 (*Hae*III restriction site present in the Sabin 2 strain but absent in both Sabin 1 and 2-IIc strains). Subsequently, by sequencing the genome of strain 2-IIc, we precisely localized the recombination junction to the region between nucleotides 5464 and 5494 (the Sabin 1 and Sabin 2 genomes differ at these nucleotide positions).

When the molecular characterization of the strains was performed, the strains of the three wild-type cases (w1, w2, and w3) presented RFLP patterns that differed from those of the reference wild-type and the homotypic vaccine strains. All of the isolates for each of the wild-type cases had an identical RFLP pattern in the two regions screened. Sequencing over at least 140 nucleotides at the beginning of the 2A region (nucleotides 3428 to 3618) revealed 100% identity between the stool and CNS isolates from the same case. Thus, for the wild-type poliomyelitis cases, the patient had been infected with a unique strain which was identical for all the isolates.

For the VAPP cases the situation was more complex (Fig. 2). In five of the VAPP cases, similarly to the wild-type poliomyelitis cases, a unique strain was present in all the isolates from stool, CNS, and other sources. For these cases, the same genotypes were detected in the multiple samples collected from a patient (Table 2). For simplification, in Fig. 2 only one stool isolate is represented.

Of these five cases, only for cases 3-I and 3-II did the stool and CNS isolates contain one viral strain closely related to one of the Sabin strains. Intertypic vaccine/vaccine recombinants were detected in both stool and CNS isolates from cases 2-II and 3-III. In these two cases, the strain isolated from the CNS presented the same recombination junction as the stool strain. This indicates that both strains originated from a common heterotypic recombinant ancestor, which initially multiplied in the gut. For case 2-I, the stool and CNS strains were identified as vaccine-derived type 2 strains by using monoclonal antibodies and RFLP (RFLP-1). Moreover, when two fragments in the capsid protein-encoding region (nucleotide positions 1004 to 1142 and 2820 to 2939) were sequenced, 100 and 99.1% nucleotide homology, respectively, was found between these strains and the corresponding Sabin 2 strain. However, the stool and CNS strains from case 2-I presented an unusual

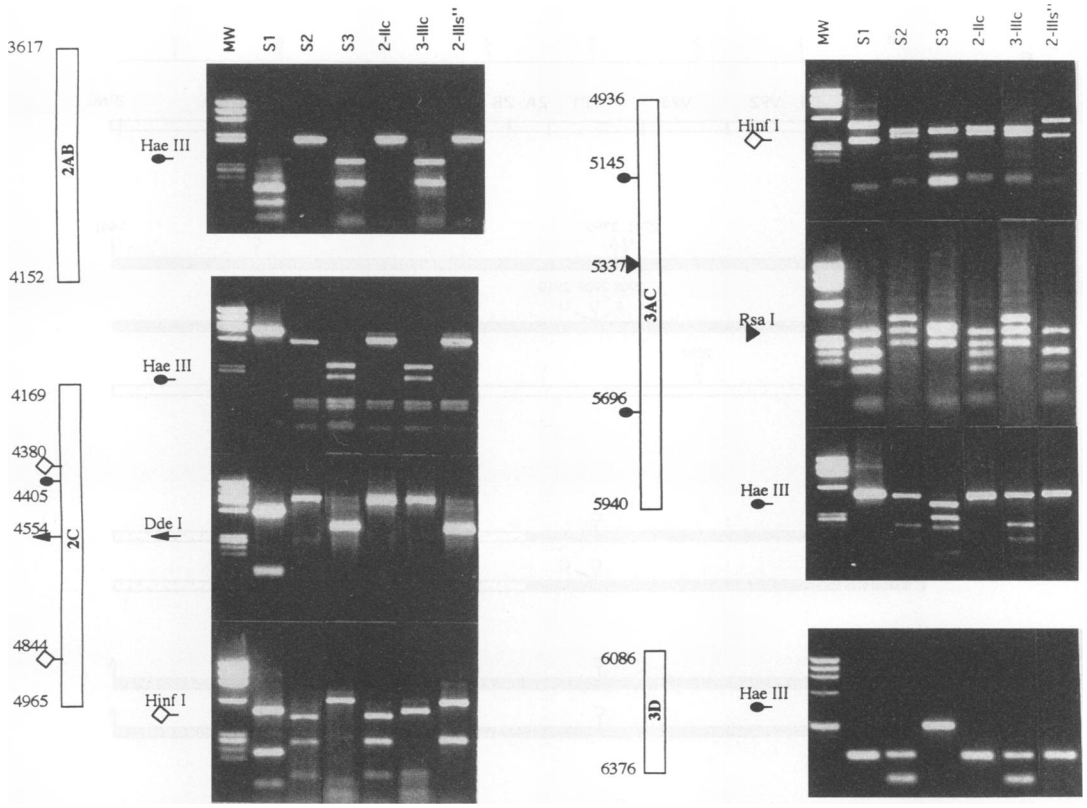

FIG. 1. Determination of recombination junctions of heterotypic recombinant strains by multiple RFLPs. Ethidium bromide-stained agarose gels showing the enzymatic restriction patterns of PCR-amplified reverse-transcribed genomic viral fragments are shown. Restriction profiles from the Sabin 1, 2, and 3 strains (S1, S2, and S3, respectively) and from three VAPP strains (2-IIc, 3-IIIc, and 2-IIIs'') are indicated. Schematic representations of each of the DNA PCR fragments analyzed are shown on the left of the photographs. Viral proteins encoded by the corresponding viral genome fragment are indicated (2AB, 2C, 3AC, and 3D; see Fig. 2 for the poliovirus genetic structure). The names of the restriction endonucleases used are given for each agarose gel. Symbols corresponding to some restriction sites and their nucleotide positions on the viral genome are given on the schematic representations of the PCR DNA fragments. A detailed analysis of results obtained with VAPP strain 2-IIc, an S2 × S1 recombinant (as illustrated by the 2AB and 3D RFLP profiles), is given as an example. The recombination junction was first matched to the segment where a transition RFLP pattern was detected (3AC region: *Hin*fI and *Hae*III restriction patterns similar to those of Sabin 2 and Sabin 1 strains, respectively, and a different *Rsa*I restriction pattern). A more restricted localization was carried out by deducing from the restriction maps of the Sabin partners the restriction sites introduced or suppressed from the transition profile of the recombinant strain (the *Rsa*I restriction site at nucleotide 5337 is present in both Sabin 2 and 2-IIc strains and the *Hae*III restriction site at nucleotide 5696 is present in Sabin 2 but absent in both Sabin 1 and 2-IIc strains). The recombination junctions for strain 3-IIIc, an S3 × S2 recombinant (between nucleotide positions 4380 and 4554), and strain 2-IIIs'', an S2 × S1 × S3 recombinant (between nucleotide positions 4405 and 4554 and positions 4844 and 5145 for the S2 × S1 and S1 × S3 junctions, respectively) were similarly localized. MW, molecular weight markers.

RFLP pattern in the 3D region compared with the patterns of the three Sabin strains. Sequencing of genomic regions from nucleotide positions 5723 to 5908, 6811 to 7021, and 7198 to 7410 gave only 87.1, 92.4, and 92.0% homology with the Sabin 2 strain genome, respectively (Table 3), nor was significant higher homology found with the Sabin 1 or 3 strains or with the three wild-type (w1, w2, and w3) strains circulating in the country when the 2-I strains were isolated. These results indicated that the 2-I strains are recombinant vaccine-derived strains with the 3′-terminal part possibly originating from a wild-type strain. As shown in Table 3, the homology between the genomes of the CNS and the stool strains of case 2-I in the sequenced region was 100%.

In three of the VAPP cases (cases 2-III, 2-IV, and 3-IV), mixtures of different strains were identified in the stool and/or CNS (Fig. 2). These cases were analyzed in detail in order to determine the origin of the CNS isolates. Serotyping techniques permitted isolation of viruses of different serotypes present in the mixture. The RFLP analysis of a given serotype allowed us for the first time to detect mixtures of recombinant strains carrying different 3′-terminal genomic ends (Fig. 3). By plaque purification of the mixture followed by RFLP analysis of the individual clones, we were able to differentiate the various strains in the absence of any selection pressure.

For VAPP case 2-IV, five different isolates were analyzed: three isolates recovered at various times from stool, one isolate from the CNS, and one isolate from a nasopharyngeal swab (Fig. 4). The first stool isolate appeared to be a mixture of type 2 and 3 strains by serotyping. The type 3 strain of this stool isolate (2-IVs''') was an S3 × S1 recombinant. Moreover, RFLP-3D analysis of the type 2 strain showed that it was actually a mixture of a nonrecombinant Sabin 2 strain (2-IVs′) and an S2 × S1 recombinant strain (2-IVs'') (Fig. 3). Surprisingly, the second stool isolate and the nasopharynx isolate contained only one nonrecombinant Sabin 2 strain. The third stool isolate was again a mixture of an S2 × S1 recombinant

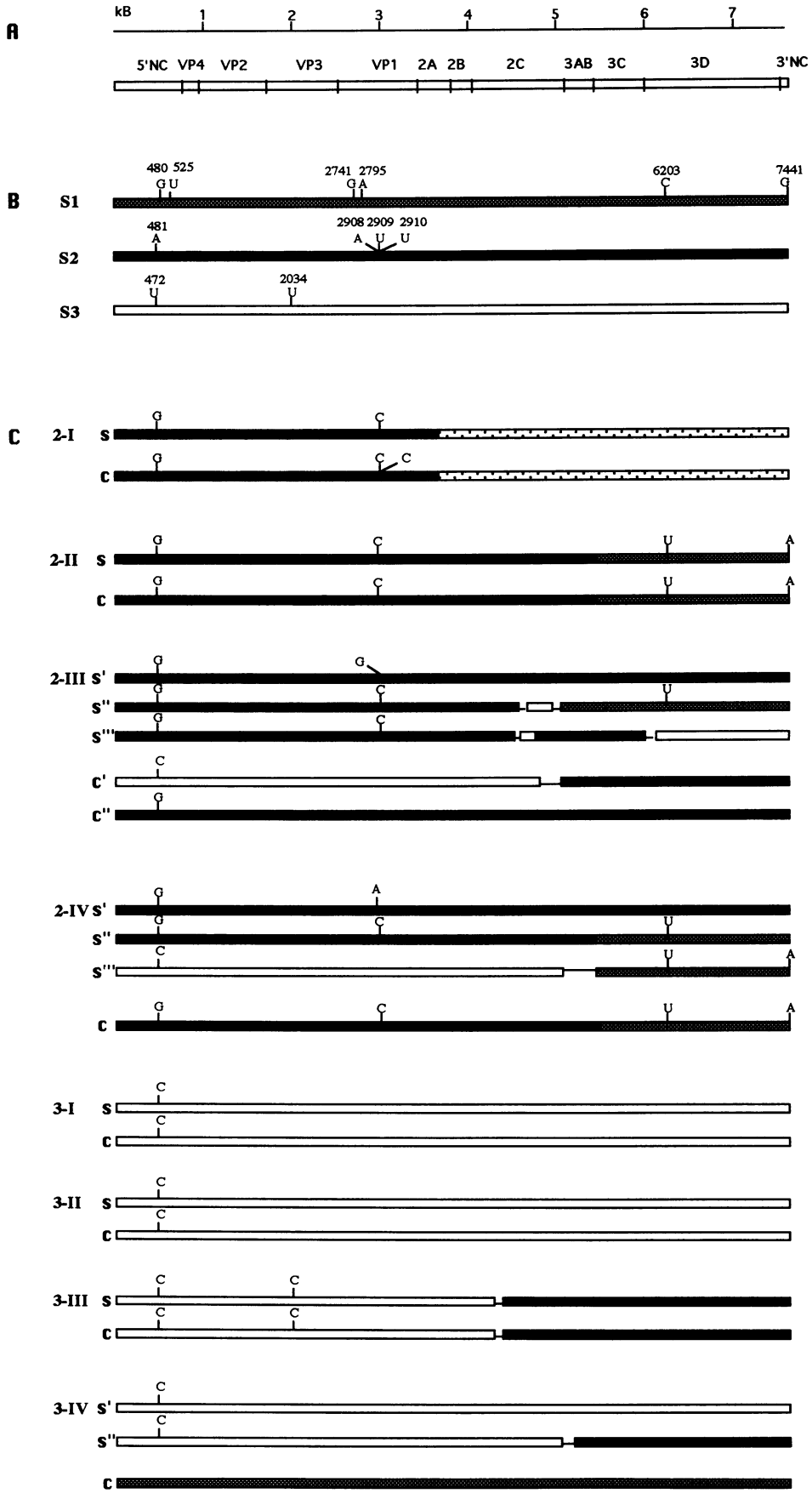

FIG. 2. Diagrammatic representation of the genetic organization of VAPP strains. (A) Schematic representation of the genetic organization of the poliovirus genome. The 5′ noncoding region (5′NC) and the 3′ noncoding region (3′NC) are indicated. Structural capsid proteins (VP4 through VP1) and nonstructural proteins (2A through 3D) are shown. The genome length is also indicated (kilobases). (B) Schematic representations of the Sabin 1, 2, and 3 strain genomes (S1 [▩], S2 [■], and S3 [□], respectively) are shown. Nucleotide positions known to be involved in the attenuation and/or to revert to the wild-type genotype are indicated for each strain (for references, see text). (C) Schematic representation of the genomes of poliovirus strains isolated from stools and CNS of patients with VAPP (2-I, 2-II, etc.; arabic numbers indicate the serotype obtained by serotyping methods, and roman numbers refer to the given VAPP case). When different strains were isolated from stool or CNS isolates from the same VAPP case, they are indicated as s′, s″, s‴, c′, or c″, corresponding to stool (s) and CNS (c) strains. The structures of the genomes were deduced from RFLP patterns and from partial genomic RNA sequences. When heterotypic recombinant genomes were identified, the part of the genome corresponding to a given Sabin strain (see panel B) or to a different strain ( ) is shown. Lines indicate undetermined junction regions of the genome. The nucleotide positions screened for reversion are indicated for the VAPP strains only if they were different from those of the Sabin strains.

strain and a nonrecombinant S2 strain. These results indicated that fluctuation may occur in the populations of viruses which replicate in the gut.

For case 2-IV, only one recombinant S2 × S1 strain (2-IVc) was recovered from the CNS. In order to compare the S2 × S1 strains appearing in the stools and in the CNS, we localized the recombination junction for the CNS strain and for one S2 × S1 virus clone isolated from each of the stool isolates. In all cases, the recombination junction was found to lie in the 3AC coding region of the virus genomes. The analysis of the RFLP pattern used to determine the recombination junction of the genomes indicated that a nucleotide mutation differentiated the genome of the CNS isolate from that of the stool isolates. This mutation was localized by RNA sequencing (nucleotides 5142 to 5371) at position 5350 (U→C) and was found not to modify the corresponding amino acid. RNA sequencing was also used to localize more precisely the recombination junction, which was found to be situated between nucleotides 5230 and 5233 for all three S2 × S1 strain genomes derived from case 2-IV. These results, indicating that the stool and CNS S2 × S1 recombinant strains are closely related, strongly suggested that these strains evolved from a common S2 × S1 ancestor initially multiplying in the gut.

For case 2-III, a mixture of serotypes (type 2 and type 3) was found in the CNS, whereas only one serotype (type 2) was detected in the stool isolate analyzed. RFLP analysis indicated that the type 2 stool isolate actually contained a mixture of different recombinant and nonrecombinant strains (S2, S2 × S1, and S2 × S3 strains) (Fig. 2). Clones of each of the two recombinant strains were isolated, and the recombination junctions of their genomes were analyzed by RFLP. The S2 × S1 recombinant (2-IIIs″) had an S2-like RFLP pattern in the 2AB region, a transition pattern in the 2C region, and an S1-like pattern in the 3AC region (Fig. 1). This result suggested that the recombination junction was in the 2C coding region of the genome. However, RNA sequencing of this part of the genome (nucleotides 4697 to 4912) detected a Sabin 3 sequence, indicating that this strain was actually an S2 × S3 × S1 recombinant. The RFLP patterns of one virus clone of the supposed S2 × S3 recombinant (2-IIIs‴) indicated that a recombination junction was present in the 3AC region of the genome. The RFLP pattern in the 2C region of the genome of this strain was slightly modified; on the basis of RNA sequencing results (nucleotides 4708 to 4917), we identified the presence of an S3 × S2 recombination junction (between nucleotides 4773 and 4799). Thus, for this strain, the RFLP and sequence data are consistent with an S2 × S3 × S2 × S3 quadruple recombinant. The presence of such multiple recombinant strains indicated that several rounds of recombination events can occur after the administration of the OPV vaccine strains.

For this patient (2-III), virus strains were also isolated from a blood clot (results not shown). Two serotypes (type 2 and type 3) were identified in this isolate. The two strains isolated from the blood clot had the same RFLP pattern as those isolated from the CNS. Actually, the type 3 strain isolated from the blood clot, as well as that isolated from the CNS, appeared to be an S3 × S2 recombinant strain whose recombination junction was located in the 2C coding region of the genome. As mentioned above, this S3 × S2 strain present in the CNS and in the blood clot isolates was not detected in the stool sample analyzed and may have disappeared from the gut at the time of the disease.

The presence in the CNS of a strain not detected in the stool

TABLE 3. Homology between genomic sequences of VAPP case 2-I strains, wild-type strains, and Sabin strains[a]

Strain	% Homology with:									
	2-Is[b]	2-Ic[b]	Sabin 1	Sabin 2	Sabin 3	Lansing	Finland	w1s	w2s	w3s
2-Is	100.0									
2-Ic	100.0	100.0								
Sabin 1	90.6	90.6	100.0							
Sabin 2	**92.0**	**92.0**	91.1	100.0						
Sabin 3	87.7	87.7	91.1	88.7	100.0					
Lansing	**92.0**	**92.0**	89.2	88.2	87.8	100.0				
Finland	88.7	88.7	88.7	88.7	84.5	91.1	100.0			
w1s	**94.1**	**94.1**	94.8	92.1	92.8	91.5	90.2	100.0		
w2s	**92.1**	**92.1**	91.5	90.2	93.4	92.8	88.2	94.8	100.0	
w3s	85.1	85.1	89.1	85.1	87.1	83.8	83.1	89.8	87.1	100.0

[a] Homology was determined for the 3D polymerase and 3′ noncoding regions over 213 nucleotides (positions 7198 to 7410) for the 2-I strains, reference Sabin strains, and reference wild-type strains (PV2/Lansing and PV3/Finland). For the wild type w1, w2, and w3 field strains, the percent homology was determined for 153 nucleotides (positions 7258 to 7410).

[b] Boldface indicates the highest percentages of homology between the genomes of the 2-I VAPP case strains and of other strains taken for comparison.

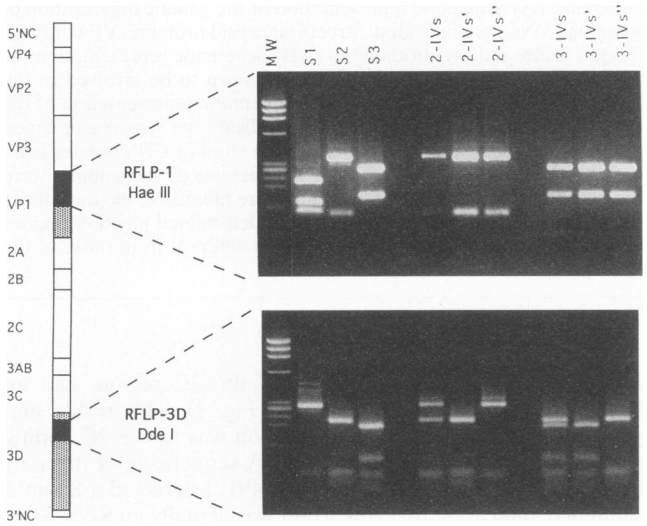

FIG. 3. RFLP analysis of mixtures of recombinant and nonrecombinant strains presenting the same serotype. The viral genomes of two VAPP stool isolates (2-IVs and 3-IVs) and of two viral clones from each of these two isolates (2-IVs′, 2-IVs″ and 3-IVs′, 3-IVs″, respectively) were analyzed by RFLP. Results were compared with those obtained for the reference Sabin 1, 2, and 3 strains (S1, S2, and S3 respectively). Two kinds of RFLP assay were performed by analyzing two different PCR-amplified DNA fragments whose locations are indicated on a schematic representation of the poliovirus genome. RFLP-1 consisted of analyzing a 480-bp DNA fragment from the VP1 region of the genome. Restriction patterns of this fragment were analyzed on an agarose gel after digestion with the restriction endonuclease *Hae*III. RFLP-3D consisted of analyzing a 291-bp fragment corresponding to part of the 3D polymerase region of the viral genome by using the restriction endonuclease *Dde*I. While in RFLP-1 the profiles for the stool isolates corresponded to a given Sabin strain profile, in RFLP-3D a superposition of restriction fragments is observed, indicating a mixture of several strains. RFLP analysis of individual clones isolated from these mixtures permitted the characterization of nonrecombinant strains (2-IVs′ and 3-IVs′) and heterotypic recombinant strains (2-IVs″ and 3-IVs″). (See Fig. 1 for the analysis of heterotypic recombinant strain genomes.) MW, molecular weight markers.

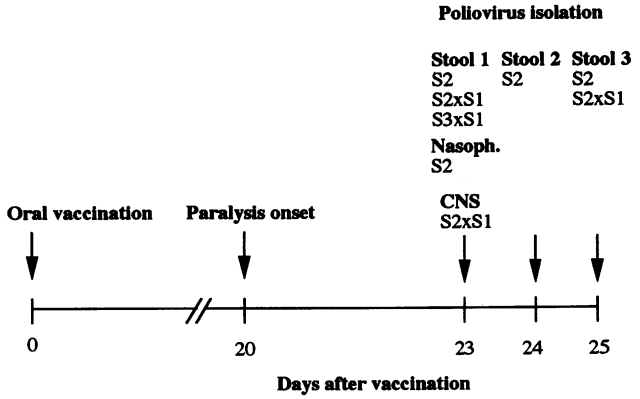

FIG. 4. Characteristics and chronology of the different isolates from VAPP case 2-IV. Dates of vaccination, paralysis onset, and virus isolation are given (arrows). The origin of each of the isolates (stool, nasopharynx [nasoph.], or CNS) is indicated. The presence of mixtures of strains and the characteristics of the strains are given for each of the isolates. Three kinds of strains have been isolated from this VAPP case: one Sabin 2-derived strain (S2) and two Sabin 2 × Sabin 1 (S2 × S1) and Sabin 3 × Sabin 1 (S3 × S1) heterotypic recombinant strains.

was also found in case 3-IV. Whereas type 3 vaccine-derived strains were identified in two stool isolates, only a type 1 strain was found in the CNS. Once more, this result strongly suggested that the ancestor of the strain identified in the CNS had disappeared from the gut when the paralysis occurred.

Phenotypic markers and neurovirulence of the strains. In order to further compare the strains isolated from stool or CNS with the original Sabin vaccine strains, we analyzed some in vitro phenotypic markers, i.e., thermosensitivity (RCT marker; see Materials and Methods) and plaque size, as well as the neurovirulence of the strains in PVR-Tg mice (Table 4).

In most of the cases, the stool isolate taken for comparison was recovered on the same day as the CNS isolate; if such a stool isolate was not available, the one taken closest in time to the CNS isolate was used (Table 2). For these experiments, only different serotypes were separated from mixtures. Therefore, strains 2-IIIs, 2-IVs, and 3-IVs were tested as mixtures of recombinant and nonrecombinant strains of the same serotype.

In contrast to the original Sabin strains, all stool isolates and five of the CNS isolates (2-Ic, 2-IIc, 2-IIIc′, 3-IIc, and 3-IIIc) from VAPP cases lost the *ts* phenotype. Surprisingly, four CNS strains (2-IIIc″, 2-IVc, 3-Ic, and 3-IVc) kept the *ts* phenotype.

As expected, the wild-type 1 and 2 strains were non-*ts*. However, for the wild-type 3 case (w3) there were *ts* strains in both the CNS and nasopharynx.

The neurovirulence of the strains was assessed in PVR-Tg mice as described in Materials and Methods. The IP-MHT test that we developed allowed the screening of a large number of strains by administration of a single intraperitoneal dose of virus. Since the differences in 50% lethal doses between attenuated and neurovirulent viruses are larger with intracerebral than with intraperitoneal inoculation (15), a single intraperitoneal dose closer to the highest nonpathogenic dose of the attenuated strains (Sabin 2 and 3 strains in this study) in the IP-MHT test permitted discrimination between attenuated, intermediary, and virulent strains but not between virulent and highly virulent strains. To confirm the reproducibility of the test, two strains (2-IVs‴ and 2-Ic) were tested in three different experiments. For each virus the three values obtained for the MHT were compared by using an F test (single-factor analysis of variance) and were found to be drawn from the same population of means (37).

As shown in Fig. 5, the neurovirulence of the vaccine-derived strains ranged from high to almost attenuated. As expected, two of the wild-type virus isolates (w1 and w2) were highly virulent. However, the type 3 wild-type strains (w3) had an intermediary neurovirulence. This finding was surprising but correlated well with the *ts* phenotype found for these wild-type strains.

As expected, the vaccine-derived strains isolated from the CNS and stools of all the patients with VAPP lost the attenuated phenotype of the parental vaccine Sabin strains. However, the strains isolated from the CNS were not more neurovirulent than the stool isolates. Actually, we observed that in most of the cases the CNS strains were less virulent than the corresponding stool isolate (Fig. 5). To see if this difference was statistically significant, we performed a paired one-tailed *t* test for eight wild-type and VAPP cases (37). We excluded from the comparison the cases presenting different serotypes (3-IV) or mixtures of strains in the stool or CNS isolate (2-III and 2-IV). We found a statistically significant difference between the neurovirulence levels of the CNS and the stool isolates from the same case ($P < 0.05$).

TABLE 4. Phenotypic markers of vaccine-derived and wild-type strains

Strain[a]	Serotype	RCT (log_{10} $TCID_{50}$/ml)		Plaque size[b] (mm)	MHT[c] (days)	No. of diseased mice/no. of inoculated mice
		39.5°C	40.1°C			
Sabin 2	2	3.00	5.50	1.69 ± 0.07	14.00	0/15
MEF1	2	−0.50	0.50	2.97 ± 0.15	5.80	4/5
Sabin 3	3	3.75	4.25	2.05 ± 0.10	14.00	0/6
Leon/37	3	0.25	0.75	1.31 ± 0.08	2.00	8/8
v2-Is	2	−0.75	0.00	1.94 ± 0.13	7.00	4/5
v2-Ic	2	0.50	0.50	2.79 ± 0.18	6.33	18/24
v2-IIs	2	0.50	1.25	1.67 ± 0.09	3.20	5/5
v2-IIc	2	0.75	1.75	1.29 ± 0.08	7.40	4/5
v2-IIIs[d]	2	0.00	1.50	1.96 ± 0.11	9.17	3/6
v2-IIIc′	2	0.00	3.00	1.61 ± 0.08	13.00	1/5
v2-IIIc″	3	3.00	5.00	1.62 ± 0.08	12.00	1/4
v2-IVs[d]	2	0.50	2.00	1.45 ± 0.09	10.60	2/5
v2-IVs‴	3	0.00	1.75	0.73 ± 0.04	1.60	23/23
v2-IVc	2	2.75	4.75	2.19 ± 0.10	11.80	1/5
v3-Is	3	0.75	1.00	2.56 ± 0.10	1.80	5/5
v3-Ic	3	3.00	4.75	1.51 ± 0.08	9.60	3/5
v3-IIs	3	0.75	0.50	2.11 ± 0.09	1.80	5/5
v3-IIc	3	0.25	0.50	1.82 ± 0.07	4.80	4/5
v3-IIIs	3	0.50	2.50	2.42 ± 0.12	4.60	4/5
v3-IIIc	3	0.25	1.00	2.85 ± 0.15	7.50	4/6
v3-IVs[d]	3	1.50	2.25	2.37 ± 0.13	5.17	6/6
v3-IVc	1	5.00	6.50	1.90 ± 0.08	10.00	3/6
w2s	2	−0.50	1.25	2.65 ± 0.18	2.80	5/5
w2c	2	−0.25	1.00	2.79 ± 0.18	5.60	4/5
w3n	3	4.00	4.75	2.52 ± 0.12	8.40	3/5
w3c	3	3.00	4.75	2.21 ± 0.11	10.40	2/5
w1s	1	1.00	1.25	2.13 ± 0.13	4.40	4/5
w1c	1	−0.25	1.00	2.23 ± 0.12	1.60	5/5

[a] Tested strains were reference strains (Sabin 2 and 3, MEF1, and Leon/37), vaccine-derived strains from VAPP cases (v), and wild-type strains (w).
[b] Values are means ± standard errors of the mean for 50 measurements.
[c] Neurovirulence as estimated by MHT.
[d] Isolate consisted of a mixture of recombinant and nonrecombinant strains having the same serotype.

When the neurovirulence of the isolated strains was compared with the in vitro phenotypic markers, no correlation between plaque size and neurovirulence was found, as previously reported (29). In contrast, in previous studies carried out with in vitro recombinant genomes (29) or type 3 vaccine-derived strains (17), a good correlation between thermosensitivity (RCT marker) and attenuated phenotype was observed. In the present study, for many field strains of different serotypes, the RCT marker also correlated very well with neurovirulence (Fig. 6). A nonparametric test of rank correlation was applied to correlate neurovirulence and thermosensitivity (Spearman's coefficient of rank correlation) (37) for the isolated strains. The best correlation observed was between the neurovirulence (MHT) and the RCT marker at 40.1°C (P = 0.004), but a good correlation between the neurovirulence and the RCT marker at 39.5°C was also obtained (P = 0.008).

Genetic structure and neurovirulence. In an attempt to correlate the neurovirulence of the VAPP strains with some molecular markers, we checked the nucleotide positions in the genomes of the VAPP strains that have been described as being involved in reversion to neurovirulence. These mutations occur in the 5′ noncoding region at nucleotide positions 480 (and 525) (4, 13), 481 (32), and 472 (42) for the Sabin 1, 2, and 3 strains, respectively. We also checked the mutations affecting the viral capsid proteins (nucleotide positions 2908 and 2034 for the Sabin 2 and 3 genomes, respectively). Moreover, some specific Sabin 1 nucleotides known to revert to the wild-type genotype were also checked in the genomes of type 1-derived VAPP strains (nucleotide positions 476 [6] and 2741, 2795, 6203, and 7441 [4]).

The genomes of all the Sabin 2- and 3-derived strains isolated from VAPP cases carried reversions to the wild-type genotype at the nucleotide positions known to affect the 5′ noncoding region (Fig. 2). In many but not all cases, reversion to the wild-type genotype also occurred in the codons affecting the capsid proteins. One of the two major molecular determinants of the attenuation of the Sabin 2 strain is the Ile residue located at amino acid position 143 of the capsid protein VP1 (32). Ten of 11 Sabin 2-derived VAPP strain genomes had substitutions at this residue (Fig. 2). The most frequent change was a U→C at position 2909, yielding a Thr instead of Ile-143. For the 2-III strain isolated from the CNS, Ile-143 was conserved as it was in the Sabin 2 strain.

For the Sabin 3-derived VAPP strains, in only two strains (CNS and stool strains of the 3-III case) had the codon corresponding to amino acid residue 91 of the capsid protein VP3 reverted to the wild-type genotype (Phe-91→Ser) (Fig. 2). The amino acid residue Phe-91 is known to be involved in the attenuation of the Sabin 3 strain and also to be a major determinant of the thermosensitivity of this strain (23). In the other VAPP cases from which highly neurovirulent and non-*ts* strains have been recovered, second-site mutations must have accounted for the non-*ts* and neurovirulent phenotype of these strains (17).

All recombinant VAPP strains carrying a 3′-terminal part of their genome from the Sabin 1 strain had reverted to the wild-type genotype at codon 73 of the polymerase coding region (His-73→Tyr). His-73 has been shown to contribute to the attenuation of poliovirus type 1 in a mouse model (40). The majority of these recombinant strains (four of six) also carried reversion to the wild-type genotype at position 7441. Surprisingly, for the nonrecombinant Sabin 1-derived strain isolated from the CNS (case 3-IV), RNA sequencing in the regions containing nucleotides 476, 480, 525, 2741, 2795, 6203, and 7441 revealed no difference from the parental Sabin 1 strain. The possible unknown mutations which could be involved in the reversion of this vaccine-derived strain to neurovirulence are being investigated.

When these results were compared with the neurovirulence of the strains, it was difficult to determine what molecular event(s), i.e., reversion to the wild-type genotype of a nucleotide marker known to be linked with attenuation and/or genomic recombination, is associated with the neurovirulence of the VAPP strains.

Reversion to the wild-type genotype of the 5′ noncoding region attenuation determinants (which are known to be highly involved in the attenuation of the Sabin strains) could explain at least part of the neurovirulence of the VAPP strains analyzed. However, this does not explain why the level of neurovirulence of the strains varied significantly, mainly between stool and CNS strains (for example, in case 3-I). Neither the reversions to the wild-type genotype affecting the capsid

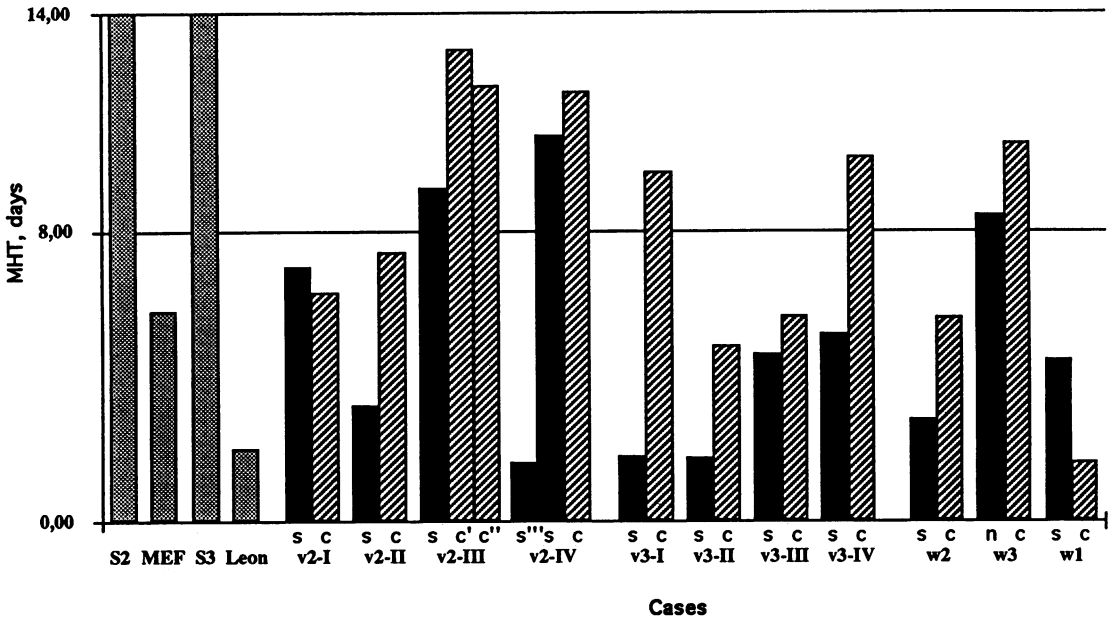

FIG. 5. Neurovirulence of reference strains and field vaccine-derived (v) or wild-type (w) isolates tested on Tg-PVR mice. The homozygous Tg21 transgenic mice (kindly provided by A. Nomoto) were inoculated intraperitoneally with a fixed amount of virus and observed daily for paralysis or death for 14 days. The lengths of the vertical bars represent the MHT, which was calculated for each virus as the mean of life spans without clinical signs for the inoculated mice. Filled bars and hatched bars indicate the stool (s) or CNS (c) origin of the isolates, respectively. Stippled bars indicate the laboratory strains used for reference. For the VAPP cases in which mixtures of recombinant and nonrecombinant strains of the same serotype were detected in stool (2-IIIs, 2-IVs, and 3-IVs), the neurovirulence of the mixture was tested.

proteins nor those of the type 1 polymerase-encoding region could be significantly linked from these results to a certain level of neurovirulence in Tg mice. For example, the recombinant S2 × S1 strains from cases 2-II and 2-IV had the same molecular markers but different levels of neurovirulence. Undetermined suppressive mutations could have been selected either in the gut or in the CNS, and this could also explain an increase in neurovirulence. The neurovirulence of the Sabin 1-derived CNS strain of case 3-IV, which was shown to have conserved all the molecular determinants so far known to affect the neurovirulence of poliovirus type 1, supports this hypothesis. In any case, with the exception of this Sabin 1-derived strain, all of the recombinant or nonrecombinant VAPP strains carried reversions to the wild-type genotype at some, if not all, loci known to affect the attenuation of the Sabin strains.

DISCUSSION

To our knowledge, this is the first time that a representative sample of strains from stool and CNS simultaneously isolated from patients with VAPP has been analyzed at both the phenotypic and molecular levels. For all eight VAPP cases studied, only vaccine-derived strains were recovered, whether from stool or CNS. These vaccine-derived strains had lost the attenuated phenotype of the parental vaccine Sabin strains, since they proved to be neurovirulent when tested on PVR-Tg mice. In five of these eight VAPP cases, unique and similar strains were detected in the stool and in the CNS of the same patient. In the remaining three cases, mixtures of vaccine-derived strains were found in the stool isolates. Among the strains recovered from the CNS, several heterotypic recombinants were identified. These were mainly vaccine/vaccine recombinant strains, but for one VAPP case, vaccine/nonvaccine recombinant strains were also isolated.

To determine which of the stool-isolated viruses is the actual etiologic agent of the neurological disease was sometimes a puzzling problem. In our study, in five of the eight VAPP cases no difficulty arose, because practically the same strains were isolated from paired stool and CNS samples. That the stool and CNS isolates shared a common origin was confirmed by characterizing the recombination patterns and the sites of the recombination junction. In addition to two recipient cases (3-II and 3-III; Table 2), all the contact cases under study (2-I, 2-II, and 3-I) fell into this category. The probability of simultaneously detecting different infecting strains is obviously lower for a contact case than for the vaccinee. This suggests that in the contact VAPP cases, a single strain isolated from stool has a high probability of being a good representative of the neuropathogenic agent.

The detection of mixtures of strains in the stool of recipient VAPP patients is not surprising because of the trivalent nature of the OPV and the simultaneous multiplication in the digestive tract of its three different strains. However, it is not clear why in some VAPP cases single strains were recovered from stools and CNS, while in others mixtures were isolated. The single strains recovered from two VAPP patients, both of whom were OPV recipients (cases 3-II and 3-III), showed a rather neurovirulent phenotype. Possibly, these neurovirulent strains were selected in the gut because they replicated efficiently and thus overgrew other strains.

For the case for which mixtures of strains were isolated, the relative proportions of the populations of strains present in the mixture fluctuated over time (Fig. 4). Time-dependent fluctuation of variants with mutations in the 5′ noncoding region of the genome (25) and the appearance of new structural vaccine-

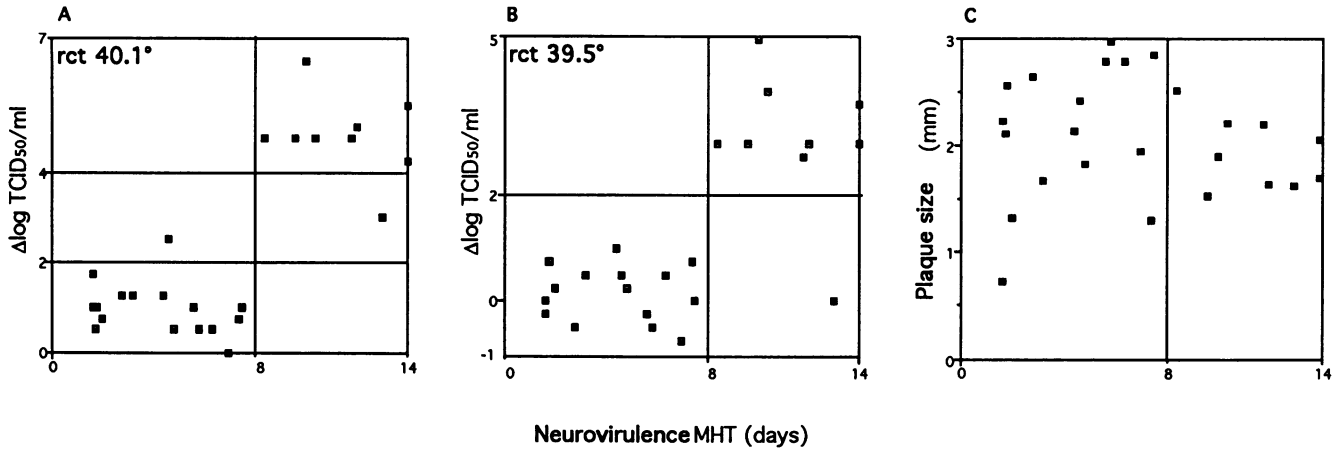

FIG. 6. Correlation between the neurovirulence and in vitro phenotypic markers for the vaccine-derived or wild-type field strains studied. RCT values between 37 and 40.1°C (A) and between 37 and 39.5°C (B) and plaque size values (C) were plotted versus MHT for PVR-Tg mice (values are given in Table 4). The vertical bar in each panel separates the highly neurovirulent (MHT, <8 days) from the intermediary (MHT, 8 to 14 days) or attenuated (MHT, 14 days) strains. The horizontal bars define, in the lower left and upper right squares, groups of RCT values correlated with neurovirulence values. The values for the reference PV2/Sabin, PV2/MEF1, PV3/Sabin, and PV3/Leon/37 strains are also included. The neurovirulence values corresponding to mixtures of strains (2-IIIs, 2-IVs, and 3-IVs) were omitted.

derived recombinants (24) have previously been reported for healthy vaccinees. The presence in the stool isolate of mixtures of strains, the presence of different strains fluctuating as a function of the day of isolation, and possibly the appearance of new strains occurred also for some of the VAPP cases presented here. These phenomena are crucial in understanding why the identification of the stool isolate does not always provide information about the actual causative strain. For instance, in the cases for which mixtures of strains were isolated from stool (2-III, 2-IV, and 3-IV), the etiologic diagnosis would have been impossible without the existence of the CNS isolate. For one case (2-IV), among other strains in the stool we detected a strain similar to that isolated from the CNS. However, if we had not identified the CNS strain, it would have been difficult to pick it out from the mixture of strains found in the stool. Moreover, since we detected in this stool isolate a vaccine-derived type 3 strain significantly more neurovirulent for PVR-Tg mice than the other type 2 strains, we would have been tempted to identify this highly neurovirulent strain as the disease-inducing agent, which obviously was not the case. This also indicated that the neurovirulence of stool strains, as determined by using PVR-Tg mice, might not be the perfect marker for the prediction of the etiologic agent of VAPP.

In another case (2-III), two different vaccine-derived strains were recovered from the CNS, showing that, as in the gut, several strains can multiply simultaneously in the CNS. It is difficult to determine whether one of them or both induced paralysis. Whereas one stool isolate in this case consisted of a mixture of type 2 recombinant and nonrecombinant strains, the blood clot isolate recovered on the same day as the CNS isolate consisted of the same strains as the CNS isolate. These findings strongly support the mechanism of virus spread to the CNS via the bloodstream (20). This case also indicated that sometimes a strain (the type 3 strain) can be recovered only from the CNS and not from the stool. In one case (3-IV), a complete discrepancy between CNS and stool isolates was found. While in the two stool isolates only type 3 strains were found, a single Sabin 1-derived strain was detected in the CNS. We cannot exclude laboratory contamination in this case. However, fluctuation of viral populations in the gut over time might explain why the CNS strain was not isolated from stool. It is obvious that the same question would have arisen for the 2-IV case if only the second of the three stool isolates, from which the CNS strain was absent, had been available (Fig. 4). Alternatively, the type 1 strain mentioned above might have been cleared from the feces early on in the infection. There is evidence that type 1 vaccine-derived strains are excreted for a shorter period of time after vaccination than type 2 or 3 strains (24).

A surprising finding was that the strains from the CNS were not more neurovirulent than the strains from stool but were even slightly less virulent (see Results). The biological meaning of this finding is difficult to explain, but the data are consistent with the results of Kawamura et al. (13), who reported that the strains recovered from the spinal cords of paralyzed monkeys were less neurovirulent than the inoculated strain. In our study, a marked difference in neurovirulence as well as in the *ts* phenotype between apparently genomic similar CNS and stool strains was recorded for one contact VAPP case (case 3-I). For this case a nasopharynx isolate with a phenotype similar to that of the CNS strain was recovered (results not shown). Since poliovirus vaccine-derived strains present in the gastrointestinal tract can undergo important phenotypic modifications during their multiplication (14), the strain recovered from the CNS can exhibit a different phenotype, depending on when it has migrated from the gut or nasopharynx to the CNS. In fact, the stool and CNS strains we investigated here are the progenies of a single initial strain, resulting from its multiplication in different tissues. In case 3-I, it seems evident that the stool strain acquired new phenotypic properties during its multiplication in the gut. For the other cases, it remains to be elucidated whether the evolution of the strains in the gut and/or in the CNS precisely accounts for the small differences in phenotype.

None of the nine vaccine-derived strains isolated from the CNS was attenuated when tested on PVR-Tg mice. However, while four strains were highly neurovirulent, the other five had an intermediary neurovirulence. More strikingly, we found that the wild-type 3 strains (w3) had a *ts* phenotype and were also intermediary in regard to neurovirulence. The reduced virulence of the CNS strains found in these cases might have four possible explanations. First, use of the animal system that we

used for testing neurovirulence, i.e., PVR-Tg mice, might overlook neurovirulence determinants operating in humans (22), although to date neurovirulence in PVR-Tg mice has correlated well with neurovirulence in monkeys (12, 31). However, even for the PVR-Tg mice, we found differences in the neurovirulence levels of some VAPP strains as a function of the inoculation route (results not shown). This also suggests that neuropathogenicity may depend on properties of the virus other than the capacity to multiply in the motor neuron, including the abilities to replicate in extraneural sites, to diffuse through the organism, and to be transported through the blood-brain barrier. Studies to test this hypothesis in Tg mice are in progress. A second interpretation could be that the strain recovered from the CNS underwent attenuation before or during its multiplication in the CNS. This is difficult to prove because, unlike for animal models (13), in humans the strain which actually initiates the CNS infection is not available for comparison. Third, the problem of the etiologic diagnosis in VAPP cases should also be discussed in terms of tissue specificity relative to virus isolation in cultured cells in vitro. The virus that we characterize and on which we base our conclusions is always isolated in vitro on cells of a different origin. It is possible that during this passage, a particular viral population which may have properties different from those of the virus in the original sample is selected. This had already been demonstrated at the earliest time of poliovirus isolation (21, 36), and it might cause errors in our image of the actual etiologic agent of the disease or of the virus multiplying in the alimentary tracts of the vaccinees. A fourth possibility is that even weakly neurovirulent viruses, whether wild type or vaccine derived, can induce poliomyelitis in humans. It is not excluded that, besides the etiologic agent, not-yet-identified host factors are involved in the clinical evolution of the disease.

In conclusion, our results strongly suggest that the vaccine-derived strains recovered from the CNS resulted from the multiplication of the virus in the CNS. In all but one of the cases studied, the strains recovered from the CNS corresponded to at least one of the strains isolated from stool or from another source from the same patient, indicating that the CNS strain is not artifactual. Moreover, for some cases, we detected small genomic differences between the otherwise very similar strains isolated from the CNS and stool of the same patient, which might indicate multiplication of the virus in the CNS.

The diversity of the genomes of the vaccine-derived viruses inducing the disease, ranging from mutated viruses to recombinants having a polio vaccine or even a non-polio vaccine donor, shows the complexity of the evolution of the Sabin vaccine strains in humans. Therefore, efforts to construct new candidates for oral poliovirus vaccines (28) are entirely justified.

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