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Characterization of a recombinant type 3/type 2 poliovirus isolated from a healthy vaccinee and containing a chimeric capsid protein VP1

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A Sabin 3/Sabin 2/Sabin 3 (S3/2/3) intertypic recombinant poliovirus was isolated from a faecal specimen from a 2-year-old healthy boy approximately 12 weeks after administration of oral poliovirus vaccine. The first recombination junction was in the genomic region encoding the VP1 capsid protein between nucleotide positions 3274 and 3285 (numbering according to Sabin 3) and the second was in the RNA polymerase region (nucleotide positions 6824 and 6825). The recombination had introduced six Sabin 2-derived amino acids into the Sabin 3 capsid environment in the carboxyl terminus of VP1. The complete genome of the recombinant virus differed from corresponding parental Sabin strains at 33 nucleotide positions, nine of them resulting in an amino acid substitution. Four substitutions were in the capsid proteins and five were in the region encoding the non-structural proteins. One amino acid was changed in the antigenic site 2B and two in site 3B. In addition, the whole antigenic site 3A was replaced by Sabin 2-specific amino acids, but the antigenic characteristics of the S3/2/3 did not show type 2-specific features. Neutralizing antibody titres in sera from Finnish children immunized with the inactivated poliovirus vaccine were not lower against the recombinant virus than against Sabin 3. Our results suggest that the chimeric virus was most likely generated by recombination events in the vaccinee, rather than representing progeny of circulating vaccine-derived virus.

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INTRODUCTION

The polioviruses, members of the *Enterovirus* genus in the *Picornaviridae* family, are important human pathogens, causing the acute paralytic disease poliomyelitis. Polioviruses have a positive-strand RNA genome about 7·5 kb long. In the virus particle the genome is surrounded by an icosahedral capsid composed of 60 protomers each comprising a single copy of each of the four capsid proteins, VP1, VP2, VP3 and VP4. In addition to the capsid proteins, the genome encodes non-structural proteins, e.g. two proteases and RNA polymerase. The protein coding region is flanked by a 750 nucleotide long 5′-untranslated region and a 3′-untranslated region ending in the poly(A) tail.

The World Health Organization (WHO)-coordinated international initiative of worldwide eradication of wild-type poliovirus has two cornerstones, intensive immunization with polio vaccine, including national immunization days, and systematic virological surveillance. Both the oral live attenuated vaccine (OPV) and the inactivated vaccine (IPV) consist of all three serotypes (Sabin & Boulger, 1973).

The GenBank accession number of the sequence reported in this paper is AF541919.

The rate of evolution of polioviruses is extremely high and considered to be mainly due to the high frequency of errors in RNA synthesis, roughly 10^{-4} per base pair per replication cycle. The genetic diversity of poliovirus strains is exploited in molecular epidemiology, a key component of poliovirus surveillance (Rico-Hesse *et al.*, 1987), currently based on sequence analysis of the VP1 coding region of the genome. Strains isolated from patients with an epidemiological connection regularly show only limited sequence divergence, while those from unrelated cases usually differ much more (Rico-Hesse *et al.*, 1987). Genomic analysis over a wider range has revealed that recombination is a frequent phenomenon in polio vaccinees, including strains isolated from cases of vaccine-associated paralytic poliomyelitis (VAPP) (e.g. Gammack *et al.*, 1989; Lipskaya *et al.*, 1991; Furione *et al.*, 1993; Georgescu *et al.*, 1994, 1995; Georgopoulou & Markoulatos, 2001).

Vaccine/vaccine and vaccine/non-vaccine recombinants have been described in many studies (e.g. Guillot *et al.*, 2000; Cuervo *et al.*, 2001). Recently, vaccine-derived polioviruses (VDPV) have been associated with three outbreaks of poliomyelitis. Type 2 VDPV circulated in Egypt for over 10 years (1983–1993) and was isolated from 32 cases (Centers

for Disease Control and Prevention (CDC), 2001a). An outbreak of poliomyelitis on the island of Hispaniola was associated with circulating type 1 vaccine-derived poliovirus (Kew *et al.*, 2002). In the Philippines, type 1 VDPV was involved in three poliomyelitis cases in 2001 (CDC, 2001b). In each of these three outbreaks, the VDPV strains were recombinants in which most of the genomic region encoding the non-structural proteins was apparently derived from a non-polio enterovirus origin.

In natural intertypic recombinant poliovirus strains characterized so far, the recombination junctions have been almost without exception in the genomic region encoding non-structural proteins. Two special genomic arrangements have been described in the intratypic recombinants. In Romania, a type 2 vaccine/non-vaccine strain with a 5′ recombination site at the beginning of the coding region was isolated from a lethal VAPP case (Georgescu *et al.*, 1995). Type 1 wild-type/vaccine recombinant polioviruses were isolated from poliomyelitis patients in China from 1991 to 1993. All these type 1 recombinants had a 367 nt block of sequence derived from Sabin 1 spanning 115 3′-terminal nucleotides of the VP1 gene and the 5′ half of the 2A gene in a wild-type poliovirus 1 environment (Liu *et al.*, 2000).

In this study, we describe a Sabin3/2/3 recombinant which was isolated from a faecal specimen from a healthy child 12 weeks after administration of the oral poliovirus vaccine. Primary characterization of the isolate revealed a chimeric Sabin 3/Sabin 2 capsid structure. This observation led us to study further the complete genomic sequence, antigenic properties and temperature sensitivity of this virus.

METHODS

Virus strains and cell lines. Reference poliovirus Sabin strains 1, 2 and 3 were originally obtained from SK–RIT, one of the ancestors of the current GlaxoSmithKline company (Rixensart, Belgium). Poliovirus 3/Leon/USA/1937 (VR-62) was obtained from the ATCC. Mouse L cells expressing human poliovirus receptor (L20B) and human rhabdomyosarcoma (RD) cells were provided by the WHO Polio Labnet. Two Green monkey kidney cell lines GMK and Vero had been maintained in the laboratory since the 1960s.

Virus isolation. Poliovirus 3 was first isolated from a faecal specimen from a 5-year-old boy. The index case, his 2-year-old brother and his 9-year-old sister stayed in Sri Lanka for 8 months and received a dose of OPV on the national immunization day, 28 October 2000. They had all been previously immunized with inactivated poliovirus vaccine (IPV) in Norway according to the usual schedule, which includes three doses of IPV at 6, 8 and 16 months of age and two revaccinations at the ages of 7–8 and 15 years. During the last week in Sri Lanka, the index case got an unexplained fever and was hospitalized soon after returning to Oslo. He had several septic peaks every day, but no other symptoms. Both siblings were healthy.

Poliovirus 3 strain PV3/NOR/00/46220, from the index case, was isolated in L20B cells, typed as poliovirus 3 in the National Institute of Public Health, Oslo, Norway, and sent to the Regional Reference Laboratory [National Public Health Institute (KTL), Helsinki, Finland] for intratypic differentiation (ITD) tests on 16 January 2001. Approximately 12 weeks (83 days) after the OPV dose, poliovirus 3 strain PV3/NOR/01/8 was also isolated from the younger brother of the index case and at the same time, PV3/NOR/01/1 was isolated from a faecal specimen from the 9-year-old sister of the index case and sent to KTL on 6 February 2001.

Confirmation of serotype and ITD. The PV3 isolates were analysed according to the protocol recommended by WHO. The serotype was confirmed by neutralization with polyclonal antisera [obtained from National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands]. ITD by enzyme immunoassay (EIA) was carried out with cross-absorbed antisera to PV3 Sabin and non-Sabin-like PV3 (RIVM). The molecular ITD was carried out by restriction fragment length polymorphism analysis of RT-PCR amplicons (RT-PCR-RFLP). A 480 nt genomic segment of the VP3/VP1 coding region was digested with three restriction enzymes, *Dde*I, *Hae*III and *Hpa*II (New England BioLabs). The patterns of the digests were analysed by agarose gel electrophoresis and compared with the patterns of the reference Sabin strains (Balanant *et al.*, 1991).

RT-PCR and sequencing. The entire VP1 protein coding region of PV3/NOR/01/8 was sequenced using two overlapping RT-PCR amplicons with the following primer sets: for the 5′ part, primers 4548 and PV1A, and for the 3′ part, PV2S and Q8 (Table 1). Both previously described and newly designed primers were used in the sequencing of the complete genome of the isolate PV3/NOR/01/8 (Table 1). The protocols for RT-PCR and sequencing were the same for all primer pairs. Briefly, RNA was extracted from 100 µl of infected cell cultures with an RNeasy Total RNA Kit (Qiagen) according to the manufacturer's instructions. RNA (1 µl) was added to RT-PCR mixtures (total volume 50 µl) containing 67 mM Tris/HCl (pH 8·8), 17 mM $(NH_4)_2SO_4$, 6 µM EDTA (pH 8·0), 200 µM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics), 1·5 mM $MgCl_2$, 10 U RNasin Ribonuclease Inhibitor (Promega), 3·6 U avian myeloblastosis virus reverse transcriptase (Finnzymes), 2·7 U Thermoperfect Taq polymerase (Integro, Leuvenheim, The Netherlands) and 50 pmol each of the forward and reverse primers. The mixtures were incubated at 50 °C for 30 min and at 94 °C for 3 min. Thirty-five cycles of amplification (94 °C for 30 s, 42 °C for 30 s and 60 °C for 30 s) were followed by a final extension step at 60 °C for 5 min.

The PCR amplicons were purified with the QIAquick Gel Extraction Kit (Qiagen) and used as templates in cycle sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) in an automated sequencer (model 310, Applied Biosystems). Electropherograms were analysed by SEQUENCING ANALYSIS, v.3.1, and SEQUENCE NAVIGATOR, v.1 (Applied Biosystems). At least two corresponding electropherograms were performed and analysed for each genomic region. The complete genome sequences of Sabin 3 and Sabin 2 used in comparisons were from GenBank (accession numbers X00925 and X00595, respectively).

Preparation of mouse antisera to the PV3/NOR/01/8 isolate. The PV3/NOR/01/8 isolate was cloned by plaque purification in GMK cells. Five separate plaques were collected in 100 µl of Eagle's minimum essential medium supplemented with 2 % foetal calf serum. One plaque was passaged twice in GMK cells and the virus was purified by precipitation with polyethylene glycol and sucrose gradient centrifugation (Abraham & Colonno, 1984). The fractions were assayed by titration in RD cells; infective fractions were combined and concentrated by ultracentrifugation. The pellets were suspended in PBS; the protein concentration was measured and the virus preparation diluted to 51·8 µg protein ml^{-1}.

Ten in-house-bred NIH mice (female, 8 weeks old) were used to

Table 1. Primers used in this study

Numbering of nucleotide positions (Sabin 3, P3/Leon 12a1b) is according to Stanway *et al.* (1983).

Primer	Position	Polarity	Sequence (5′–3′)	Reference
72437	001–020	Sense	TTA AAA CAG CTC TGG GGT TG	Mulders *et al.* (1999)
216615	446–463	Sense	TCC TCC GGC CCC TGA ATG	Blomqvist *et al.* (1999)
216616	545–565	Antisense	GAA ACA CGG ACA CCC AAA GTA	Blomqvist *et al.* (1999)
294010	779–804	Sense	CAC GAR AAT TCW AAC MGA GCC TA	This study
72283	1712–1728	Sense	ATG TGC TGC GAG TTC AA	Mulders *et al.* (1999)
294011	1776–1795	Antisense	GTT ACT MCC WGG AGT GTT CA	This study
4548	2399–2419	Sense	TTT GTG TCA GCG TGT AAT GA	Balanant *et al.* (1991)
72284	2399–2419	Antisense	TCA TTA CAC GCT GAC ACA AA	Mulders *et al.* (1999)
PV2S	2847–2866	Sense	CIT AIT CIM GIT TYG AYA TG	Kilpatrick *et al.* (1996)
4547	2852–2872	Antisense	GAA TTC CAT GTC AAA TCT AGA	Balanant *et al.* (1991)
PV1A	2906–2925	Antisense	TTI AII GCR TGI CCR TTR TT	Kilpatrick *et al.* (1996)
71935	3206–3222	Sense	GTC AAT GAT CAC AAC CC	Mulders *et al.* (1995)
Q8	3476–3495	Antisense	AAG AGG TCT CTR TTC CAC AT	modified from Rico-Hesse *et al.* (1987)
294017	4367–4386	Sense	ATY CAR TCC AAG AGR TTY GC	This study
244334	4460–4474	Antisense	GGT TCA ATA CGG TGT TTG CT	Caro *et al.* (2001)
294019	5096–5115	Sense	TTC CAR GGA CCA CTS CAG TA	This study
294018	5180–5199	Antisense	ACT TCC TGG GAR TCA ACT GC	This study
294021	5951–5972	Sense	AAG CGG TCA TAC TTC ACT CAG A	This study
294020	5990–6009	Antisense	TCY TTT GAT GGT YTC ATC CA	This study
92369	6827–6846	Sense	GGI GGI ATG CCM TCN GGN TG	This study
294022	6903–6925	Antisense	TAT GCC CTT GTA GGT TTT CAG TA	This study
92370	7432–7452	Antisense	TTT TTT TTT TTT TTT TTT TTT TTC	This study

produce antisera to the PV3/NOR/01/8 virus. Three inoculations of virus (each 5·18 μg per mouse) were given at 0, 2 and 4 weeks. The antisera were prepared from blood drawn 1 week after the last injection.

Micro-neutralization assay. Neutralizing antibodies to Sabin 1, Sabin 2, Sabin 3 and PV3/NOR/01/8 were assayed by a standard micro-neutralization test. Fifty human serum specimens were selected from routine diagnostic material from the Department of Virology, University of Helsinki. Specimens had been collected during 1998 from healthy IPV-immunized children under 5 years of age. Twofold dilution series, starting from a 1 : 4 dilution, were made in duplicate in a 96-well plate from each human and mouse serum specimen. One-hundred $TCID_{50}$ units of virus were added and the mixtures were incubated for 1 h (mice sera) or overnight (human sera) at 36 °C. After neutralization, 2×10^5 Vero cells were added to each well. The plates were sealed, incubated at 36 °C for 7 days and stained with crystal violet. The reciprocal of the dilution showing 50 % inhibition of virus-induced CPE was taken as the end-point titre.

Determination of neutralization indices. Polyclonal antisera for Sabin 2 and Sabin 3 were diluted 1 : 20 and viruses Sabin 2, Sabin 3 and PV3/NOR/01/8 1 : 10. Equal volumes of virus and antiserum dilutions were mixed and incubated for 2 h at 36 °C for neutralization. The titres of remaining infectious viruses were determined in 96-well cultures of Vero cells at 36 °C. The neutralization indices of the sera were calculated as the logarithm of the ratio of the titre of the virus without antiserum to that with the tested antiserum.

Assay for temperature sensitivity. Temperature sensitivity of the isolate PV3/NOR/01/8 was assayed in monolayer cultures of RD cells. Two 24-well plates were inoculated with 50 μl of undiluted virus stocks (Sabin 3, PV3/NOR/01/8 and PV3/Leon). After adsorption for 1 h at 36 or 40 °C, the unadsorbed virus inoculum was removed, maintenance medium was added and the plates were incubated at 36 or 40 °C. Wells were harvested at 8 and 24 h post-infection and total virus concentration was determined by the end-point dilution method in RD monolayer cultures in 96-well plates at 36 °C.

RESULTS

Preliminary characterization of the isolate

The serotype of poliovirus PV3/NOR/01/8 was confirmed as type 3 by complete neutralization with polyclonal antisera specific for type 3. ITD was carried out by two different methods. RFLP analysis with three enzymes revealed a typical Sabin 3 restriction pattern. However, in the EIA test with cross-absorbed antibodies to Sabin-like (SL) and non-Sabin-like (NSL) poliovirus 3, the PV3/NOR/01/8 strain reacted with both antisera, the absorbance being definitely higher with the NSL than the SL antiserum and at the borderline for being classified as NSL (Table 2).

Due to the discrepant EIA results in the two ITD tests, the complete VP1 sequence of the isolate was determined. Strikingly, the last 91 nucleotides at the 3′ end of the VP1 gene were found to be 100 % identical to Sabin strain 2, while the rest of the gene was almost identical to Sabin 3.

To verify that the virus had a recombinant genome and was not a mixture of two distinct serotypes, it was plaque-purified. Five different plaques were collected and genomic

Table 2. Intratypic differentiation of PV3/NOR/01/8 by EIA with cross-absorbed Sabin-like and non-Sabin-like antisera

The test was carried out according to the WHO (2001) Polio Laboratory Manual. NSL control is a non-infectious control material. Two assays based on two parallel cultures are shown.

		Absorbance		
Test material	**Antiserum...**	**Sabin-like**	**Non-Sabin-like**	**Non-absorbed serotype-specific**
Test 1				
Sabin 3		1·161	0·177	1·175
PV3/NOR/01/8		0·179	0·704	1·387
NSL control		0·128	1·318	2·348
Test 2				
Sabin 3		1·434	0·229	1·381
PV3/NOR/01/8		0·544	1·249	2·058
NSL control		0·217	1·682	2·537

sequences in the VP1/2A junction region determined. All five revealed the same recombination junction in the capsid coding region.

PV3 was also isolated, in Norway, from faecal specimens from the two siblings of the 2-year-old boy. The isolate PV3/NOR/00/46220 was typically Sabin-like in the ITD tests, while PV3/NOR/01/1 was confirmed as Sabin-like PV3 in an EIA test, but in RT-PCR-RFLP the virus gave an extra band with one of the restriction enzymes (*Hpa*II). The sequence of the VP1-encoding gene of PV3/NOR/01/1 was 99·8 % identical to Sabin 3 without any sign of recombination. No further studies were performed with this virus.

Sequence analysis of the complete genome

The PV3/NOR/01/8 isolate was sequenced from nucleotide 11 to the 3′ end of the genome. Two recombination junctions were identified (Fig. 1). The first recombination crossover point was in the VP1 capsid protein-coding gene from nucleotides 3274 to 3285. This recombination event led to the change in the genome from Sabin 3 to Sabin 2 and resulted in an insertion of six amino acid 'substitutions' in the carboxyl terminus of VP1 (amino acids 279, 286, 287, 288, 290 and 293 of VP1). The second crossover point, a change from the Sabin 2 genome to Sabin 3, was in the polymerase coding region, and included nucleotides 6824 and 6825 (numbering of Sabin 3).

The comparison of the S3/2/3 genome to respective Sabin strains revealed 33 point mutations evenly distributed throughout the genome (Table 3). The nucleotide substitution T→C in position 472 in the 5′ non-coding region was a reversion to the sequence of the neurovirulent precursor of Sabin 3, P3/Leon/USA/1937. Nine of the mutations led to a change in amino acid, four in the capsid and five in the region coding for the non-structural proteins. Three of the four amino acid substitutions in the capsid region were located at known antigenic epitopes (Minor *et al.*, 1986). One substitution was in site 2B (VP2, Ala-165→Thr) and two were in site 3B (VP3, Ser-59→Asn; VP3, Asp-77→Asn). The fourth substitution (VP1, Ala-34→Val) has been described previously in a Sabin 3 strain with reduced temperature sensitivity (Minor *et al.*, 1989).

Antigenic characteristics of PV3/NOR/01/8

Isolate PV3/NOR/01/8 was neutralized in the standard poliovirus serotyping procedure (WHO) with polyclonal antisera to PV3, but not with PV2 antisera. To confirm this result, we determined the neutralization indices of the two antisera against PV3/NOR/01/8 and Sabin 2 and 3.

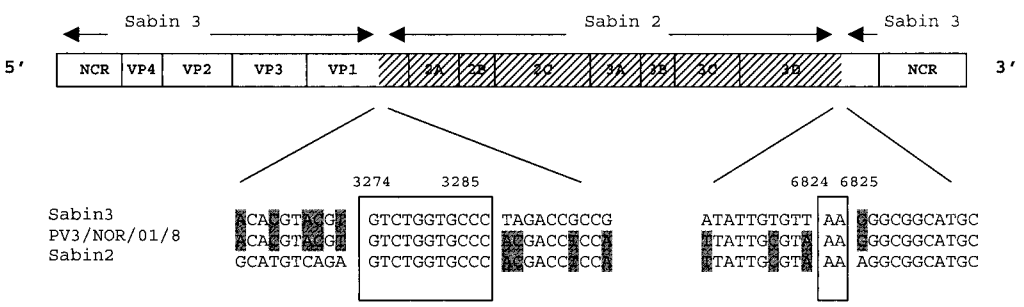

Fig. 1. Scheme of genomic structure of PV3/NOR/01/8. The proposed recombination sites are shown in detail.

Antiserum to PV2 had no effect on the infectivity of the PV3/NOR/01/8 isolate (data not shown).

Ten mice were immunized with the PV3/NOR/01/8 virus. All mice produced neutralizing antibodies to both the immunogen strain and Sabin 3 (Table 4). Nine mice out of ten produced antibodies with slightly better neutralizing capacity to the PV3/NOR/01/8 than to Sabin 3. Sabin 1 and Sabin 2 were not neutralized by the immune sera.

Fifty sera drawn from IPV-vaccinated children were tested for neutralizing antibodies to PV3/NOR/01/8 and the standard Sabin strains. All sera had antibodies to all virus strains and the titres against PV3/NOR/01/8 were similar to those against Sabin 3 (median titres 320 and 448, respectively).

Temperature sensitivity

The PV3/NOR/01/8 virus was compared to Sabin 3 and the parental PV3/Leon strain as regards replication capacity at an elevated temperature (40 °C). Sabin 3 was temperature sensitive as expected while in the case of the PV3/NOR/01/8 strain a definitely lesser effect was seen (Table 5).

DISCUSSION

We have demonstrated in this paper that natural recombination among enteroviruses may result in viable viruses with intertypic hybrid capsid proteins. In this case, the inserted type 2/Sabin amino acid motif did not provide the recombinant virus with type 2 antigenic characteristics.

Table 3. Nucleotide and amino acid substitutions in the PV3/NOR/01/8 recombinant virus compared to Sabin 3 and Sabin 2

The nucleotide and amino acid positions are given as in the parental Sabin strains.

	Sequence position		Sabin 3		PV3/NOR/01/8		Sabin 2	
	nt	aa	nt	aa	nt	aa	nt	aa
5′ NCR	472	–	T	–	C	–	–	–
	652	–	T	–	G	–	–	–
VP2	1243	167	C	–	T	–	–	–
	1442	234	G	Ala	A	Thr	–	–
	1505	255	C	–	T	–	–	–
VP3	1938	399	G	Ser	A	Asn	–	–
	1991	417	G	Asp	A	Asn	–	–
	2410	556	C	–	G	–	–	–
VP1	2577	612	C	Ala	T	Val	–	–
	2638	632	A	–	G	–	–	–
	3115	791	G	–	C	–	–	–
	3241	833	A	–	G	–	–	–
	3259	839	A	–	G	–	–	–
2A	3717	990	–	–	T	–	C	–
2B	3996	1083	–	–	C	–	T	–
	4044	1099	–	–	A	–	T	–
2C	4117	1124	–	–	A	Lys	G	Glu
	4147	1134	–	–	A	Thr	G	Ala
	4290	1181	–	–	T	–	G	–
	4437	1230	–	–	A	–	G	–
	4719	1324	–	–	C	–	T	–
	4974	1405	–	–	G	–	A	–
3C	5586	1613	–	–	T	–	C	–
	5802	1685	–	–	G	–	A	–
3D	6136	1797	–	–	G	Gly	A	Arg
	6543	1932	–	–	T	–	C	–
	6609	1954	–	–	C	–	T	–
	6744	1999	–	–	A	–	C	–
	6937	2065	C	–	T	–	–	–
	7020	2093	A	Lys	G	Arg	–	–
	7231	2163	T	–	C	–	–	–
	7281	2180	A	Lys	G	Arg	–	–

Table 4. Neutralization titres in sera from mice immunized with purified virus PV3/NOR/01/8

End-point titres of the sera against Sabin 3 and PV3/NOR/01/8 are compared.

	Virus strain	
Serum	Sabin 3	PV3/NOR/01/8
01	128	512
02	128	512
03	128	512
04	64	128
05	1024	2048
06	128	256
07	256	512
08	256	4096
09	512	>8192
10	512	512

The virus strain characterized was not isolated from a patient with suspected poliomyelitis but from a healthy child, who had been examined because of having visited a region that was considered to be a risk area for wild-type poliovirus circulation. In fact, Sri Lanka has been free of wild-type polio since 1993. Bearing in mind the recent outbreaks caused by circulating vaccine-derived poliovirus strains (cVDPV) (CDC, 2001a, b; Kew *et al.*, 2002), careful analysis of the characteristics of poliovirus strains isolated from any location is recommended by WHO. Intratypic differentiation tests are performed on every poliovirus isolate to distinguish between vaccine and wild-type viruses. In this case, the preliminary molecular analysis (RT-PCR-RFLP) did not reveal any wild-type characteristics but, according to antigenic analysis (EIA), the isolate appeared to be on the borderline of Sabin-like and non-Sabin-like. The growth characteristics at elevated temperature supported the wild-type nature of this virus. Further sequencing revealed the novel composition of a Sabin recombinant poliovirus with a chimeric capsid protein VP1. While we were preparing the manuscript, we learnt that a similar recombinant virus with one of the recombination junctions within the VP1-coding region had also been observed and characterized by Martin *et al.* (2002).

Recombination is a frequent event in polioviruses, occurring at a rate of 10^{-4} per base pair per replication cycle at the RNA level (Jarvis & Kirkegaard, 1992). There may be a selective advantage for recombination to increase the fitness of the strain for replication in the human gut by preventing the accumulation of harmful mutations. Tripartite recombinant polioviruses with homotypic 5′- and 3′-terminal regions of type 3 have been described (e.g. Cuervo *et al.*, 2001). Usually, the first recombination site is in the P2 region and the second in P3, often within the polymerase gene. Structural constraints in the capsid proteins are considered to restrict enrichment of intertypic recombinants with chimeric capsid proteins. Indeed, the previously described recombinants in this category did not result in any amino acid substitutions. Survival of the current strain with a chimeric VP1 might be based on the fact that the inserted type 2-specific motif is located at the virion surface, allowing more freedom for aberrant folding. This was also consistent with the observation that the virus had altered type 3-specific antigenic properties but had not acquired any type 2-specific characteristics.

In our case, it seems likely but cannot be proven that the virus had been generated in the sampled vaccinee. The median divergence of the nucleotide sequence is 0·44 %, which is more than one would generally expect after 12 weeks of replication, but because of selection and chance, divergent lineages are occasionally generated rapidly (Kinnunen *et al.*, 1991). We have previously proposed that many of these lineages may have a reduced fitness for transmission, as the divergence generated during a widespread epidemic did not strikingly exceed that seen between sequential specimens collected from a given individual (Huovilainen *et al.*, 1988; Kinnunen *et al.*, 1991).

Isolate PV3/NOR/01/8 had a nucleotide substitution of T for C at position 472 in the 5′ non-coding region, which is a reversion to the sequence of the neurovirulent precursor of Sabin 3, P3/Leon/USA/1937. This substitution is found often in vaccine strains isolated from healthy vaccinees and VAPP cases, consistent with rapid reversion of this nucleotide in the human gut (Evans *et al.*, 1985; Minor & Dunn, 1988). Another sequence variation frequently associated with increased neurovirulence (nucleotide

Table 5. Temperature sensitivity of poliovirus PV3/NOR/01/8

Values are $\log_{10}$ $TCID_{50}$ ml^{-1} virus produced in RD cells at 36 and 40 °C.

	Growth temperature and virus titre					
	36 °C		40 °C		Log titre reduction 36/40 °C	
Virus strain	8 h p.i.	24 h p.i.	8 h p.i.	24 h p.i.	8 h p.i.	24 h p.i.
Sabin 3	5·50	6·63	2·38	2·25	3·13	4·38
PV3/NOR/01/8	7·25	7·13	6·13	5·50	1·13	1·63
PV3/Leon	7·75	7·63	7·75	6·88	0	0·75

2493) (Chumakov *et al.*, 1992) was not seen in our PV3/NOR/01/8 strain.

Three amino acid changes at known antigenic epitopes, in addition to the exchange of the C terminus of VP1, may explain why PV3/NOR/01/8 reacted as non-Sabin-like with cross-absorbed intratype-specific antisera. The mutations in VP2 at amino acid 165 and in VP3 at amino acid 77 were previously reported to be common both in healthy vaccinees and vaccine-associated poliomyelitis cases, possibly reflecting immune pressures in vaccinees (Macadam *et al.*, 1989). The mutations in VP3, Ser-59→Asn and Asp-77→Asn, were also common in the Sabin 3 strains imported into The Netherlands. At least one of these mutations was seen in a set of Sabin 3 isolates which were double-reactive in the ITD assay (Reimerink *et al.*, 1999).

In spite of the altered antigenic epitopes, the serotype of the isolate was not altered, as shown by the virus being totally neutralized by polyclonal type 3 antisera and not at all by type 2 antisera. Despite the mutations in two of the four antigenic sites, the virus was readily neutralized with sera from IPV-vaccinated children. It seems that there is no risk of circulation of this virus in well-vaccinated populations.

Strain PV3/NOR/01/8 had almost entirely lost the temperature-sensitive phenotype of the parent Sabin viruses. The mutation that results in temperature sensitivity in type 3 Sabin virus involves the substitution of a phenylalanine for a serine residue at amino acid 91 in VP3 (Minor *et al.*, 1989). This amino acid was Sabin-like in our virus. A number of Sabin 3 strains with reduced temperature sensitivity have been isolated from vaccine-associated cases. Besides amino acid 91 in VP3, eleven other amino acid substitutions have been suggested to be related to temperature sensitivity; among these is the mutation alanine for valine at position 34 in the VP1 capsid protein, also found in PV3/NOR/01/8.

In conclusion, we have characterized a recombinant poliovirus type 3 with a tripartite genome, in which the first recombination site was in the VP1 capsid region and the second in the polymerase gene. In spite of the amino acid changes in antigenic sites 2B and 3B, and the replacement of site 3A by Sabin 2-specific amino acids, the recombinant was neutralized by sera from IPV-vaccinated children as well as the parental Sabin 3 strain and did not acquire type 2-specific antigenic characteristics. Because of limited divergence from the parental Sabin strains, it seems that the recombinant virus had evolved in the vaccinee and does not represent a circulating VDPV.

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