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Retrospective molecular and phenotypic analysis of poliovirus vaccine strains isolated in Greece

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Abstract

The live oral poliovirus vaccine (OPV) strains are genetically unstable, causing, in rare cases, vaccine-associated paralytic poliomyelitis. Reversions of the known attenuating mutations in OPV strains and intertypic recombination have been identified as the underlying causes of the increased neurovirulence of poliovirus isolates. In this study, three OPV isolates (one non-recombinant and two recombinants) were tested in order to correlate phenotypic traits such as temperature sensitivity (Rct test) and growth kinetics (one-step growth curve test) with mutations and recombination events of the viral genome. Moreover, the immunity level of the western Greek population aged 1–40 years was evaluated against OPV isolates and Sabin vaccine strains, with a microneutralization assay. Members of the 1–40-year age group (both pooled and individual sera) showed no significant differences in neutralization test (NT) titres against OPV isolates in comparison with the Sabin vaccine strains. However, all three OPV isolates showed reverted phenotypic traits in Rct or one-step growth curve assays. The results of our study revealed a significant decrease in immunity level from the 1–10-year age group to the 21–30-year age group (pooled sera) for both poliovirus types 1 and 3. For both poliovirus types, the highest NT titres were observed in the 1–10-year age group, and the lowest NT titre was observed in the 21–30-year age group, towards poliovirus type 3. Our study underlines the need for immunological studies in all age groups, in order to allow reconsideration of the current vaccination policies and to avoid epidemics caused by the circulation of highly evolved OPV derivatives.

Keywords: Greece, immunity, mutations, neurovirulence, oral poliovirus vaccine isolates, polioviruses, recombination
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Introduction

Polioviruses are the aetiological agents of acute paralytic poliomyelitis, belong to the genus *Enterovirus* of the family *Picornaviridae*, and exist as three immunologically defined serotypes (P1, P2 and P3). The genome of polioviruses is a 7.5-kb positive-sense single-stranded polyadenylated RNA. The viral RNA contains a long open reading frame flanked by a 5′-noncoding region (5′-NCR) and a 3′-noncoding region, which are involved in viral replication and translation. The structural (VP4, VP2, VP3 and VP1) and non-structural (2A, 2B, 2C, 3A, 3B, 3C and 3D) viral proteins are produced by translation of the coding region and proteolytic cleavage of the polyprotein [1]. The poliovirus capsid consists of 60 copies of each of the four structural virion proteins. Surface-exposed loops of structural proteins VP1, VP2 and VP3 contain the four antigenic sites designated AgS1–AgS4 [2].

Since 1960, poliomyelitis has been effectively controlled by the use of inactivated poliovirus vaccine (IPV) or live oral poliovirus vaccine (OPV). OPV consists of attenuated and thermosensitive strains of each of the three serotypes (Sabin 1, 2 and 3). The molecular determinants of attenuation and temperature sensitivity have been intensively studied, to elucidate the mechanisms involved and to improve the safety of Sabin vaccine strains [3–5].

OPV was the vaccine adopted throughout most of the world by 1964, because it multiplies actively in the gut of vaccinees, eliciting a strong, long-lasting immune response, and local immunity induced by OPV prevents or limits re-infection of humans. However, the selection of variants

with increased neurovirulence, caused by genetic instability, constituted a real problem with respect to OPV safety [6–8]. In rare cases (one case per 750 000 primary vaccinees), OPV strains have been implicated in vaccine-associated paralytic poliomyelitis (VAPP). Genetic recombination and mutations at attenuating sites of the genome have been associated with the emergence of VAPP cases [9,10]. Moreover, changes are frequently observed in the antigenic properties of OPV strains, representing a selection of viral variants that are less prone to be neutralized by human antibodies [11].

OPV was introduced into Greece in 1964, and was administrated to 2 million persons aged <18 years. A standard vaccination schedule of four doses at the ages of 2, 4, 6 and 18 months, with a booster dose at 4–6 years, led to the elimination of indigenous cases of poliomyelitis after 1982 [12]. However, Greece has switched to the exclusive use of IPV since 2005, as is also the case in most polio-free countries.

In this study, three OPV isolates (one non-recombinant and two recombinants) were tested in order to correlate the reverted phenotypic traits, such as temperature sensitivity and growth kinetics, with mutations and recombination events in the viral genome. Moreover, the immunity level of the western Greek population aged 1–40 years was evaluated against OPV isolates and Sabin vaccine strains.

Materials and Methods

Virus isolation

Two viral strains were isolated from faecal samples of a VAPP patient or a healthy vaccinee and one from the environment during the time period 1978–1985. They were recultured for the purpose of the present study in Hep-2 cells (Table 1), as previously described [13]. The vaccine strains (Sabin 1, 2 and 3) that were used in this study were provided by the WHO.

Genotyping and identification of recombinant OPV isolates

Primer pair UG52/UC53 was used for identification of vaccine origin and for genotyping of the isolates in the 5′-NCR [14]. Also, the genotype of isolates according to VP1 capsid protein was determined by sequencing. The primer pairs used for complete VP1 gene sequencing were UG1/222 [15,16] for the first half of the VP1 gene and serotype-specific primer pairs $S_1$37/$S_1$688 and $S_3$26/$S_3$651 for Sabin 1 and Sabin 3 isolates, respectively, for the second half of the VP1 gene [17].

Identification of recombination sites of OPV isolates was initially performed by restriction fragment length polymorphism screening analysis followed by sequencing, as previously described [18,19].

Extraction of viral RNA

Two hundred microlitres of frozen and thawed viral stocks were subjected to viral RNA extraction as previously described [20].

Sequencing of OPV isolates

Reverse transcription and PCR reactions for OPV isolates were performed with an MJ Research Minicycler, as previously described [19].

Complete genomic sequencing of isolate MB1 and sequencing of VP1–VP4 capsid genes of isolates 522 and 742 were performed. Also, the partial 5′-NCR sequences of isolates 522 and 742 were determined with primer pair UG52/UC53. The primer pairs used in this study are shown in Table 2. For all primer pairs, PCR conditions were as described in the original publications (Table 2). Analysis of PCR products was performed by agarose gel electrophoresis (2% agarose; 1 mg/L ethidium bromide in Tris–boric acid–EDTA buffer), with the 100-bp DNA ladder as molecular mass marker (Invitrogen Life Technologies, Paisley, UK). PCR products were cleaned up with a PCR gel extraction kit (Qiagen, Hilden, Germany). All PCR products were sequenced at Macrogen Inc.

Computational analysis/computer graphics

ClustalW was used for alignment of nucleotide and amino acid sequences of OPV isolates with those of the reference strains cited in GenBank (Sabin 1, AY184219; Sabin 2,

TABLE 1. Available data for oral poliovirus vaccine isolates

Isolate	5′-NCR and VP1 genotype	Source	Recombination site/type/junction	Accession numbers[a] 5′-NCR/Capsid/2A–3D	Reference
522	S1	Healthy vaccinee	Non-recombinant	FJ609754/HM537000/–	[21]
742	S1	Environment	2A S1/S3 3461–3465 2C S3/S2 4511–4527	EU598472/HM537002/EU598488	[19]
MB1	S3	VAPP	2C S3/S1 4880–4887	GU180608	This study

5′-NCR, 5′-noncoding region.
[a]The accession number of isolate MB1 refers to the complete genome. The 2A–3D genomic region of isolate 522 was not sequenced.

TABLE 2. Primer pairs used for amplification of the complete genome of isolate MB1 and of the partial 5′-noncoding region (5′-NCR) and capsid region of isolates 522 and 742

Primer[a]	Polarity	Sequence (5′ → 3′)[b]	Reference
72437	Sense	001-TTAAAACAGCTCTGGGGTTG-020	[31]
216616	Antisense	545-GGAACACGGACACCCAAAGTA-565	[32]
UG52*	Sense	162-CAAGCACTTCTGTTTCCCCGG-182	[14]
UC53*	Antisense	577-ACCGACCAATACCACTGTT-595	[14]
$S_3$309	Sense	309-GTAGCTTGGGCCGATGAGT-327	[33]
$S_3$044	Antisense	1025-ACTGAATTTGCTGCCTCCTG-1044	[33]
$S_3$936	Sense	936-CTCCAGCACTCAATTCACCA-955	[33]
$S_3$510	Antisense	1491-TCCCAGTAACACCCCACATC-1510	[33]
$S_3$78	Sense	1378-TTATGCAAATGCGAATCCAG-1397	[33]
$S_3$864	Antisense	1844-AATAGGCGGAGTGACATCAAA-1864	[33]
$S_3$709	Sense	1709-CCCAATGTGTAGCGAGTTCA-1728	[33]
$S_3$218	Antisense	2200-GTGCCCAACATAGCCTCCT-2218	[33]
$S_3$40	Sense	2140-TACGGGGAAAATCCTAGTGG-2159	[33]
$S_3$659	Antisense	2640-CGTTTGAACTGTGTCGGATG-2659	[33]
UG1	Sense	2402-TTTGTGTCAGCGTGTTAATG-2421	[15]
222	Antisense	2982-CICCIGGIGGIAYRWACA-2999	[16]
$S_3$26	Sense	2826-GCCGTAAGTTGGAGTTTTTCAC-2847	[17]
$S_3$651	Antisense	3451-CTCCTTAGTGGCGAGATGGTAG-3430	[17]
$S_1$23*	Sense	323-GAGTCTGGACATCCCTCACC-342	[34]
$S_1$976*	Antisense	957-TACCCGCAAGCCTCTATGTT-976	[34]
$S_1$82*	Sense	882-CTCAAGACCCTTCCAAGTTCA-902	[34]
$S_1$542*	Antisense	1523-ATTATCTGGTGCGGGAACAC-1542	[34]
$S_1$350*	Sense	1350-GGGATAGCAACACCACTACCA-1370	[34]
$S_1$693*	Antisense	1675-TGGAATCTCTGGGGAGGAC-1693	[34]
$S_1$476*	Sense	1476-CGGTGGATTACCTCTTTGGA-1495	[34]
$S_1$154*	Antisense	2136-AGTTTGCCAGTTGCCATCA-2154	[34]
$S_1$013*	Sense	2013-TCTGCCTGTCACTCTCTCCA-2032	[34]
$S_1$669*	Antisense	2648-TGGTTTGCACTGTATCAGAAGG-2669	[34]
$S_1$37*	Sense	2837-AGGAAATTGGAGTTCTTCACC-2857	[17]
$S_1$688*	Antisense	3488-ACATGACGTTCACTGCGTTTT -3468	[17]
71935	Sense	3206-GTCAATGATCACAACCC-3222	[35]
EUC2	Antisense	4454-TTTGCACTTGAACTGTATGTA-4474	[36]
UG23	Sense	4169-AAGGGATTGGAGTGGGTGTC-4188	[37]
UC15	Antisense	4948-CATCTCTTGAAGTTTGCT-4965	[37]
$S_3$141	Sense	4741-AGGCATTCTGTTCACATCCAAC-4763	[18]
$S_3$368	Antisense	5239-ATGTTGACGATCCATCCTTTCT-5217	[18]
$S_2$48	Sense	4968-TCCTTTAGTGTGTGGCAAGG-4988	[18]
$S_2$911	Antisense	5812-GTTTGTCGTCCACCGAGATT-5832	[18]
UG16	Sense	5921-GTTGGTGGGAACGGTTCACA-5940	[37]
UC12	Antisense	6516-TCAATTAGTCTGGATTTTCCCTG-6494	[37]
$S_1$70	Sense	5769-TATGTATGTTCCTGTCGGTGCT-5791	[18]
$S_1$450	Antisense	6851-ATTGAAGTGCCTGAGCAACC-6871	[18]
$S_1$198	Sense	6728-GCACTAAAGATGGTGCTTGAGAAA-6752	[18]
$S_1$885	Antisense	7415-CTACAACAGTATGACCCAATCCAA-7391	[18]
$S_2$107	Sense	6647-TCCCAGTGCTAATGGAAGAGA-6668	[18]
$S_2$872	Antisense	7393-ACAACAGCATGACCCAATCC-7413	[18]

[a]Primer pairs marked with an asterisk were used for amplification of the partial 5′-NCR and capsid region of isolates 522 and 742. Primer pair UG1/222 was used for amplification of all three isolates.
[b]Positions refer to Sabin types according to primer notification (accession numbers: S1, Sabin 1 strain, AY184219; S2, Sabin 2 strain, AY184220; S3, Sabin 3 strain, AY184221). For all other primers, positions refer to Sabin 1 strain.

AY184220; and Sabin 3, AY184221). Amino acid sequences of OPV isolates were obtained with Gene-Runner V. 3.05.

Rasmol V. 2.7.1.1 (http://www.umass.edu/microbio/rasmol) was used for depiction of the amino acid substitutions, which were identified in the capsid proteins of OPV isolates. For visualization of the observed amino acid substitutions, the capsid protomer structure of the poliovirus type 1 Mahoney strain (1ASJ.pdb) was used for Sabin 1 isolates, and the structure of the poliovirus type 3 Sabin strain (1VBE.pdb) was used for Sabin 3 isolates.

Rct marker test

Reproductive capacities at different temperatures (Rct marker) were evaluated with the Rct test. The Rct value is defined as the difference between the $\log_{10}$ virus titre of a viral stock measured at the optimal temperature (37°C) and that measured at the supraoptimal temperature (40°C). Rct values of OPV isolates and Sabin vaccine strains were determined as previously described [13]. Viruses were considered to be thermosensitive if the Rct value (between 37 and 40°C) was greater or equal to 2.00, and thermoresistant if the Rct value was lower than 2.00.

One-step growth curve experiment

One-step growth curve experiments were performed in Hep-2 cells at 37°C and 40°C for 0–16 h, with a multiplicity of infection of 10 (determined by titration of virus stocks on Hep-2 cells), as previously described [13].

Neutralization assay with human sera

The level of immunity of the human population against poliovirus was determined with a microneutralization assay, as previously described [21]. Pooled sera (ten mixed serum samples) were used from each age group (1–10, 11–20,

21–30 and 31–40 years) for the neutralization test (NT). The serum samples were provided by volunteers from regions of western Greece during the time period 2009–2010.

Moreover, the immunity level against poliovirus of two individuals randomly selected from each age group (1–10, 11–20, 21–30 and 31–40 years) was determined with the same microneutralization assay.

Statistical analysis

Student's *t*-test (paired samples test) was used to compare the mean values of $\log_{10}$ reciprocal NT titres of four age groups (1–10, 11–20, 21–30 and 31–40 years; pooled sera of ten individuals for each) against each Sabin vaccine strain (Sabin 1 or Sabin 3) with those against each OPV isolate of the same serotype. It was also used to compare the mean values of $\log_{10}$ reciprocal NT titres of four age groups (1–10, 11–20, 21–30 and 31–40 years; two individual sera for each) against each Sabin vaccine strain (Sabin 1 or Sabin 3) with those against each OPV isolate of the same serotype.

A one-way ANOVA (Duncan's multiple range test) was used to compare the mean values of $\log_{10}$ reciprocal NT titres against all polioviruses of the same serotype between the 1–10-year, 11–20-year, 21–30-year and 31–40-year age groups (pooled sera of ten individuals each). It was also used to compare the mean values of $\log_{10}$ reciprocal NT titres against all polioviruses of the same serotype between the 1–10-year, 11–20-year, 21–30-year and 31–40-year age groups (sera of two individuals for each).

Student's *t*-test was used to compare the mean values of $\log_{10}$ reciprocal NT titres of four age groups (1–10, 11–20, 21–30 and 31–40 years) against each poliovirus strain shown in the neutralization assay of pooled sera with those of individual sera.

Results

Genotypes and recombination types of OPV isolates

The 5′-NCR and VP1 genotypes of isolates were identified. Two strains were genotyped as Sabin 1 and one strain as Sabin 3 (Table 1). Among the three isolates, one (522) was identified as non-recombinant, and two, MB1 and 742, as recombinant in one or two sites, respectively. The recombination type was S3/S1 for isolate MB1, and the recombination site was located in the 2C genomic region, from nucleotides 4880 to 4887. Isolate 742 was identified previously as S1/S3/S2 recombinant, with the first recombination site located in the 2A region from nucleotides 3461 to 3465 and the second in the 2C region from nucleotides 4511 to 4527 (Table 1).

Temperature sensitivity of OPV isolates

The non-recombinant Sabin 1 isolate 522 and the recombinant Sabin 3 isolate MB1 showed a thermosensitive phenotype, with Rct values of 3.5 and 4.5 units, respectively. The bi-recombinant Sabin 1 isolate 742 showed partial reversion to a thermoresistant phenotype, with an Rct value of 2.00 units. The Sabin vaccine strains (Sabin 1 and Sabin 3) exhibited, as expected, Rct values greater than 2.00 units (Table 3).

One-step growth curve experiment

The growth rates and virus yields of each isolate were compared with those of the corresponding genotype of the Sabin vaccine strain (Sabin 1 or Sabin 3) in one-step growth experiments in Hep-2 cells at 37°C and 40°C.

The non-recombinant Sabin 1 isolate 522 showed different growth kinetics from that of its progenitor Sabin 1 vaccine strain (Fig. 1). More specifically, isolate 522 showed 1.5–2.5 $\log_{10}$ units higher viral yield than the Sabin 1 vaccine strain at both 37°C and 40°C during the infectious life cycle.

TABLE 3. Location of mutations in the 5′-noncoding region and capsid region sequences of oral poliovirus vaccine (OPV) isolates, and comparison with the corresponding nucleotide and amino acid positions in Sabin vaccine strains

Origin	Region	Isolate	Mutations[a]	Δ log Rct-40
S1	5′-NCR	522	U344C G480A C583G	3.5
S1	5′-NCR	742	G480A C583G	2.0
S3	5′-NCR	MB1	U472C	4.5
S1	VP2	522	C1119A, Asp56 → Glu G1207A G1479A C1695U	
S1	VP2	742	C1441U	
S3	VP2	MB1	–	
S1	VP3	522	G1775U, Val4 → Ile C1941A, Ala59 → Glu G2003A, Asp80 → Asn	
S1	VP3	742	–	
S3	VP3	MB1	A2207U U2208G, Met149 → Trp	
S1	VP1	522	A2623G A2749G, Ile90 → Met A3248G, Ile257 → Val	
S1	VP1	742	G2502A, Ser8 → Asn G2776U, Lys99 → Asn A2833G U2904G, Phe142 → Cys	
S3	VP1	MB1	C2493U, Thr6 → Ile C2855A U2857A C2863A, Asp129 → Glu U2865G, Met130 → Arg G2882U, Val136 → Leu A3248G, Ile258 → Val	

Numbering according to vaccine strains Sabin 1 (AY184219) and Sabin 3 (AY184221). Reproductive capacity values of OPV isolates and Sabin vaccine strains at different temperatures (Rct marker) are also indicated.
[a]The first nucleotide or amino acid refers to the Sabin vaccine strain. Amino acid substitutions situated near or in antigenic sites are underlined. No mutations were identified in VP4 capsid protein of all three isolates.

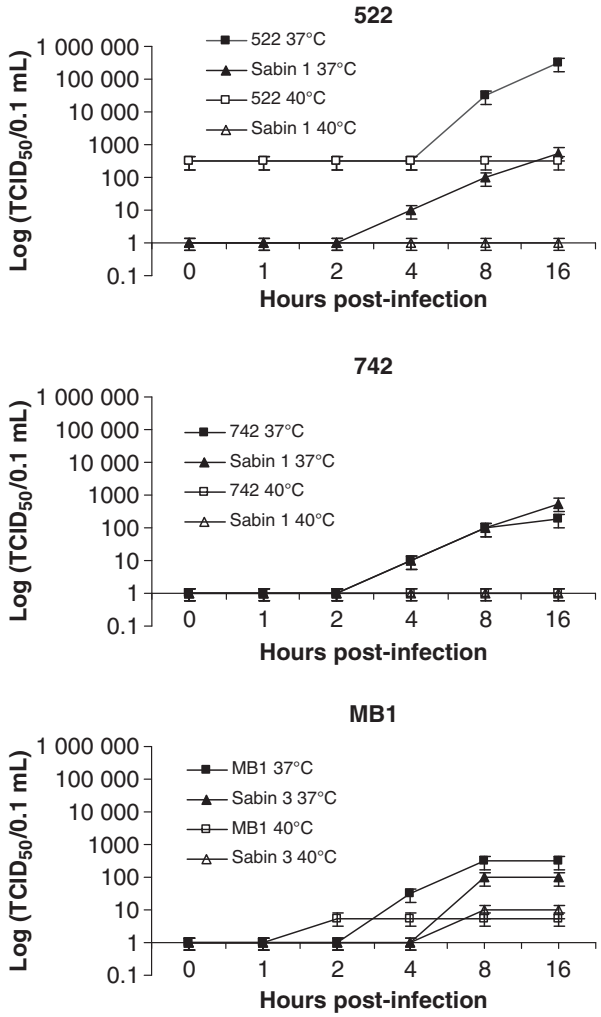

FIG. 1. One-step growth curve analysis of oral poliovirus vaccine isolates 522, 742 and MB1 in comparison with Sabin vaccine strains in Hep2 cells. Cells were infected at a multiplicity of infection of 10, and incubated at 37°C or 40°C. Total virus production at different times (0–16 h) post-infection was determined by $TCID_{50}$ assay on Hep2 cells. Each point represents the mean (± standard deviation) of virus titres from five different experiments.

However, the growth of isolate 522 showed a flat curve at 40°C, like that of the Sabin 1 vaccine strain. The bi-recombinant Sabin 1 isolate 742 replicated with kinetics similar to those of its progenitor Sabin 1 vaccine strain, showing that the virus yield rose 2 h post-infection at 37°C (Fig. 1), whereas at 40°C its growth was blocked, as it was also for the Sabin 1 vaccine strain.

The recombinant Sabin 3 isolate MB1 showed different growth kinetics from that of its progenitor Sabin 3 vaccine strain (Fig. 1). More specifically, the viral yield of isolate MB1 rose 2 h post-infection at 37°C, whereas that of the Sabin 3 vaccine strain rose 4 h post-infection. This initial difference resulted in a 1.5 $\log_{10}$ units higher viral yield of isolate MB1 than of the Sabin 3 vaccine strain 4 h post-infection. However, isolate MB1 showed a similar viral yield to that of the Sabin 3 vaccine strain at the final stages of the infectious life cycle (8 and 16 h post-infection). At 40°C, the growth of isolate MB1 was blocked, as was that of the Sabin 3 vaccine strain.

Nucleotide and amino acid sequence analysis

Table 3 shows the 5′-NCR and capsid sequence differences of OPV isolates with respect to Sabin vaccine strains. Both Sabin 1 isolates (522 and 742) displayed the mutation G480A in the 5′-NCR, which has been correlated with reversion to neurovirulence and thermoresistance [7]. They also displayed the mutation C583G, which has been observed in isolates of previous studies [13,22], and may confer a selective advantage. In VP1 of isolate 522, the amino acid substitution Ile90 → Met is situated in the exterior of the capsid, near AgS1 [2]. The amino acid substitution Ile257 → Val is partially exposed at the outer surface of the virion, situated on the last strand of VP1, which is located next to strand B, where residue 90 is located (data not shown). The amino acid substitution Lys99 → Asn in VP1 of isolate 742, which is highly exposed in the exterior of the capsid, is located in AgS1 as well as in the VP1 BC loop, which participates in the formation of the north rim of the canyon and is involved in the binding of receptor CD155 to the virus [2,23]. The amino acid substitution Phe142 → Cys is exposed on the external surface of the virion in the DE loop (data not shown), and may contribute to restoring neurovirulence, like residue 143, a well-known determinant of neurovirulence in Sabin 2 isolates [6]. The amino acid substitutions Ala59 → Glu and Asp80 → Asn in VP3 of isolate 522 are located in AgS3 and near AgS4, respectively [2]. The amino acid substitutions Asp56 → Glu in VP2 and Val4 → Ile in VP3 of isolate 522, as well as Ser8 → Asn in VP1 of isolate 742, are situated in the N-terminus of capsid proteins, which participates in hydrophobic interactions contributing to the stability of the virion [24].

The Sabin 3 isolate MB1 displayed the mutation U472C in the 5′-NCR, which has been correlated with reversion to neurovirulence and thermoresistance [8,10]. In the VP1 coding region, it displayed the reversion at nucleotide 2493 (C → U (Thr6 → Ile)), which is a determinant of attenuation [25]. The amino acid substitutions Asp129 → Glu and Met130 → Arg are situated in the bottom of the canyon (data not shown), and may be involved in the interaction of receptor CD155 with the virus. The amino acid substitutions Val136 → Leu and Ile258 → Val are partially exposed at the outer surface of the virion, and are situated in the hydrocarbon-binding pocket of VP1, interacting with a sphingosine molecule (data not shown). The amino acid substitution

Met149 → Trp in VP3 is located in the inner surface of the capsid (data not shown), where the N-termini of capsid proteins interact, contributing to the integrity of the virion [24].

Nucleotide and amino acid substitutions were also identified in the 2A–3D non-structural genomic region of isolates 742 and MB1 (data not shown). The reversion at nucleotide 6203 (C → U (His73 → Tyr)) of the 3D polymerase coding region, which has been correlated with the attenuated and thermosensitive phenotype of the Sabin 1 vaccine strain [4], was not identified in the 3D region of isolate MB1. Other mutations in 2A–3D coding regions appeared rarely and with no obvious preferences, and will not be considered further.

Neutralizing capacity with human antisera

The levels of immunity towards all three OPV isolates and Sabin vaccine strains of the western Greek population of the 1–10-year, 11–20-year, 21–30-year and 31–40-year age groups were measured. Pooled (Table 4) or individual (data not shown) sera were tested against each poliovirus strain. Table 4 shows the statistical analysis of the $\log_{10}$ reciprocal NT titres against OPV isolates and Sabin vaccine strains.

Members of the 1–40-year age group (both pooled and individual sera) showed no significant differences in NT titres against OPV isolates in comparison with the Sabin vaccine strains.

A significant decrease in NT titre was observed from the 1–10-year age group to the 21–30-year age group for both poliovirus types (P1 and P3). An increase in NT titre was observed from the 21–30-year age group to the 31–40-year age group for poliovirus types 1 and 3. However, this increase was significant only for poliovirus type 1 (Table 4).

In the neutralization assay with individual sera, no significant differences in NT titres were observed for poliovirus type 1 between the 1–10-year, 11–20-year, 21–30-year and 31–40-year age groups. For poliovirus type 3, the results are consistent with those of pooled sera. Sequential decreases in NT titre were observed from the 1–10-year age group to the 11–20-year and 21–30-year age groups, with the first being significant.

The mean values of NT titres of the four age groups against each poliovirus strain with pooled sera showed no significant differences in comparison with those of individual sera (data not shown).

Discussion

The OPV strains are genetically unstable. Thermosensitivity of reproduction (the Rct-40 marker), antigenic modifications and one-step growth curve experiment have been used as indirect markers of reversion to neurovirulence [4,26,27].

Reversions of the known attenuating mutations in OPV strains and intertypic recombination have been identified as the underlying causes of the phenotypic changes and increased neurovirulence of poliovirus isolates [6–8]. Natural recombinants of Sabin vaccine origin have been isolated from VAPP patients [9,18]. Administration of trivalent OPV provides optimal conditions for multiple infections of human intestinal target cells, thus favouring the possibility of intermolecular recombination between heterotypic viral genomes [28].

In this study, we determined phenotypic traits such as thermosensitivity, growth kinetics and neutralizing capacity with human antisera of three OPV isolates, and attempted to correlate them with genomic modifications.

Two Sabin 1 isolates, one non-recombinant (522) and one bi-recombinant (742), were tested. The presence of mutations at known determinants of attenuation and thermosensitivity and of the rare recombination event (S1/S3/S2) did not affect the growth phenotype of isolate 742 at either 37°C or 40°C, as it replicated similarly to the Sabin 1 vaccine strain. However, it showed partial reversion to a

TABLE 4. Statistical analysis of the $\log_{10}$ reciprocal neutralization test (NT) titres (pooled sera) against Sabin vaccine strains and oral poliovirus vaccine (OPV) isolates

Serotype	Virus strain	Mean values of $\log_{10}$ reciprocal NT titres of four age groups, 1–10, 11–20, 21–30 and 31–40 years (each consisting of the pooled sera from ten individuals) against each poliovirus strain[a]	Mean values of $\log_{10}$ reciprocal NT titres against all polioviruses of the same serotype in each age group: 1–10, 11–20, 21–30 or 31–40 years (each consisting of the pooled sera from ten individuals)[b]			
			1–10	11–20	21–30	31–40
1	Sabin 1	2.50	*2.50* (p 1.0)	*2.50* (p 1.0)	2.20 (p 1.0)	2.70 (p 1.0)
	522	2.50				
	742	2.42 (p 0.391)				
3	Sabin 3	2.42	2.80 (p 0.275)	*2.05* (p 0.068)	*1.90* (p 0.068)	*2.50* (p 0.068)
	MB1	2.20 (p 0.215)				

[a]Mean values of all three OPV isolates have no significant differences from the respective values of Sabin vaccine strains according to Student's *t*-test.
[b]Mean values in italic for each poliovirus serotype have no significant differences according to ANOVA.

thermoresistant phenotype (Rct value = 2.00). The discrepancy in the results between the two assays may correlate with the fact that they show viral multiplication for different time periods. Isolate 522 showed a thermosensitive phenotype in both the Rct test (Rct value = 3.5) and the one-step growth curve test (flat curve at 40°C). However, it showed a higher viral yield than the Sabin 1 vaccine strain at both 37°C and 40°C throughout the infectious life cycle (0–16 h post-infection). In general, the presence of many determinants of thermosensitivity scattered along the whole genome of the Sabin 1 vaccine strain makes the reversion of Sabin 1 isolates to a thermoresistant phenotype difficult [4]. It is noteworthy that isolate 522 showed a higher virus titre (2.5 $\log_{10}$ unit) than the Sabin 1 vaccine strain at the initial stages of the infectious life cycle (0, 1 and 2 h post-infection). This initial difference leads to a higher virus titre of isolate 522 than of the Sabin 1 vaccine strain throughout the infectious life cycle, and may originate from the different adsorption and/or penetration properties of isolate 522 from those of the Sabin 1 vaccine strain. The presence of mutations in antigenic sites of isolate 522 (Ala59 → Glu and Asp80 → Asn in VP3, and Ile90 → Met in VP1) may be responsible for the difference in properties between isolate 522 and the Sabin 1 vaccine strain. Because of the structural proximity and even overlapping of antigenic sites and the capsid regions that interact with poliovirus receptors, it seems reasonable to hypothesize that the amino acids likely to be replaced in antigenic sites may interfere with optimal virus–cell recognition. Moreover, the mutation Ile257 → Val, which is situated in the hydrocarbon-binding pocket of VP1 that interacts with a sphingosine molecule, may contribute to the different adsorption and/or penetration properties of isolate 522.

The recombinant Sabin 3 isolate MB1 showed a thermosensitive phenotype in both the Rct test (Rct value = 4.5) and the one-step growth curve test (blocking of its growth at 40°C). Moreover, isolate MB1 showed a similar viral yield to that of the Sabin 3 vaccine strain at the final stages of the infectious life cycle (8 and 16 h post-infection). Consequently, the presence of mutations at known determinants of attenuation and thermosensitivity (U472C in the 5′-NCR, Thr6 → Ile in VP1) does not affect its phenotype. However, the amino acid substitutions Asp129 → Glu, Met130 → Arg, Val136 → Leu and Ile258–Val in VP1 may be responsible for the earlier increase in titre of isolate MB1 (2 h post-infection) than of the Sabin 3 vaccine strain (4 h post-infection), resulting in a difference in titre of 1.5 $\log_{10}$ units at 4 h post-infection. These residues, which are situated in the canyon and in the hydrocarbon-binding pocket of VP1, may lead to different adsorption and/or penetration properties of isolate MB1 from those of the Sabin 3 vaccine strain.

The capacity of human antisera to neutralize OPV isolates was also evaluated. More specifically, the levels of immunity towards all three OPV isolates and Sabin vaccine strains of the western Greek population of the 1–10-year, 11–20-year, 21–30-year and 31–40-year age groups was investigated. No significant differences from the homotypic Sabin strains were observed in the capacity of OPV isolates to be neutralized in both assays with pooled or individual sera. The presence of mutations in antigenic sites of isolates 522 and 742 did not affect their antigenic properties. The above reinforces the notion that the driving force behind antigenic alterations of OPV is not immune evasion, but negative selection of unfavourable mutations interfering with optimal virus–cell recognition [11].

The results of our study showed a significant decrease in immunity level from the 1–10-year age group to the 21–30-year age group (pooled sera) for both poliovirus types 1 and 3. These findings are consistent with those reported in previous studies [21,29]. An increase in NT titre was observed from the 21–30-year age group to the 31–40-year age group for both poliovirus types 1 and 3, which is consistent with the results of our previous study [21].

For both poliovirus types, the highest NT titres were observed in the 1–10-year age group, indicating a good response to vaccination. The lowest NT titre was observed in the 21–30-year age group towards poliovirus type 3, indicating an unsatisfactory level of immunity against poliovirus type 3 in young adults. These results are consistent with those of previous studies [21,29,30]. The presence of the lowest NT titre in the 21–30-year age group towards poliovirus type 3 suggests the need for a booster dose of monovalent Sabin 3 vaccine to ensure personal and herd immunity.

In the post-eradication era of wild polioviruses, the only remaining sources of poliovirus infection worldwide will be vaccine-derived polioviruses. As the majority of countries certified as being polio-free have switched from OPV to IPV, and taking into consideration the fact that IPV does not induce the same immunity levels as OPV, importation of recombinant evolved derivatives of vaccine strains would have serious implications for public health. Our study underlines the need for poliovirus surveillance. The repeated occurrence of highly divergent pathogenic poliovirus strains that have retained Sabin-like antigenic properties presents a serious challenge to the current poliovirus surveillance strategy. Phenotypic markers such as one-step growth curve analysis and the Rct assay, along with genomic analysis, are effective markers for the estimation of neurovirulence of OPV isolates. Additionally, there is a vital need for immuno-

logical studies in all age groups, in order to allow reconsideration of the current vaccination policies and to avoid epidemics caused by the circulation of highly evolved OPV derivatives.

Transparency Declaration

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