Molecular and Antigenic Characterization of a Highly Evolved Derivative of the Type 2 Oral Poliovaccine Strain Isolated from Sewage in Israel

LESTER M. SHULMAN,*1 YOSEF MANOR,1 RACHEL HANDSHER,1 FRANCIS DELPEYROUX,2 MICHAEL J. MCDONOUGH,1 TOVA HALMUT,1 ILANA SILBERSTEIN,1 JACKLYN ALFANDARI,1 JACQUELINE QUAY,3 TAMAR FISHER,1 JANA ROBINOV,1 OLEN M. KEW,3 RADU CRAINIC,3 AND ELLA MENDELSON1

Central Virology Laboratory, Public Health Laboratories, Chaim Sheba Medical Center, Tel-Hashomer 52621, Israel; Molecular Epidemiology of Enteroviruses, Institut Pasteur, 75724 Paris, France; and Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

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An unusual, highly diverged derivative of the Sabin type 2 oral poliovaccine (OPV) strain was recovered from environmental samples during routine screening for wild polioviruses. Virus was cultivated in L20B cells and then passaged on BGM cells at 40°C (RCT [reproductive capacity at supraoptimal temperature]-positive marker) to select against most OPV strains. All but 1 of 25 RCT-positive OPV-derived environmental isolates were antigenically and genetically (>99.5% VP1 sequence match) similar to the respective Sabin strains. However, isolate PV2/4568-1/ISR98 (referred to below as 4568-1) escaped neutralization with Sabin 2-specific monoclonal antibodies and cross-adsorbed sera, and had multiple nucleotide substitutions (220 of 2,646; 8.3%) in the P1 capsid region. Fourteen of the 44 associated amino acid substitutions in the capsid mapped to neutralizing antigenic sites. Neutralizing titers in the sera of 50 Israeli children 15 years old were significantly lower to 4568-1 (geometric mean titer [GMT], 47) than to Sabin 2 (GMT, 162) or to the prototype wild strain, PV2/MEF-1/EGY42 (GMT, 108). Two key attenuating sites had also reverted in 4568-1 (A481 to G in the 5′ untranslated region and the VP1 amino acid I143 to T), and the isolate was highly neurovirulent for transgenic mice expressing the poliovirus receptor (PVR-Tg21 mice). The extensive genetic divergence of 4568-1 from the parental Sabin 2 strain suggested that the virus had replicated in one or more people for ∼6 years. The presence in the environment of a highly evolved, neurovirulent OPV-derived poliovirus in the absence of polio cases has important implications for strategies for the cessation of immunization with OPV following global polio eradication.

Rapid evolution is characteristic of both wild and vaccine-derived polioviruses (4, 5, 11, 12, 15; A. Heim, A. Bellmunt, G. May, P. Pring-Akerblom, and W. Verhagen, Abstr. Eur. Soc. Clin. Virol. Prog. Clin. Virol. IV, abstr. 350, p. 72, 1998). For wild polioviruses, nucleotide substitutions accumulate at a rate of approximately 1% per year and consist primarily of changes at synonymous codon positions (10; L. De, J. Jorba, J. Boshell, R. Salas, and O. Kew, Abstr. 17th Annu. Meet. Am. Soc. Virol., abstr. W36-3, p. 123, 1998), i.e., do not result in amino acid changes at those loci. In contrast, the mutations initially appearing and fixed into the genomes of the Sabin vaccine strains upon administration of oral poliovaccine (OPV) are frequently associated with reversion of the attenuated phenotype and alteration of the neutralizing antigen (NAg) sites of the OPV strains (1, 12). Reversion of the OPV strains to increased neurovirulence is one key factor for the occurrence of cases of vaccine-associated paralytic poliomyelitis (VAPP), which occur at a rate of ∼1 per 500,000 first doses of OPV in immunocompetent individuals (23) and at a ∼1,000-fold-higher rate for immunodeficient patients (24). Poliovirus replication is restricted to about 2 months in immunocompetent persons (1, 2) but may be prolonged up to 10 years in patients with deficiencies in antibody production (11). Because poliovirus genomes evolve rapidly, the duration of replication of an OPV-derived virus may be estimated by the extent of its nucleotide sequence divergence from its respective prototype OPV strain (11; De et al., Abstr. 17th Annu. Meet. Am. Soc. Virol.).

Routine environmental surveillance of wastewater for polioviruses was initiated in Israel and the Palestinian Authority following the last poliomyelitis outbreak in 1988 (14, 22). This approach has proven to be a powerful method to detect wild poliovirus circulation in communities where no poliomyelitis cases have been reported (14). Concentrated environmental samples are cultured using a double-selective cultivation technique (13) that selects against the growth of most OPV-derived strains by use of a supraoptimal temperature of incubation (40°C). Polioviruses that grow at 40°C are said to be positive for the RCT (reproductive capacity at supraoptimal temperature) marker. A small number (3 to 25) of RCT-positive isolates are recovered each year from the environment. Most of these isolates have been otherwise typical OPV-derived viruses that have lost their temperature-sensitive phenotypes; some have been imported wild polioviruses (14; L. M. Shulman, Y. Manor, R. Handsher, A. Vonsover, O. M. Kew, E. Mendelson, et al., Abstr. Xth Int. Cong. Virol., abstr. W54-6, p. 79, 1996); and one, PV2/4568-1/ISR98, isolated in 1998, proved to be a highly divergent, neurovirulent derivative of the Sabin type 2 OPV strain. In this report, we describe the molecular, antigenic, and neurovirulence properties of this unusual isolate. We estimate from the high degree of nucleotide divergence of
from Sabin 2 that the initiating OPV dose was given approximately 6 years before recovery of the virus from the environment. The observations cannot distinguish between chronic infection of a single individual and person-to-person transmission of an OPV-derived poliovirus. In either event, the detection in the environment of a highly evolved derivative of Sabin 2 has important implications for development of the optimal strategy for cessation of immunization with OPV following the eradication of all wild poliovirus transmission (4).

MATERIALS AND METHODS

Reference poliovirus. Nonattenuated Salk inactivated poliovaccine (IPV) reference strains of type 1 (PV1/Mahoney/USA42), type 2 (PV2/MEF-1/EGY42), and type 3 (PV3/Saukett/USA52) and attenuated Sabin OPV type 1 (L2a 2b), type 2 (P712 ch 2a), and type 3 (Leon 12 alb) strains were obtained from Radu Cramic, Institut Pasteur, Paris, France.

Isolation of polioviruses from sewage samples. Sampling and extraction of sewage samples were performed as described in detail previously (14), except that processed sewage extracts (15 to 24 ml) were first applied to monolayers of L20B cells (mouse L cells expressing the human poliovirus receptor [PV-R]) (16, 20), kindly provided by David Wood, National Institute for Biological Standards and Control, London, England. Individual isolates have been named according to the following convention: PV (poliovirus) followed by a number denoting the type/isolate number/strain letter word, each as described previously (6) except that Lumi Phos 530 was replaced by disodium octaethylporphyrin (Applied Biosystems, Foster City, Calif.). Reaction mixtures were analyzed on Applied Biosystems model 373 DNA Automatic Sequencing Systems.

The Wisconsin Genetics Computer Group (GCG) gene analysis programs (version 9, 1994) were used for comparing nucleotide and amino acid sequences from isolates and the GenBank/EMBL database. Both strands of the entire VP1 gene and the 5' -UTR of the first five isolates were sequenced. All subsequent sequences referred to have the following accession numbers: PV2/Sabin 2 (AF249265), PV3/Sabin 3 (X09835), and PV1/Mahoney/USA42 (AF249260) for the type 1 viral genome, PV2/Sabin 2 (X09835), PV3/Sabin 3 (X09825) for type 2, and PV1/Mahoney/USA42 for type 3.

An additional measure of genetic relatedness is the proportion of transversional differences between 4568-1 and MEF-1 (19.8 and 22.4% VP1 nucleotide differences, respectively) (Fig. 1).

An additional measure of genetic relatedness is the proportion of substitutions that are transversions (substitutions be-

Sequence properties of isolate 4568-1. Analysis of the VP1 nucleotide sequence of isolate 4568-1 was extended to include back-titrated to confirm the quantity of virus injected per mouse. Mice were monitored daily for as long as 14 days after inoculation, and clinical symptoms (paresis, paralysis, or death) were recorded for each mouse. The mean healthy time (MHT) was determined for each virus by calculating the mean number of days before the appearance of any clinical symptom for individual mice inoculated with the corresponding virus.

Nucleotide sequence accession numbers. The sequences of the RCT-positive isolates described in this article have been deposited in the EMBL/GenBank data library and have been assigned accession no. AF237871 to AF237885. Additional sequences referred to have the following accession numbers: PV2/Sabin 2, X09835; PV3/Sabin 3, X09825; and AF249260 to AF249265 for the type 2 clinical isolates as described in the legend to Fig. 2.

RESULTS

Properties of the poliovirus isolates from sewage. Twenty-five RCT-positive sewage isolates were obtained in 1998, of which 20 were type 2 and 5 were type 3 (Table 1). Three of the isolates were found to be antigenically distinct from the parental vaccine strains: 4568-1 (type 2), which was not neutralized by Sabin 2-specific monoclonal antibodies and cross-adsorbed sera; 4625-1 (type 2), which was neutralized by only a Sabin 2-specific monoclonal antibody; and 4745-3 (type 3), which was neutralized only by Sabin 3-specific cross-adsorbed sera. Twenty of the 25 sewage isolates were partially sequenced, and 19 of these (including 4625-1 and 4745-3) were found to be very closely related (＞99.5% nucleotide sequence similarity in the VP1 region) to their respective OPV strains (Table 1). One of the type 2 antigenic variants, 4568-1, differed from Sabin 2 at 8.6% (78 of 903) of VP1 nucleotides (Table 1) and did not form stable hybrids with the Sabin 2-specific RNA probe (Fig. 1).

Relationships of sewage isolates to other type 2 polioviruses. To assess the genetic relationships of 4568-1 to other type 2 polioviruses, VP1 sequences of 4568-1 were compared with those of the Sabin 2 vaccine strain; the wild type 2 reference strain, MEF-1; a wild type 2 isolate (7570) from a 1998 polio case in India (the only country in which type 2 wild poliovirus was known to be endemic in 1998 [3]); and 10 Sabin 2-derived isolates. The other Sabin 2-derived isolates included five additional 1998 type 2 sewage isolates from Israel (4588-5, 4588-3, 4623-1, and the antigenic variant 4625-2), two isolates (3739A and 70715) from Argentina, and a divergent poliovirus isolate (7834) from an Italian patient in Peru, and two recent isolates (7089 and 7717) from children with acute flaccid paralysis in Brazil. The sequence relationships, summarized in a tree (Fig. 2) constructed using the DNA maximum-likelihood program of PHYLIP and rooted to the sequence of MEF-1, confirmed that 4568-1 was more closely related to the Sabin 2 group than to the 3rd viruses. Very similar relationships were obtained by quartet puzzling using the PUZZLE program (data not shown). Isolate 4568-1 was more divergent in VP1 sequences from Sabin 2 (8.6%) than were 7834 (6.0%) and 70715 (1.4%), but was less divergent than 3739A (10.2%). Isolate 4568-1 was unrelated to MEF-1 and 7570 (19.8 and 22.4% VP1 nucleotide differences, respectively) (Fig. 1).

An additional measure of genetic relatedness is the proportion of substitutions that are transversions (substitutions between a purine and a pyrimidine). The proportion of transversional differences between closely related polioviruses is ~5 to 15% but rises to >30% with increased genetic distance (10). The proportion of transversional differences between 4568-1 and Sabin 2 (15.5%) was significantly lower than the proportion of transversional differences between 4568-1 and MEF-1 (27.5%) or 7570 (45.9%).

Sequence properties of isolate 4568-1. Analysis of the VP1 nucleotide sequence of isolate 4568-1 was extended to include
Fifty serum samples, collected from a cohort of 15-year-old high school students who had received at least three OPV doses in early childhood and a booster dose during the 1988 Israeli outbreak (22), were tested for titers of neutralizing antibodies to 4568-1, Sabin 2, and MEF-1 (the type 2 IPV strain). While all 50 serum samples contained protective levels of neutralizing antibody to all three type 2 polioviruses, the geometric mean titers (GMT) of antibody to 4568-1 were sig-

<table>
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<th>Sampling site</th>
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Homology to the corresponding region of the respective Sabin strain.

Antigenic drift of 4568-1. Because isolate 4568-1 was initially recognized as an antigenic variant when tested with highly specific animal sera, we sought to determine whether the antigenic differences between 4568-1 and Sabin 2 would also be evident when they were tested against human immune sera. Fifty serum samples, collected from a cohort of 15-year-old high school students who had received at least three OPV doses in early childhood and a booster dose during the 1988 Israeli outbreak (22), were tested for titers of neutralizing antibodies to 4568-1, Sabin 2, and MEF-1 (the type 2 IPV strain). While all 50 serum samples contained protective levels of neutralizing antibody to all three type 2 polioviruses, the geometric mean titers (GMT) of antibody to 4568-1 were sig-

FIG. 1. Intratypic differentiation of type 2, RCT-positive poliovirus isolates by blot hybridization. Viral RNA was bound to nylon filters and hybridized with a DIG-labeled Sabin 2-specific VP1 probe (left blot) or a panenteroviral 5'-UTR probe (right blot). Hybrids were detected by chemiluminescence (6). Controls: A, Sabin 1; A2, Sabin 2; A3, Sabin 3; B1, Mahoney; B2, PV2/MEF-1/EGY42; B3, PV3/Saukett/USA52. RCT-positive isolates: C1, PV2/4568-1/ISR98; C2, PV2/4586-5/PAL98; C3, PV2/4588-3/PAL98; D1, PV2/4623-1/ISR98; D2, PV2/4625-1/ISR98.
Neurovirulence of 4568-1.

The neurovirulence of 4568-1 was examined in PVR-Tg21 transgenic mice (7, 21) in comparison with Sabin 2 and the nonattenuated reference strains MEF-1 and Mahoney (type 1). Under our assay conditions, inoculation with 10^8 PFU/mouse and follow-up for 14 days after challenge with virus, the original Sabin 2 strain (n = 10 mice tested) showed maximum attenuation, with an MHT of 14.0 ± 0.0 days (mean ± standard error of the mean). The equivalent challenge dose of the nonattenuated polioviruses Mahoney (n = 10) and MEF-1 (n = 10) gave MHTs of 4.7 ± 1.14 and 9.6 ± 1.49 days, respectively. The Sabin 2-derived field isolate 4568-1 (n = 20) had clearly lost the attenuated phenotype, since it had a shorter MHT: 3.1 ± 0.07 days, than either of the two reference nonattenuated polioviruses.

Estimation of the duration of replication of the 4568-1 lineage. The duration of replication of an OPV-derived isolate after the initiating vaccine dose can be estimated from the degree of its sequence divergence from its parental Sabin strain. The VP1 evolution rate for type 1 poliovirus, whether a vaccine-derived strain replicating in an immunodeficient patient (11) or a wild poliovirus circulating over a period of 10 years (De et al., Abstr. 17th Annu. Meet. Am. Soc. Virol.) or 1 year (22), is ~3% third-codon-position substitutions/year. Similar values have been obtained for the entire P1 capsid region (4) and for a Sabin 3-derived poliovirus (15). The observed number of third-codon-position differences between 4568-1 and Sabin 2 over the entire P1 capsid region was 166 of 882 codons (18.8%). By assuming that the evolution rate for the P1 region of 4568-1 was constant throughout the entire period of replication and similar to the rates observed for other polioviruses (3% third-position substitutions/year), and without correcting for the small effects of multiple substitutions at a site, we estimate that the total period of replication was about 6 years.

DISCUSSION

Environmental sampling has been a powerful tool for poliovirus surveillance in Israel and the Palestinian Authority (14). Several independent introductions of wild polioviruses (type 1 in 1991, 1994 to 1995, and 1996; type 3 in 1990) have been detected since the appearance of the last polio cases in Israel, Gaza, and the West Bank in 1987 to 1988 (14, 22; Shulman et al., Abstr. Xth Int. Cong. Virol.). Because OPV is widely used (in combination with IPV [25, 27] by both Israeli and Palestinian health authorities (14), OPV-derived viruses are prevalent in local wastewater. The key to detecting polioviruses that have properties distinct from most OPV-derived strains is the use of a double-selective culture technique (13) that favors the growth of RCT-positive isolates. The yield of RCT-positive isolates has increased since the replacement of BGM cells in

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**FIG. 2.** Phylogenetic tree of type 2 RCT-positive sewage isolates. VP1 sequences of 4568-1 were compared with those of Sabin 2, five other Sabin 2-derived RCT-positive sewage isolates from Israel (including the type 2 antigenic variant VP2/4625-1/ISR98), two isolates from immunodeficient VAPP patients in the United States (PV2/0715/USA91 and PV2/3739A/USA92), a divergent type 2 isolate from a patient in Peru (PV2/7834/PERS83), two recent isolates from children with acute flaccid paralysis in Brazil (PV2/7089/BRAG96 and PV2/7717/ BRA97), the reference wild type 2 IPV strain, PV2/MEF-1/EGY42, and a wild type 2 isolate from a 1998 polio case in India (PV2/7570/IND98).

**FIG. 3.** Amino acid substitutions in NAg sites of 4568-1. Nonhomologous amino acids are indicated below the consensus sequence for each NAg site. Amino acid positions are numbered according to that for Sabin 2 (26).
the culture system with L20B cells that express the human gene for the PVR (16, 20). All RCT-positive isolates are subse-
quently characterized by molecular and antigenic methods.

Isolate 4568-1 was unusual because it was highly divergent from the prototype Sabin 2 OPV strain. A nonvaccine origin for 4568-1 could be ruled out because it is genetically much closer to Sabin 2 than to any of the wild polioviruses found in the Middle East or elsewhere since 1980 (10), and because the only known remaining foci of endemicity for wild type 2 poliovirus in 1998 were in northern India (3), and those wild viruses were unrelated to 4568-1. On the other hand, 4568-1 was similar to, but not derived from, highly divergent Sabin 2-derived polioviruses isolated from immunodeficient patients in the United States (13).

The extent of sequence divergence of 4568-1 from Sabin 2 suggests that the virus had replicated in one or more people for about 6 years since the administration of the initiating OPV dose. This estimate of the duration of replication is only approxi-
mate and is based upon the assumptions that rate of VP1 evolution for poliovirus type 2 is essentially constant over the period of replication and similar to the rates observed for types 1 and 3 (11, 15, 22; De et al., Abstr. 17th Annu. Meet. Am. Soc. Virol.; Heim et al., Abstr. Eur. Soc. Clin. Virol.; C.-F. Yang, S.-J. Yang, M. A. Pallansch, and O. M. Kew, Abstr. 15th Annu. Meet. Am. Soc. Virol., 1996). We cannot distinguish from the existing evidence whether virus replication was restricted to a single chronically infected individual (such as an immunodeficient patient) or whether a succession of people were infected through continuous transmission. The largely urban communities sampled at the waste-
water collection site for isolate 4568-1 have a combined pop-
ulation of 1.3 million and include both long-term resident and recent immigrant populations. Additional sampling sites have been selected within this community to better localize the source of the unusual type 2 virus. Although it is possible that we have detected a unique infection, it appears more likely that viruses related to 4568-1 were more widely distributed in the community (8). Moreover, we do not know whether the initial infection occurred within Israel or came from some external source. One factor which might potentially permit the spread of the unusual vaccine strain derivative is the reduced titers of neutralizing antibodies to 4568-1 relative to Sabin 2 and MEF-1 in the sera of children immunized with OPV. This factor may be negligible in communities with high OPV coverage but could be important in communities with lower OPV coverage and where the immunogenicity of OPV is reduced (25, 27).

It appears unlikely that the presence of a neuroviral, highly diverged Sabin 2 derivative currently presents any sig-
nificantly increased risk to a well-immunized community over that already presented by the frequent excretion of neuroviru-
lus in Gaza in 1996 (14; Shulman et al., Abstr. Xth Int. Cong. Virol.).

Neurovirulent OPV-derived polioviruses could present a potentially serious global health risk if they were present in any community after the cessation of immunization with OPV fol-
lowing certification of the interruption of all wild poliovirus transmission (4). Little information is currently available on the environmental prevalence of highly divergent OPV-derived polioviruses. Environmental studies in most countries have been impeded by the continuous presence of typical OPV-
derived strains in wastewaters. However, the availability of efflcient methods to select for atypical OPV-derived environ-
mental isolates (13) opens the way to assess the possible broader significance of the findings reported here.

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