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Antigenic Evolution of Vaccine-Derived Polioviruses: Changes in Individual Epitopes and Relative Stability of the Overall Immunological Properties

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The Sabin oral poliovirus vaccine (OPV) readily undergoes changes in antigenic sites upon replication in humans. Here, a set of antigenically altered descendants of the three OPV serotypes (76 isolates) was characterized to determine the driving forces behind these changes and their biological implications. The amino acid residues of OPV derivatives that lie within or close to the known antigenic sites exhibited a marked tendency to be replaced by residues characteristic of homotypic wild polioviruses, and these changes may occur very early in OPV evolution. The specific amino acid alterations nicely correlated with serotype-specific changes in the reactivity of certain individual antigenic sites, as revealed by the recently devised monoclonal antibody-based enzyme-linked immunosorbent assay. In comparison to the original vaccine, small changes, if any, in the neutralizing capacity of human or rabbit sera were observed in highly diverged vaccine polioviruses of three serotypes, in spite of strong alterations of certain epitopes. We propose that the common antigenic alterations in evolving OPV strains largely reflect attempts to eliminate fitness-decreasing mutations acquired either during the original selection of the vaccine or already present in the parental strains. Variability of individual epitopes does not appear to be primarily caused by, or lead to, a significant immune evasion, enhancing only slightly, if at all, the capacity of OPV derivatives to overcome immunity in human populations. This study reveals some important patterns of poliovirus evolution and has obvious implications for the rational design of live viral vaccines.

The live oral poliovirus vaccine (OPV) made from attenuated strains of three poliovirus serotypes (42, 56) rightly takes a place among the most efficacious and safest vaccines. Its worldwide use over the past 50 years has resulted in the almost complete eradication of the disease (28). The vaccine induces lifetime protective immunity and can spread to and immunize contacts of primary vaccine recipients. The latter property was always considered one of the added benefits of a live vaccine. It was thought that this transmission is limited to immediate contacts and that, therefore, unlike the wild-type viruses, Sabin strains cannot establish chains of transmission but, rather, rapidly disappear from circulation (66). The problem of transmissibility is important because the vaccine strains are known to revert rapidly to neurovirulence during replication in both cell cultures and vaccine recipients (18, 28, 42). The commonly accepted view is that even though Sabin strains do gain neurovirulence by accumulating mutations that restore the ability of the virus to replicate in neurons of the central nervous system, the determinants of limited transmissibility remain stable. This view implies that there are separate genetic determinants of neurovirulence and transmissibility, even though the latter were never convincingly identified or localized.

In recent years, however, it became increasingly clear that in some instances OPV polioviruses are capable of fully regaining the ability to circulate in human populations and even cause small outbreaks of paralytic poliomyelitis (28, 30). A high degree of nucleotide sequence divergence of some vaccine-derived (VDPV) poliovirus strains isolated from paralytic cases as well as from healthy individuals and environments (5, 7, 10, 11, 27, 29, 37, 38, 55, 58, 59, 69, 71) suggests that they might have circulated in communities for years. The solid body of evidence accumulated recently clearly demonstrates that the transformation of vaccine poliovirus into virulent strains is not an exception but, rather, a consistent pattern of their natural evolution.

It has long been known that OPV strains may change their immunological reactivity, sometimes even in the primary vaccinees or their immediate contacts (13, 44). Such changes are exploited in poliovirus surveillance to detect modified OPV strains by using an intratypic differentiation test (ITD), in particular, by using different panels of monoclonal antibodies (13) or cross-absorbed polyclonal (CAP) antisera (63, 64). According to the standard CAP-enzyme-linked immunosorbent assay (CAP-ELISA), polioviruses exhibiting different reactivity from the reference Sabin strains are classified as non-Sabin-like (NSL) if they have antigenic properties similar to wild-type

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reference polioviruses, nonreactive (NR) if their properties differ from both vaccine and wild-type reference strains, or double reactive (DR) in the case of a similar reaction with both types of antibodies (63). The World Health Organization (WHO) guidelines require a more detailed investigation of a poliovirus isolate if it exhibits such antigenic alterations.

It is tempting to assume that changes in antigenic properties frequently observed in OPV derivatives result from the immune evasion response and therefore represent selection of viral variants less prone to be neutralized by human antibodies. If so, these changes may have very important epidemiological consequences, contributing to enhanced viral transmissibility and conversion to epidemic strains.

Prompted by the above considerations, we undertook a detailed investigation of the nature and biological implications of antigenic changes in OPV derivatives. To this end, a genomic segment encoding capsid proteins of a collection of such derivatives was sequenced, and the reactivity of some of the viral isolates toward monoclonal antibodies and polyclonal antisera was investigated. The results allowed us to correlate specific nucleotide replacements with changes in individual epitopes. More importantly, the overall results are consistent with the conclusion that the antigenic changes observed even in highly diverged OPV derivatives clearly do not arise primarily as a result of immune evasion. Therefore, they do not appear to represent a major factor of enhancement of viral transmissibility, if such enhancement takes place at all.

Besides the obvious practical implications for the polio eradication program, these findings also raise interesting questions about the nature of the forces driving the evolution of vaccine viruses. Extensive surveillance of poliovirus strains performed during the last decade resulted in the accumulation of a large database of nucleotide sequences of the OPV derivatives. Millions of children were given the same polioviruses in the form of OPV, and changes occurring during vaccine reproduction can be detected through analysis of the excreted viruses. This presents a unique opportunity to follow consistent patterns of the natural evolution of attenuated viruses in the human organism. Factors governing viral evolution in human organisms differ from those operating in tissue culture in several aspects (2, 17). The results presented here provide deeper insights into mechanisms of natural poliovirus evolution.

MATERIALS AND METHODS

Virus isolation and typing. Virus isolation from stool samples was done by standard methods (68). The viruses were passaged once on monolayers of human rhabdomyosarcoma or L20B cell lines and were typed in microneutralization tests with type-specific rabbit antisera (RIVM, Bilthoven, The Netherlands).

ITD. ITD was performed by CAP-ELISA (RIVM, Bilthoven, The Netherlands) and PCRs used Sabin-specific primers following published procedures (68).

Reverse transcription, PCR, and sequencing of poliovirus RNAs. RNA was extracted from cell lysates with TRIzol Reagent (Life Technologies) and reverse transcribed using random hexamer primers (Boehringer Ingelheim) with avian myeloblastosis virus reverse transcriptase (Promega) at 42°C for 1 h. The DNA copies of the genomic regions coding for the capsid proteins VP1, VP2, and the N-terminal half of VP3 (corresponding to amino acid residues 3001 to 3120; hereinafter the amino acid residues of VP1, VP2, and VP3 are numbered starting at 1001, 2001, and 3001, respectively) were amplified by PCR (the primer sequences are available upon request). The PCR products were purified with a Wizard PCR Preps DNA Purification System (Promega) and directly sequenced either manually using an fmol DNA Cycle Sequencing System (Promega) or automatically using an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Comparative analysis of nucleotide and corresponding amino acid sequences. The obtained nucleotide sequences were compared with those of Sabin vaccine strains determined by direct sequencing of PCR products (51, 54, 61). Multiple alignments of these sequences were carried out with the program CLUSTAL W, version 1.83 (62). The estimation of the degree of synonymous nucleotide divergence was performed according to the method of Li et al. (33). To assess the similarity of deduced amino acid sequences to all of the corresponding wild-type poliovirus sequences available in the GenBank database, the protein-protein BLAST search program was used (http://www.ncbi.nlm.nih.gov/BLAST/).

MAP-ELISA method. A monoclonal antibody (MAb) reactivity profile ELISA (MAP-ELISA) of antigenic profiles of polioviruses has been described in detail elsewhere (53) and was based on the use of biotin-labeled polyclonal immunoglobulin G (IgG) and panels of MAbs (52). Briefly, poliovirus antigen captured on ELISA plates coated with polyclonal anti-polio IgG was incubated with a neutralizing MAb specific to individual antigenic sites. The residual antigenic reactivity was determined by treatment with polyclonal biotin-IgG conjugate and avidin peroxidase. The decrease in conjugate binding caused by the blocking of the surface of poliovirus antigen with the MAb reflects the immunoreactivity of this particular MAb. This reactivity is expressed as a ratio to the level of blocking activity of the same MAb with reference samples (wild-type and Sabin strains). Data on the blocking activity of a panel of MAbs specific to different antigenic sites represent a MAP, i.e., antigenic composition, of a sample.

NT with human sera. The neutralization test (NT) was performed in the microneutralization format with 100 50% tissue culture infective doses of challenge virus according to the standard protocol (67). The sera were collected from 2001 to 2003 from donors of different ages (from 11 days to 46 years old), health (vaccine-associated paralytic poliomyelitis [VAPP] cases and non-polio conditions and healthy persons), and vaccination status (vaccinated and nonvaccinated). For each serum sample, titers of neutralizing antibodies against the given VDPV strain were compared with titers against homotypic Sabin strain and wild-type poliovirus, i.e., type 1 strain Mahoney or unrelated strain 537 (from the collection of wild polioviruses of the Regional Reference Laboratory in Moscow), type 2 strain MEF-1, and type 3 strain Saukett. Regression analysis was used to calculate the correlation coefficient (r) between the NT titer values against the given VDPV strain and corresponding vaccine or wild-type poliovirus strain.

NT with polyclonal rabbit sera. The polyclonal rabbit sera raised against one virus in each pair of Sabin-1 or Mahoney, Sabin-2 or MEF-1, or Sabin-3 or Saukett were obtained as described elsewhere (26). For each antiserum the NT was performed using standard procedures (67), and NT titers against homological poliovirus as well as homotypic VDPV strains were defined.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper are available from the GenBank nucleotide sequence database under accession numbers DQ264247 through DQ264389.

RESULTS

Collection of viruses analyzed. The WHO-coordinated epidemiological surveillance of polioviruses involves their ITD by both serological (CAP-ELISA) and genetic (PCR with Sabin-specific primers) methods (63, 64, 70). During the last decade, such monitoring in Russia and other NIS countries was performed, and more than 1,000 poliovirus isolates collected from cases of VAPP and their contacts, patients with other diagnoses, and healthy children, as well as from sewage, were analyzed by the WHO Regional Reference Laboratory in Moscow. Although almost half of the strains had a vaccine origin as judged by the results of the PCR test, they exhibited different antigenic characteristics (NSL, NR, or DR) in the CAP-ELISA (unpublished data). To identify the genetic changes underlying these discordant results, we have selected 76 strains (42, 21, and 13 of types 1, 2 and 3, respectively) isolated from various sources (Fig. 1). Three slightly diverged isolates of type 2 (PV2/3d, PV2/60d, and PV2/78d) from a paralytic case in an immunodeficient child as well as two highly diverged OPV derivatives (type 1 strain 14 and type 3 strain 11264) isolated from a VAPP case and contact person, respectively, have been described in detail elsewhere (10–12). For clarity, type 1 strain 14 and type 3 strain 11264 will be named henceforth VDPV-1 and VDPV-3, respectively.

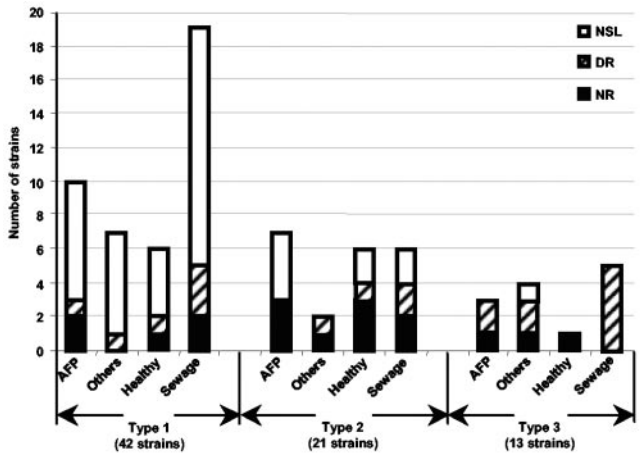


FIG. 1. Collection of the studied antigenically altered OPV derivatives. The numbers of strains isolated from acute flaccid paralysis (AFP) cases including VAPP, patients with other diagnoses, and healthy children as well as from sewage are presented according to their antigenic characteristics in CAP-ELISA.

Special remarks should be made on two type 2 VDPV strains, 19288 and 19890 (hereinafter VDPV-2a and VDPV-2b, respectively), isolated and characterized during this study. They were isolated in Kazakhstan in 2002 from stool samples of a 2-year-old poliomyelitis patient on day 3 after the appearance of the signs of flaccid paralysis and 4.5 months later, respectively. The patient had received three doses of OPV (the last one was given 1 month before the onset of the disease) but developed no relevant antibodies. This fact, along with the long-term excretion of poliovirus, suggested that the patient was immunodeficient. Unfortunately, no appropriate immunological investigation was done. The VP1-coding sequences of these strains exhibited a marked nucleotide divergence (2.5%) from Sabin-2. Although the strains were closely related to each other (99.7% identity in the VP1-coding sequence), both were included in the present study because of differences in the antigenic sites.

Mutations in the antigenic regions of the isolates. The antigenic structure of poliovirus has been extensively studied, primarily by using MAbs in neutralization experiments with escape mutants. Four major epitopes were identified, designated as antigenic sites 1 through 4 (AgS1 to AgS4) (39). In serotypes 1 and 2, AgS1 is thought to include amino acids 1091 to 1102; AgS2 consists of residues 2164 to 2166, 2168 to 2170, 2270, 1221 to 1224, and 1226; AgS3 includes amino acids 3058 to 3060, 3071, and 3073; and AgS4 is represented by residues 2072 and 3076. In serotype 3, AgS1 is composed of the residues 1089 to 1100; AgS2 is thought to consist of amino acids 2164, 2166 to 2167, 2172, and 1221 to 1224, 1226; AgS3 includes of residues 3058 to 3060, 3071, 3073, and 1286 to 1290; AgS4 is a complex of amino acids 2072 and 3076 to 3077 and 3079 (39).

To identify mutations that might lead to antigenic changes, partial nucleotide sequences of antigenically altered OPV derivatives were determined. At a minimum, the regions coding for VP1, VP2, and the N terminus-adjacent half of VP3 (residues 3001 to 3120) were sequenced. The positions of amino acids that underwent mutations are presented in Fig. 2. To illustrate the time course of the acquisition of the alterations, the isolates were classified according to their "age," i.e., the time of divergence from the parental strains. The age of each OPV derivative was calculated on the basis of the K_s value, the ratio of observed synonymous nucleotide mutations to all synonymous positions (those alterations which could potentially result in a synonymous replacement). The synonymous replacements in the VP1-coding region are known to accumulate at a rate of $\sim 3 \times 10^{-2}$ substitutions per synonymous site per year (17). The set of investigated viruses was arbitrarily divided into three "age groups": (i) those with a K_s of 0, considered "newborn" viruses acquiring no synonymous mutations in the VP1-coding region; (ii) those with a K_s between 0.3 and 3.0, or "young"; and (iii) those with a K_s above 3, assumed to independently circulate for at least 1 year, or "old."

The results presented in Fig. 2 demonstrate that the alterations were clearly serotype specific. Although mutations were detected in numerous positions (50, 34, and 27 in serotypes 1, 2, and 3, respectively), the most commonly affected positions (found in >50% of isolates) were positions 3060 and 1106 in type 1 (Fig. 2A), 3075 and 1143 in type 2 (Fig. 2B), and 3059 and 1054 in type 3 (Fig. 2C). Importantly, the most typical alterations could occur very early in OPV evolution, even when there were no synonymous mutations in the VP1 sequence.

The mutations were scattered over the entire genomic segment investigated, but they predominantly affected portions encompassing, or closely flanking, the known antigenic site. For the sake of brevity, the amino acids corresponding to all antigenic sites (as defined above), each with five flanking amino acid residues, will be henceforth called the antigenic region (AgR). This definition does not imply that all the residues of an AgR are necessarily directly involved in the interaction with antibodies, but we assume that changes within the AgR may modulate this process. It should also be kept in mind that neutralizing MAbs used to define antigenic sites have been selected randomly and do not at all represent the full set of possible variants. Moreover, only a subset of antigenic sites is involved in neutralization.

Remarkably, a significant proportion of frequently acquired mutations in the AgR of OPV derivatives affected the amino acid residues that distinguish the Sabin strains from either their predecessors or wild homotypic viruses. This is especially the case with the type 1 polioviruses. The AgR of Sabin-1 differs from that of Mahoney in seven positions (Fig. 3A, thick arrows). All of these Sabin-specific amino acid residues except one (Ser1095) are unique among known wild polioviruses of type 1 strain, and they are prone to be changed in OPV derivatives. For some positions this tendency was especially strong: Lys3060 was eliminated invariably, either reverting to the wild-type consensus Thr or being replaced by another amino acid. Similarly, frequent reversions or elimination of Sabin-specific mutations were observed at other positions, especially Thr1106, Ile1090, and Lys1099. Replacements at other AgR positions were rare, but Ala1088, when changed, obeyed the same rule.

A somewhat different pattern of changes was observed in derivatives of Sabin-2 (Fig. 3B). The total number of sequence differences between Sabin-2 and its parental strain P712 is uncertain. Moreover, no reliable consensus of the VP2 and VP3 capsid regions for wild polioviruses of type 2 strain is known, because the relevant sequences of only a few relatively

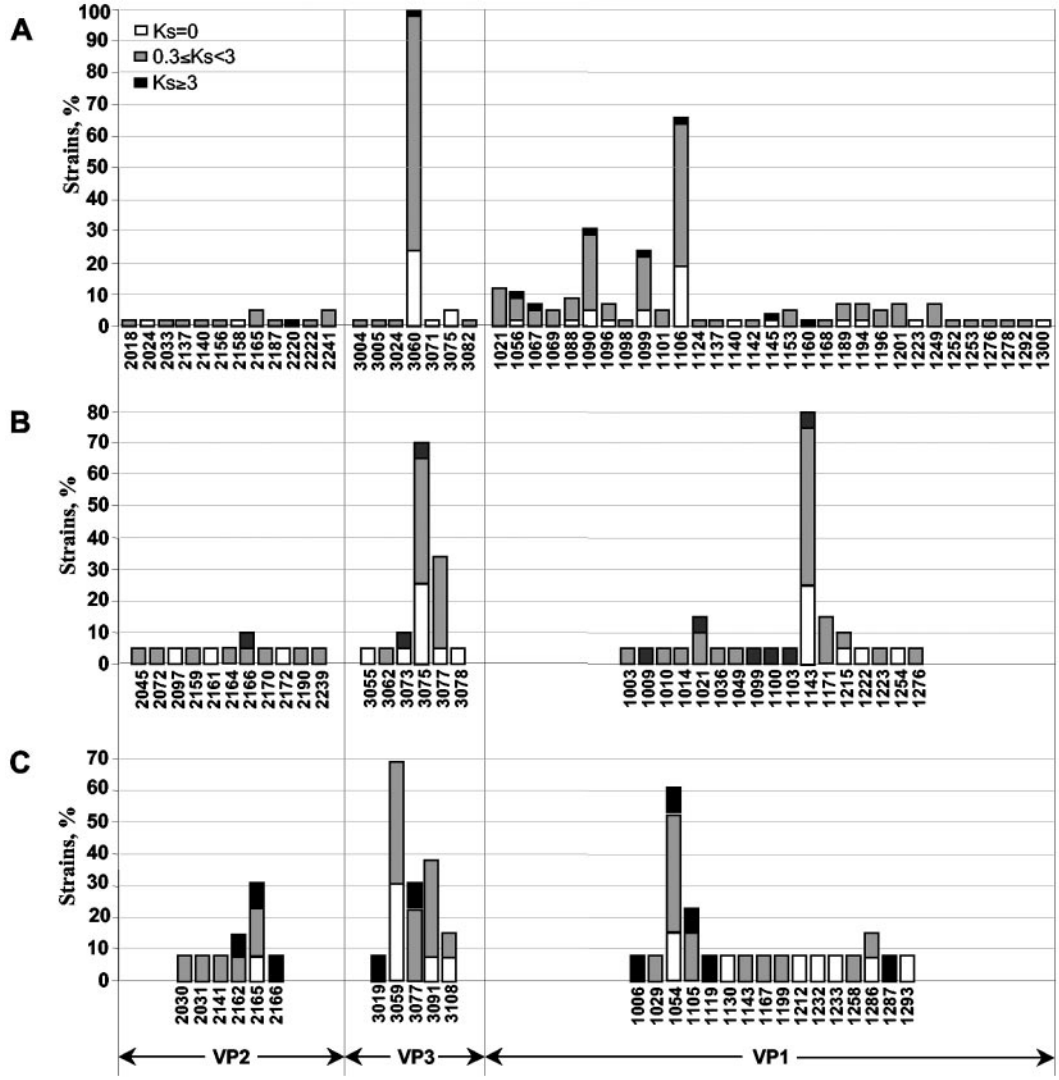


FIG. 2. Capsid alterations in type 1 (A), type 2 (B), and type 3 (C) vaccine derivatives. The proportions of strains containing a mutation at a given amino acid position are shown. The data for only VDPV-2b (out of the two related type 2 VDPV strains investigated) were included in this analysis. K_s values are calculated for the VP1 genomic region.

similar wild polioviruses of type 2 strain, Lansing (32), W-2 (48), and MEF-1 (14), are available. This made interpretation of the alterations in Sabin-2 derivatives more difficult. As already mentioned, the only AgR mutation consistently found (in two-thirds of the isolates) was a replacement of Thr3075 by another amino acid, in particular Ala, the wild-type consensus residue (Fig. 3B). Also, residues at positions 3073 and 1103 in some Sabin-2 derivatives are replaced by amino acids characteristic of wild viruses of type 2.

The AgR of Sabin-3 differs from that of its predecessor Leon only by having Arg at position 1286 (Fig. 3C). A direct reversion of this amino acid to Lys (a consensus residue for wild viruses of type 3) was sometimes observed. Also, Ser3059 and Asp3077 in the descendants of Sabin-3 were not infrequently replaced by Asn, which represents at both positions the consensus residue characteristic of wild homotypic polioviruses. A similar replacement by "wild" Thr was observed at Met1105, though more rarely.

Some amino acid substitutions in the capsid proteins were also recorded outside AgR. Among them, mutations known to be involved in restoring neurovirulence or eliminating the temperature-sensitive phenotype of vaccine derivatives (34, 36, 43, 49, 65) were relatively frequently observed, in particular Ile1143→Thr, Val, Asn, or Ser in type 2 (Fig. 2B) as well as Phe3091→Ser and/or Ala1054→Thr or Val in type 3 polioviruses (Fig. 2C). Other mutations in the sequenced RNA segments appeared rarely and with no obvious preferences (Fig. 2). They will not be considered further here.

Thus, a major trend within the AgR of OPV derivatives consisted in the replacement of certain capsid residues by those found in the predecessor strains and wild-type homotypic polioviruses.

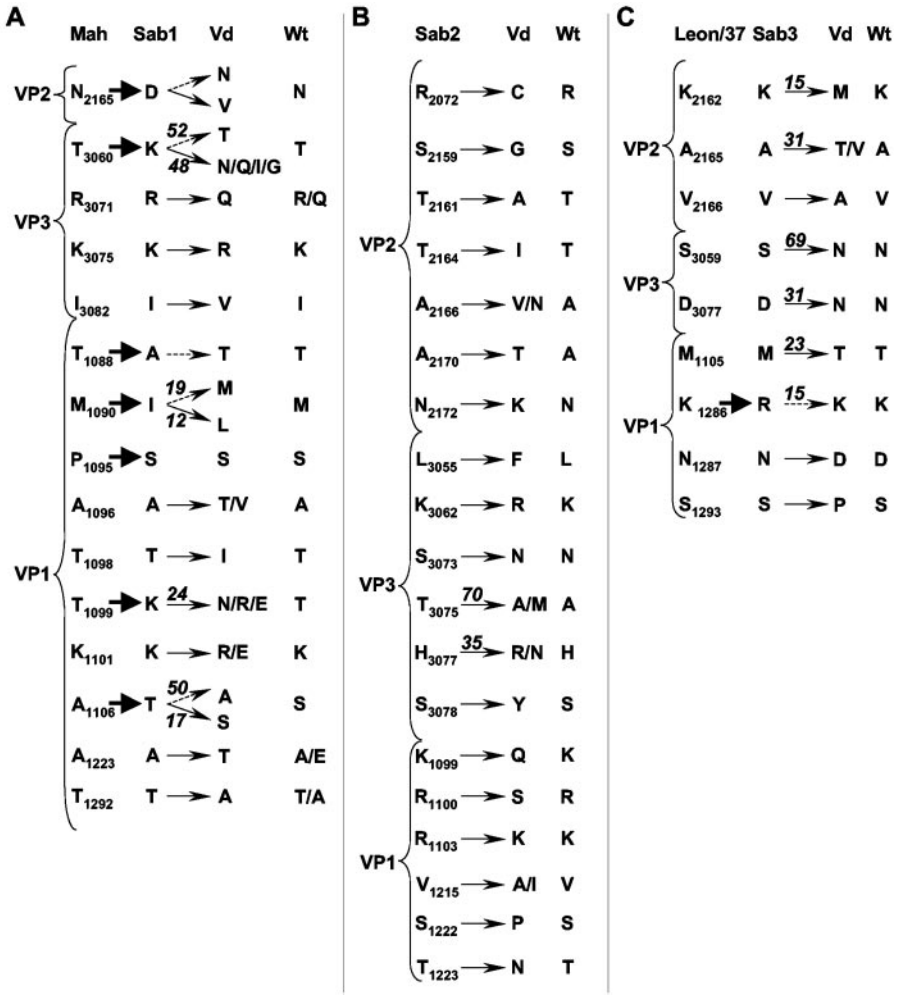


FIG. 3. Variations in the AgRs of OPV strains, their predecessors, vaccine derivatives (Vd), and wild-type polioviruses (Wt). The AgR differences between Sabin strains and their predecessors are marked by thick arrows. The alterations observed in type 1 (A), type 2 (B), and type 3 (C) OPV derivatives are marked by thin arrows (in the case of true reversion, by thin dotted arrows). Numbers adjoining these arrows represent the percentages of strains with the relevant mutations (the values are given only for the mutations present in more than 10% of vaccine derivatives). For wild-type, the consensus amino acids are presented. Mah, Mahoney; Sab1, Sabin-1.

Alterations in individual epitopes of OPV derivatives. Next, alterations in individual epitopes were studied immunologically. Recently, we developed a new approach based on the blocking reaction with a panel of MAbs, which produces a detailed profile of the MAb reactivity of an antigen (53) (see Materials and Methods). The advantage of this method is that it provides a way to quantitatively compare the reactivity of poliovirus antigens to a variety of MAbs and to identify minor differences in the antigenic properties of polioviruses. The panels of available MAbs (53) were used to analyze some OPV derivatives.

The set of type 1 viruses studied by MAP-ELISA also included, in addition to OPV derivatives classified by CAP-ELISA as antigenically altered, a Sabin-like (SL) strain St2, which possessed a mutation in AgS1. This strain was isolated from a healthy vaccinee on the seventh day after the first OPV dose and had a direct reversion of Lys1099→Thr in AgS1 but no mutations in other sites (31). The MAP of type 1 wild-type reference strain Mahoney was clearly different from that of Sabin-1 in AgS1 and AgS3 (Fig. 4, left panels). In Fig. 4, high bars indicate significant reactivity with a particular MAb, while low bars show that the MAb did not react with the strain under study. The reactivity of AgS1 dramatically depended on the nature of the amino acid residue at position 1099. The replacement of the Sabin-1-specific Lys by other amino acids, e.g., Thr (the consensus residue for homotypic wild polioviruses including Mahoney), Asn, Glu, and even positively charged Arg was sufficient to qualitatively modify recognition by several MAbs. Mutations at positions 1088, 1096, 1098, and 1106 individually and especially in combination influenced the reactivity to the Mahoney-specific MAb C3.

The reactivity of AgS3 appeared to be largely controlled by the residue at position 3060 (Fig. 4, Site 3, left), in agreement with previous findings (8). The reversion of Lys3060→Thr led to a Mahoney-like MAP, while the replacement of this critical residue by Asn resulted in a nearly complete loss of reactivity with any of the MAbs used. The replacement Lys3060→Ile produced opposite changes in reactivity, depending on the

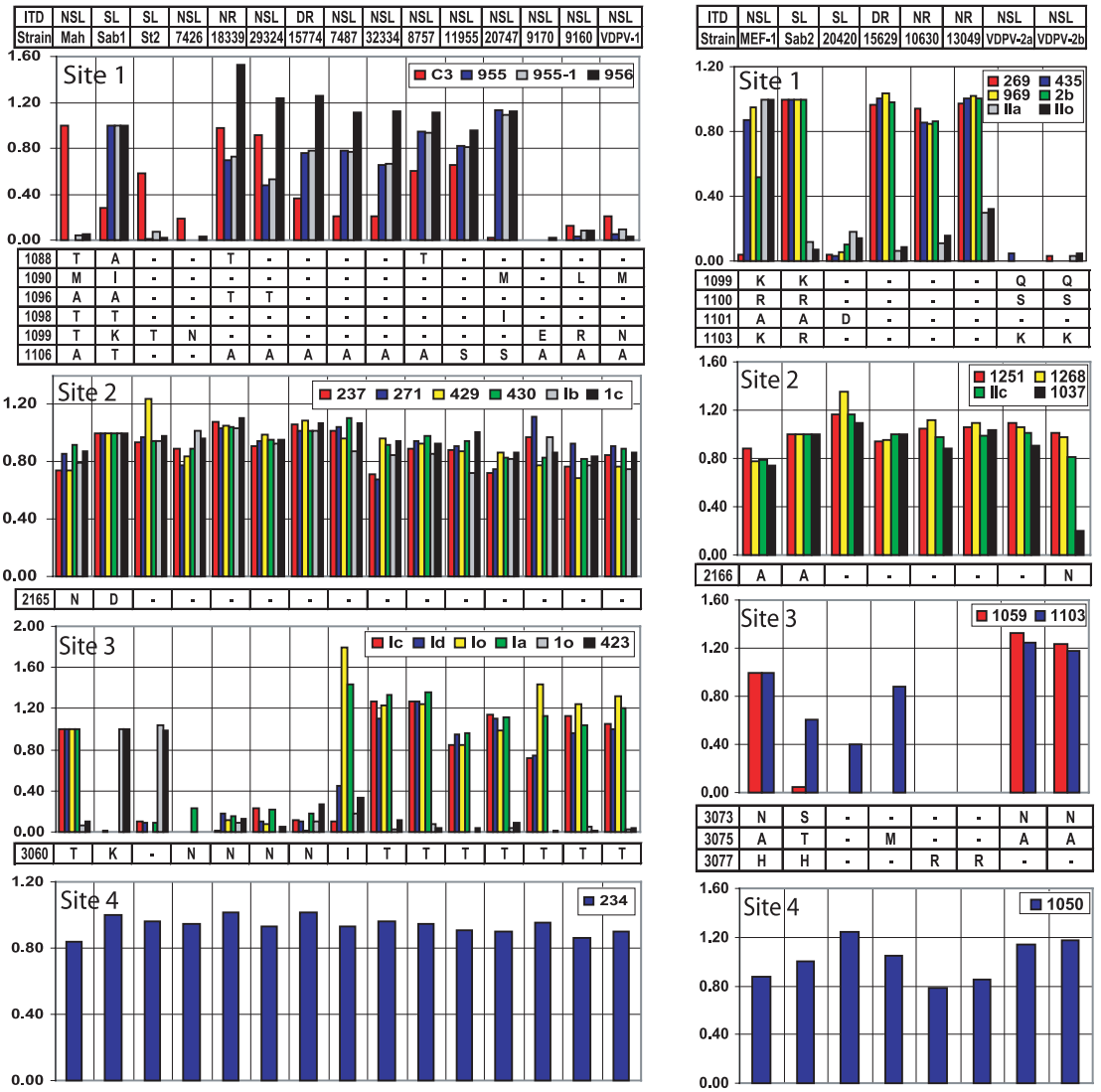


FIG. 4. The MAPs of type 1 (left) and type 2 (right) OPV derivatives. The antigenic characteristics revealed by CAP-ELISA as well as alterations in the AgR are presented for each strain. Amino acid identities to the parental Sabin strain are marked by dashes. The profiles of reference strains, Sabin-1 (Sab1)and Mahoney (Mah; left panels) and Sabin-2 (Sab2) and MEF-1 (right panels) are given for comparison. The bars and the corresponding MAb (framed) have identical colors.

antibody. Only marginal alterations in the reactivity of AgS2 and AgS4 were observed.

The set of type 2 vaccine-derived strains included isolate 20420, which, though exhibiting the SL phenotype in CAP-ELISA, possessed an Ala1101→Asp mutation within AgS1. This strain was isolated from an immunodeficient patient, who developed paralysis on day 128 after the third OPV dose. With regard to AgS1, the OPV-derived viruses could be divided into two groups with three strains in each (Fig. 4, right panels). The AgS1 MAPs of one group were more or less similar to that of Sabin-2, in line with the identity of the structures of this antigenic site. Representatives of the other group (which included the SL isolate 20420) failed to generate a significant signal in samples treated with any of the MAbs used. The failure could be traced to either a single substitution of Ala1101→Asp or changes in one or both positions of Lys1099→Gln and Arg1100→Ser (an Arg1103→Lys replacement present in the both relevant isolates could hardly be responsible for the effect because Lys1103 is also present in wild-type reference strain MEF-1).

All of the studied type 2 isolates exhibited roughly similar MAPs of AgS2, with the exception of VDPV-2b, demonstrating a decreased reactivity to a single MAb (Fig. 4, Site 2, right). This peculiarity was likely due to the Ala2166→Asn substitution, the only mutation at AgS2 among the isolates studied by this assay.

The MAPs of AgS3 divided the isolates into three groups, those with some similarity to Sabin-2 (reacting with only one of the two MAbs used), those exhibiting a MEF-1-like pattern, and those failing to generate appreciable signals with any of the two MAbs (Fig. 4, right). Sabin-2 and MEF-1 differ from one another at this site in two positions, Ser instead of Asn at position 3073 and Thr instead of Ala at position 3075, respec-

tively. Not surprisingly, the isolates with a Sabin-like MAP possessed Ser at position 3073 and Thr (or Met) at position 3075, whereas the isolates with a MEF-1-like MAP had Asn3073 and Ala3075, suggesting that one or both of these residues is responsible for the specific pattern of reactivity. The replacement His3077→Arg resulted in a complete loss of the signal with the antibodies used.

No significant alteration of interaction with MAb 1050 specific to AgS4 of type 2 was observed (Fig. 4, Site 4, right).

Due to the unavailability of an appropriate set of MAbs against serotype 3, we were unable to perform the MAP-ELISA for OPV derivatives belonging to this serotype.

It should be noted that during the above description of the MAP-ELISA results, we ignored relatively small differences between the reactivity of different isolates toward different MAbs. For example, AgS2 of Sabin-2, MEF-1, and OPV derivatives (with the exception of VDPV-2b) were identical in their amino acid structures, whereas their MAPs differed slightly but reproducibly (Fig. 4, right panels), suggesting that minor differences in the MAP may reflect long-range conformational changes induced by alterations outside a given antigenic site.

A comparison of the CAP-ELISA and MAP-ELISA in their ability to distinguish antigenic epitopes showed that the CAP-ELISA allowed us to identify those vaccine derivatives that have a mutation in the VP3 component of AgS3 leading to an NSL, NR, or DR result. For Sabin-1 derivatives, this is a change at position 3060, and in the case of type 2 the key positions are 3075, 3077, and possibly 3073. On the other hand, the CAP-ELISA was completely insensitive to AgS1 mutations when they were present alone (e.g., type 1 strain St2 and type 2 strain 20420), and therefore these strains were classified as SL. Therefore, the MAP-ELISA method proved to be more powerful for the detection of antigenically altered poliovirus derivatives.

Neutralizing capacity with polyclonal antisera. The results presented in the previous section unambiguously identified significant changes occurring in antigenic epitopes of OPV-derived viruses as revealed by MAbs. Under natural conditions, however, viruses are confronted with polyclonal antibody responses. To evaluate whether the observed antigenic changes could ensure immune evasion from natural immunological defense mechanisms, the capacity of three highly evolved OPV derivatives to be neutralized by human antisera was investigated (Table 1). The sera were collected from donors of different ages, health, and vaccination status. Notably, the circulation of wild-type polioviruses has not been detected in Russia since 1996, allowing us to assume that the immune donors were recipients of either OPV or its derivatives. The sera generally contained higher levels of antibodies to type 1 poliovirus than to types 2 and 3.

About 40% of human sera demonstrated a significant (greater that fourfold) difference between neutralization of Sabin-1 compared to VDPV-1 (Fig. 5A, symbols in shaded area). The ability of sera to distinguish VDPV-1 from Sabin-1 did not seem to be clearly related to either the titer of the neutralizing antibodies they contain or to the donor's age (data not shown). Somewhat surprisingly, the most differentiating serum was obtained from an 11-day-old healthy child, who obviously possessed maternal antibodies.

TABLE 1. Polioviruses and human sera investigated by the microneutralization assay

Serotype	Poliovirus strain			No. of sera tested
	VDPV	Vaccine reference strain	Wild reference strain	
1	VDPV-1	Sabin-1	Mahoney	9
			Strain 537	19
2	VDPV-2a	Sabin-2	MEF-1	19
3	VDPV-3	Sabin-3	Saukett	18

To assess the effect of different amino acid changes in antigenic sites on the decrease in the capacity to neutralize VDPV-1, some other type 1 OPV-derived isolates were also tested with one of the most discriminating sera (Fig. 6). All the derivatives containing any mutation at position 3060 possessed a capacity to be neutralized comparable to that of Mahoney, suggesting a key role of this position. The contribution of replacements at positions 1106 and perhaps 1090 could not be unequivocally ruled out on the basis of the data shown in Fig. 6, but on the basis of the MAP (Fig. 4, left panels) this contribution was hardly decisive. Mahoney was neutralized by this serum as weakly as was VDPV-1. Likewise, no appreciable difference in the capacity to neutralize VDPV-1, on the one hand, and wild polioviruses of type 1, either parental Mahoney or unrelated strain 537, on the other, was observed when the whole set of human sera was investigated (Fig. 5B).

Interestingly, there was no appreciable difference in the capability of the human sera to neutralize Sabin-1 and strain St2 (Fig. 6), which possessed a reversion at position 1099, despite a marked effect of this mutation on the MAP of AgS1 (Fig. 4, Site 1, left). This observation suggested that AgS1 of type 1 poliovirus provided a minor contribution, if any, to the capacity to be neutralized by polyclonal antibodies.

No significant decrease compared to the homotypic Sabin strains was observed in the capacity of type 2 and type 3 VDPV strains to be neutralized (Fig. 5C and E). Nor were the human sera able to clearly distinguish between the VDPV strains of types 2 and 3, on the one hand, and homotypic wild-type polioviruses, MEF-1 and Saukett, respectively, on the other (Fig. 5D and F).

To ascertain whether the described peculiarities in the capacity of VDPV strains to be neutralized were due to the polyclonal nature of the sera used or to their human origin, experiments with polyclonal rabbit sera were carried out. The rabbit antisera raised against Sabin-1 and Sabin-2 showed a slightly higher activity against these strains compared to the homotypic VDPV strains, whereas no such difference was observed with the pair Sabin-3 and VDPV-3 (Fig. 7A). No discrimination between VDPV strains and the respective wild-type strains could be demonstrated with rabbit polyclonal sera raised against the wild-type viruses, which supports the notion about their close antigenic similarity (Fig. 7B).

DISCUSSION

Although OPV is a really excellent vaccine, it is not devoid of certain shortcomings, which especially surfaced now, when optimal endgame strategies in the worldwide effort to elimi-

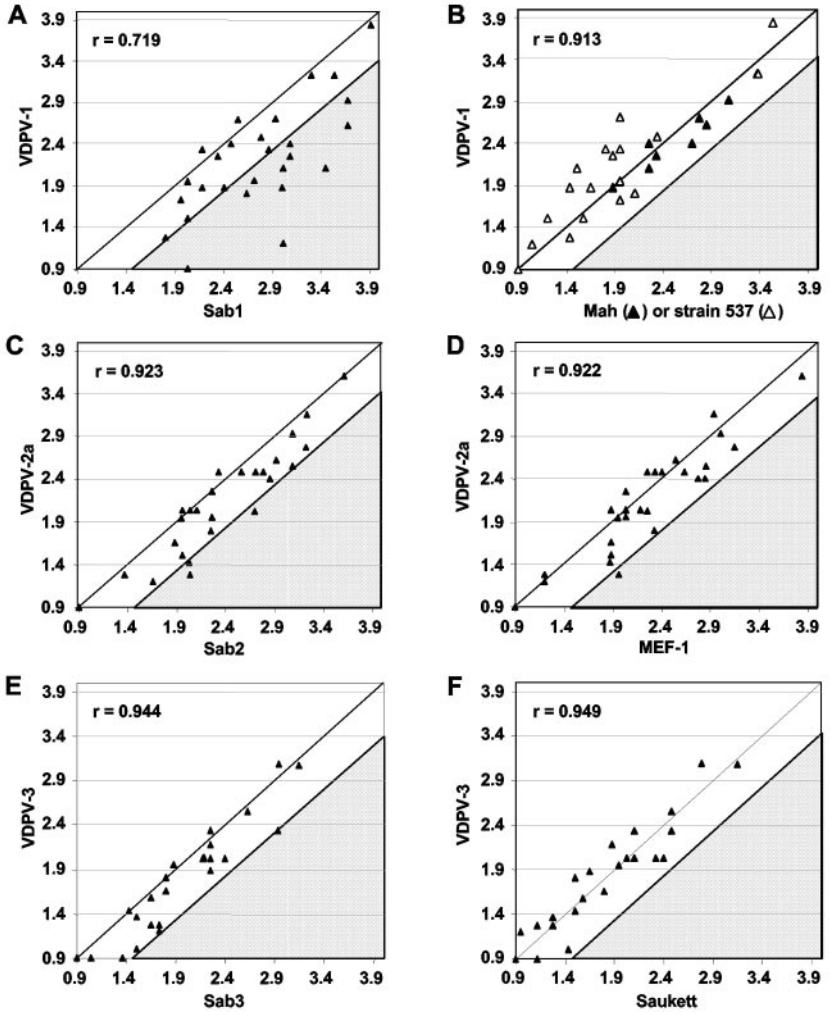


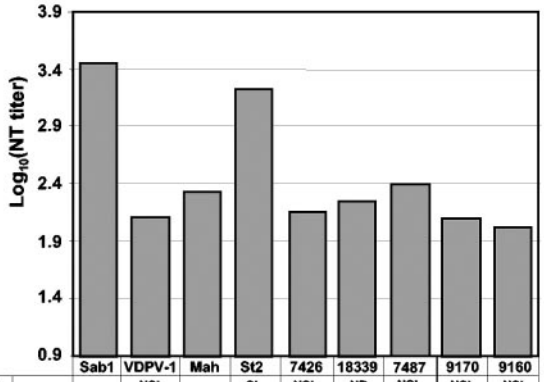
FIG. 5. The capacity of the VDPV strains to be neutralized by human antisera. The $\log_{10}$(NT titer) of individual sera for VDPV strains (*y* axis) are compared with the corresponding values (*x* axis) for homotypic reference strains, either Sabin (A, C, and E) or wild-type (B, D, and F). The gray area indicates a significant (greater than fourfold) difference between the NT titers. Mah, Mahoney; Sab1, Sabin-1; Sab2, Sabin-2; Sab3, Sabin-3.

nate wild-type polioviruses are hotly discussed. Putting aside economical and logistical issues, there are two major problems associated with OPV use: the occurrence, though admittedly very rare, of VAPP cases, and the possibility of long-term circulation and evolution of OPV strains toward transmissible pathogenic agents qualitatively indistinguishable from wild-type polioviruses (28).

Both of these adverse features are intimately associated with the intrinsic genetic instability of OPV. As a result of this instability, attenuating mutations tend to be eliminated by point mutations and recombination (2). Another common observation is that there are changes in the antigenic properties of OPV derivatives (28). Consequently, the WHO-adopted poliovirus surveillance program requires that the antigenic properties of each polio isolate be investigated in order to ascertain whether or not they have changed. However, in spite of widespread use of this analysis throughout the world (many thousands of isolates annually), neither the driving force(s) underlying antigenic changes nor their biological significance (e.g., their putative contribution to transmissibility and neurovirulence) is well understood. The present study aimed at shedding some additional light on both these issues.

Changes in capsid composition. Although amino acid replacements could occur in numerous positions of the capsid proteins, only a few of them were found fairly consistently: two positions were changed in more than half of the representatives of each serotype. In addition, depending on the serotype, from one to four positions were altered in more than 20% of the isolates (Fig. 2). Remarkably, the overwhelming majority of these mutational "hot spots" were serotype specific. An important feature of these mutations was that they could be fixed very early after the onset of vaccine reproduction in humans, even when no synonymous mutations, the hallmarks of viral evolution, could be detected. The early and consistent fixation of these mutations strongly argues that they were due to adaptive selection rather than stochastic drift.

Nearly all of the commonly found mutations affected capsid regions corresponding to the known antigenic sites or their flanking amino acid residues (i.e., AgR as defined above). In derivatives of type 1 OPV, alterations tend to occur predom-



		Sab1	VDPV-1	Mah	St2	7426	18339	7487	9170	9160
ITD			NSL		SL	NSL	NR	NSL	NSL	NSL
Site 1	1088	A	-	T	-	-	T	-	-	-
	1090	I	M	M	-	-	-	-	-	L
	1096	A	-	-	-	-	T	-	-	-
	1099	K	N	T	T	N	-	-	E	R
	1106	T	A	A	-	-	A	A	A	A
Site 2	2165	D	-	N	-	-	-	-	-	-
Site 3	3060	K	T	T	-	N	N	I	T	T

FIG. 6. The contribution of individual amino acid residues to the capacity of different OPV derivatives of type 1 to be neutralized by a human serum. The *y* axis shows values of $\log_{10}$(NT titer). The antigenic characteristics revealed by CAP-ELISA as well as alterations in the AgR are presented for each strain. Amino acid identities to the parental Sabin strain are marked by dashes. Mah, Mahoney; Sab1, Sabin-1.

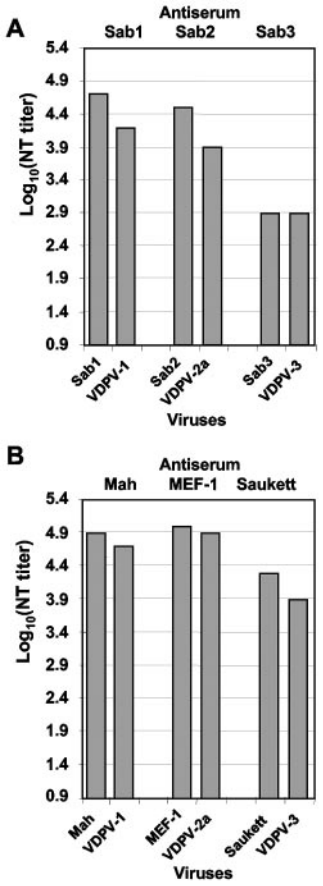


FIG. 7. The capacity of VPDV to be neutralized with rabbit antisera raised again Sabin (A) or reference wild-type (B) viruses. In both panels, the *y* axis shows $\log_{10}$(NT titer) values. Mah, Mahoney; Sab1, Sabin-1; Sab2, Sabin-2; Sab3, Sabin-3.

inantly in the Sabin-specific amino acid residues. Thus, no antigenically altered Sabin-1 derivatives investigated retained Lys3060, which either had reverted to Thr or, equally often, was replaced by another (not positively charged) residue. Likewise, Sabin-specific Thr1106 tended to be replaced by either Ala (true reversion to Mahoney) or Ser (a consensus residue for homotypic wild polioviruses). Though somewhat more rarely, other Sabin-1-specific amino acids within the AgR (e.g., Ile1090 and Lys1099) also reverted or were replaced by other residues. In line with this reasoning, Ser1095, differing from the relevant Mahoney residue but corresponding to the wild-type consensus, proved to be fairly stable among the OPV derivatives investigated. The only residue in the AgR distinguishing Sabin-3 from its predecessor, Arg1286, also reverted, albeit less frequently.

Thus, the amino acid mutations most frequently fixed in capsid proteins of OPV derivatives were represented by either reversion to the parental structure or replacement of a vaccine-specific residue by another one, which usually corresponded to the consensus for wild-type polioviruses.

Changes in antigenic properties. The use of a recently devised MAP-ELISA enabled a detailed characterization of changes in individual epitopes of OPV derivatives of type 1 and 2 (no adequate panel of MAbs for antigenic sites of type 3 was available). In both serotypes, the most conspicuous changes occurred at AgS1 and AgS3. Compared with the commonly used ITD assay, CAP-ELISA, the MAP-ELISA exhibited at least two important advantages. First, it proved to be more sensitive, being able to reveal antigenic alterations even in isolates with the SL phenotype, e.g., isolates St2 of type 1 (Fig. 4, left) and 20420 of type 2 (Fig. 4, right). Since the collection of strains in this study was selected on the basis of the CAP-ELISA, it did not fully represent all possible antigenically altered polioviruses but was enriched in strains with mutations in AgS3 while possibly missing mutations at AgS1. Obviously, screening for vaccine-derived polioviruses might be better performed either by using a MAP-ELISA or direct nucleotide sequencing or microarray hybridization. Second, and perhaps more important, results of the MAP-ELISA exhibited a clear correlation with specific amino acid substitutions in the AgR. For example, single specific amino acid replacements, such as Lys3060→Asn or His3077→Arg resulted in complete loss of the signal for AgS3 in derivatives of Sabin-1 and Sabin-2, respectively. Some other modifications of MAPs as revealed by MAP-ELISA could also be traced to changes in one or two specific amino acids.

The marked alterations in the reactivity of individual epitopes were not, however, accompanied by significant alterations in the capacity to be neutralized by polyclonal human sera. More accurately, about 40% of these sera proved to be somewhat more active against Sabin-1 compared to VDPV-1. When the neutralizing activity of the most differentiating serum was tested with several Sabin-1 derivatives, the major responsible capsid alteration was traced to the already discussed mutation of the Sabin-specific Lys3060. Highly diverged VDPV-2a and VDPV-3 exhibited nearly the same sensitivity to neutralization with human sera as their vaccine counterparts. Importantly, only marginal differences, if any, in the capacity of OPV strains

and highly diverged VDPV strains to be neutralized could be observed with rabbit polyclonal antisera. These facts suggest that poliovirus determinants relevant to its natural transmission may be represented by capsid structures other than those defined by the use of MAbs. Moreover, these biologically relevant epitopes appeared to be very stable genetically.

Possible driving forces of changes in antigenic sites. The fact that identical mutations are consistently fixed in independent lineages of OPV descendants strongly suggests that these alterations have conferred a selective advantage and increased viral fitness. The location of frequently occurring mutations in OPV derivatives within, or close to, the antigenic sites as well as marked alteration of the specificity of individual epitopes might suggest that these phenomena were the results of immune response evasion. Although attempts to escape the immune response of vaccine recipients might indeed contribute to the observed changes (see below), some of our data (discussed below) suggest that this contribution, if any, was hardly a decisive factor. To consider other possibilities, one should remember that derivation of the OPV strains involved passages in nonnatural hosts, multiple cloning, and deliberate selection of attenuated (in other words, less fit) variants (41, 56). As a result, the Sabin strains had acquired point mutations (especially numerous in Sabin-1, apparently because of the more intensive selection protocol) in both coding and noncoding regions of their genome (45, 60). As is well known, viral RNA-dependent RNA polymerases are devoid of proofreading activity, and therefore replication of RNA viruses is usually accompanied with numerous errors (reviewed in references 1 and 2). Such error-prone replication, if complemented with a selective pressure, can readily eliminate any fitness-decreasing point mutations. The loss of attenuating mutations in polioviruses isolated from the primary vaccinees and their contacts is a well-established and generally accepted fact (28, 40).

The replacements, which were found relatively often in this study but were not obviously related to antigenicity, appeared to belong only to the class of deattenuating fitness-increasing alterations, as previously reported for mutations at position 1143 in type 2 (36, 49) and positions 1054 and 3091 in type 3 (15, 34, 43, 65). The latter appeared to increase the efficiency of virion maturation (35).

We propose that not only the known attenuating mutations exemplified above but also mutations within the AgR of OPV derivatives may often have the same nature and represent attempts to eliminate fitness-decreasing replacements acquired during the selection of the vaccine or already present in the parental strains. This hypothesis is supported by the following arguments.

As already noted, elimination from the AgR of the Sabin-specific mutations is a characteristic feature of the evolving OPV derivatives. This removal, however, does not appear to confer on OPV derivatives a marked advantage in the capacity to overcome a normal (polyclonal) immune response at least in vitro, challenging the notion of immune evasion as a major factor contributing to the antigenic changes. It may also be noted that mutations at positions 1090, 1099, and 1106 could be observed as early as the seventh day after administration of OPV (31; our unpublished data), i.e., before a strong immune response could have been developed.

The adverse effects on fitness might be conferred not only by strictly Sabin-specific mutations. Certain amino acids present in the OPV predecessors may also be suboptimal. Thus, P712, the progenitor of Sabin-2, appeared to be somewhat attenuated (56), suggesting that it already had some fitness-decreasing load. Some fitness-decreasing mutations may be present in wild-type viruses as "passengers" (2).

Thus far, little is known about the possible molecular mechanism of the adverse effects of the Sabin-specific and other frequently changing amino acid residues in the capsid proteins of OPV strains. One such Sabin-1-specific amino acid, Lys1099, is known to confer on virions sensitivity to trypsin (16). Due to the structural neighboring and even overlapping of AgRs and the capsid regions that interact with poliovirus receptors (6, 21, 22), it seems reasonable to hypothesize that the amino acids likely to be replaced in AgR may interfere with optimal virus-cell recognition. It should be admitted that this hypothesis, while explaining some of the described changes, appears to have limitations. Some of the amino acid residues involved in antigenicity changes, for instance, 1090 and 1099 in type 1, 3075 and 3077 in type 2, and 3077 in type 3, do not seem to directly participate in at least the first steps of the virion-receptor interaction, as judged by crystallographic data (6, 21, 22). Importantly, the type 1 and type 3 Sabin-specific amino acid residues exhibiting a strong tendency to change in vaccinees appear to be quite stable upon passaging in tissue cultures (51, 54), though relevant changes may take place under certain conditions (46, 47). To reconcile the hypothesis on the optimization of receptor recognition as a cause of changes in the antigenic sites with the stability of these sites in tissue culture, one might consider the possibility that the requirements for optimal virion-receptor interactions in tissue culture and in gut may be different.

The mutations in question may possibly also affect other steps of viral reproduction, such as release of RNA from the incoming virions (24) or virion maturation (50), or may modulate some other step of virus reproduction.

The idea that negative selection of unfavorable mutations is a major driving force behind antigenic alterations of OPV does not necessarily rule out some contribution of immunological mechanisms as well. Immunological factors may be of some significance at the very early steps of OPV reproduction in human gut, when some capsid changes may help the virus to escape from what is still a very low level of specific antibodies. Some contribution of immunological factors cannot be ruled out also at very late stages of the evolution of OPV derivatives. Thus, it was reported that the capacity of highly evolved type 2 (59) and type 3 (7) VDPV strains to be neutralized appeared to be somewhat less efficient compared to that of appropriate Sabin strains. It may be noted that these two OPV derivatives were much "older" than those studied here. However, even these strains, despite some antigenic alterations, were fully neutralized by human sera in vitro. Thus, as noted previously (20), the general biologically relevant antigenic structure of polioviruses appears to be extremely stable, likely because of the overlapping of antigenic and receptor-recognizing determinants. In other words, immune evasion does not appear to play a decisive part in poliovirus evolution.

General remarks on evolution of polioviruses. Wild-type polioviruses, when passaged in cultured cells under constant

conditions and at a relatively high multiplicity of infection, exhibit a remarkable genetic stability. Taking into account that poliovirus replication is inherently error prone (reviewed in reference 1), the stability of viruses cultivated in vitro could be explained by strong negative (purifying) selection that eliminates less fit variants. On the other hand, natural circulation of wild-type polioviruses is associated with permanent changes in the nucleotide sequence of their genomes, which occurs at a relatively constant rate of $\sim 3 \times 10^{-2}$ substitutions per synonymous site per year and is characterized by predominant accumulation of silent (synonymous), mostly neutral mutations (17). This evolution is based primarily on stochastic drift caused by bottlenecking events during person-to-person transmission of small, not necessarily representative, sets of variants picked from intrinsically heterogeneous poliovirus populations (1, 2). This evolution may include fitness fluctuations caused by fixation of detrimental mutations (Muller's ratchet) and their reversion or suppression by second-site mutations. Some minor adaptive fitness fluctuations may be due to variability of host susceptibility (e.g., associated with the polymorphism of human receptors for poliovirus) (57). True fitness-increasing mutations in wild polioviruses are so uncommon that they have never been convincingly documented.

With OPV strains, the situation is markedly different. As already mentioned, the history of these strains included passages in nonoptimal hosts, multiple cloning (that is bottlenecking events), and deliberate selection of less fit (attenuated) variants (56). The error-prone replication generates variants devoid of these fitness-decreasing mutations, and the (pseudo)revertants are readily selected for. As a result, evolution of OPV is a biphasic process (3). The first phase occurs in the organisms of vaccinees and their immediate contacts very early after vaccine ingestion. Essentially, it consists of a burst of mutations, mostly represented by nonsynonymous replacements within coding sequences and/or alterations of important control elements in the noncoding portions of the viral genome. Most of these mutations are of an adaptive nature and aim at reducing the adverse effects of the fitness-decreasing mutations accumulated during the original selection of the vaccine strains by Albert Sabin. The improvement of the phenotype takes some time, with more detrimental mutations being eliminated faster than the less damaging ones. The major alterations in antigenic epitopes of OPV derivatives take place during this phase.

Concomitant with the restoration of fitness (equivalent to the loss of at least the majority of attenuating mutations), the second phase of OPV evolution is beginning. Essentially, it is analogous to the evolution of wild-type polioviruses (17) and consists in predominant accumulation of synonymous substitutions.

This work was done with a subset of antigenically altered vaccine-derived strains and, therefore, focused on the capsid coding region. It is clear that if some attenuated mutations are located in genomic regions coding for nonstructural proteins, similar processes of deattenuation by purging fitness-lowering mutations can occur outside of the capsid region. At this point the database of respective sequences is very limited, but it would be interesting to perform a similar study to reveal possible patterns and driving forces.

Implications for development of new vaccines. Notwithstanding the error-prone character of replication of the RNA genome, this genome, as exemplified by poliovirus, is remarkably robust, sustaining different kinds of alterations (1, 2). This sustainability is a basis for the development of live viral vaccines. Generally, it is a feasible task to select a highly attenuated viral variant lacking the ability to grow in or damage specific organs or tissues. A much harder problem is to get such attenuated variants in a genetically stable form. The lesson from OPV is that point mutations, even if there are several of them, can readily revert. Another approach is to achieve attenuation by bigger alterations of the genome, e.g., deletions (4, 25) or domain shuffling with other picornaviruses (19). Although seemingly logical and even perhaps workable under certain conditions, this approach also has a drawback: the attenuating portion of the genome can be replaced by recombination. In the case of poliovirus, there are plenty of naturally circulating enterovirus recombination partners, especially belonging to the so-called cluster C, ready to help to purge the fitness-decreasing portion of the genome (9, 28).

In the light of the above considerations, a new poliovirus vaccine, which is a highly desirable endgame strategy in the effort to eradicate poliovirus (23), will unlikely be a live vaccine.

Obviously, the above general consideration should apply also to live vaccines against other RNA viruses, but the variations among these viruses with regard to the fidelity of the relevant viral RNA-dependent RNA polymerases, the possibility of the restoration of virulence by recombination, modes of transmission, the severity of the inflicted disease, etc., may significantly modify the situation in each particular case.

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