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Alteration in Oligonucleotide Fingerprint Patterns of the Viral Genome in Poliovirus Type 2 Isolated from Paralytic Patients

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A close relationship was demonstrated by oligonucleotide fingerprinting between genomes of the poliovirus type 2 Sabin vaccine strain and recent isolates from paralytic cases associated with vaccination in Japan. The oligonucleotide maps of isolates from an agammaglobulinemic patient, who continued to excrete poliovirus type 2 for 3.5 years after the administration of oral vaccine, showed that the genomic alteration proceeded gradually, retaining the majority of the oligonucleotides characteristic of the vaccine strain for a long period, indicating vaccine origin for the isolates. The final isolate at month 41, however, lost the majority of these oligonucleotides. The heterologous antigenic relationship between the final isolate and the previous isolates was also observed. The serial alteration in electrophoretic mobility of the major structural proteins (VP1, VP2, and VP3) was observed throughout the excreting period. These results indicate that the population of the virus in this individual changed markedly during the last short period (about 3 months), in which the treatment with secretory immunoglobulin A was carried out. Genome comparisons in oligonucleotide maps show that some oligonucleotides in the genome of the vaccine strain are highly mutable after passage in humans.

The poliovirus vaccine strains (Sabin) currently in use originate from wild-type strains (21), and it is well known that the phenotypic markers of vaccine strains are unstable during passage in the human intestine (9, 12). Among methods for examination of the change of markers, antigenic marker tests have been applied to determine whether the isolate is derived from the vaccine strains (15). Serological methods such as neutralization tests by cross-absorbed sera (23) or monoclonal antibodies (Arita, manuscript in preparation) have also been used for the determination of the origin of isolates. The results obtained with these serological methods are similar (Hara et al., manuscript in preparation). The antigenic drift of the virus during passage in human intestine sometimes brings about difficult problems for determination of the origin of isolates in Japan, where wild-type poliovirus is considered to have been eliminated by the use of Sabin vaccine (7, 22). Thus, it became necessary to determine the origin of recent isolates with non-vaccine-like antigenicity. The fingerprinting technique of resolving RNase T_1-resistant oligonucleotides on two-dimensional gel electrophoresis has been established as a sensitive method for determination of viral genomes in several RNA viruses (3, 14, 25). Recently the technique has been applied to poliovirus (10, 11, 13, 18, 19). Poliovirus type 2 has been the major type isolated from paralytic cases among vaccinees and their contacts in the last 10 years in Japan (24). Among these isolates, only one strain (strain Hokkaido), isolated in 1977 from a paralytic patient with agammaglobulinemia in Sapporo, Hokkaido, showed non-vaccine-like antigenicity and typical neurovirulence in monkeys (1, 7). This patient continued to excrete poliovirus type 2 for 3.5 years after the second vaccination. Since such a long persistent infection with poliovirus in the same individual is rather rare, it is of interest to examine the genomic alteration of the Hokkaido strains in one immunodeficient patient. Therefore we applied the oligonucleotide fingerprinting analysis to genomes of poliovirus type 2 isolates.

In this report, we show that recent isolates from paralytic and nonparalytic cases were originated from the vaccine strain, that the Hokkaido strains from a paralytic patient with agammaglobulinemia showed the genomic alteration gradually retaining majority of the oligonucleotides characteristic of the vaccine strain during a long period, and that the virus population of the final isolate showed marked alteration in the oligonucleotide maps of the viral genome as well as in antigenicity of virus and in virion polypeptides.

TABLE 1. Recent poliovirus type 2 isolates in Japan

Strain	Original code[a]	Vaccination history	Days after vaccination when isolated	Clinical status
12	Hokkaido-72-1	Contact	59	Paralytic
20	Osaka-80-1	Contact	61	Paralytic
26	Toyama-77-2	Vaccinee	62	Healthy
27	Akita-79-1	Vaccinee	72	Healthy

[a] Original code: place of isolation-year of isolation-isolate number.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of a continuous line of cynomolgus monkey kidney (CMK) cells in Eagle minimal essential medium containing 10% bovine serum was used. Poliovirus type 2 isolates were derived from stool specimens and not plaque purified to avoid arbitrary selection. Virus stocks were grown once in CMK cell cultures at 35°C after isolation and antigenic characterization on primary CMK cell cultures. Wild-type strains of poliovirus type 2 were isolated from paralytic patients before oral poliovaccine had been widely used and were designated as 58-Y-8 (isolated in Yamaguchi in 1958) and 61-E-1 (isolated at Ebara Hospital in Tokyo in 1961). Isolated strains with vaccine-like antigenicity are listed in Table 1. Isolates (Hokkaido strains) from a paralytic patient with agammaglobulinemia over a period of 41 months are listed in Table 2. Type 2 Sabin vaccine strain p712, ch, 2ab was originally sent from the Connought Laboratory, Toronto, Canada.

Virus purification. Infected CMK cell cultures in roller bottles (1,600 cm^3) were incubated in serum-free Eagle minimal essential medium at 35°C. When severe cell degeneration was seen (usually between 16 and 24 h after infection), the cultures were frozen and thawed three times. The cultures were clarified at 10,000 rpm for 5 min in a Hitachi 20RP-5 centrifuge to remove cell debris. Virus was precipitated from the supernatant by adding polyethylene glycol 6000 (80 g/liter) and NaCl (23 g/liter). The virus precipitate was collected by centrifugation at 10,000 rpm for 5 min. It was suspended in Dulbecco phosphate-buffered saline and loaded on 2 ml of a 40% (wt/vol) sucrose cushion. After centrifugation for 3 h at 4°C (38,000 rpm in a Beckman SW41 rotor), the virus precipitate was suspended and then purified by isopicnic centrifugation in CsCl (38,000 rpm overnight in a Beckman SW50.1 rotor). The virus was further purified by 15 to 45% (wt/vol) sucrose gradient centrifugation for 4 h at 4°C (25,000 rpm in an SW27 rotor). The visible virus band was collected and stored at −70°C.

Extraction of poliovirus RNA. Sodium dodecyl sulfate (SDS, 1%) was added to the purified poliovirus fraction from a sucrose gradient. RNA was extracted three times with an equal volume of phenol mixture (50 parts phenol, 48 parts chloroform, and 2 parts isoamyl alcohol saturated with water and containing 1 mg of 8-hydroxyquinoline per ml). The RNA in the aqueous phase was precipitated with ethanol at −20°C.

Oligonucleotide mapping. Details of the oligonucleotide mapping technique (20) were kindly provided by K. Nakajima. Briefly, poliovirus RNA (about 5 μg) was digested with 20 U of T_1 RNase (Sankyo Ltd., Tokyo, Japan). The resulting oligonucleotides were 5′ end labeled with 50 μCi of [γ-^{32}P]ATP (>2,000 Ci/mmol; Radiochemical Centre, Amersham, England) by using 2.5 U of polynucleotidekinase (Boehringer Mannheim Corp., New York, N.Y.) and separated by two-dimensional gel electrophoresis. The first-dimensional electrophoresis was done at pH 3.5 in 10% polyacrylamide–6 M urea gel until the bromophenol blue had migrated 20 cm. The second-dimensional electrophoresis was done at pH 8.3 in 21.8% polyacrylamide gel in 40 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA until the bromophenol blue had migrated 19 cm from the bottom of the first-dimensional gel. Gels were exposed to Sakura A or AO X-ray film with an appropriate intensifying screen at 4°C.

Labeling and electrophoresis of virion proteins. CMK cell cultures in 60-mm plastic dishes were infected at about 20 PFU/cell. After 1 h of adsorption at 35°C, 5 ml of Eagle minimal essential medium was added. Cultures were labeled after 3 h of incubation at 35°C by the addition to each dish of 50 μCi of [^{3}H]leucine (136 Ci/mmol; Radiochemical Centre) in minimal essential medium lacking leucine. After incubation for an additional 16 h, the virus was released from cells by freezing and thawing. Virions were partially purified by pelleting through a 40% sucrose cushion and isopicnic centrifugation in CsCl. The virions were heated at 100°C for 2 min in disrupting mixture (1.5% SDS, 1.5% 2-mercaptoethanol, and 1.5 M urea in 0.01 M phosphate buffer, pH 7.0) for electrophoresis.

SDS-polyacrylamide gel electrophoresis was carried out on a 10 to 30% linear gradient polyacrylamide slab gel as described previously (24). After electrophoresis,

TABLE 2. Poliovirus type 2 Hokkaido strains isolated from a paralytic patient with agammaglobulinemia after vaccination[a]

Strain	Date of isolation	Months after:	
		Onset of paralysis	Second vaccination
18-BA	15 November 1976	−11	7
18-A[b]	4 November 1977	1	19
18-G	8 June 1979	20	38
18-V	3 September 1979	23	41

[a] The patient (male) was born on 10 December 1974. Because of increased susceptibility to infection, he was examined in a hospital. He was found to be agammaglobulinemic and was treated with human immunoglobulin. His past history revealed that he had received two doses of trivalent oral poliovaccine on 17 May 1975 and 16 April 1976. On 28 October 1977 he showed signs of paralysis at the age of 2 years and 10 months. From 5 to 11 July 1979, he was treated with secretory IgA (150 mg/day). Stool specimens were taken on the indicated days, and isolated strains were designated as shown in the table. After the last isolation (18-V), no virus was isolated in spite of isolation trials. The clinical studies of this patient were reported previously (1).

[b] Original code: Hokkaido-77-1.

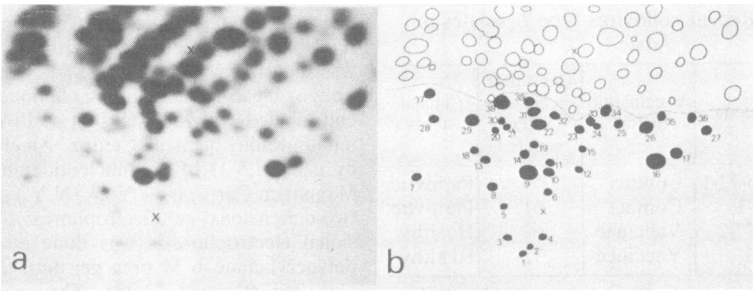

FIG. 1. Oligonucleotide map of poliovirus type 2 vaccine strain. a, RNA of the vaccine strain (Sabin); b, diagram of a. The RNase T_1-resistant oligonucleotides of the vaccine strain were 5′ end labeled and separated by two-dimensional polyacrylamide gel electrophoresis (see text). The large oligonucleotide spots below dashed line (black spots) of the vaccine strain were numbered 1 through 39 (b) and used for comparison. Faint spots were not included in analysis. The position of the dye markers xylene cyanol and bromophenol blue are noted by Xs.

the polypeptide bands were visualized by fluorography as described by Bonner and Laskey (2).

RESULTS

Comparison of the oligonucleotide maps of genomes of the vaccine strain and isolates. The oligonucleotide map of the genome of the vaccine strain was prepared (Fig. 1a) and showed a pattern basically similar to that shown by others (10, 18). The map pattern did not change during six passages of the vaccine strain in CMK cell cultures. To identify the oligonucleotides in RNAs of isolates, large oligonucleotide spots of the vaccine strain were numbered 1 through 39 (Fig. 1b) for comparison. Figure 2 shows the maps prepared from two wild-type strains isolated from paralytic patients before oral polio vaccine had been used widely in Japan. The maps of wild-type strains were completely different from the pattern of the vaccine strain given in Fig. 1. In contrast, the maps of recent isolates from paralytic patients and healthy vaccinees were similar to that of the type 2 vaccine strain (Fig. 3). Overlapping spots common to both the isolates and the vaccine strain are shown as black spots in Fig. 3b, d, f, and h. The number of overlapping spots was 33 in strains 12, 20, and 27 (Fig. 3b, d, and h). Thirty-two overlapping spots were counted in strain 26 (Fig. 3f). These results show that most of oligonucleotide spots characteristic of the vaccine strain are retained in these recent isolates and suggests that these isolates are originated from the vaccine strain, in agreement with their antigenic properties. However, they were not identical to the vaccine strain. Several spots were missing in the maps of isolates compared with that of the vaccine strain (Sabin). Such genome differences were accompanied by changes in the viral structural protein (VP1) detected as a shift in electrophoretic mobility as previously described (24).

Alteration of the oligonucleotide maps in genomes of the Hokkaido strains isolated from a paralytic patient with agammaglobulinemia. Among recent isolates of poliovirus type 2 in Japan, only the Hokkaido strains isolated from a paralytic patient with agammaglobulinemia showed non-vaccine-like antigenicity. Since this patient excreted non-vaccine-like viruses for 3.5 years after the second vaccination, the maps of the serial isolates were represented in the time course of the isolation (Fig. 4). The map of strain

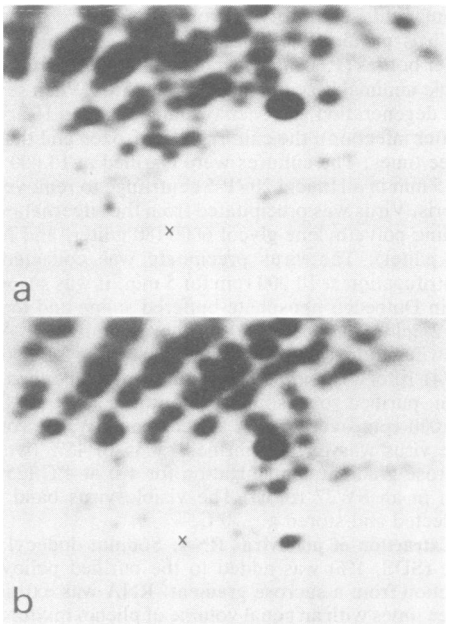

FIG. 2. Oligonucleotide maps of the wild-type strains of poliovirus type 2. RNAs of the wild-type strains were analyzed as in Fig. 1. Panels: a, 58-Y-8; b, 61-E-1.

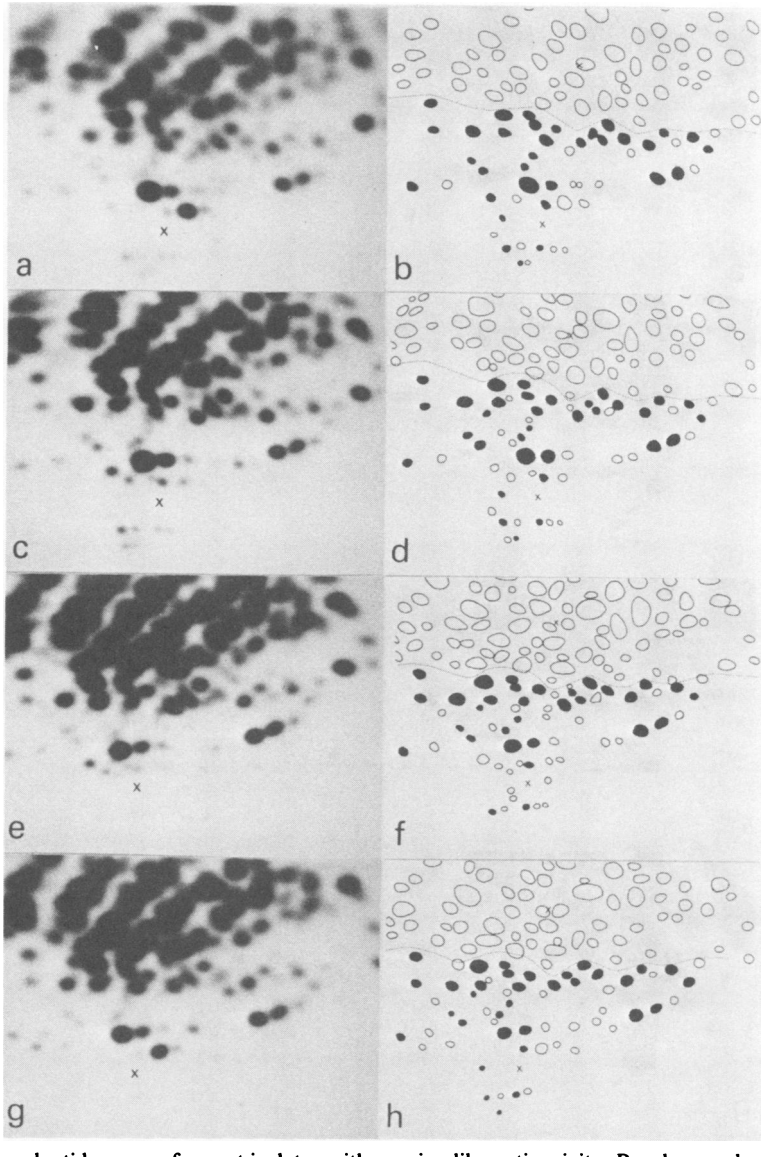

FIG. 3. Oligonucleotide maps of recent isolates with vaccine-like antigenicity. Panels: a and c, strains 12 and 20, respectively, isolated from paralytic cases; b and d, diagrams of a and c, respectively; e and g, strains 26 and 27, isolated from healthy vaccinees; f and h, diagrams of e and g, respectively. Black spots in diagrams are spots overlapping those of the vaccine strain.

18-BA, isolated 1 year before the onset of paralysis, showed a map pattern similar to that of the vaccine strain (Fig. 4a). Thirty-three overlapping spots were counted (Fig. 4b), and 6 spots were missing at 7 months after vaccination. The map of strain 18-A, isolated 1 week after the onset of paralysis, was a little different from those of the vaccine strain and the previous isolate (18-BA) (Fig. 4c). However, 28 oligonucleotide spots of strain 18-A overlapped those of the vaccine strain (Fig. 4d). The result indicates that during 1 year before the onset of paralysis, changes of the viral genome proceeded, and 5 spots overlapping those of the vaccine strain were lost. The map of strain 18-G, isolated 20 months after the onset of paralysis, was also a little different from those of the vaccine strain and the previous isolates, 18-BA and 18-A (Fig. 4e). However, 27 oligonucleotide spots were still overlapping those of the vaccine strain (Fig. 4f). These serial maps of the Hokkaido strains show that missing spots increased gradually and that these strains originated from the vaccine strain. The map of the final isolate, 18-V is shown in

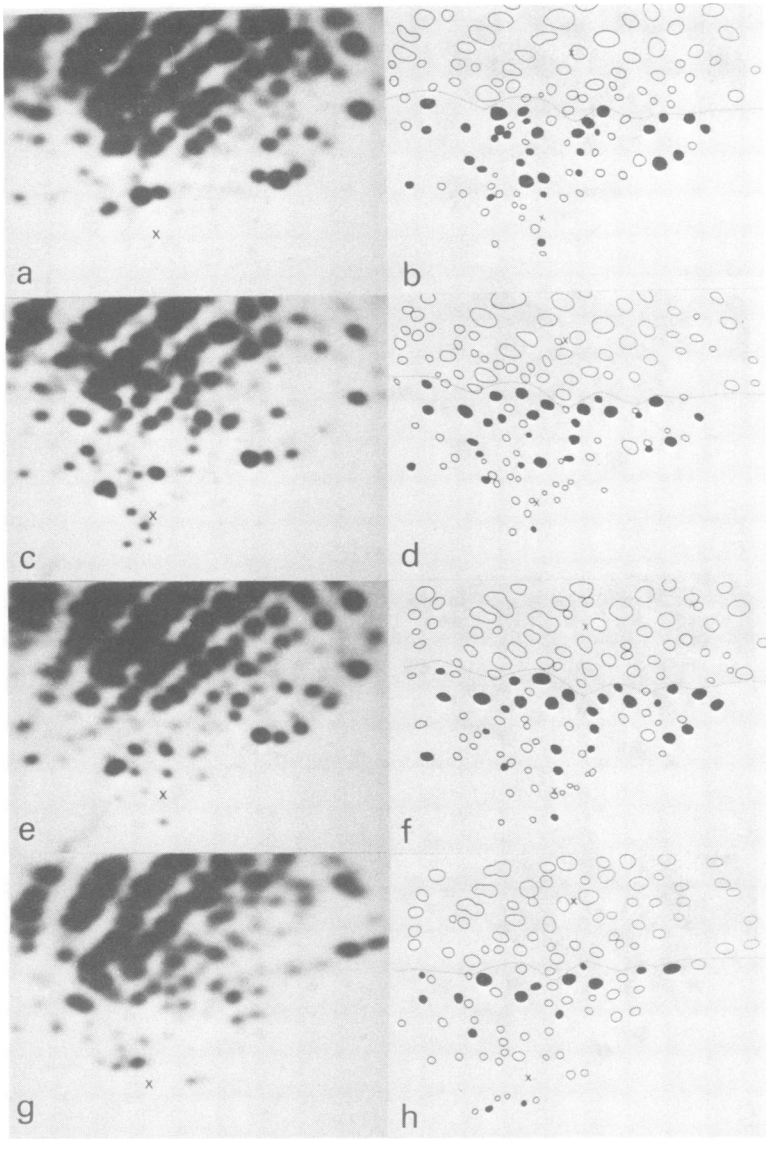

FIG. 4. Oligonucleotide maps of the Hokkaido strains from a paralytic patient with agammaglobulinemia. Panels: a, 18-BA; c, 18-A; e, 18-G; g, 18-V; b, d, f, and h, diagrams of a, c, e, and g, respectively. Black spots in diagrams are spots overlapping those of the vaccine strain. The overlapping spots in the diagram h were confirmed by coelectrophoresis.

Fig. 4g. The map pattern was markedly different from those of the vaccine strain and the previous isolates (18-BA, 18-A, and 18-G). The oligonucleotide spots characteristic of the vaccine strain were lost so extensively that the origin of the strain could not be estimated without knowing the serial isolation processes. To confirm the similarity between the oligonucleotide spots of the vaccine strain and 18-V strain, coelectrophoresis was carried out (data not shown); 15 overlapping spots were identified (Fig. 4h). Thus, strain 18-V has lost 12 spots overlapping those of the vaccine strain during the last 3 months in the 3.5-years virus-excreting period. The results indicate that the viral genome altered markedly during the last 3 months. The clinical report showed that the patient received the immunological treatment with immunoglobulin, but the treatment had no effect on excretion of the virus for a long period (1). After the isolation of strain 18-G, the patient received treatment with secretory immunoglobulin A (IgA) (Chiba et al., personal communication). Polivirus type 2 became undetectable in his stool specimens at

TABLE 3. Antigenic relationships among Hokkaido strains[a]

Strain	Antigenic character (NK value)[b] with antiserum[c] against:	
	Sabin 2[d]	18-V
Vaccine strain Sabin 2	Isologous (100)	Heterologous (27)
Hokkaido strain 18-BA	Heterologous (44)	Heterologous (21)
Hokkaido strain 18-A	Heterologous (37)	Heterologous (30)
Hokkaido strain 18-G	Heterologous (31)	Heterologous (29)
Hokkaido strain 18-V	Heterologous (29)	Isologous (100)

[a] Antigenic characterization was carried out by the modified McBride test (17).
[b] Normalized K value: ≦60 (heterologous).
[c] Antiserum was prepared in rabbits as described previously (7).
[d] The results with antiserum against Sabin 2 were from reference 7.

about 2 months later. The final isolate after this treatment was strain 18-V. It is suggested that the viral genome changed markedly in response to the treatment with secretory IgA.

Phenotypic changes of the Hokkaido strains during persistent infection. To confirm the change in the viral genome, antigenic analysis and structural polypeptide analysis by SDS-polyacrylamide gel electrophoresis were carried out. The McBride test (17) was carried out with serum against strain 18-V. As shown in Table 3, antigenic differences were observed between the final isolate (18-V) and the previous isolates (18-BA, 18-A, and 18-G) as well as the vaccine strain. The result showed that the virus population in this individual changed during the last 3 months after the treatment with secretory IgA. Since previously the antigenic variation proceeded gradually and slowly, but not markedly, when antisera against early isolates (18-BA and 18-A) were used (7), the antigenic difference between 18-G and 18-V was not expected until the result of the fingerprinting map was obtained. Thus, the antigenic alteration agrees with the alteration in the viral genome by fingerprinting.

Structural polypeptides of the Hokkaido strains were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). In the course of 3.5 years, the electrophoretic mobility of VP1 increased; in contrast, that of VP2 and VP3 decreased. The mobility differences in the structural polypeptides between the Hokkaido strains can probably be attributed to amino acid substitutions (5, 8). Serial substitutions of amino acid may be accumulated on each structural polypeptide during the long persistent infection. The marked alteration between strain 18-G and 18-V was not observed by using SDS-polyacrylamide gel electrophoresis.

Alteration of RNAs in isolates. It is known that poliovirus consists of heterologous populations (16, 19). RNAs prepared from plaque-purified clones from strain 18-BA showed maps slightly different from each other and slight differences in oligonucleotides characteristic of the vaccine strain (Yoneyama et al., manuscript in preparation). Additional spots in RNAs of isolates appeared, possibly due to mutation in oligonucleotides of the vaccine strain. Sometimes some of them, however, overlapped with faint spots of the vaccine strain. These faint spots were not used for analysis. Since the addition of these faint spots complicated the analysis, the analysis was restricted to the presence or absence of 39 oligonucleotide spots of the vaccine strain in maps of isolates.

The alteration of RNAs of isolates is summarized in Table 4. Larger oligonucleotides (no. 1 through 21) of the vaccine strain are likely to be missing in isolates. In contrast, smaller oligonucleotides (no. 22 through 39) are not. Hot spots (highly mutable oligonucleotides) were also observed. Oligonucleotides no. 2 and 11 were never observed in RNAs of isolates. The alteration of oligonucleotides no. 8, 12, 15, and 21 was observed at low frequency. Successive oligonucleotide maps of the Hokkaido strains show that once oligonucleotides became missing, most of them did not reappear, but a few of them reappeared (Table 4).

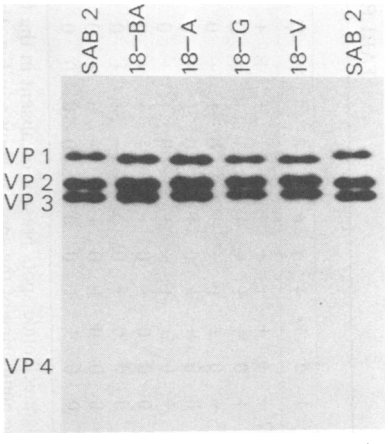

FIG. 5. Fluorography of a 10 to 30% gradient slab gel of [^{3}H]leucine-labeled structural polypeptides of the Hokkaido strains and the vaccine strain (SAB 2).

TABLE 4. Alteration in oligonucleotides of poliovirus isolates[a]

Strain	Oligonucleotide spots[b]																																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
Sabin 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	0	+	+	+	+	+	+	+	+	0	0	+	+	0	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	0	+	+	+	+	+	0	+	+	0	0	+	+	0	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26	0	0	0	0	0	+	+	0	+	+	0	0	+	+	0	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27	0	+	0	0	+	+	+	0	+	+	0	0	+	+	0	+	+	+	+	+	0	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18-BA	0	0	0	+	0	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	0	0	+	+	+	0	+	+	+	+
18-A	0	0	0	+	0	0	+	+	+	+	0	+	+	0	+	+	+	+	0	0	+	+	+	0	+	+	+	+	0	0	0	+	+	+	+	+	+	+	+
18-G	0	0	0	+	0	+	+	+	0	+	0	+	0	0	+	0	0	0	0	0	0	+	0	0	+	0	0	+	+	0	0	0	+	+	+	+	+	+	+	+
18-V	0	0	+	+	0	0	0	0	0	0	0	0	+	0	+	0	0	0	0	0	0	+	0	0	+	0	0	+	+	0	0	0	+	+	+	+	+	+	+	0

[a] Oligonucleotide spots present or absent in the fingerprint of type 2 isolates are noted by + or 0, respectively.

[b] The numbering of the spots is shown in Fig. 1b.

DISCUSSION

The present results show that fingerprinting analysis by in vitro labeling, as well as the in vivo labeling method previously described (10, 18), can be applied to determine the origin of poliovirus isolates. The in vitro method requires only a small amount of viral RNA and less radioisotope than does the in vivo method (6). In our experiments usually about 5 μg of viral RNA and 50 μCi of [γ-^{32}P]ATP were employed. The oligonucleotide fingerprinting pattern was highly reproducible in duplicate analyses of the same RNA preparation or among different preparations of the same virus strain.

In general, the serological differences between strains of poliovirus are in agreement with differences in base sequences of the viral genome as revealed by their RNase T_1-resistant oligonucleotides. However, the serological differences detected by intratypic serodifferentiation methods reflect only a small part of the genome information responsible for the base sequences of the structural polypeptides. The origin of the Hokkaido strains was determined by oligonucleotide maps, but not by serological methods. All recent isolates of poliovirus type 2 in Japan, except the Hokkaido strains, had close relationships to the vaccine strain by serological methods (7, 24); this supports the conclusion that wild-type strains of poliovirus type 2 have been eliminated in Japan.

There have been several clinical reports on vaccine-associated paralysis in immunodeficient patients (1, 4, 26, 27). In such cases, usually the poliovirus infection persists for a long time. The isolation of poliovirus type 2 from an agammaglobulinemic patient over a period of 41 months is described here. The difference in oligonucleotide maps between the vaccine strain and the Hokkaido late isolates was greater than that between the vaccine strain and the early isolates (Fig. 4). This suggests that alterations in the map patterns of the Hokkaido strains could be due to the generation of new mutants in the virus population during long-term, persistent infection. However, the majority of oligonucleotides characteristic of the vaccine strain were retained for a long period (38 months), suggesting that there was rather mild selection pressure on the virus population in this immunodeficient patient, except for during the last 3 months. After immunotherapy with secretory IgA, genomic and antigenic characters of the final isolate (18-V) changed markedly in comparison with the previous isolates, suggesting that such a marked change in the virus population was caused by the immunotherapy, and that under a particular condition the population of poliovirus may change easily. The electrophoretic mobility of the major structural polypeptides (VP1, VP2, and VP3) of

the Hokkaido strains shifted gradually and serially in parallel with genomic and antigenic changes. The Sabin type 2 strain easily altered in the electrophoretic mobility of VP1 during human intestinal passage (24). In general, poliovirus can hardly be isolated at 3 months or later after administration of oral vaccine. The present result shows that the longer period of virus excretion enabled us to detect the alteration in the electrophoretic mobility of not only VP1 but also VP2 and VP3.

This immunodeficient patient received immunological treatment with immunoglobulin, which had no effect on excretion of type 2 poliovirus for a long period. After the treatment with secretory IgA, the virus population markedly changed, and type 2 poliovirus became undetectable. It is possible that the disappearance of the virus may be due to some effect of secretory IgA. However, it is difficult to say whether the disappearance of the virus is due to direct effect of secretory IgA, because it took about 2 months before the virus disappeared.

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