Articles

Analysis of the reversion to the neurovirulent genotype in attenuated polio vaccine viruses passaged in cultivation cells derived from the human alimentary tract

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Background It is known that the attenuated polio vaccine viruses derived from oral poliovirus vaccine undergo neurovirulent reversion during repeated replication in the human alimentary tract, and some paralytic cases caused by the revertants have been reported. Furthermore, the revertants are excreted with the feces into the environment, and the viruses have caused new epidemics of poliomyelitis in the world. It is an obstacle for the achievement of polio eradication program.
Objective In this study, to elucidate the reversion mechanism of polio vaccine virus to the neurovirulent genotype in the human alimentary tract, an accumulation of the reversion of the vaccine viruses passaged in cultivation cells which were derived from the human alimentary tract was analyzed.
Methods Polio vaccine viruses were passaged three times in Caco-2 cells derived from human colon carcinoma. The reversion of the passaged viruses was analyzed by the MAPREC method designated to estimate the ratio of revertants in a virus population.
Results The accumulation of reversion in the vaccine viruses increased rapidly with viruses passaged at a temperature of 37° C compared with those at 34° C. However, it was hardly observed in viruses passaged in HEp-2 cells which were derived from human laryngeal carcinoma at a temperature of 37° C.
Conclusion A large difference was observed in the frequency of reversion between Caco-2 and HEp-2 cells though both cells were derived from human carcinoma. It is important to elucidate the cellular factors which take part in the reversion frequency of the virus genome. Such research is expected to lead to the elucidation of the reversion mechanism and to the development of a controlling expedient for the neurovirulent reversion of the polio vaccine virus.
Key words poliovirus, oral poliovirus vaccine, vaccine-derived poliovirus, polio eradication program, neurovirulent reversion

Introduction

Paralytic poliomyelitis was a common disease in Japan during the 1950s, as in many other countries. Following the introduction of the two-dose administration of the oral poliovirus vaccine (OPV), which was imported from Canada and the Soviet Union, to children in 1961-1963, the number of patients decreased markedly, while 1,000 to 5,000 paralytic cases of poliomyelitis were reported annually before the introduction of OPV (Takatsu et al.; 1973, Shimojo et al.;1984). A two-dose administration of domestic OPV to infants at intervals longer than 6 weeks has been performed routinely since 1964. A wild poliovirus was isolated from one

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patient with poliomyelitis in 1980, and from two patients with non-acute flaccid paralysis in 1984 and 1993, but since then no wild poliovirus has been isolated from patients with poliomyelitis in Japan (Infectious Agents Surveillance Center; 1997).

The global polio eradication program has progressed by the initiative of the World Health Organization (WHO) (World Health Organization; 2003). OPV has been used as a major tool for the eradication program. The result of the program is remarkable, since at present in July, 2007, epidemics caused by wild poliovirus are reported only in Africa and the southern area in Asia (Global Polio Eradication Initiative; 2007). Although OPV confers a dramatic efficacy on the prevention of poliomyelitis, some paralytic cases (vaccine-associated paralytic poliomyelitis cases: VAPP) have been reported to have occurred in vaccinees or in individuals who have been in contact with vaccinees (World Health Organization Consultative Group; 1982, Nkowane et al.; 1987). It is considered that paralytic cases are caused by vaccine viruses which undergo neurovirulent reversion during repeated replication in the human alimentary tract. Moreover, as long as OPV is in use, the revertants (vaccine-derived poliovirus: VDPV) continue to be excreted with the feces into the environment, and there is a possibility that VDPV may cause a new epidemic of poliomyelitis. Actually, epidemics of poliomyelitis caused by VDPV have been reported in Egypt, Hispaniola (Dominican Republic and Haiti), the Philippines, Madagascar, China, Indonesia and Cambodia, as shown in Fig.1 (Centers for Diseases Control and Prevention; 2006).

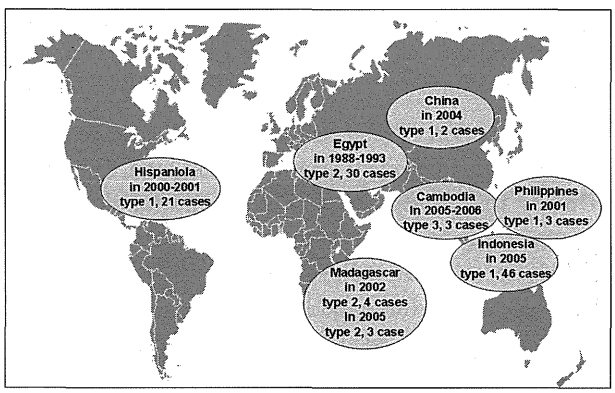

Fig. 1. Epidemics caused by VDPVs in the world between 1988 and 2006.

The poliovirus has a single-stranded RNA genome, which is known to be highly mutable during replication (Holland et al.; 1982, Ward et al.; 1988). It was demonstrated that neurovirulence increases when the following changes take place in the base positions of the vaccine viruses: in the case of the type 1 virus, position 480 in the 5' noncoding region changes from G to A (Kawamura et al.; 1989, Horie et al.; 1994); position 525, which is base paired with position 480 in a stem and loop structure, changes from U to C (Muzychenko et al.; 1991) ; for type 2, position 481 changes from A to G (Pollard et al.; 1989, Macadam et al.; 1991); and for type 3, position 472 changes from U to C (Evans et al.; 1985, Westrop et al.; 1989). Furthermore, Chumakov et al. (1991, 1992) developed a method designated "mutant analysis by PCR and restriction enzyme cleavage (MAPREC)" to estimate the ratio of revertants in a virus population. The results of MAPREC analysis were correlated with neurovirulence in monkeys (Rezapkin et al.; 1994, Taffs et al.; 1995, Chumakov et al.; 1999). MAPREC is useful for monitoring the reversion of the polio vaccine virus genome.

In this study, to elucidate the reversion mechanism of polio vaccine viruses to the neurovirulent genotype in the human alimentary tract, an accumulation of the reversion of the vaccine viruses passaged in cultivation cells derived from the human alimentary tract was analyzed by using the MAPREC method.

Methods

1) Cultivation of cells and viruses

Caco-2 cells (RCB0988) derived from human colon carcinoma were obtained from Riken Cell Bank, Tsukuba, Japan. The cells were propagated in Minimum Essential Medium (MEM, Invitrogen Gibco, Grand Island, NY) containing 20% fetal bovine serum at a temperature of 37℃. HEp-2 cells derived from human laryngeal carcinoma were propagated in MEM containing 10% fetal bovine serum at a temperature of 37℃.

Poliovirus Sabin type 1 and 3 attenuated vaccine strains were used in this study. The viruses were serially passaged three times in confluent cultures of the Caco-2 or HEp-2 cells in a cell culture flask

(cultivation area; 25 cm^2, Nalge Nunc International). The viruses were infected with a concentration of approximately $10^{-1.5}$~10^{-2} $CCID_{50}$/cell, incubated at a temperature of 37℃ or 34℃. After all cells caused virus-specific cytopathic effects (CPE), the viruses were harvested (frozen at -30℃).

2) MAPREC

Base changes for the type 1 Sabin vaccine virus with a virulent base A at position 480 (480-A reversion) and base C at position 525 (525-C reversion), and for the type 3 Sabin virus with a virulent base C at position 472 (472-C reversion) were examined by the MAPREC method. MAPREC was performed according to the method of Chumakov et al. (1991, 1994) and Rezapkin et al. (1994). The procedure of MAPREC for the type 3 virus is summarized in Fig.2. Briefly, poliovirus RNA was extracted from a virus suspension using buffer-saturated phenol (Invitrogen, Carlsbad, CA) after adding sodium dodecyl sulfate (SDS) to a final concentration of 1%. The extract was treated with isopropanol and ethanol to effect precipitation. The RNA precipitate was resuspended with a buffer containing a hexadeoxinucleotide random primer (Invitrogen) and M-MLV reverse transcriptase (Invitrogen) and incubated for 1 h at 37℃ to achieve synthesis of cDNA. PCR amplification using sense and antisense primers labeled with ^{32}P as shown in Table 1 and Taq DNA polymerase (Takara Taq™ Hot Start Version; Takara, Tokyo) was conducted to create recognition sites for restriction enzyme *Alu* 1 (for 480-A in the type 1 virus), *ScrF* 1 (for 525-C in the type 1 virus) and *Mbo* 1 (for 472-C in the type 3 virus). After treatment of the amplified DNA product with the restriction enzyme, the digested material was separated by polyacrylamide gel

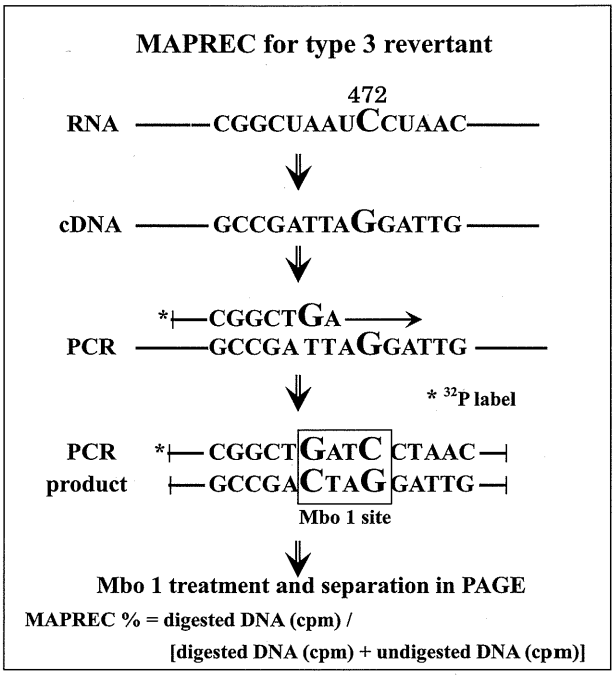

Fig. 2. MAPREC procedure for detection of 472-C reversion in type 3 vaccine viruses.

Table 1 PCR primers and restriction enzymes used to quantify mutations by MAPREC

Nucleotide	Primer name	Primer sequence	Restriction enzyme	Mutation type
Type 1				
480	pS-1/453	(421) AGCCTATTGG GCTACATAAG AATCCTCCGG CCC (453)	*Alu* 1	480-A
	pA-1/482	(513) CGACAGGCCA ATCACTGGTT TGTGACCACC AG (482)		
525	pS-1/516	(485) GTGGTCACAA ACCAGTGATT GGCCTGTCGT AA (516)	*ScrF* 1	525-C
	pA-1/527	(560) AACACGGACA CCCAAAGTAG TCGGTTCCGC TCCG (527)		
Type 3				
472	pS-3/471	(440) TGAGAGTCCT CCGGCCCCTG AATGCGGCTG AT (471)	*Mbo* 1	472-C
	pA-3/502	(532) ACGGACTTGC GCGTTACGAC AGGCTGGCTGC (502)		

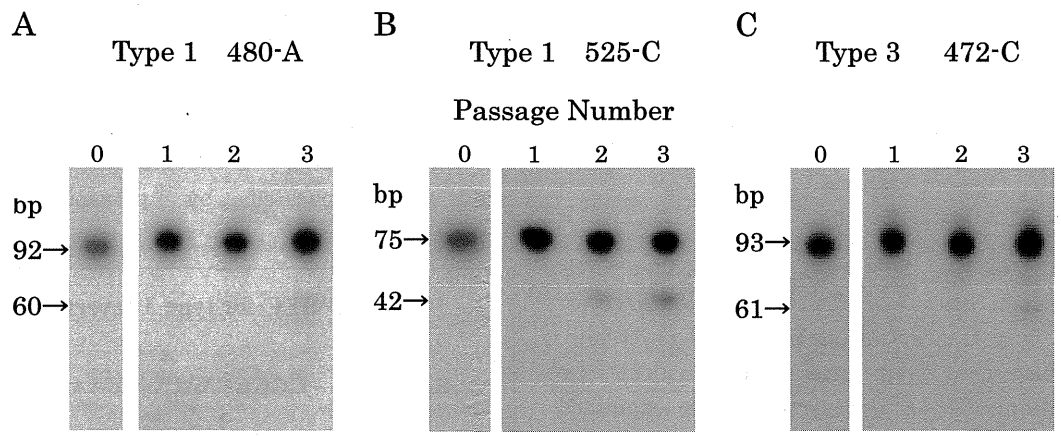

Fig. 3. Autoradiography of a polyacrylamide gel containing Alu 1 (A), ScrF 1 (B) and Mbo 1 (C) -digested PCR products of the type 1 and 3 viruses passaged in Caco-2 cells at the temperature of 37℃. PCR products (92, 75 and 93bp) and restriction enzyme-digested PCR products (60, 42 and 61bp) are shown by arrows.

electrophoresis. The MAPREC percentages of 480-A, 525-C and 472-C were calculated by measuring the radioactivity in counts per minute (CPM) of digested and undigested DNA bands with *Alu* 1, *ScrF* 1 or *Mbo* 1, using the equation: digested DNA (CPM)/ [digested DNA (CPM) + undigested DNA (CPM)].

Results

1) Electrophoresis patterns of passaged viruses on MAPREC

The Sabin vaccine viruses were passaged in Caco-2 cells with a virus concentration of approximately $10^{-1.5}$～10^{-2} $TCID_{50}$ /cell at a temperature of 37℃. The proportions of type 1 passaged viruses containing A at position 480 (480-A reversion), C at position 525 (525-C reversion), and type 3 passaged viruses containing C at position 472 (472-C reversion) were analyzed by MAPREC. The electrophoresis patterns of type 1 and 3 passaged viruses by MAPREC are shown in Fig. 3. After amplification of cDNA by PCR with synthetic primers modified to create the restriction enzyme *Alu* 1 or *ScrF* 1 recognition site in the specific region containing nucleotide position 480 or 525 in type 1 passaged viruses, the proportions of 480-A reversion and 525-C reversion were estimated by the *Alu* 1 or *ScrF* 1 digestion of PCR products (92 and 75 base pairs, respectively) and separation in a polyacrylamide gel (Fig.3A,B). The restriction enzyme-digested DNA band appears intensively when the revertants on 480-A or 525-C are contained abundantly in the passaged viruses. Although the *Alu* 1-digested DNA band (60 base pairs) hardly appeared in passaged viruses (Fig.3A), the *ScrF* 1-digested DNA band (42 base pairs) appeared intensively in viruses passaged the second time and third time (Fig.3B). It was demonstrated that the revertants on 525-C are contained abundantly in the passaged viruses. Also the proportion of 472-C reversion was estimated by the *Mbo* 1 digestion of PCR products (93 base pairs) in type 3 passaged viruses (Fig. 3C). The *Mbo* 1-digested DNA band (61 base pairs) appeared in viruses passaged the second time and third time.

2) Accumulation of revertants in vaccine viruses passaged in Caco-2 cells

Accumulations of 480-A and 525-C reversions in type 1 and 472-C reversion in type 3 vaccine viruses during passaging in Caco-2 or HEp-2 cells were analyzed by the MAPREC method (Fig.4). Percentages of reversions (%480-A, %525-C and %472-C) were calculated by measuring the radioactivity of digested and undigested DNA bands with *Alu* 1, *ScrF* 1 or *Mbo* 1, as described in the Methods section. The accumulation of reversion in type 1 vaccine viruses increased rapidly with passaging in Caco-2 cells at the temperature of 37℃ (Fig.4A). However, the accumulation was hardly observed at

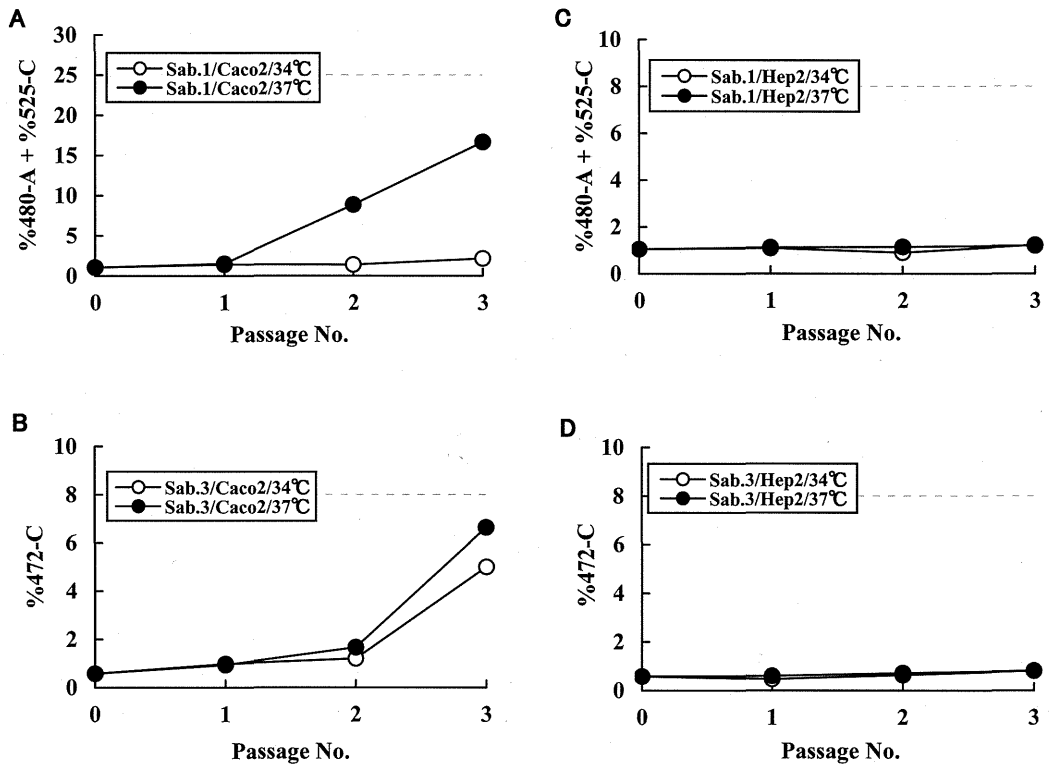

Fig. 4. Accumulations of 480-A and 525-C reversions (%480-A + %525-C) in type 1 (A and C) and 472-C reversions (%472-C) in type 3 (B and D) vaccine viruses during passaging in Caco-2 cells (A and B) or HEp-2 cells (C and D) at the temperature of 34℃ (solid circles) or 37 ℃ (open circles).

the temperature of 34℃. On the contrary, the accumulation of 472-C reversion in type 3 vaccine viruses increased with passaging in Caco-2 cells at both temperatures (37 ℃ and 34 ℃) (Fig.4B). Rezapkin et al. (1994) and Chumakov et al. (1999) demonstrated that a stipulated cut-off percentage of the type 1 (%480-A + %525-C) or type 3 (%472-C) vaccine virus for passing or failing the monkey neurovirulence test was approximately 5% and 1%, respectively. The percentages of reversions of the type 1 and 3 vaccine viruses passaged in Caco-2 cells easily exceeded the stipulated cut-off, particularly, at the cultivation temperature of 37℃.

Furthermore, accumulation of reversion in vaccine viruses during passaging in HEp-2 cells was analyzed by MAPREC. The accumulation of reversion in type 1 and 3 vaccine viruses was hardly observed at either cultivation temperature (37℃ and 34 ℃) (Fig.4C,D). A large difference was demonstrated in the accumulation of reversion between Caco-2 and HEp-2 cells.

Discussion

In the three serotypes of polio vaccine viruses, it has been reported that VAPP is caused frequently by the type 3 virus (Nkowane et al.; 1987) and epidemics of poliomyelitis by VDPV have occurred frequently due to the type 1 virus (see Fig.1). Therefore, analysis of the accumulation of reversion of type 1 and 3 polio vaccine viruses was performed in this study. Polio vaccine viruses cause the rapid reversion to the neurovirulent genotype on the virus genome during repeated replication in the human alimentary tract, and the revertants (virulent VDPV) continue to be excreted with the feces into the environment. The problem of VDPV is an obstacle for the achievement of the polio eradication

program. Fortunately, poliomyelitis cases caused by VDPV have not been reported in Japan. However, virulent VDPVs have been easily isolated from river and sewage water in Japan (Yoshida et al.; 2000, 2002, Horie et al.; 2002). It is considered that the reason why an epidemic caused by VDPV has not occurred in Japan is due to high vaccine coverage of more than 80%. Actually, the efficacy of Japan's vaccination policy against virulent VDPV has been confirmed (Iwai et al.; 2006, 2008). The epidemics caused by VDPV will be prevented as long as sufficient herd immunity is maintained. In other words, an outbreak of poliomyelitis caused by VDPV will occur if the viruses infect individuals who have insufficient antibody titers, or when the vaccination coverage decreases. Actually, most epidemics of poliomyelitis caused by VDPV occur in regions where the vaccine coverage decreased (Centers for Diseases Control and Prevention; 2006).

In this study, accumulation of the reversion of polio vaccine viruses to the neurovirulent genotype was analyzed by the MAPREC method. MAPREC was established by Chumakov et al. (1991, 1992); a non-RI method was later developed by Horie et al. (1998) to estimate the ratio of revertants in a virus population. MAPREC can be used as an effective method for a quality control test for OPV production and the characterization of cultured polio vaccine viruses on cells. Actually, MAPREC has been described in WHO requirements for OPV production as one of the quality control tests (World Health Organization; 2002). It has been reported that the ratio of nucleotide change in the bases at position 480 and 525 (MAPREC percentage of 480-A and 525-C) for the type 1 poliovirus correlates with neurovirulence in monkeys (Rezapkin et al.; 1994). Also, the type 3 poliovirus provides sound evidence that a single nucleotide change in the base at position 472 (MAPREC percentage of 472-C) in the genome correlates directly with neurovirulence (Chumakov et al.; 1999). These bases are contained in the region designated "internal ribosomal entry site (IRES)", and the stability of this region has a considerable influence on the translation efficiency of the virus-specific protein and neurovirulence (Pilipenko et al.; 1989, Skinner et al.; 1989). In the case of the type 1 vaccine virus, position 525, which is base paired with position 480 in a stem and loop structure of the F-domain in the IRES region, becomes an unstable base pair (480-G-525-U) which may alter a stable pair, virulent bases 480-A (480-A-525-U) or 525-C (480-G-525-C), during multiplication in the human alimentary tract. The stable base pairs are more effective in promoting translation initiation of virus-specific protein as compared with the unstable base pair (Muzychenko et al.; 1991).

It has been reported that the accumulation of reversion of polio vaccine viruses to the neurovirulent genotype on cultured cells occurs easily at a virus cultivation temperature of 37℃ (Chumakov et al.; 1994, Rezapkin et al.; 1994, Taffs et al.; 1995, Horie et al.; 1999). In this study, a similar tendency was observed in Caco-2 cells. However, accumulation of reversion was hardly observed in HEp-2 cells even at the cultivation temperature of 37℃. It is extremely interesting that a difference was shown in the frequency of the reversion according to cell type even though both cells derive from human carcinoma. If the cellular factors which take part in the reversion of the vaccine virus genome are elucidated, it is expected to lead to the development of a controlling expedient for the reversion of the vaccine virus.

In the near future, the eradication of the wild poliovirus will be achieved globally as a result of the polio eradication program. However, VDPV exists in the environment as long as OPV is used. It is extremely important to analyze the properties of VDPV to evaluate the risk of outbreak after OPV administration is stopped in the final phase of the eradication program. Although it is considered that the introduction of an inactivated poliovirus vaccine (IPV) which does not have the VDPV problem is necessary, it is extremely difficult to supply IPV to all developing countries considering the manufacturing capacity and the cost. Research on the development of a controlling expedient for the reversion of the vaccine virus will be important for the achievement of the polio eradication program in the future.

Conclusion

In this study, a large difference was observed in the frequency of neurovirulent reversion of polio

vaccine viruses between Caco-2 and HEp-2 cells even though both cells were derived from human carcinoma. In the future, to elucidate the cellular factors which take part in the reversion frequency of the vaccine virus genome is extremely important, because such research is expected to lead to the elucidation of the reversion mechanism and to the development of a controlling expedient for the neurovirulent reversion of the polio vaccine virus. Research on the development of a controlling expedient for the reversion will contribute to the achievement of the global polio eradication program by WHO.

Acknowledgement

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(Summary)

ヒト消化管由来細胞におけるポリオワクチンウイルスの毒力復帰変異の解析

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背景　経口生ポリオワクチンとして使用されている弱毒ポリオワクチンウイルスは、ヒトの消化管において毒力復帰変異を起こし易いことが知られており、そのウイルスが原因と考えられるポリオ（急性灰白髄炎）の発症例も報告されている。更に、その変異ウイルスは、糞便とともに環境中に排泄されることで、世界中でポリオの新たな流行を引き起こしており、ポリオ根絶計画達成の妨げとなっている。

目的　本稿では、ヒト消化管におけるポリオワクチンウイルスの毒力復帰変異機構を明らかにすることを目的として、ワクチンウイルスをヒトの消化管由来培養細胞を用いて継代培養し、継代によってどの程度変異ウイルスが出現（蓄積）するか解析を行った。

方法　ヒトの大腸ガン由来細胞を用いて37℃あるいは34℃でポリオワクチンウイルスを3代継代培養し、その培養ウイルス液中の毒力復帰変異ウイルスの混入割合（変異ウイルスの蓄積）について、遺伝子レベルでウイルスの神経毒力の強さを測定することができるMAPRECと呼ばれる方法で解析した。

結果　継代による毒力復帰変異ウイルスの蓄積は、37℃で培養した方が34℃で培養したときと比べて明らかに高かった。しかしこの変異ウイルスの蓄積は、ヒトの喉頭ガン由来細胞であるHEp-2細胞においてはほとんど観察されなかった。

結論　本研究より、細胞の種類や培養温度により毒力復帰変異ウイルスの出現頻度（蓄積の程度）に大きな違いが見られた。今後は、どのような細胞側因子がこの違いに関与しているかを解明することが重要である。そしてそのような研究は、ワクチンウイルスの毒力復帰変異機構の解明と、その抑制手段の開発に結びつくことが期待される。

キーワード　ポリオウイルス、経口生ポリオワクチン、ワクチン由来ポリオウイルス、ポリオ根絶計画、毒力復帰変異