CONSTRUCTION OF A RECOMBINANT VIRUS BETWEEN POLIOVIRUS AND COXSACKIE A VIRUS 11

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ABSTRACT

Recent outbreaks of circulating vaccine-derived poliovirus (cVDPV) revealed the possibility of recombination between vaccine strains poliovirus (PV) and cluster C enterovirus. Based on genetic analysis, it is assumed that coxsackie A virus 11 (CAV-11), one of the cluster C enterovirus, may naturally recombine with PV. To elucidate this hypothesis, the chimeric virus between PJ156, a type 1 cVDPV isolate isolated from an acute flaccid paralysis (AFP) case in the Philippines in 2001, and CAV-11 (PJ156/CAV-11) was constructed by using long-PCR method. As the results, PJ156/CAV-11 was viable in HEp-2 cell line. The PJ156/CAV-11 exhibited mostly similar phenotype with parental PJ156 in term of plaque size, viral growth and neurovirulence. These results suggested that recombination between PV and CAV-11 might naturally occur during transmission of vaccine strains in the community. The effect of recombination on the viral phenotype is significantly depending on the counterpart virus.

Key words: Poliovirus, recombination, CAV-11

ABSTRAK

Wabah polio yang disebabkan oleh virus keturunan galur vaksin (circulating vaccine-derived poliovirus, cVDPV) yang terjadi belakangan ini memperlihatkan kemungkinan rekombinasi antara virus polio galur vaksin dengan virus entero klaster C. Berdasarkan analisa genetika, diperkirakan virus coxsackie A 11 (CAV-11), salah satu virus entero klaster C, berekombinasi dengan virus polio secara alami. Untuk membuktikan hipotesa ini, virus kimera antara PJ156, tipe 1 cVDPV yang diisolasi dari pasien polio di Filipina tahun 2001, dan CAV-11 (PJ156/CAV-11) dikonstruksi secara artifisial dengan teknik long-PCR. Sebagai hasilnya, PJ156/CAV-11 bisa hidup dan berkembangbiak di dalam sel HEp-2. Virus rekombinan PJ156/CAV-11 memiliki karaktersitik yang mirip dengan PJ156 dalam hal ukuran plague, kecepatan pertumbuhan, dan virulensi. Hasil ini membuktikan bahwa rekombinasi antara virus polio dan CAV-11 bisa terjadi secara alami pada saat galur vaksin menyebar dalam suatu komunitas. Efek rekombinasi terhadap karakter virus sangat ditentukan oleh virus lawan rekombinasi.

Kata kunci: Poliovirus, rekombinasi, CAV-11

INTRODUCTION

Immunization with the oral poliovirus vaccine (OPV) is the cornerstone of the World Health Organization's program for the global eradication of poliomyelitis. The attenuated OPV strain of the three poliovirus serotypes (Sabin 1, 2, and 3) replicate in the gut of OPV recipients and can efficiently induce type-specific humoral and mucosal immunity (Sutter *et al.*, 2003). However, replication of OPV in humans is frequently accompanied by genetic change of the vaccine virus, including reversion mutations (Minor and Dunn, 1988; Bouchard *et al.*, 1995) and intertypic recombination among OPV strains (Cammack *et al.*, 1988; Furione *et al.*, 1993). The phenotypic reversion of the OPV strains to neurovirulence is the underlying mechanism for the rare cases of vaccine-associated paralytic poliomyelitis (VAPP) among OPV recipients or

their close contacts (Minor and Almond, 2002; Strebel *et al.*, 1992; Sutter *et al.*, 2003).

The large majority of OPV isolates from healthy individuals, the environment, or patients with VAPP are closely related to the original OPV strain (Sabin-like), diverging by <1.0% of nucleotide sequences encoding the major capsid protein VP1 (Matsuura *et al.*, 2000). The low nucleotide sequence diversities from the respective OPV strains are consistent with the short duration of most poliovirus infections (Alexander *et al.*, 1997) and the usually restricted spread of OPV virus (Benyesh-Melnick *et al.*, 1967). In fact, no paralytic poliomyelitis outbreak associated with such recombinant viruses has occurred up to now. However, incidents of paralytic poliomyelitis outbreaks due to circulating VDPV (cVDPV) were reported recently; respectively in Dominican Republic and Haiti (Kew *et al.*, 2002), the Philippines (Shimizu *et al.*, 2004), Egypt (Yang *et al.*, 2003), and Madagascar (Rousset *et al.*, 2003). Sequence analysis showed that those cVDPVs were recombinant viruses between PV and unidentified enterovirus that underwent the recombination in non-capsid region. Although the significance and mechanism of natural genetic recombination are not still obvious, four recombinant cVDPV outbreaks might suggest a biological role of the genetic recombination in poliovirus evolution, especially for the prolonged circulation of OPV-related PV.

Detail mechanism of PV recombination is not well known. However, based on the current knowledge, the recombination occurs by copy choice mechanism with homologous genome templates (Wimmer *et al.*, 1993). Accordingly, PV prefers to recombine with viruses belong to cluster C enterovirus, which composed by PV and some coxsackie A viruses (CAVs). Furthermore, PV is assumed to only recombine with some specific CAVs which genetically closely related to them rather than with all CAVs. Recent study demonstrated that among CAVs within cluster C enterovirus, CAV-11, CAV-17, and CAV-20 have a high homology with PV (>95%) in between 2B and 3D regions (Brown *et al.*, 2003). From these results, it is suggested that PV might recombine with CAV-11, CAV-17 or CAV-20 in between 2B and 3D regions. So far, however, there is no direct evidence to prove this hypothesis. In this study, we artificially constructed and characterized the chimeric virus between PV and CAV-11.

MATERIALS AND METHODS

Viral RNA extraction and construction of chimeric cDNA

PJ156 isolate, isolated from an AFP case in the Philippines in 2001 (Shimizu *et al.*, 2004), and CAV-11 stored in our laboratory were used as parental viruses. Viral RNAs were extracted from freeze-thaw lysates of infected cells culture supernatants using High Pure Viral RNA Kit (Roche, Germany). Chimeric cDNAs between PJ156 and CAV-11 was constructed by long PCR method (Fig. 1) (Dekker et al., 2000; Morita *et al.*, 2001). The 5' and 3' end of the genome of both viruses were separately amplified by RT-PCR reaction using Titan One Tube RT-PCR System (Roche). The primers were designed to produce fragments contained a small overlapping sequence. The primers were as follows; P3A, 5'-GAGGCATGCTAATACGACTCACTATA GGTTAAAACAGCTCTGGG-3' (the underline is T7 promoter); P4, 5' GGGA ATTCT TTTTTTTTTTTTTTTTTTTT CTC CGAATTAAAG-3'; A, 5'-CCGC-CAAGGAGA TAGCTGGCTCAAG AAGTTCACCGAGG CGTGCA ACGC-3'; B, 5'- GCGTTGCACGC CTCGGTGA ACTT CTTGAG CCAGCTATC TCCTTG GCGG-3'. Twenty five cycles of PCR reaction was performed after 40 min of RT reaction. Amplified cDNA fragments were gel-purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and concentrated by ethanol precipitation. These fragments were then fused to each other in a PCR reaction using Expand™ Long Template PCR System (Roche, Germany) without primers to obtain a target of chimeric full-length cDNA. The fusion products were further amplified in another PCR reaction with 5'- and 3'-end primers by the same PCR kit.

RNA transcription and transfection

Full-length chimeric cDNAs were transcribed, and were mixed with 100 µl of 0.5 mg/ml DEAE dextran in 1 × HeBSS buffer (5 g/L of HEPES, 8 g/L of NaCl, 0.36 g/L of KCl, 0.125

g/L of $Na_2HPO_4 \cdot 2H_2O$, 10 g/L of dextrose) (Lu *et al.*, 1995). The transcript-DEAE dextran mixtures were spread over HEp-2 cells in 24-well plates. The cells were then rocked on a shaker for 30 min at room temperature. The mixtures were aspirated off, and the cells were incubated at 35.0°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% bovine calf serum (maintenance medium). Cytophatic effect (CPE) was observed until 7 days. Recombinant viruses which showed CPE were subsequently infected to HEp-2 cells to confirm the infectivity of the viruses. If CPE was not observed, a three time's blind passage was performed to confirm that no virus recovered from the RNA transfection.

Plaque purification of the recombinant virus

All recovered viruses were cloned by plaque assay on HEp-2 cells monolayer (Arita *et al.*, 2005). Tenfold serial dilution of viruses prepared in maintenance medium were inoculated in HEp-2 cells at 6-well plates, and incubated at 35.0°C for 30 min. The cells were covered with 2 ml of 0.5% Agarose-ME in DMEM with 5% bovine calf serum. After incubation at 35.0°C for 3 days, plates were stained with 2 ml of 0.5% neutral red in maintenance medium contained 0.5% Agarose-ME. Plaque size was measured, and plaque numbers were calculated after incubation at 35.0°C for another 1 day.

One-step growth curve and temperature sensitivity analyses

One-step growth curve experiments were conducted by infecting monolayer of HEp-2 cells with viruses at a multiplicity of infection (MOI) of 10 $CCID_{50}$ per cell (Shimizu *et al.*, 2004). At different time post infection, the cells and supernatant were collected, frozen and thawed three times, and centrifuged to remove cell debris. Virus titers in the supernatants were determined by the end-point dilution method in HEp-2 monolayer cultures in 96-well plates at 35.0°C. To test the temperature sensitivity, one-step growth experiments were carried out at 35.0°C and 39.5°C, respectively.

Neurovirulence test

Groups of eight (four male and four female) 5-week-old PV receptor-transgenic (TgPvr) mice were inoculated intracerebrally with 30 µl virus (Shimizu *et al.*, 2004). Tenfold dilutions of virus were made in the maintenance medium, and each mouse received approximately $10^{1.3}$ to $10^{6.3}$ of $CCID_{50}$. Mice were observed for 14 days for paralysis and/or death. The amount of virus that caused 50% paralysis and/or death dose (PD_{50}) was calculated.

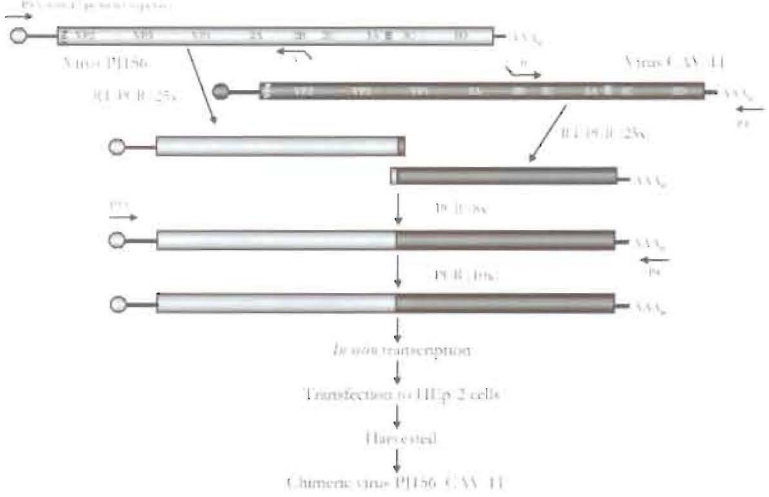


Figure 1. Strategy for construction of chimeric virus PJ156/CAV-11

RESULTS

Construction chimeric virus PJ156/CAV-11

Chimeric cDNAs between PJ156 and CAV-11 (PJ156/CAV-11) with crossover site in 2C-coding region (amino acid no. 8 of 2C) was constructed. After RNA transfection to HEp-2 cells following by incubation at 35.0°C, PJ156/CAV-11 showed CPE, implied that the virus was viable. These results suggested that recombination could occur between PJ156 and CAV-11 in 2C region. After plaque purification, DNA sequence of VP1, 3D, and surrounding crossover site of the recombinant virus were determined. Sequence analysis showed that no mutation was found in VP1 region of PJ156/CAV-11 (Table 1). One mutation was found in recombination site; however this mutation was silent mutation. Only one amino acid substitution was found in 3D region of PJ156/CAV-11. None of these amino acid substitutions was crucial for viral growth, as well as for other phenotype including neurovirulence.

Plaque assay of recombinant PJ156/CAV-11

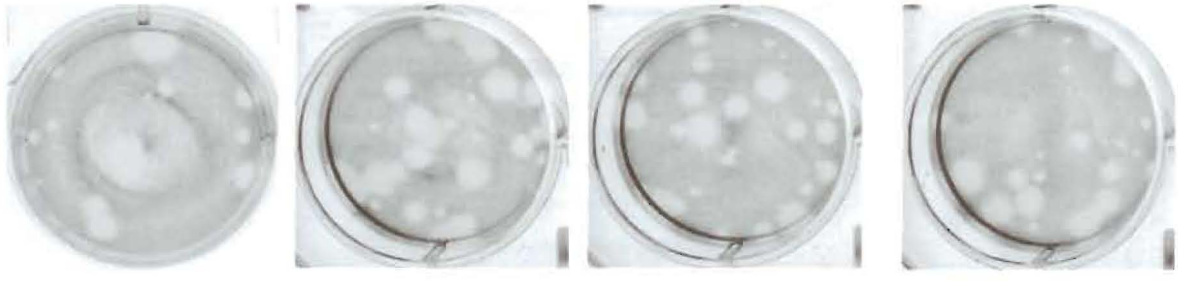
Plaque assay of recovered recombinant PJ156/CAV-11 virus along with parental PV such as Sabin 1, Mahoney, and PJ156 was performed to analyze the effect of recombination on viral phenotype. As the result, the recombinant PJ156/CAV-11 expressed similar plaque size (1-5 mm) with parental PJ156, recombinant viruses PJ156/Sabin 1 and PJ156/Mahoney (Fig. 2). The recombinant virus also showed similar virus titer with parental PJ156 and both PJ156/Sabin 1 and PJ156/Mahoney recombinant viruses (1.4×10^8 pfu/ml) (Table 1).

Table 1. Plaque phenotype and amino acid substitution in some regions of each recombinant virus

Virus	Location of crossover site[a]	Plaque size (mm)	Titer (pfu/ml)	Nucleotide mutation in		
				VP1[b]	Crossover site[b]	3D[b]
PJ156	-	1-5	6.6×10^8	-	-	-
PJ156/Sabin 1	2B (39)	1-5	1.5×10^8	0 (0)	2 (0)	1 (1)
PJ156/Mahoney	2B (39)	1-5	1.4×10^8	1 (0)	5 (2)	2 (0)
PJ156/CA-11	2C (8)	1-5	1.4×10^8	0 (0)	1 (0)	5 (1)

[a]No. in parenthesis describes no. of amino acid in each protein

[b]No. in parenthesis describes no. of amino acid substitution in entire VP1 and 3D regions, and 1.52 kb of surrounding crossover site, [c]Not determined, [d]Not applicable



PJ156 *PJ156/Sabin 1* *PJ156/Mahoney* *PJ156/CAV-11*

Figure 2. Plaque sizes of the parental and recombinant viruses.

One-step growth of recombinant viruses

One-step growth experiment of the recombinant viruses along with parental PJ156 virus at 35.0°C and 39.5°C was conducted to identify the effect of recombination on viral growth and temperature sensitivity. PJ156/CAV-11 showed mostly similar growth compared parental PJ156 both at 35.0°C and 39.5°C (Fig. 3). No significant difference of growth between two different temperatures. These results implied that recombinant PJ156/CAV-11 was not temperature sensitive.

Neurovirulence test

To analyze the effect of recombination on viral neurovirulence, groups of 8 PV receptor-expressed transgenic mice (TgPvr mice) were intracerebrally inoculated with the recombinant virus. As shown in Table 2A, PJ156/Mahoney exhibited 50% paralysis and/or death dose (PD_{50}) of 3.3, which mostly similar with Mohaney strain. In another experiment, it was shown that parental PJ156 possessed a similar PD_{50} value with Mahoney strain (Table 2B). These results suggest that recombination of PJ156 with Mahoney did not cause any pathogenic change.

PJ156/Sabin 1 showed PD_{50} values of 4.8, indicating lower neurovirulence than that of Mahoney strain (Table 2A). The recombinant PJ156/CAV-11 showed a PD_{50} of 5.3, which higher than that of PJ156/Sabin 1 (Table 2A). These results suggested that recombination with CAV-11 make PJ156 become less neurovirulence, even though if compared with recombination with Sabin 1 (vaccine strain). These results also suggested that the neurovirulence of recombinant viruses were determined by the counterparts of recombination.

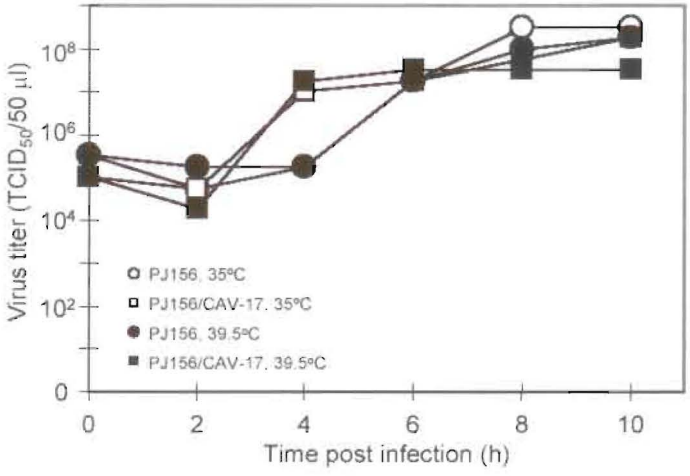


Figure 3. One-step growth of the recombinant virus

Table 2. Neurovirulence of recombinant viruses on Tg-mouse

(A)

Virus	Dose (log $CCID_{50}$/mouse)							PD_{50}[c]
	7.28	6.28	5.28	4.28	3.28	2.28	1.28	
Sabin 1	2/8[a]	ND[b]	ND	ND	ND	ND	ND	>7.53
Mahoney	ND	ND	ND	7/8	3/8	1/8		3.41
PJ156/CA-11	ND	8/8	4/8	0/8	0/8	0/8	ND	5.28

(B)

Virus	Dose (log $CCID_{50}$/mouse)							PD_{50}[c]
	8.18	5.78	4.78	3.78	2.78	1.78	0.78	
Sabin 1	5/8[a]	ND[b]	ND	ND	ND	ND	ND	>8.06
PJ156	ND	ND	ND	5/8	0/8	0/8	0/8	3.66

[a] No. of paralyzed or dead mice/no. of total mice, [b] Not determined,
[c] PD_{50} was calculated by the Karber formula

DISCUSSION

Analysis of four independent incidents of paralytic poliomyelitis outbreaks due to circulating VDPVs (cVDPVs) in Dominican Republic and Haiti, Egypt, Philippines and Madagascar revealed that those cVDPVs were recombinant viruses between vaccine strains and non-polio enterovirus that underwent recombination in non-capsid region (Yang *et al.*, 2003; Rousset *et al.*, 2003; Shimizu *et al.*, 2004). However, the exact counterparts of recombination were not identified for those recombinant viruses. Because the recombination occurs by copy choice mechanism with homologous genome templates (Wimmer *et al.*, 1993), PV including vaccine strain prefers to recombine with viruses which belong to cluster C enterovirus, which composed by PV and some coxsackie A viruses (CAVs).

The complete genomic sequence data of most CAVs which belong to cluster C enterovirus, which recently determined (Brown *et al.*, 2003), allows us to compare the homology between PVs, including cVDPVs, and those CAVs. Similarity plot has demonstrated that CAV-11 have a high homology with cVDPVs in between 2B and 3D regions. It is suggested that vaccine strains might recombine with CAV-11 in between 2B and 3D regions; however, there is no direct evidence to prove this hypothesis. By using long PCR method, a recombinant virus between PV (cVDPV) and CAV-11 was constructed. As the result, recombinant virus PJ156/CAV-11 was viable. Sequence analysis of resulted recombinant virus showed very few mutation in VP1 and 3D regions, as well as surrounding crossover site, suggesting that viability of viruses did not affected by those mutation. These results also suggest that recombination can occur between PJ156 and CAV-11 in this region in nature, consistent with the initial hypothesis. These are the first direct evidence that confirm recombination between PV and CAV-11.

No significant plaque size difference between recombinant PJ156/CAV-11 and parental PJ156, and recombinants PJ156/Sabin 1 and PJ156/Mahoney as well (Table 1 & Fig. 2). Likewise, the PJ156/CAV-11 expressed similar virus titer (Table 1) and viral growth (Fig. 3) with PJ156 and recombinant viruses PJ156/Sabin 1 and PJ156/Mahoney.

The effect of recombination counterpart sequences also can be seen in neurovirulent characteristics. Recombinant PJ156/Mahoney exhibited PD_{50} which was mostly similar with Mohaney strain (Table 2B). This result suggested that recombination of PJ156 with Mahoney did not cause any pathogenic change. PJ156/Sabin 1 showed PD_{50} values of 4.6, indicating lower neurovirulence than that of Mahoney strain. Moreover, the PJ156/CAV-11 did showed PD_{50} values of 5.3. These results indicated that recombination with Sabin 1, as well as with CAV-11, made parental PJ156 become less neurovirulence. It was assumed that by recombining with other enteroviruses, VDPV may acquire the neurovirulence and transmission characteristics similar with the wild type PV.

Since PJ156/CAV-11 did not expressed extreme neurovirulence on TgPvr mice, it is suggested that not all recombination results in acquirement of wild type characteristics. However more studies need to be done in order to elucidate the contribution of recombination on PV phenotype.

ACKNOWLEDGEMENTS

This research was supported by the Japanese Society for Promotion of Sciences (JSPS) Postdoctoral grant to AU.

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