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ASSESSMENT OF EFFICACY OF A LIVE ORAL POLIOVIRUS VACCINE FOR VIRULENT SABIN-LIKE POLIOVIRUS 1 STRAINS IN JAPAN

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Summary. – Virulent Sabin-like poliovirus (VSLP) was isolated from river and sewage waters between October 1993 and September 1995 in Toyama Prefecture, Japan (Yoshida *et al.*, *Lancet* **356**, 1461–1463, 2000). In this study, to assess the possibility of an epidemic of poliomyelitis caused by a VSLP in Japan under the current vaccination policy of administration of live attenuated oral poliovirus vaccine (OPV), we determined titers of serum neutralizing antibodies to poliovirus 1 (PV-1) strains Sabin (vaccine strain), Mahoney (wild-type strain) and G4-12 (VSLP) in various groups of residents of Toyama Prefecture, Japan. The seropositivity and geometric mean neutralizing antibody titers against these strains in the individuals who obtained two doses of OPV were 99.1%, 94.5% and 95.5%, respectively, and 564, 186 and 194, respectively. Although the antibody titers to G4-12 were lower compared with those to Sabin, these results indicate that the OPV vaccination policy in Japan has been effective in preventing poliomyelitis caused by VSLPs. These results also suggest that (i) an epidemic of poliomyelitis caused by a VSLP has not occurred in Japan due to herd immunity, and (ii) the possibility of reemergence of VSLPs will be prevented if sufficient herd immunity is acquired immediately after completion of the OPV vaccination in accordance with the poliomyelitis eradication program.

Key words: poliovirus 1; poliomyelitis; oral poliovirus vaccine; inactivated poliovirus vaccine; virulent Sabin-like poliovirus

Introduction

Live OPV containing Sabin's attenuated strains of PV-1, PV-2 and PV-3, has been used as a major tool for worldwide eradication of poliomyelitis. It is usually given to an individual in three subsequent doses. It confers a high seropositive rate (seropositivity) against all three polioviruses (Lago *et al.*, 1994; Ramsay *et al.*, 1994; World Health Organization Collaborative Study Group on Oral and Inactivated Poliovirus Vaccines, 1995). Ninety-five or more percent of the recipients probably develop a life-long immunity to all three polioviruses after the vaccination (Recommendation of the Advisory Committee on Immunization Practices, 1997). 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 OPV, which was imported from Canada and Soviet Union, to children of 3 months to 12 years of age in 1961–1963, the number of patients declined dramatically; before the use of OPV, 1,000–5,000 paralytic cases of poliomyelitis were reported annually (Takatsu *et al.*, 1973; Shimojo, 1984). A two-dose administration of domestic OPV to infants of 3–48 months (in 1964–1994) and 3–90 months (since 1995 till now) of age at intervals longer than 6 weeks has been performed routinely since 1964. Till now, wild-type polioviruses were isolated from one patient with poliomyelitis in 1980, and from two patients

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Abbreviations: IPV = inactivated poliovirus vaccine; OPV = oral poliovirus vaccine; PV-1 = poliovirus 1; VDPV = vaccine-derived poliovirus; VSLP= virulent Sabin-like poliovirus

with non-acute flaccid paralysis in 1984 and 1993, respectively (Infectious Agents Surveillance Center, 1997). The two-dose administration of OPV therefore appears effective in preventing poliomyelitis caused by wild-type polioviruses in Japan.

The poliomyelitis eradication program of WHO is close to the final stage of replacing virulent wild-type polioviruses with OPV strains (World Health Organization, 2003a). Recently, epidemics of poliomyelitis by vaccine-derived polioviruses (VDPVs) have been reported worldwide (Kew *et al.*, 2004). According to WHO criteria a VDPV is defined as a strain, provided there is more than a 1% drift in the VP1 capsid protein at nucleotide level (World Health Organization, 2003b; Kew *et al.*, 2004). Fortunately, poliomyelitis cases caused by VDPVs have not been reported in Japan yet. However, a total of 78 VDPV or VSLP isolates (16 PV-1, 31 PV-2, and 31 PV-3) were obtained from river and waste waters sampled between October 1993 and September 1995. As a result of VP1 gene nucleotide sequence analysis of the isolates, their divergence from Sabin strains was less than 1.4% (Matsuura *et al.*, 2000).

Furthermore, using PCR and restriction analysis (Chumakov *et al.*, 1991, 1994), we have found virulent genotype in some isolates of PV-1 (Horie *et al.*, 2002a), PV-2 (Yoshida *et al.*, 2002) and PV-3 (Yoshida *et al.*, 2000) In particular, one of the PV-1 VSLPs, G4-12, displayed a phenotype similar to that of wild-type Mahoney strain in terms of neurovirulence, temperature sensitivity, plaque-forming ability and neutralization by monoclonal antibodies (Horie *et al.*, 2002b). Nevertheless, G4-12 was categorized as a VSLP because of its VP1 nucleotide divergence from that of the Sabin strain of 0.6% only. Earlier, we have suggested the possibility of existence of VSLPs in the community as a result of environmental surveillance (Yoshida *et al.*, 2000; Horie *et al.*, 2002a). We believe that the reason why an epidemic of poliomyelitis caused by a VSLP has not occurred in Japan is the herd immunity.

To confirm this idea, we determined neutralizing antibody titers against PV-1 strains Sabin, Mahoney and G4-12 in the sera of various groups of residents of Toyama Prefecture, Japan. In this way, we attempted to assess retrospectively the efficacy of the current poliovirus vaccination policy, namely the two-dose administration of OPV in preventing epidemics of poliomyelitis caused by VSLPs in Japan.

Materials and Methods

Viruses. The strains Sabin and Mahoney of PV-1 were obtained from the National Institute of Infectious Diseases, Tokyo, Japan. The G4-12 strain of PV-1 (VSLP) was originally isolated from a sewage disposal plant located downstream of the Oyabe River, Toyama Prefecture, Japan in 1993. Its divergence from the Sabin strain of PV-1, as determined for the VP1 sequence, was 0.6% at nucleotide and 1.0% at amino acid level (Matsuura *et al.*, 2000).

Sera. A total of 244 serum samples from residents of Toyama Prefecture, Japan, of up to 76 years of age, collected in 1998, were tested. The vaccination history of individual groups of residents is shown in Table 1.

Neutralization test was performed in 96-well microtiter plates using Vero cells in standard manner (World Health Organization, 1995). Cytopathic effect was read on day 7 post infection. A sample with a neutralizing antibody titer ≥8 was regarded as positive. Geometric means of titers were used for comparison of different groups of individuals.

Results and Discussion

In the individuals who were born after the enforcement of the domestic OPV vaccination but were not vaccinated (8 sera), the seropositivity against the strains Sabin, Mahoney and G4-12 was in the range 50.0–62.5% (individual values 62.5%, 50.0% and 62.5%, respectively) (Table 1). In contrast, in the individuals of the same group (110 sera),

Table 1. Vaccination history and seropositivity against PV-1 strains Sabin, Mahoney and G4-12 of residents of Toyama Prefecture, Japan

Vaccination history (No. of OPV doses)		No. of serum samples	Seropositivity (%)		
			Sabin	G4-12	Mahoney
Residents born after 1964 (the introduction of OPV)[a]	Non-vaccinated	8	62.5	62.5	50.0
	Vaccinated[d] (1)	14	85.7	85.7	85.7
	Vaccinated[d] (1 or 2)	10	90.0	80.0	80.0
	Vaccinated[d] (2)	110	99.1	95.5	94.5
	Vaccinated[d] (unknown)	33	90.9	78.8	81.8
Residents born before 1964 (the introduction of OPV)[b]	0[c]	69	92.8	89.9	89.9

[a]Below 35 years of age in 1998.
[b]Over 35 years of age in 1998.
[c]A small number of individuals received imported OPV in 1961–1963.
[d]The OPV, prepared by the Japan Poliomyelitis Research Institute, Tokyo, Japan, contained $10^{6.0\pm0.5}$, $10^{5.0\pm0.5}$ and $10^{5.5\pm0.5}$ $TCID_{50}$ of Sabin strains of PV-1, PV-2 and PV-3, respectively, in one dose.

standard vaccination with two doses of OPV resulted in a 94.5–99.1% seropositivity (individual values 99.1%, 94.5% and 95.5%, respectively), and even the vaccination with one dose (14 sera) gave a 85.7% seropositivity. All individual values represent geometric means. These results validate the efficacy of OPV vaccination.

In the individuals who were born before the enforcement of the domestic OPV vaccination and, as a rule, were not vaccinated (69 sera), the seropositivity was 89.9–92.8% (individual values 92.8%, 89.9% and 89.9%, respectively). This relatively high seropositivity, close to that found in the vaccinated group, may be explained by natural infection, as there were still occurring epidemics of poliomyelitis caused by poor public health conditions at that time in Japan.

Neutralizing antibody titers (geometric means) against the strains Sabin, Mahoney and G4-12 of the individuals vaccinated with two doses of OPV as compared with non-vaccinated individuals were significantly higher, namely neutralizing antibody titers for these strains for vaccinated individuals were 564, 186 and 194, respectively; corresponding titers for non-vaccinated individuals were 181, 74.0 and 62.5, respectively (Fig. 1). Within the group of vaccinated individuals, the antibody titers against Mahoney and G4-12 did not differ but were lower than those against Sabin. Even though they were lower, they were still sufficiently high (about 180) to prevent poliomyelitis caused by those virulent viruses (Mahoney and G4-12). These results indicate that the Japan's OPV vaccination policy has been effective in preventing poliomyelitis caused not only by wild-type poliovirus, but also by a VSLP. Although a similar investigation of the situation with PV-2 and PV-3 VSLPs is necessary, the results with PV-1 VSLPs strongly suggest that the absence of an epidemic of poliomyelitis caused by a VSLP in Japan is due to the herd immunity.

Serum neutralizing antibody titers of all 244 individuals regardless of their grouping, presented as pairwise strain comparisons, are plotted in Fig. 2. The titers against Mahoney were much lower than those against Sabin and their correlation was low (r = 0.223). Similarly, he titers against G4-12 were lower than those against Sabin; however, they did not correlate at all (r = 0.124). On the other hand, the titers against G4-12 were very similar to those against Mahoney and their correlation was high (r = 0.933). Previously, we found that G4-12, in reacting with various monoclonal antibodies, had properties similar to Mahoney (Horie *et al.*, 2002b). The results of this study support the view that both viruses are antigenically quite similar.

In this study, we presented the concept of VSLP for the first time. A VSLP, like the G4-12 strain, with altered antigenicity and neurovirulence has existed in the environment though its divergence from the vaccine Sabin strain, based on VP1 gene, was less than 1%. VDPV/VSLP would not be eliminated from the environment as long as

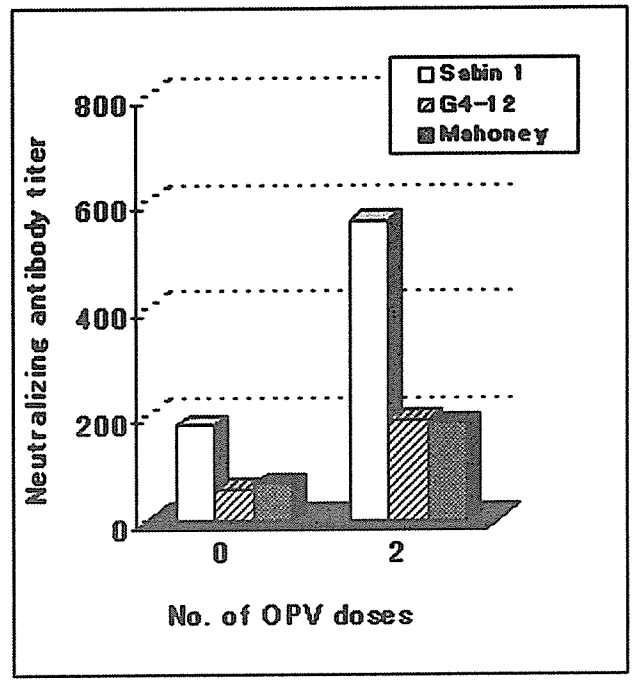

Fig. 1

Serum neutralizing antibody titers against PV-1 strains Sabin, Mahoney and G4-12 in vaccinated and non-vaccinated residents of Toyama Prefecture, Japan

Geometric means of titers in vaccinated (2 doses of OPV) and non-vaccinated individuals (no OPV dose) are indicated. Titers of $\geq 2^{10}$ and $<2^2$ were used to calculate in regard as 2^{10} and 2^2, respectively.

the use of OPV is continued. Introduction of an inactivated poliovirus vaccine (IPV) would be effective in eliminating the abovementioned viruses. After replacing OPV with IPV, VDPV/VSLP would probably remain in the environment for short time. These viruses might exist in the environment for a longer time due to immunodeficient patients excreting them for periods of approximately ten years (Kew *et al.*, 1998). Moreover, mutations in the excreted viruses may accumulate (Kew *et al.*, 1998; Bellmunt *et al.*, 1999; Martin *et al.*, 2000). It is unclear whether IPV would be effective against VDPV/VSLP, which incurs mutations in neutralizing antigenic sites. In fact, an outbreak of poliomyelitis caused by virulent variant of PV-3, comprising 9 paralytic cases and 1 non-paralytic case, occurred in Finland where IPV had been used between August 1984 and January 1985 (Hovi *et al.*, 1986). In particular, 8 of these cases had been vaccinated with 3–5 doses of IPV. Although the epidemic strain was not a VDPV/VSLP, its antigenic properties were slightly different from those of the Saukett strain, the PV-3 of IPV. The epidemics were obstructed by OPV administration. It is known that OPV induces a wider range of neutralizing antibodies compared to IPV, as the

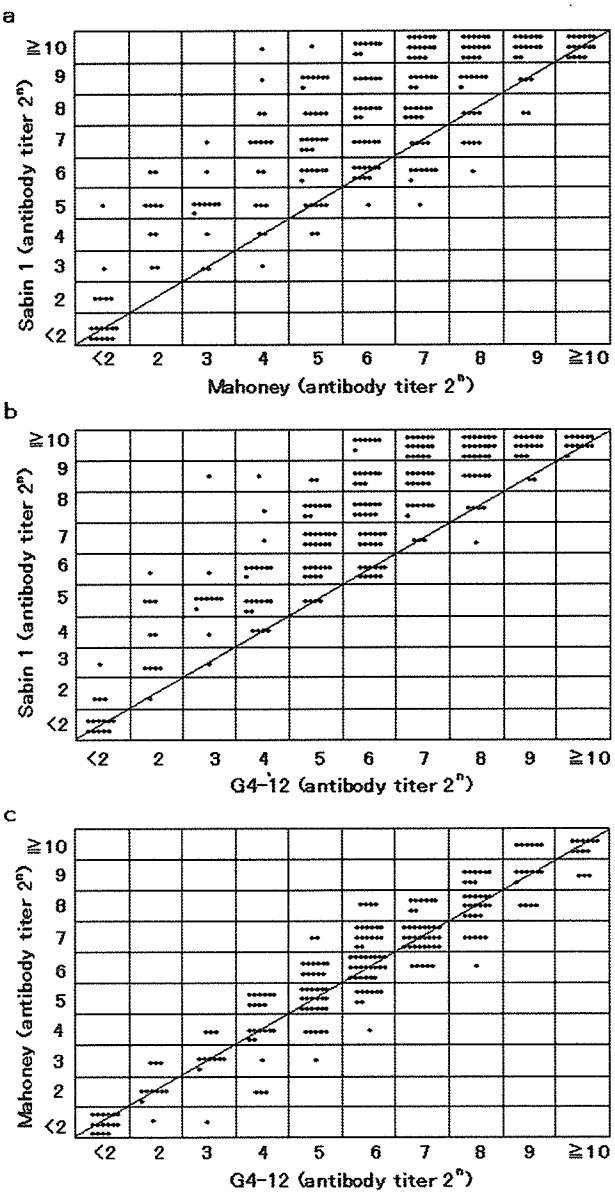

Fig. 2

Pairwise strain comparisons of serum neutralizing antibody titers for residents of Toyama Prefecture, Japan

Plots of neutralizing antibody titers for the strain pairs Sabin/Mahoney (a), Sabin/G4-12 (b), and Mahoney/G4-12 (c) for all 244 serum samples regardless of their grouping is shown in Table 1 are indicated.

vaccination with OPV is closer to the natural infection with poliovirus.

In eliminating VDPV/VSLP by replacement of OPV with IPV, it would be extremely important to continue for certain time period the investigation of the polioviruses in the form of surveillance of the environment and the population as well. Our results strongly suggest that the reason why an epidemic of poliomyelitis has not occurred in Japan is the herd immunity despite the persistence of VSLPs in the environment and/or population. They also suggest the possibility that a reemergence of VSLPs would be prevented if sufficiently strong herd immunity is acquired immediately after ending the OPV vaccination in accordance with the poliomyelitis eradication program.

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Quantitative analysis of poliomyelitis-like paralysis in mice induced by a poliovirus replicon

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Poliovirus (PV) infection causes severe paralysis, typically of the legs, by destruction of the motor neurons in the spinal cord. In this study, the relationship between PV replication in the spinal cord, damage in the motor neurons and poliomyelitis-like paralysis was analysed in transgenic mice expressing the human PV receptor (TgPVR21). First, a PV replicon encoding firefly luciferase in place of the capsid genes (PV-Fluc mc) was *trans*-encapsidated in 293T cells and the *trans*-encapsidated PV-Fluc mc (TE-PV-Fluc mc) was then inoculated into the spinal cords of TgPVR21 mice. TE-PV-Fluc mc was recovered with a titre of $6{\cdot}3 \times 10^7$ infectious units ml^{-1}, which was comparable to those of PV1 strains. TgPVR21 mice inoculated with TE-PV-Fluc mc showed non-lethal paralysis of the hindlimbs, with severity ranging from a decline in grip strength to complete flaccid paralysis. The replication of TE-PV-Fluc mc in the spinal cord reached peak levels at 10 h post-inoculation (p.i.), followed by the appearance of paralysis at as early as 12 h p.i., reaching a plateau at 16 h p.i. Histological analysis showed a correlation between the lesion and the severity of the clinical symptoms in most mice. However, severe paralysis could also be observed with an apparently low lesion score, where as few as $5{\cdot}3 \times 10^2$ motor neurons (1·4 % of the susceptible cells in the lumbar cord) were infected by TE-PV-Fluc mc. These results indicate that PV replication in a small population of the motor neurons was critical for severe residual poliomyelitis-like paralysis in TgPVR21 mice.

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INTRODUCTION

Poliovirus (PV) is a small, non-enveloped virus with a single-stranded, positive-sense genomic RNA; it is known as the causative agent of poliomyelitis and belongs to the family *Picornaviridae*. The motor neurons are the major target of PV infection in the central nervous system (CNS) (Bodian, 1949). The tropism of PV to the motor neurons is attributable in part to the expression of the PV receptor (PVR) (Crotty *et al.*, 2002; Ida-Hosonuma *et al.*, 2002; Koike *et al.*, 1994; Ren & Racaniello, 1992).

Tissues susceptible to PV infection in the CNS are limited: the brainstem, the roof nuclei of the cerebellum, the precentral gyrus of the cerebrum and the spinal cord (the cervical and lumbar cords) (reviewed by Minor, 1997). Among these tissues in the CNS, the spinal cord seemed to have a high susceptibility to PV infection: PV can adapt to the spinal cord with an increased tropism (Nathanson & Bodian, 1961) and a PV mutant that has a tropism for the spinal cord, but not for the brain, has been isolated (Jia *et al.*, 1999). The adaptation of PV was partly supported by an enhanced efficiency in the uncoating step with decreased thermostability in the virion (Couderc *et al.*, 1996). The lumbar cord supported stable replication of a PV mutant with a severe defect in viral protein synthesis, which showed unstable replication in *in vitro*-cultured cells and also in the brain (Arita *et al.*, 2004). Experimental infections of other enteroviruses, including coxsackievirus A21 (Dufresne & Gromeier, 2004) and enterovirus 71 (Arita *et al.*, 2005), also suggested that the spinal cord provides a niche for enterovirus infection.

The properties of PV infection in neurons remain controversial. Sabin vaccine strains show decreased levels of viral protein synthesis in a neuroblastoma cell line (SH-SY5Y) or in the cell lysate, compared with the parental virulent strains (Gutiérrez *et al.*, 1997; Haller *et al.*, 1996; Svitkin *et al.*, 1985, 1990). Primary hippocampal neurons of mice produce 100-fold fewer infectious particles than do fibloblasts (Daley *et al.*, 2005), although the growth of PV in a neuroblastoma cell line (SK-N-SH) (Yanagiya *et al.*, 2005) or in 293 cells (Campbell *et al.*, 2005), which retained some properties of the neuronal lineage (Shaw *et al.*, 2002), was almost identical to that in HeLa cells. The sensitivity of SK-N-SH cells to cell death was different from that of HeLa cells, and multiple rounds of PV infection were required to cause cytopathic effects (CPE) in SK-N-SH cells (Yanagiya *et al.*, 2005). Mice inoculated with PV replicons did not show noticeable

pathogenesis, despite the occurrence of replication and the expression of foreign gene products (luciferase and green fluorescent protein) (Bledsoe *et al.*, 2000), even after repetitive inoculations via the intrathecal route (Jackson *et al.*, 2001). Viral RNA was detected for at least 12 months in the spinal cord of mice in a persistent-infection model of PV (Destombes *et al.*, 1997). Therefore, neurons or cells derived from neural origins could show partial resistance to cell death caused by PV infection.

In this study, we analysed the relationship between PV replication in the spinal cord, damage in the motor neurons and poliomyelitis-like paralysis in transgenic mice expressing human PVR (TgPVR21). We performed both biological and histological analyses and estimated the number of critical motor neurons required for severe residual poliomyelitis-like paralysis in TgPVR21 mice.

METHODS

Cells, viruses and antibodies. 293T cells (human embryonic kidney cell line 293 expressing the large T antigen of *Simian virus 40*) (DuBridge *et al.*, 1987) and HEp-2c cells (a human larynx epidermoid carcinoma cell line) were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS). HEp-2c cells were used for virus titration and measurement of replication kinetics of the PV replicon. 293T cells were used for propagation of PV1 (Sabin strain) and *trans*-encapsidation of the PV replicon. Rabbit hyperimmune serum against the PV 2C protein (aa 68–329) was a kind gift from Dr Naokazu Takeda, Department of Virology II, National Institute of Infectious Diseases, Japan.

General methods of molecular cloning. *Escherichia coli* strain XL10gold (Stratagene) was used for the preparation of plasmids. Ligation of DNA fragments was performed by using a Quick Ligation kit (New England Biolabs). DNA sequencing was performed by using a BigDye Terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems) and then analysed by using an ABI PRISM 3100 genetic analyser (Applied Biosystems).

Construction of an expression vector for PV capsid proteins. For the construction of an expression vector for PV capsid proteins, we first fused the enhanced green fluorescent protein (EGFP) gene to the PV capsid protein coding region. The EGFP coding region was amplified by PCR with primers *Sac*I-EGFP+ (5′-CTCAGAGCTCTGAGCAAGGGCGAGGAGCTGTTCACC-3′) and EGFP-2A− (5′-TACGGAGCTCCGTAGGTGGTCAGGCCCTTCTTGTACAGCTCGTCCATGC-3′), using pIRES2-EGFP (Clontech) as the template. The PCR product was digested by *Sac*I and then cloned into the infectious clone of PV, pMah-*Sac*I (Arita *et al.*, 2004). The resultant plasmid was named pEGFP-Mah. Next, the EGFP gene and PV capsid protein coding region fusion was amplified by PCR with primers *Eco*RI-EGFP+ (5′-GGTGAATTCACCATGGGAGCTCTGAGCAAG-3′) and *Sal*I-PV3378− (5′-TAAGTCGACTTAATATGTGGTCAGATCCTTGG-3′), using pEGFP-Mah as the template. The PCR product was digested by *Eco*RI and *Sal*I and then cloned into the corresponding sites (*Eco*RI site and *Xho*I site) of expression vector pKS435 (a generous gift from Dr Koji Sakai, AIDS Research Center, National Institute of Infectious Diseases, Japan). pKS435 is a derivative of expression vector pKS336 (Saijo *et al.*, 2002), which expresses the inserted gene under the control of the human elongation factor-1α (HEF-1α) gene promoter (Kim *et al.*, 1990). pKS435 has the puromycin-resistance gene (*pur*) as a selection marker instead of the blasticidin S deaminase gene in pKS336. The resultant plasmid was designated pKS435-EGFP-PV CAPSID and was used for the transient expression of PV capsid proteins in 293T cells.

Construction of the PV replicon. A plasmid encoding the PV replicon with a luciferase reporter was constructed from plasmid PV-139(−) mc (Arita *et al.*, 2004). A cDNA fragment of the PV IRES (internal ribosome entry site) was amplified by PCR with primers PV110+ (5′-GCGTGAATTCACGCACAAAACCAAGTTC-3′) and PV-*Sma*I− (5′-TAACCCCGGGGTTAAAAGTCATTATGATACAATTG-3′), using plasmid pMah-*Sac*I as the template. The PCR product was digested by *Eco*RI and *Xma*I and then cloned into the corresponding sites of PV-139(−) mc. The resultant construct, encoding a PV luciferase replicon (PV-Fluc mc), was designated pPV-Fluc mc.

DNA transfection. A six-well plate (Falcon) with a 30 % confluent monolayer of 293T cells was transfected with 1 μg pKS435-EGFP-PV CAPSID DNA per well by using Effectene transfection reagent (Qiagen) and then incubated at 37 °C in 2 ml 10 % FCS/DMEM per well. The cells were washed with 10 % FCS/DMEM at 24 h post-transfection and then used for *trans*-encapsidation of the PV replicon.

RNA transfection. RNA transcripts were obtained by using a RiboMAX large-scale RNA production system – T7 kit (Promega) with *Dra*I-linearized DNA of pPV-Fluc mc as the template. RNA transcripts were transfected into a monolayer of 293T cells, which were transiently expressing PV capsid proteins, by the DEAE/dextran method (van der Werf *et al.*, 1986).

Western blot analysis. Western blot analysis was performed by using rabbit hyperimmune serum against the PV1 (Mahoney) virion (Arita *et al.*, 1998), which was a kind gift from Dr Akio Nomoto, Department of Microbiology, Graduate School of Medicine, The University of Tokyo, Japan. The samples were subjected to 5–20 % polyacrylamide gradient gel electrophoresis (e-PAGEL; Atto Corporation) in a Laemmli buffer system (Laemmli, 1970). The proteins in the gel were transferred to a PVDF filter (Immobilon; Millipore) and blocked in PBS containing 0·1 % Tween 20 and 5 % non-fat dry milk. The filters were incubated with rabbit hyperimmune serum against PV1 (Mahoney) (1 : 1000 dilution in PBS containing 0·1 % Tween 20 and 0·5 % non-fat dry milk) at room temperature for 1 h. The filters were washed by PBS containing 0·1 % Tween 20 three times for 5 min each and then incubated with donkey anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Amersham Biosciences) (1 : 2000 dilution in PBS containing 0·1 % Tween 20 and 0·5 % non-fat dry milk) at room temperature for 1 h. The filters were washed by PBS containing 0·1 % Tween 20 three times for 5 min each, and then treated with the ECL Western blotting analysis system (Amersham Biosciences) for detection of the signal.

***trans*-Encapsidation of the PV replicon.** For the preparation of seed stocks of *trans*-encapsidated PV-Fluc mc (TE-PV-Fluc mc), 293T cells in a six-well plate (Falcon) were transfected with pKS435-EGFP-PV CAPSID DNA followed by transfection of the RNA transcript of PV-Fluc mc at 24 h post-transfection, and then incubated at 37 °C in 2 ml 10 % FCS/DMEM per well. Cells were harvested when all of the cells showed CPE. For the preparation of TE-PV-Fluc mc, 293T cells transiently expressing PV capsid proteins in a 10 cm diameter dish (Falcon) were inoculated with 100 μl seed stock in 10 ml 10 % FCS/DMEM per dish, and were harvested when all of the cells showed CPE [around 48 h post-inoculation (p.i.)]. Virus stocks were stored at −70 °C.

For Western blot analysis, TE-PV-Fluc mc was purified from the cell supernatant of infected 293T cells by using DEAE/Sepharose CL-6B (Amersham Biosciences) (Arita *et al.*, 1998), followed by centrifugation at 35 000 r.p.m. for 2·5 h at 4 °C in a Beckman SW41 rotor with 1 ml of

a 30 % sucrose cushion. The pellet was washed three times with distilled water and then dissolved in 100 µl PBS at 4 °C overnight. Any remaining pellet was disrupted by pipetting and then stored at −70 °C.

Luciferase assay. For the measurement of luciferase activity in *in vitro*-cultured cells, HEp-2c cells in 96-well plates (Falcon) ($2{\cdot}8 \times 10^4$ cells per well) were infected with 100 µl of the indicated dilution or titre of TE-PV-Fluc mc. The cells were harvested at the time indicated by adding 50 µl passive lysis buffer (Promega) and 10 µl lysate was used for the measurement of luciferase activity. For the measurement of luciferase activity in the spinal cords of TgPVR21 mice, the spinal cords of inoculated mice were collected around the lumbar area (1·5–2·0 cm) at the time indicated and stored at −70 °C. After freezing and thawing of the collected spinal cords, samples were homogenized with 250 µl passive lysis buffer (Promega) and then subjected to centrifugation at 20 000 *g* for 1 min at 4 °C. Part of the supernatant (2 or 10 µl) was used for the measurement of luciferase activity with the Luciferase Assay system (Promega) and a TR717 Microplate luminometer (Applied Biosystems), according to the manufacturers' instructions.

Electron microscopy. Purified TE-PV-Fluc mc was subjected to negative staining in uranyl acetate as described previously (Utagawa *et al.*, 2002). Samples were examined by transmission electron microscopy (JEM-1220; JEOL DATUM) at an acceleration voltage of 80 kV and images were obtained at a magnification of × 50 000.

Virus titration. Virus titre was determined by measuring the 50 % cell culture infective dose ($CCID_{50}$) by the microtitration assay in HEp-2c cells (Nagata *et al.*, 2002), and also by measuring the infectious units (IU) by counting the number of infected cells stained by indirect immunofluorescence against the viral antigen (Barclay *et al.*, 1998). For the measurement of $CCID_{50}$, virus solution was inoculated into a HEp-2c cell suspension on 96-well plates (Falcon) and then incubated at 37 °C for 1 week for the observation of CPE. The $CCID_{50}$ value was calculated according to the Behrens–Kärber method (Kärber, 1931). For the measurement of IU, virus solution was diluted with 10 % FCS/DMEM and inoculated into HEp-2c cell monolayers on 96-well plates (Falcon) ($2{\cdot}8 \times 10^4$ cells per well). The cells were incubated at 37 °C for 8 h and then fixed with 3 % paraformaldehyde. The cells were stained by indirect immunofluorescence with rabbit hyperimmune serum against the PV 2C protein and Hoechst 33258 (Molecular Probes) for counterstaining (Arita *et al.*, 1999). The numbers of infected cells were counted for the calculation of IU (Jackson *et al.*, 2001).

Intraspinal inoculation and histological analysis of TgPVR21 mice. All animal procedures were approved by the Committee for Biosafety and Animal Handling and the Committee for Ethical Regulation of the National Institute of Infectious Diseases, Japan. Animal care, breeding, virus inoculation and observation were performed in accordance with the guidelines of the Committees.

Human PVR-expressing transgenic mice, ICR TgPVR21 (TgPVR21) (Central Institute of Experimental Animals, Kanagawa, Japan), 4–5 weeks old, were inoculated with 5 µl TE-PV-Fluc mc or Sabin 1 via the intraspinal route as described by Abe *et al.* (1995). Inoculated mice were observed for up to 2 months for clinical symptoms (paralysis and death). The severity of paralysis was classified into four levels according to the symptoms observed in the hindlimb: (i) a decline in grip strength, (ii) weakness of the hindlimb, (iii) partial flaccid paralysis and (iv) complete flaccid paralysis. Fifty per cent paralytic doses (PD_{50}) were calculated according to the Behrens–Kärber method (Kärber, 1931). The replication kinetics of the PV replicon were determined by measuring the luciferase activity in the spinal cords of inoculated mice collected at the time indicated (2–14 h p.i.).

For the histological analysis, the spinal cords of inoculated mice were collected at day 3 p.i. and sections around the lumbar cord were prepared (Nagata *et al.*, 2001). Lesions on the sections were observed after haematoxylin and eosin staining or after Luxol fast blue/cresyl violet staining (Klüver–Barrera method). The lesion scores of the spinal cords were determined according to the procedure recommended by the World Health Organization for the quality control of oral PV vaccine strains (WHO, 1990).

RESULTS

trans-Encapsidation of the PV replicon in 293T cells

For quantitative analysis of PV replication in the spinal cord of TgPVR21 mice, we *trans*-encapsidated a PV replicon encoding firefly luciferase in place of the capsid genes (TE-PV-Fluc mc) in 293T cells. 293T cells were transfected with expression vector pKS435-EGFP-PV CAPSID PV, which expresses the PV capsid proteins as a fusion protein with EGFP under the control of the HEF-1α promoter (Fig. 1). The RNA transcript of PV-Fluc mc was then transfected into the 293T cells expressing PV capsid proteins, resulting in the production of pseudovirions (Fig. 1c). The composition of the capsid proteins of *trans*-encapsidated PV-Fluc mc (TE-PV-Fluc mc) was similar to that of PV1 (Mahoney) virions. However, the VP2 and VP3 proteins in TE-PV-Fluc mc particles were slightly smaller than those of PV1 (Mahoney) (Fig. 1d). These results indicated that the PV replicon was *trans*-encapsidated efficiently in 293T cells transiently expressing PV capsid proteins.

We measured the titre of TE-PV-Fluc mc by counting the numbers of infected cells stained by indirect immunofluorescence using rabbit hyperimmune serum against the PV non-structural protein 2C (Fig. 2a). The titre of TE-PV-Fluc mc was $6{\cdot}3 \times 10^7$ IU ml^{-1} and the estimated $CCID_{50}$ was $3{\cdot}0 \times 10^8$ ml^{-1}, which was calculated from that of the PV1 (Sabin) strain ($6{\cdot}7 \times 10^7$ IU ml^{-1} and $3{\cdot}2 \times 10^8$ $CCID_{50}$ ml^{-1}, 4·8 $CCID_{50}$ IU^{-1}). PV1 (Mahoney) showed a fourfold higher titre than PV1 (Sabin); however, the $CCID_{50}$ IU^{-1} value was similar to that of PV1 (Sabin) (data not shown). Therefore, the estimated $CCID_{50}$ values of TE-PV-Fluc mc from the titres of attenuated or virulent strains were the same.

Next, we aimed to detect viable viruses that could emerge during *trans*-encapsidation in the preparations of TE-PV-Fluc mc. We performed virus isolation from $6{\cdot}3 \times 10^6$ IU (corresponding to $3{\cdot}0 \times 10^7$ estimated $CCID_{50}$) TE-PV-Fluc mc. Quantitative control of viable virus in TE-PV-Fluc mc was performed by adding different titres of PV1 (Sabin) (from 3·2 to $3{\cdot}2 \times 10^5$ $CCID_{50}$) to the preparation of TE-PV-Fluc mc (Fig. 2b). We observed substantial CPE in the inoculated cells at the first passage of all samples (Fig. 2b). During the second passage, no CPE was observed in cells inoculated with TE-PV-Fluc mc, in contrast to the complete CPE observed in the cells inoculated with TE-PV-Fluc mc mixed with PV1 (Sabin) at a titre as low as 3·2 $CCID_{50}$. In the third passage of TE-PV-Fluc mc, the cells showed no CPE. We

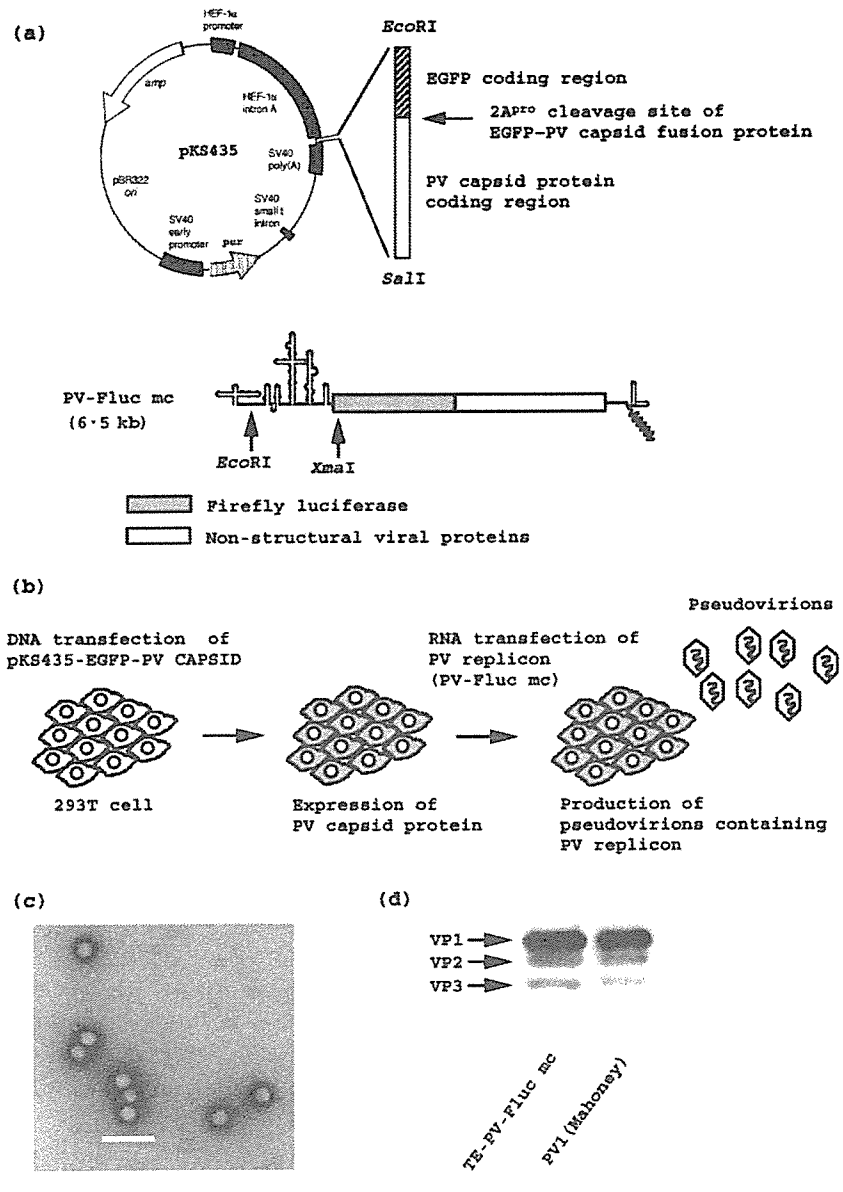

Fig. 1. *trans*-Encapsidation of a PV replicon encoding firefly luciferase. (a) Schematic view of expression vector pKS435 (upper panel) and PV replicon (lower panel) used for the *trans*-encapsidation. PV capsid was expressed as a fusion protein with EGFP in the N terminus, with a $2A^{pro}$ cleavage site at the C terminus of EGFP. In the PV replicon (PV-Fluc mc), the firefly luciferase gene was encoded on the monocistronic replicon. Restriction-enzyme sites used for the construction are shown. (b) Scheme of the *trans*-encapsidation of PV replicon in 293T cells. (c) Electron microscopy observation of purified pseudovirions. Bar, 100 nm. (d) Western blot analysis of pseudovirions by using rabbit hyperimmune serum against PV1 (Mahoney) virions. Left lane, TE-PV-Fluc mc; right lane, PV1 (Mahoney). The positions of viral capsid proteins VP1, -2 and -3 are indicated by arrows.

further examined the samples collected in the virus isolation for viable virus (Fig. 2c). Complete cell lysis was only observed in the cells inoculated with PV1 (Sabin) or with a high m.o.i. of TE-PV-Fluc mc (at an m.o.i. of 22), but not with diluted TE-PV-Fluc mc. Residual luciferase activity was observed in the samples of the first and second passages, but not in those of the third passage (Fig. 2c). The capsid proteins were not detected in these samples by Western blot analysis (data not shown). Consequently, we could not detect viable virus in the preparation of TE-PV-Fluc mc produced in 293T cells, as reported in the previously established *trans*-encapsidation system of PV replicon (Jackson *et al.*, 2001).

Replication of TE-PV-Fluc mc in HEp-2c cells and in the spinal cord of TgPVR21 mice

We measured the replication kinetics of TE-PV-Fluc mc in *in vitro*-cultured cells (HEp-2c cells) and in the spinal cord of TgPVR21 mice (Fig. 3). For the measurement of replication kinetics *in vitro*, HEp-2c cells were infected with TE-PV-Fluc mc at an m.o.i. of 0·024, 0·24 or 24. Luciferase activity in HEp-2c cells reached a peak level at as early as 6–10 h p.i., depending on the inoculated titre. The number of infected cells inoculated at an m.o.i. of 0·024, 0·24 and 24 was measured by indirect immunofluorescence and was $6{\cdot}1 \times 10^2$, $4{\cdot}8 \times 10^3$ and $2{\cdot}6 \times 10^4$, respectively. For the measurement of replication kinetics in spinal cords, TgPVR21 mice were inoculated with $3{\cdot}2 \times 10^5$ or $4{\cdot}1 \times 10^6$ IU TE-PV-Fluc mc via the intraspinal route. Maximum luciferase activities in the spinal cords were observed at 10 h p.i. (Fig. 3b). A close correlation between the inoculated titre and the maximum luciferase activity at 10 h p.i. in the spinal cords was observed for a range of titres from 10^3 to 10^7 IU TE-PV-Fluc mc (Fig. 3c). These results suggested that the properties of replication of TE-PV-Fluc mc in the

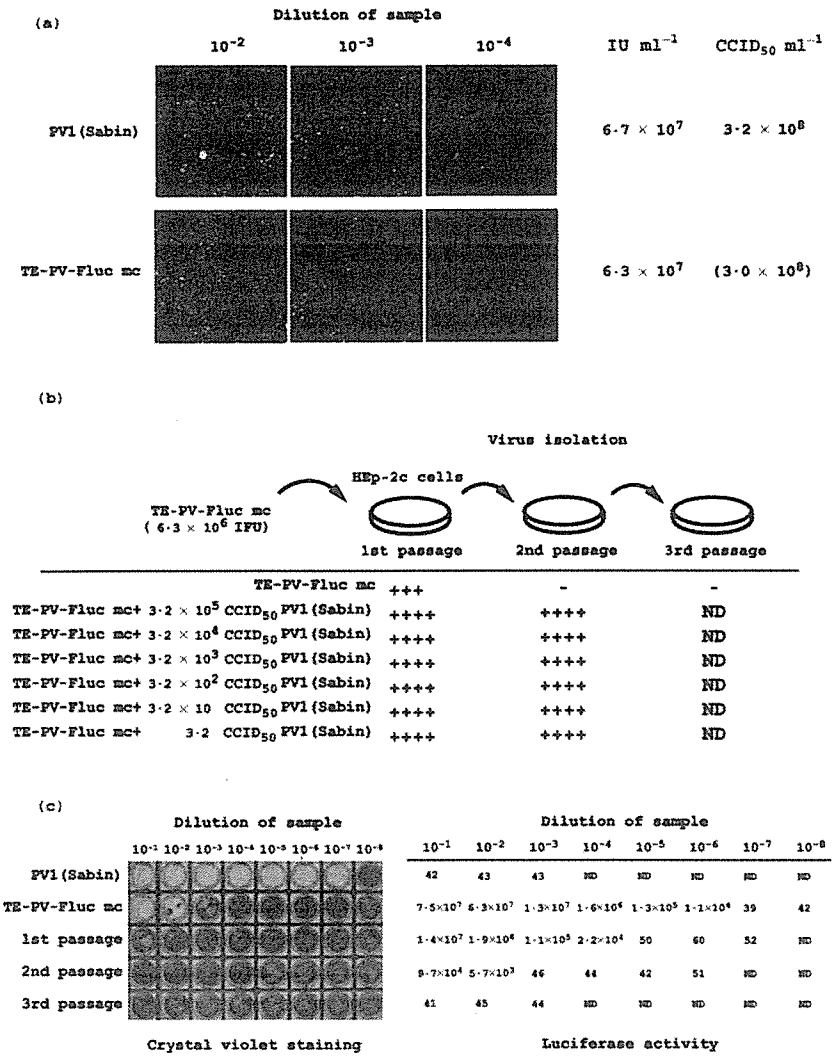

Fig. 2. Characterization of the *trans*-encapsidated PV replicon. (a) Titration of TE-PV-Fluc mc. The corresponding $CCID_{50}$ value of TE-PV-Fluc mc was estimated from that of PV1 (Sabin). (b) Isolation of viable viruses from the preparation of TE-PV-Fluc mc. TE-PV-Fluc mc alone or mixed with fixed amounts of PV1 (Sabin) was subjected to virus isolation in HEp-2c cells. +, Each 25 % of cells showing CPE; ND, not determined. (c) Infectivity of TE-PV-Fluc mc in HEp-2c cells. Cells were fixed at 48 h p.i. and then stained with crystal violet. Luciferase activity was measured at 8 h p.i. as described in Methods. The maximum luciferase activity observed per single HEp-2c cell was 14 600 relative light units.

spinal cord of TgPVR21 mice were similar to those in HEp-2c cells, although with a slight delay.

Poliomyelitis-like paralysis in TgPVR21 mice inoculated with TE-PV-Fluc mc

We then prepared a range of titres of TE-PV-Fluc mc for inoculation into TgPVR21 mice via the intraspinal route and characterized the induced paralysis of the inoculated mice. At 1 day p.i., flaccid paralysis of the hindlimb of TgPVR21 mice inoculated with $3{\cdot}2 \times 10^5$ IU (estimated $CCID_{50}$ $1{\cdot}5 \times 10^6$) TE-PV-Fluc mc was observed (Fig. 4a). TgPVR21 mice inoculated with the PV1 (Sabin) strain via the intraspinal route showed paralysis at day 1, 2 or 3 p.i., dependent on the titres of inoculated virus (Abe *et al.*, 1995) (Fig. 4b). All of the TgPVR21 mice inoculated with PV1 (Sabin) succumbed to lethal paralysis, in contrast to non-lethal paralysis of those inoculated with TE-PV-Fluc mc.

The paralysis induced by the inoculation of TE-PV-Fluc mc was mostly limited to the left hindlimb, corresponding to the inoculated site in the spinal cord (Fig. 5a). However, some mice inoculated with high doses of TE-PV-Fluc mc ($>3{\cdot}2 \times 10^6$ IU) showed paralysis of both hindlimbs and this could even result in lethal paralysis (four out of 18 inoculated mice; Table 1), although no viable viruses were isolated from the CNS (data not shown). Severity of the induced paralysis was classified into four levels according to the symptoms observed in the hindlimb: (i) a decline in grip strength, (ii) weakness of the hindlimb, (iii) partial flaccid paralysis and (iv) complete flaccid paralysis (Table 1). Paralysis could be observed at as early as 10 h p.i. in the mice inoculated with $3{\cdot}2 \times 10^6$ IU TE-PV-Fluc mc and the number of mice showing severe paralysis (i.e. partial to complete flaccid paralysis of the hindlimb) reached a plateau at 16 h p.i. (Fig. 4c). The PD_{50} value of TE-PV-Fluc mc in TgPVR21 mice was determined as $3{\cdot}2 \times 10^4$ IU ($1{\cdot}5 \times 10^5$ estimated $CCID_{50}$). The lowest titre of TE-PV-Fluc mc required for the induction of severe paralysis was $1{\cdot}6 \times 10^5$ IU, where half of the inoculated mice showed severe paralysis (Table 1). We observed mild paralysis (i.e. a decline in grip strength and weakness of the hindlimb) in

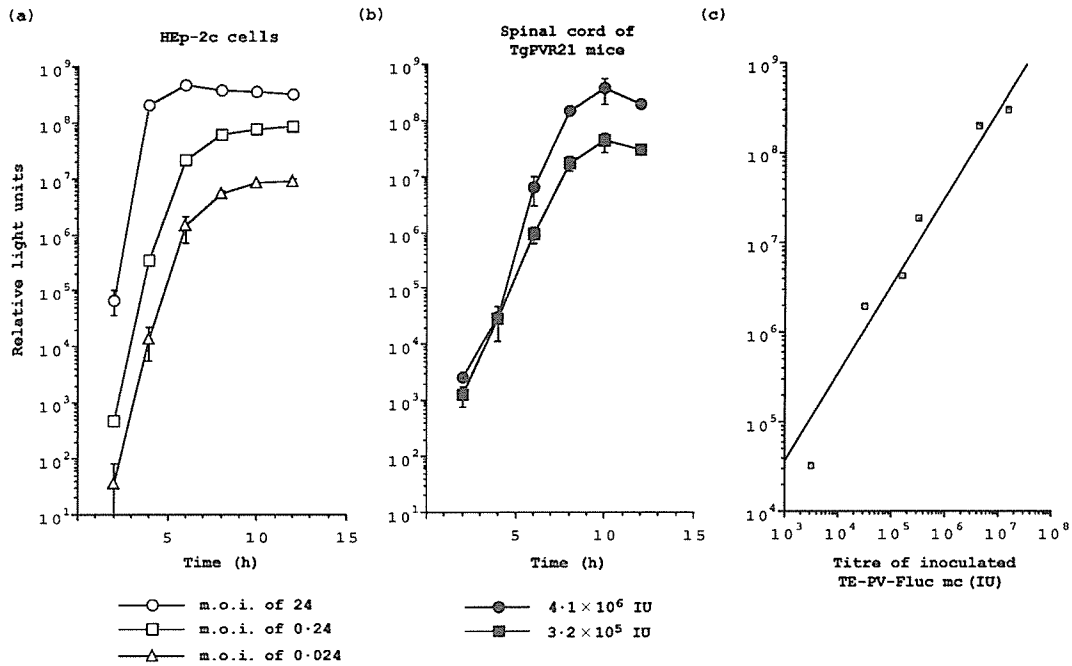

Fig. 3. *In vitro* and *in vivo* replication kinetics of TE-PV-Fluc mc. (a) Replication kinetics in HEp-2c cells. HEp-2c cells ($2{\cdot}6 \times 10^4$) were infected with $6{\cdot}3 \times 10^5$, $6{\cdot}3 \times 10^3$ or $6{\cdot}3 \times 10^2$ IU TE-PV-Fluc mc. (b) Replication kinetics in the spinal cords of TgPVR21 mice. TgPVR21 mice were inoculated with $3{\cdot}2 \times 10^5$ or $4{\cdot}1 \times 10^6$ IU TE-PV-Fluc mc via the intraspinal route. Total luciferase activity in the spinal cords is shown with SD. (c) Relationship between the maximum luciferase activity in the spinal cord of TgPVR21 mice and the inoculated titre of TE-PV-Fluc mc. Total luciferase activity in the spinal cords (*y* axis) and the inoculated titre of TE-PV-Fluc mc (*x* axis) are shown.

mice inoculated with $3{\cdot}2 \times 10^4$ IU, but no apparent clinical symptoms were observed with inoculation of $3{\cdot}2 \times 10^3$ IU TE-PV-Fluc mc. Therefore, transient replication of TE-PV-Fluc mc in the spinal cord of TgPVR21 mice caused non-lethal poliomyelitis-like paralysis with different severity in a dose-dependent manner.

Under the conditions examined, we estimated the number of infected cells in the spinal cords, from a value of the maximum luciferase activity per single HEp-2c cell infected by TE-PV-Fluc mc, to be at most $3{\cdot}9 \times 10^4$ cells (14 600 relative light units; Fig. 2c; Table 1). The number of neurons in the lumbar cord of mouse has been determined as $8{\cdot}9 \times 10^4$ ($\pm 1{\cdot}0 \times 10^4$) cells (Bjugn *et al.*, 1997), suggesting that a substantial population of the motor neurons was infected by TE-PV-Fluc mc under the conditions examined.

Histological analysis of the spinal cord of TgPVR21 mice inoculated with TE-PV-Fluc mc

We performed histological analysis of the neuronal damage in the spinal cord of TgPVR21 mice showing paralysis with different severity (Fig. 5; Table 2). We examined sections of the lumbar cord surrounding the inoculated sites and measured the lesion score for each section (Fig. 5a).

In the mice showing severe paralysis of the left hindlimb (partial or complete flaccid paralysis of the hindlimb) at day 3 p.i., where substantial loss of the motor neurons by infection of TE-PV-Fluc mc was expected (Table 1), a complete loss of the motor neurons was observed in the left side of the anterior horn (the inoculated side), whereas intact motor neurons were retained in the opposite side (Fig. 5b). In the mice showing mild paralysis of the left hindlimb (a decline in grip strength), where at most 6·1 % of the neurons in the lumbar cord were infected by TE-PV-Fluc mc (Table 1), partial loss of the motor neurons and some neuronophagia were observed (Fig. 5c). We found a close relationship between the inoculated titre of TE-PV-Fluc mc and lesion scores and also between the clinical symptoms and the observed lesions in most mice (Table 2). However, partial flaccid paralysis of the hindlimb was also observed in a mouse (number 7) with an apparently low lesion score. Therefore, a limited lesion of the lumbar cord could be critical for severe paralysis in inoculated mice.

DISCUSSION

In this study, we developed a new *trans*-encapsidation system of a PV replicon in 293T cells. To date, *trans*-encapsidation systems of PV by helper PV (Barclay *et al.*, 1998; Hagino-Yamagishi & Nomoto, 1989), by recombinant vaccinia virus expressing the capsid proteins (Ansardi *et al.*, 1993) or by plasmid expression vector in combination with recombinant vaccinia virus expressing T7 RNA polymerase

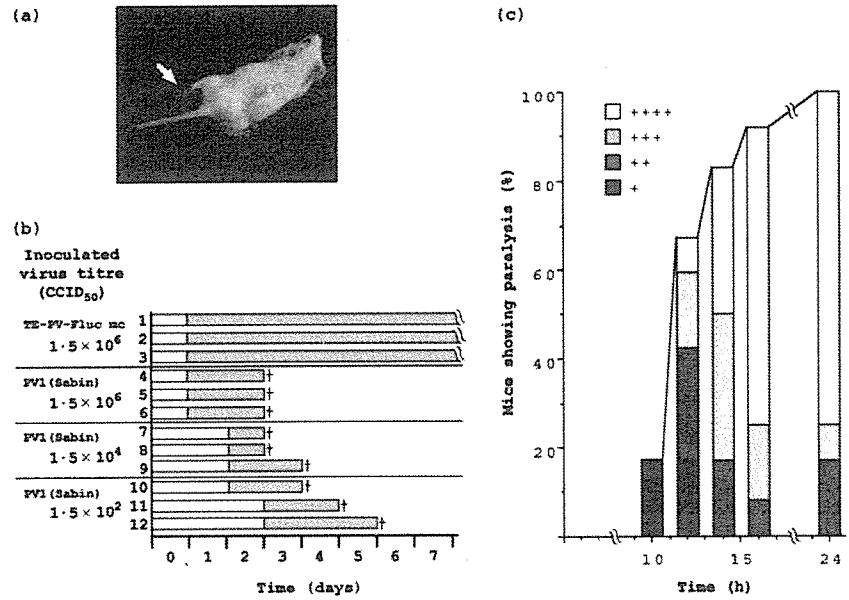

Fig. 4. Characterization of the paralysis of TgPVR21 mice inoculated with TE-PV-Fluc mc. (a) Paralysis of a TgPVR21 mouse inoculated with TE-PV-Fluc mc ($3{\cdot}2 \times 10^5$ IU) via the intraspinal route at 24 h p.i. Arrow indicates a paralysed left hindlimb. (b) Time course of the appearance of paralysis of TgPVR21 mice inoculated with TE-PV-Fluc mc or PV1 (Sabin). TgPVR21 mice were inoculated with $3{\cdot}2 \times 10^5$ IU ($1{\cdot}5 \times 10^6$ estimated $CCID_{50}$) TE-PV-Fluc mc or with $1{\cdot}5 \times 10^2$ to $1{\cdot}5 \times 10^6$ $CCID_{50}$ PV1 (Sabin) via the intraspinal route. Empty and shaded boxes indicate asymptomatic conditions and paralysis of the inoculated mice, respectively. †, Death of inoculated mice due to virus infection. (c) Time course of the appearance of paralysis of TgPVR21 mice inoculated with TE-PV-Fluc mc. Twelve TgPVR21 mice were inoculated with $3{\cdot}2 \times 10^6$ IU ($1{\cdot}5 \times 10^7$ estimated $CCID_{50}$) TE-PV-Fluc mc via the intraspinal route. The percentage of mice with indicated severity of paralysis is shown. +, Decline in grip strength; + +, weakness of the hindlimb; + + +, partial flaccid paralysis of the hindlimb; + + + +, complete flaccid paralysis of the hindlimb.

(Jia *et al.*, 1998), have been established. 293T cells allow a high-level expression of protein (DuBridge *et al.*, 1987) and have been utilized for *trans*-encapsidation systems of retrovirus (Evans *et al.*, 2004; Pear *et al.*, 1993) and also for increasing the titre of papillomavirus (Pyeon *et al.*, 2005).

TE-PV-Fluc mc particles had a similar composition of capsid proteins to that of wild-type virus; however, apparent sizes of the VP2 and VP3 proteins were smaller than those of wild-type virus (Fig. 1c). TE-PV-Fluc mc was neutralized completely by anti-PV1 antiserum, but not by anti-PV2 or -PV3 antisera (data not shown), suggesting that TE-PV-Fluc mc particles retained the antigenicity of the original PV1. The properties of capsid proteins in pseudovirions remain to be further studied.

The replication kinetics of TE-PV-Fluc mc in the spinal cord of TgPVR21 mice were similar to those in HEp-2c cells, but with a slight delay, as observed previously (Fig. 3) (Bledsoe *et al.*, 2000; Porter *et al.*, 1998). The maximum number of viral genomes of PV found in degenerating motor neurons of cynomolgus monkeys was comparable to that observed in HEp-2c cells (Couderc *et al.*, 1989). However, the efficiency of TE-PV-Fluc mc infection in the spinal cord was lower than that observed in HEp-2c cells, probably because of the limited accessibility of the virion to the target neurons (Table 1). We estimated that a mean of $1{\cdot}9 \times 10^2$ IU (or $8{\cdot}9 \times 10^2$ $CCID_{50}$) TE-PV-Fluc mc was required for the infection of a single susceptible cell in the spinal cord from the maximum luciferase activity observed in a single infected HEp-2c cell. The PD_{50} values of PV1 (Sabin) and PV1 (Mahoney) in TgPVR21 mice by intraspinal inoculation were $10^{3{\cdot}3}$ $CCID_{50}$ (Abe *et al.*, 1995) and $< 10^{1{\cdot}3}$ $CCID_{50}$ (N. Nagata, unpublished result), respectively. Assuming that PV infection in a single cell results in the paralysis of inoculated mice, the infectivity of TE-PV-Fluc mc was intermediate between those of the virulent and attenuated strains.

TgPVR21 mice inoculated with TE-PV-Fluc mc showed a wide range of paralysis symptoms (from a decline in grip strength to complete flaccid paralysis of the hindlimb), with histological features typical of PV infection (infiltration of neutrophils, neuronophagia and neuronal loss) (Bodian, 1949; Bodian & Howe, 1941) (Fig. 4; Table 1). The pathological features of TE-PV-Fluc mc were virus-specific, because UV-treated TE-PV-Fluc mc did not cause any clinical symptoms in inoculated mice (data not shown). These observations are inconsistent with previous reports on PV replicons, where no clinical symptoms or pathological features were observed in inoculated mice (Bledsoe *et al.*, 2000; Jackson *et al.*, 2001). Differences in the structure of the replicon [e.g. form of the luciferase protein,

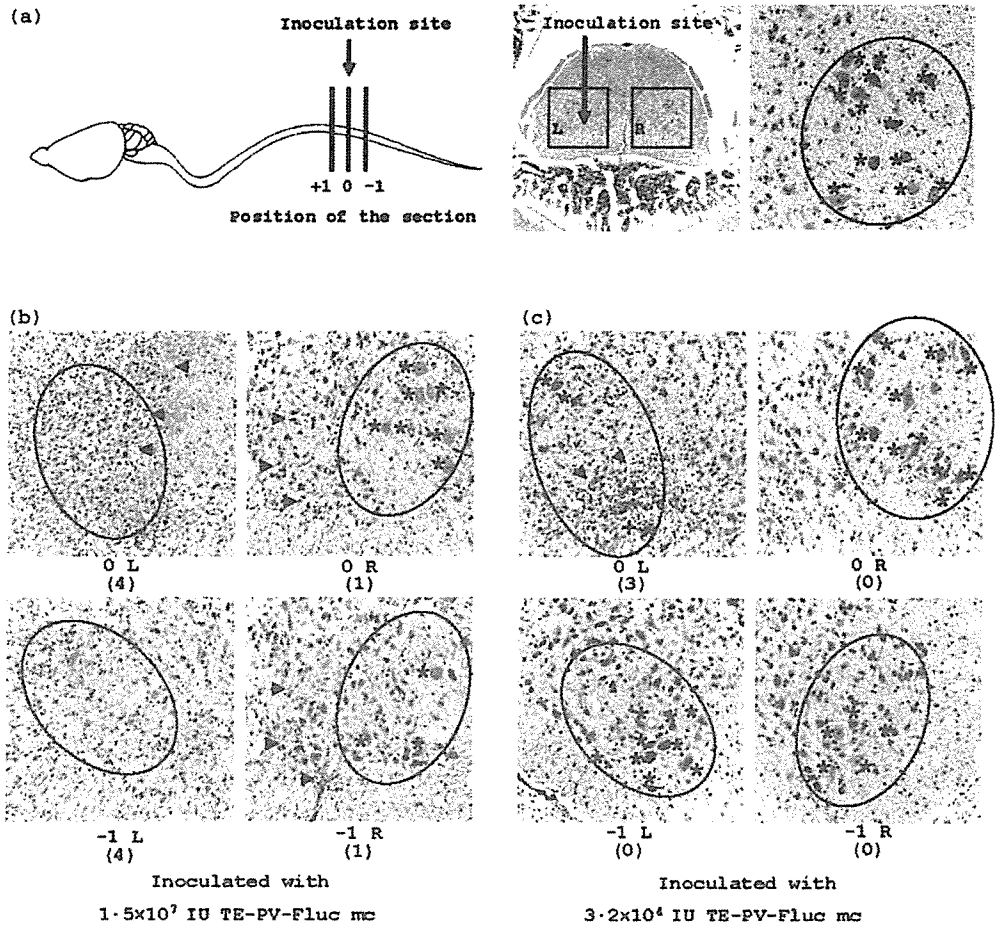

Fig. 5. Histological analysis of spinal cords in TgPVR21 mice inoculated with TE-PV-Fluc mc. TgPVR21 mice inoculated with TE-PV-Fluc mc via the intraspinal route were subjected to histological analysis at day 3 p.i. In each section, the areas of the anterior horn are encircled by ellipses, the cell bodies of the motor neurons are indicated by asterisks, the sites of inflammation are indicated by arrows and the motor neurons in a neuronophagic form are shown by +. Numbers in parentheses represent the lesion score of the section. L, Left side; R, right side. (a) Inoculation site of TE-PV-Fluc mc in the spinal cord of TgPVR21 mice. In the left panel, the positions of examined sections are shown as 0 (inoculation site), +1 (3 mm cephalad from the inoculation site) and −1 (3 mm caudad from the inoculation site) in a schematic view of the CNS of mice. In the middle panel, the inoculation site is shown in a full view of a section of the lumbar cord. The right panel shows a view of a mock-infected anterior horn. (b) A view of the lumbar cords of TgPVR21 mice inoculated with a high dose of TE-PV-Fluc mc ($1{\cdot}5 \times 10^7$ IU). A view of an area in the section around the anterior horn is shown. (c) A view of the lumbar cords of TgPVR21 mice inoculated with a low dose of TE-PV-Fluc mc ($3{\cdot}2 \times 10^4$ IU). A view of an area in the section around the anterior horn is shown.

genomic structure of the replicon, length of the poly(A) tail, restriction-enzyme sites and/or unidentified epigenetic modifications] (Brown *et al.*, 2005; DeJesus *et al.*, 2005; Porter *et al.*, 1998), the transgenic mice (e.g. strain and age) (Abe *et al.*, 1995; Crotty *et al.*, 2002) and the titration procedure of the *trans*-encapsidated PV replicon could be critical determinants of the apparent pathogenicity of the PV replicon. TE-PV-Fluc mc showed faster replication kinetics (peak at as early as 6–10 h p.i. with an m.o.i. ranging from 0·024 to 24) compared with those of a previously reported PV replicon (peak at 12 h p.i. at an m.o.i. of 10) (Porter *et al.*, 1998). The replication efficiency of PV was proportional to the size of the deletion in the genome (Kaplan & Racaniello, 1988), and coding sequences of the reporter gene could affect protein-synthesis activity in *Hepatitis C virus* (reviewed by Lemon & Honda, 1997). The factors required for the pathogenesis of the PV replicon system remain to be further elucidated.

We observed a time lag between the peak of replication of TE-PV-Fluc mc in the spinal cord (10 h p.i.) and the appearance of paralysis (which reached a plateau at 16 h p.i.) (Figs 3b, 4c). PV-induced apoptosis has been observed both *in vitro* and *in vivo* (Girard *et al.*, 1999; Romanova *et al.*, 2005; Tolskaya *et al.*, 1996), and Couderc *et al.* (2002) showed a time lag between the peak of virus growth (8 h p.i.) and the development of apoptosis (28 h p.i.) in a mixed primary nerve-cell culture. These findings suggest a direct

Table 1. Clinical symptoms of TgPVR21 mice inoculated with TE-PV-Fluc mc

Inoculated titre of TE-PV-Fluc mc (IU)	Severity of paralysis of the hindlimb*					Estimated no. infected cells†	Inoculated titre (IU) per infected cell
	−	+	++	+++	++++		
$1{\cdot}5\times10^{7}$	0	1/6	0	1/6	4/6 (1)	$3{\cdot}9\times10^{4}$ (100)	$3{\cdot}8\times10^{2}$
$4{\cdot}1\times10^{6}$	0	0	0	3/8	5/8 (3)	$2{\cdot}6\times10^{4}$ (66)	$1{\cdot}6\times10^{2}$
$3{\cdot}2\times10^{6}$	0	0	2/12	1/12	9/12	ND	ND
$3{\cdot}2\times10^{5}$	0	3/7	1/7	1/7	2/7	$2{\cdot}4\times10^{3}$ (6·1)	$1{\cdot}3\times10^{2}$
$1{\cdot}6\times10^{5}$	1/6	0	2/6	2/6	1/6	$5{\cdot}3\times10^{2}$ (1·4)	$3{\cdot}0\times10^{2}$
$3{\cdot}2\times10^{4}$	3/6	3/6	0	0	0	$3{\cdot}6\times10^{2}$ (0·94)	$8{\cdot}9\times10$
$3{\cdot}2\times10^{3}$	3/3	0	0	0	0	$6{\cdot}6\times10$ (0·17)	$4{\cdot}9\times10$

*−, No symptoms; +, decline in grip strength; ++, weakness of the hindlimb; +++, partial flaccid paralysis; ++++, complete flaccid paralysis. Number of mice showing indicated symptoms over total number of inoculated mice is shown. Number of mice with lethal paralysis is shown in parentheses.

†Number of infected cells was estimated from the total luciferase activity recovered from the spinal cord of inoculated mice. Number in parentheses represents percentage of infected cells, where the highest number of infected cells observed under the examined conditions ($3{\cdot}9\times10^{4}$ cells) is taken as 100 %. ND, Not determined.

link between *in vivo* apoptosis and functional loss of motor neurons during the transient replication of TE-PV-Fluc mc. The biological characteristics of the *in vivo* cell death induced by the PV replicon remain to be further studied.

Histological analysis showed a correlation between the severity of the clinical symptoms and the lesion scores in most mice inoculated with TE-PV-Fluc mc (Table 2). However, for mice with a partial loss of the motor neurons

Table 2. Clinical symptoms and lesion score of the spinal cords of TgPVR21 mice inoculated with TE-PV-Fluc mc

Mouse no.	Inoculated titre of TE-PV-Fluc mc (IU)	Paralysis*	Lesion score of the spinal cord†					
			Position +1		Position 0		Position −1	
			L	R	L	R	L	R
1	$1{\cdot}5\times10^{7}$	L+++, R+	2	1	4	3	2	2
2	$1{\cdot}5\times10^{7}$	L++++	3	1	4	1	4	1
3	$3{\cdot}2\times10^{5}$	L+	1	0	2	0	1	0
4	$3{\cdot}2\times10^{5}$	L+	1	0	1	0	1	0
5	$3{\cdot}2\times10^{5}$	L+	1	0	3	1	1	0
6	$1{\cdot}6\times10^{5}$	L+	2	0	3	0	1	0
7	$1{\cdot}6\times10^{5}$	L+++	1	0	2	0	1	0
8	$3{\cdot}2\times10^{4}$	L+	1	0	3	0	0	0
9	$3{\cdot}2\times10^{4}$	−	0	0	1	0	0	0
10	$3{\cdot}2\times10^{4}$	−	0	0	0	0	0	0
11	$3{\cdot}2\times10^{3}$	−	0	0	0	0	0	0
12	$3{\cdot}2\times10^{3}$	−	0	0	0	0	0	0

*Mice inoculated with the indicated titre of TE-PV-Fluc mc were subjected to histological analysis at day 3 p.i. −, No symptoms; +, decline in grip strength; ++, weakness of the hindlimb; +++, partial flaccid paralysis; ++++, complete flaccid paralysis; L, left hindlimb; R, right hindlimb.

†Positions of the section examined are represented as 0 (inoculation site), +1 (3 mm cephalad from the inoculation site) and −1 (3 mm caudad from the inoculation site). Lesion of the spinal cord was scored as: 0, no lesion; 1, inflammation (represented by an infiltration of neutrophils); 2, inflammation with partial degeneration of the motor neurons; 3, inflammation with severe degeneration and a partial loss of the motor neurons; 4, severe inflammation and complete loss of the motor neurons. L, Left side of anterior horn; R, right side of anterior horn.

(with a lesion score of <3), it was difficult to make a correct inference for the severe paralysis from the overall lesion scores only. We estimated that the proportion of critical motor neurons was, at most, 1·4 % of susceptible neurons in the lumbar cord (Table 1). Limbs affected by poliomyelitis in humans showed a mean of 40·8 % remaining motor units (McComas *et al.*, 1997). Therefore, a small population of the motor neurons in the lumbar cord seemed to be critical for severe paralysis in TgPVR21 mice.

In summary, we have developed a *trans*-encapsidation system for a PV replicon in 293T cells and analysed the poliomyelitis-like paralysis of TgPVR21 mice induced by the PV replicon. This model would be useful for the analysis of *in vivo* cell death induced by PV infection and for the development of effective therapies for poliomyelitis (Dodd *et al.*, 2005).

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Role of the Alpha/Beta Interferon Response in the Acquisition of Susceptibility to Poliovirus by Kidney Cells in Culture

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Replication of poliovirus (PV) is restricted to a few sites, including the brain and spinal cord. However, this neurotropism is not conserved in cultured cells. Monkey kidney cells become susceptible to PV infection after cultivation in vitro, and cell lines of monolayer cultures from almost any tissue of primates are susceptible to PV infection. These observations suggest that cellular changes during cultivation are required for acquisition of susceptibility. The molecular basis for the cellular changes during this process is not known. We investigated the relationship between PV susceptibility and interferon (IFN) response in primary cultured kidney and liver cells derived from transgenic mice expressing human PV receptor and in several primate cell lines. Both kidneys and liver in vivo showed rapid IFN response within 6 h postinfection. However, monkey and mouse kidney cells in culture and primate cell lines, which were susceptible to PV, did not show such rapid response or showed no response at all. On the other hand, primary cultured liver cells, which were partially resistant to infection, showed rapid IFN induction. The loss of IFN inducibility in kidney cells was associated with a decrease in expression of IFN-stimulated genes involved in IFN response. Mouse kidney cells pretreated with a small dose of IFN, in turn, restored IFN inducibility and resistance to PV. These results strongly suggest that the cells in culture acquire PV susceptibility during the process of cultivation by losing rapid IFN response that has been normally maintained in extraneural tissues in vivo.

Poliovirus (PV), belonging to the *Picornaviridae*, is the causative agent of poliomyelitis (44). The replication of PV is limited to a few sites, including the brain and spinal cord. Severe pathological lesions are not observed in most extraneural tissues, despite the presence of virus in many tissues during the viremic phase of infection (2, 52). PV was initially isolated by Landsteiner and Popper (32) in 1908 as a transmittable pathogen. In those days, PV could be transferred by inoculating a suspension of the spinal cord of a paralyzed monkey into another, because of the neurotropic nature of PV. In 1949, Enders et al. (8) for the first time succeeded in propagating PV in primary cultured cells from various human embryonic tissues. Later, Dulbecco and Vogt (7) found that monkey kidney developed permissivity to infection after cultivation in vitro and that PV titers could be quantified by plaque assay. PV, therefore, is able to replicate in monolayer cells in primary culture and in cell lines derived from almost any tissue of primates, although PV cannot replicate well in the extraneural tissues in vivo. This new technology for propagating PV in cell cultures in vitro had a great impact on virology and allowed revolutionary progress in PV studies. Attenuated Sabin strains were developed by a number of passages of virulent PV strains in cultured cells (53). Large-scale production of PV vaccines has been done using monkey kidney cells. The molecular mechanisms of PV replication have been studied using cultured cells with strict control of the experimental conditions (49). Although we have benefited greatly from this, the molecular basis for this paradoxical change in susceptibility is still unknown.

The factors that control PV susceptibility have been studied. The research focused on identifying the determinants of tissue and cell tropism. Holland and colleagues thought that susceptibility was determined at the level of virus entry into the cell. They proposed that the PV receptor (PVR) is a major determinant of tissue and cell tropism based on observations that a single round of replication occurred in nonpermissive mouse cells after transfection of PV genomic RNA (20) and that PV was adsorbed by a homogenate of neural tissues (19). Molecular cloning of the human *PVR* gene revealed that PVR is a membrane protein that belongs to the immunoglobulin superfamily (29, 40). Transgenic (tg) mice that carry the human *PVR* gene were produced. PV selectively replicated in the central nervous system (CNS), and the mice showed paralytic disease that resembled human poliomyelitis upon PV infection (31, 51). If Holland's hypothesis were correct, one could expect that PVR would not be expressed in the extraneural tissues at high levels in vivo but would be expressed in the cells after cultivation in vitro. However, PVR mRNA was detected in various tissues of human and PVR-tg mice (43). Furthermore, when Ren and Racaniello (50) investigated the distribution of PVR

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transcripts in the kidneys of tg mice by in situ hybridization, they showed that epithelial cells in the Bowman's capsule, podocytes in the glomerulus, and some of the tubular epithelial cells in the medulla expressed human PVR mRNA at high levels, although these cells were not susceptible to PV in vivo. They also observed that the freshly dispersed kidney cells expressed PVR at the cell surface, as judged by a PV binding assay, but susceptibility developed after cultivation in vitro for 24 h. They concluded that expression of PVR is not sufficient for PV susceptibility and predicted that other factors that might change during the cultivation process are also needed.

Another possibility was that susceptibility was determined at the level of translation initiation of the viral protein. Translation initiation of picornaviruses is controlled by an internal ribosome entry site (IRES) located in the 5′ noncoding region of the genome (45). The PV IRES interacts with canonical translation initiation factors and noncanonical translation initiation factors, IRES *trans*-activating factors (ITAFs), to achieve efficient translation initiation (17). To date, polypyrimidine tract binding protein (PTB) (18, 46), neural cell-specific PTB (27, 35, 37, 48), La autoantigen (38, 39), poly(rC) binding protein 2 (PCBP-2) (1), and upstream of N-ras (Unr) (3) have been identified as ITAFs for PV. There is some evidence for tissue-specific or cell-type-specific translation initiation mediated by the IRES in picornaviruses. Chimeric PVs containing replacements of IRES sequences with corresponding sequences from human rhinovirus type 2 or hepatitis C virus did not propagate in the CNS of PVR-tg mice (14, 63). The foot-and-mouse disease virus IRES was not active in neurons because it required $ITAF_{45}$, which was expressed only in proliferating cells (47). The PV Sabin 3 strain did not grow efficiently in the CNS, in which PTB was not expressed at high levels, and a translation deficit of the Sabin 3 IRES was rescued by increased expression of PTB in the CNS (15). These results may be good examples demonstrating that a lack or shortage of ITAFs resulted in tissue- or cell-specific failure of replication of viruses. If the expression of ITAFs determines susceptibility, we would expect that IRES activity would not be observed in the kidneys in vivo. Kauder and Racaniello (26) reported contradictory results. They constructed a recombinant adenovirus that expressed biscistronic reporter luciferase genes under the control of PV IRESs. They infected mice intravenously with the recombinant adenovirus vector and determined reporter gene expression by luciferase assay. They showed that the PV IRES was active in neural, as well as in extraneural, tissues, including the kidneys. This implies that kidney cells in vivo express sufficient levels of all ITAFs for PV. Furthermore, they did not observe the neural-tissue-specific reduction of translational activity of the attenuated Sabin IRES. Sabin 3 virus and hepatitis C virus/PV recombinant virus propagated efficiently in the CNS of neonatal PVR-tg mice and caused paralysis. They then concluded that PV tropism and attenuation are determined after internal ribosomal entry. Since direct measurement of expression profiles of ITAFs in adult and developing tissues has not been reported, the contribution of the ITAFs to tissue- or cell-specific infection of PV is still controversial (56; see reference 55 for a review).

Finally, we have recently demonstrated that PV tissue tropism is strongly influenced by antiviral activity mediated by alpha/beta interferons (IFN-α/β) (21). PVR-tg mice deficient in the alpha/beta IFN receptor 1 (*Ifnar-1*) gene were produced by crossing PVR-tg mice and *Ifnar* knockout mice (41). Although PV replication sites were restricted to the CNS in the wild-type PVR-tg mice, extensive PV replication was observed in a wider range of tissues in the PVR-tg/*Ifnar* knockout mice. This result suggests that extraneural tissues, such as the liver, spleen, and pancreas, are potentially susceptible to PV infection and that they are normally protected by the IFN response. In the wild-type PVR-tg mice, neural tissues expressed very low levels of IFN-stimulated genes (ISGs) and did not show rapid IFN response upon PV infection. However, extraneural tissues expressed slightly higher levels of ISGs, even in the uninfected mice. They showed sufficient IFN response and were protected from PV infection. From these results, we consider that the difference in IFN responses among the tissues influences differential PV susceptibility.

We hypothesized that the acquisition of PV susceptibility by kidney cells after cultivation may be a consequence of changes in either the IFN response, PVR expression, or ITAF expression. Using primary cultured kidney cells and liver cells from PVR-tg mice, we investigated the relationship between PV susceptibility and expression of the factors mentioned above. Here, we present evidence that the loss of rapid IFN inducibility associated with the decrease in expression of ISGs involved in IFN response during the cultivation process plays an important role in the change in susceptibility of kidney cells.

MATERIALS AND METHODS

Mice. Six-week-old C57BL/6 mice, B6.PVR-Tg21 mice, and B6.PVR-Tg21/*Ifnar* knockout mice, described by Ida-Hosonuma et al. (21), were used in this study. All experiments using mice were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Tokyo Metropolitan Institute for Neuroscience.

Preparation of mouse kidney cells. Mice were deeply anesthetized with diethyl ether and perfused with 10 ml of phosphate-buffered saline and 10 ml of 0.25% trypsin in Eagle's minimum essential medium (MEM). The kidneys were then removed, sliced into small pieces, and incubated in 0.25% trypsin in MEM at 37°C for 20 min. The cells were dispersed in MEM with 10% fetal calf serum (FCS) by pipetting, washed twice, and seeded on a plate at a density of 3×10^4 cells/cm^2. After 1 week, cells grown as a monolayer culture were used for the experiments.

Preparation of mouse liver cells. Mice were deeply anesthetized with diethyl ether and perfused with 20 ml of Hanks' balanced salt solution (HBSS; Sigma) containing 5 mM EGTA and then with 200 ml of HBSS containing 0.5 mg/ml collagenase (Wako Pure Chemical Ltd.) and 0.05 mg/ml trypsin inhibitor. The liver was removed and sliced in Gey's balanced salt solution (Sigma). The dispersed cells were passed through a 100-μm cell strainer (Falcon). The cells were then washed with 20 ml of Gey's balanced salt solution three times. The cells were suspended in William's medium E (Sigma) containing 10% FCS and seeded on a collagen-coated plate at a density of 1.25×10^5 cells/cm^2. The cells were used the following day.

Preparation of African green monkey kidney (AGMK) cells. The kidneys of an African green monkey were removed, sliced into small pieces, and digested with 0.3% trypsin in HBSS at 37°C or at 4°C with continuous stirring for an appropriate period. The dispersed cells were collected and washed twice with HBSS. The cells were suspended in Dulbecco's modified MEM supplemented with 10% FCS, seeded on a plate, and incubated at 37°C. The cells grown as a monolayer culture were passaged, and the cells at passages 3 to 5 were used in the experiments.

Cell lines. An African green monkey kidney cell line, JVK-03 (30), and human embryonic kidney 293 (HEK293) (12), COS-7 (11), HepG2 (28), and HeLa (55) cells were maintained in MEM containing 5% FCS.

Virus. PV type I Mahoney strain derived from infectious cDNA pOM (58) were used. The virus titer was determined by a plaque assay on JVK-03 cells.

Quantitative real-time PCR. Isolation of total RNA, DNase I treatment, and cDNA synthesis were performed as described previously (21). Total human

kidney and liver RNAs were purchased from BD Biosciences. Real-time PCR was performed using an ABI PRISM 7500. The quantification of mouse 18S rRNA, IFN-α mRNA, and human IFN-α mRNA was performed by the SYBR green method using 18S-rRNA-F (5′-GTA ACC CGT TGA ACC CCA TT-3′), 18S-rRNA-R (5′-CCA TCC AAT CGG TAG TAG CG-3′), mIFNα-F (5′-TCC TGA ACC TCT TCA CAT CAA A-3′), mIFNα-R (5′-ACA GGC TTG CAG GTC ATT GAG-3′), hIFN-α-F (5′-GTA CTG CAG AAT CTC TCC TTT CTC CTG-3′), and hIFN-α-R (5′-GTG TCT AGA TCT GAC AAC CTC CCA GGG CAC A-3′) as primers. The mIFNα-F and mIFN-α-R primers amplified all mouse IFN-α mRNA species, and hIFN-α-F and hIFN-α-R primers amplified all human IFN-α mRNA species. The quantification of mouse IFN-β, 2′-5′ oligoadenylate synthetase (OAS), RIG-I, MDA-5, IRF-3, IRF-7, IRF-9, STAT-1, STAT-2, TBK-1, IKKε, IFNAR-1, La, PTB, PCBP-2, UNR, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), PVR mRNAs, and 18S rRNA was performed using Assay-on-Demand PCR probes (Applied Biosystems). The amounts of mRNAs were determined by comparison with the standard templates of cloned cDNAs of known copy number. The expression levels were then normalized to the levels of 18S rRNA. The data are represented as the copy number of mRNA per 10^7 copies of rRNA.

Immunohistochemistry. Detection of PV antigens in infected mice was performed as described previously (31).

Effect of IFN treatment of kidney cells. Mouse kidney cells were treated with recombinant mouse IFN-β (Toray) at concentrations of 0.1 to 100 IU/ml for 6 h. The cells were washed with MEM three times. The IFN-treated cells were infected with PV (at a multiplicity of infection[MOI] of 0.01 to 10) in the presence or absence of anti-mouse IFN-α (17.5 μg/ml) and anti-mouse IFN-β (1.8 μg/ml) monoclonal antibodies (Yamasa Shoyu Co. Ltd, Choshi, Japan). The antibodies at the above concentrations can block 10 and 1,000 IU of IFN-α and IFN-β, respectively. The expression levels of the IFN-α, IFN-β, and Oas1a mRNAs at the indicated times were determined by reverse transcription (RT)-PCR. The PV titer was determined by plaque assay. Mouse IFN activity was measured by the cytopathic effect (CPE) dye uptake method using L929 cells (22, 67). The NIH research reference reagent for mouse IFN-β (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.) was used as the standard for unit definition.

RESULTS

PV susceptibility and IFN response in the kidneys and liver in vivo. We previously reported that most of the extraneural tissues in the PVR-tg mice are potentially susceptible to PV infection but that they are normally protected by the IFN response. We investigated PV replication and IFN response in the kidneys and liver in vivo in detail. Mice were intravenously inoculated with 10^7 PFU of PV and were sacrificed daily. Virus titers in the tissues were determined. The PV titers in the kidneys (Fig. 1A) and livers (Fig. 1B) of PVR-tg mice decreased similarly to those of non-tg mice, which suggests that PV cannot replicate well in these tissues of PVR-tg mice. However, the virus titers in the kidneys of the PVR-tg/*Ifnar* knockout mice increased over time (Fig. 1A). The PV titer in the livers of the PVR-tg/*Ifnar* knockout mice remained at approximately 10^7 PFU/g tissue until 3 days postinfection (p.i.), with only a slight decrease observed (Fig. 1B).

We inoculated 10^8 PFU of PV intravenously into PVR-tg and PVR-tg/*Ifnar* knockout mice and detected PV antigens by immunohistochemistry. PV antigens were detected in the glomeruli of the kidneys obtained from the PVR-tg/*Ifnar* knockout mice (Fig. 1D), but not from those of PVR-tg mice (Fig. 1C). This indicates that at least cells in the glomeruli of kidneys in vivo are also potentially susceptible to PV infection. These cells may be exposed most easily to PV circulating in the blood. Consistent with the profile of the PV titer, a few PV antigen-positive cells were detected in the livers of the infected PVR-tg mice (Fig. 1E), but a large number of PV antigen-positive cells were detected in those of the PVR-tg/*Ifnar* knockout mice (Fig. 1F). These results indicate that PV can replicate in these sites if IFN signaling is disrupted.

In order to determine the IFN response in the kidneys and livers of the PVR-tg mice, we examined the expression of IFN-α, IFN-β, and OAS1a mRNAs after intravenous inoculation of PV (10^8 PFU) (Fig. 1G to J). We detected very low levels of IFN-α and IFN-β mRNAs in the uninfected kidneys and in the kidneys at 4 h p.i. However, at 6 h p.i., we observed expression of IFN-α and IFN-β mRNAs and induction of OAS1a mRNA by 14.4-fold compared to the value for the uninfected mice, suggesting that an antiviral state had been established by that time (Fig. 1G and H). In the liver, expression of IFN-α and IFN-β mRNAs and induction of OAS1a mRNA were clearly detected at 6 h p.i. (Fig. 1I and J). It should be noted that both kidneys and liver establish the antiviral state within 6 h p.i. Considering the number of cells in the living animal and the amount of PV inoculated, infection in the tissues must occur at a very low MOI. Therefore, only a small number of cells are infected at the beginning. IFN detected before 6 h p.i. might have been produced by these cells. Although the amounts of IFN mRNAs detected by RT-PCR are small, the IFN mRNA per infected cell must be significant. Since a single round of PV replication in the cells takes approximately 6 h, it is likely that the multiple rounds of PV replication are strongly inhibited in the kidneys and liver due to the IFN response.

PV susceptibility and IFN response in kidney cells in vitro. We prepared primary cultured cells by dispersing the mouse kidneys with trypsin. Approximately 6×10^7 viable cells per mouse, as judged by trypan blue staining, were obtained after trypsinization. The cells were seeded in plates at a density of 3×10^4 cells/cm^2. Approximately 1% of the cells began to grow and formed a monolayer 1 week later. As shown in Fig. 2B, C, and D, we observed at least three kinds of cells with different morphologies. When PV was inoculated into cultured cells at an MOI of 0.001, the PV titer increased rapidly (Fig. 2A), and all types of cells showed CPE by 24 h p.i. (Fig. 2E and F). When cells were infected with PV at an MOI of 10, rounding and detachment of the cells were observed at 6 h p.i. The results indicate that the kidney cells derived from the PVR-tg mice were susceptible to PV, although the kidneys in vivo are not the sites of PV replication. This result further shows that the kidneys of the PVR-tg mice acquire PV susceptibility after cultivation in vitro, which is the same as monkey kidneys. We then prepared kidney cells in culture from PVR-tg/*Ifnar* knockout mice. The increase in the PV titer and the appearance of CPE in the kidney cells derived from PVR-tg/*Ifnar* knockout mice were indistinguishable from those in the kidney cells derived from wild-type PVR-tg mice (Fig. 2A, E, and F). This, in turn, suggests that replication of PV in kidney cells in culture derived from wild-type PVR-tg mice is not affected by the IFN response.

In order to confirm the conclusions mentioned above, we infected kidney cells with PV at an MOI of 10 and measured the IFN response (Fig. 2G and H). We detected very low levels of IFN-α and IFN-β mRNAs in uninfected kidneys and in the kidneys at 4 h p.i. At 6 h p.i., expression of IFN-β mRNA increased slightly. However, we did not detect induction of OAS1a mRNA or IFN activity in the supernatants of the infected cells by a standard IFN assay (data not shown). It is