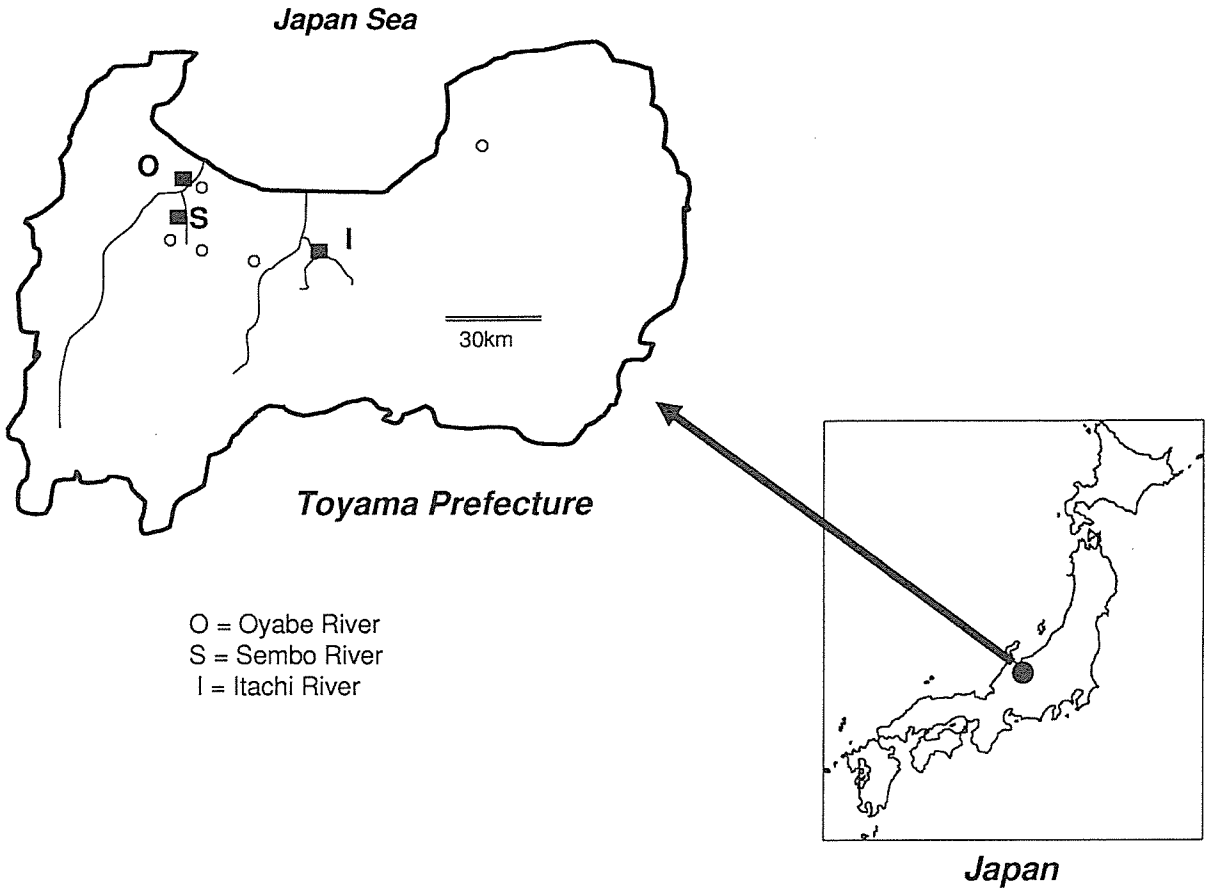

FIG. 1. Locations of the three rivers (Itachi [I], Oyabe [O], and Sembo [S]) in Toyama Prefecture. Squares (■) indicate the fixed points for water sampling. Circles (O) indicate the locations of hospitals where aseptic meningitis patients were admitted. Double lines indicate distance.

strains were isolated from May to December 2002. Nineteen E11 strains were isolated from September 2002 to January 2003. Most reoviruses were isolated in the latter periods, although they were isolated throughout the season.

Type 2 poliovirus was isolated in November 2002 after a routine immunization scheduled during the previous month. Differentiation of poliovirus isolates was performed by PCR-restriction fragment length polymorphism and sequencing methods as described previously (34), and isolates were characterized as vaccine type (data not shown).

TABLE 1. Virus isolation from three rivers in the Toyama Prefecture, Japan, between April 2002 and March 2003

Virus[a]	No. of virus isolates			
	Itachi River	Sembo River	Oyabe River	Total
E7		3		3
E11	4	14	1	19
E13	1	8	3	12
CB2	1	1		2
CB3		4	1	5
CB4		2		2
Poliovirus type 2		1[b]		1
Reovirus type 1	3		1	4
Reovirus type 2	33	48	38	119
Reovirus type 3	1			1
Not typed				3
Total	43	84	44	171

[a] E, echovirus; CB, coxsackievirus type B.
[b] A result of intratypic differentiation was Sabin 2.

Virus isolation from patients with aseptic meningitis. Clinical specimens (stool, cerebral spinal fluid, and throat swab) from seven aseptic meningitis patients diagnosed in Toyama in 2002 were used for virus isolation as described previously (13, 31). Five E13 viruses were also isolated from one stool specimen, two cerebral spinal fluid specimens, and two throat swabs from seven patients with aseptic meningitis in June and July 2002. Eight E11 isolates from aseptic meningitis patients collected between 1993 and 1998 in Hyogo Prefecture, Japan, were also used for analysis.

RT-PCR and nucleotide sequence analysis. E13 and E11 isolates were used for sequencing analysis. The viral RNA was extracted from virus fluid using a QIAamp Viral RNA Mini kit (QIAGEN, MD) and was then used for reverse transcription-PCR (RT-PCR) (Access RT-PCR system; Promega, WI). For amplification of the partial VP1 region of the viral capsid protein, two sets of panenterovirus degenerate primers described previously by Oberste et al. were used (24). Briefly, for amplification of the region upstream of VP1, sense primer 187

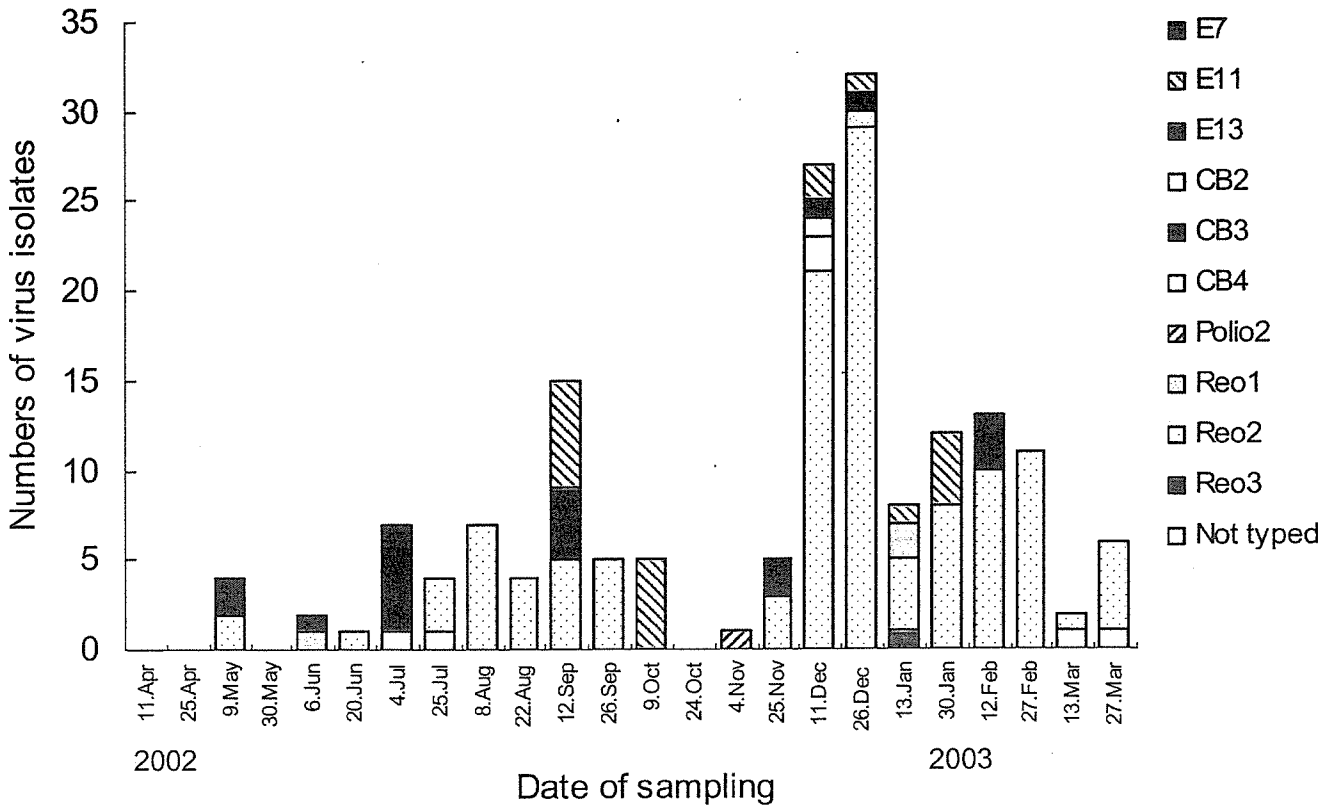

FIG. 2. Total numbers of virus isolates from three rivers from April 2002 (Apr) to March 2003 (Mar). Waters from fixed points of three rivers were collected on the indicated dates. CB2, coxsackievirus type B2; Polio2, poliovirus type 2; Reo1, reovirus type 1.

and antisense primer 222 were used, and for amplification of the downstream region, sense primer 012 and antisense primer 011 were used (24). RT-PCR was carried out under the following conditions: reverse transcription at 48°C for 45 min, inactivation at 94°C for 2 min, and 35 cycles of annealing at 50°C for 10 s, polymerization at 65°C for 1 min, and denaturation at 94°C for 10 s. After 35 cycles, an additional elongation step of 65°C for 1 min was done. The PCR product was purified by using a QIAquick PCR purification kit (QIAGEN) and directly sequenced using a PRISM Big-Dye Terminator cycle sequencing reaction kit on an automated DNA sequencer (Perkin-Elmer Applied Biosystems) as described previously (31).

Genetic relationships among E13 isolates or E11 isolates were analyzed by MEGA 3.1 software (16) using the partial VP1 region of E13 (703 bp; positions 2579 to 3281 on strain Del Carmen) and of E11 (561 bp; positions 2765 to 3325 on strain Gregory). Phylogenetic trees were constructed by neighbor-joining methods after estimation of genetic distance using the Kimura two-parameter method (14). The transition/transversion rate was set at 2.0, and a bootstrapping test was performed 1,000 times (8).

Sixty-one- and 51-nucleotide sequences of E13 and E11, respectively, were available in GenBank. The strains are represented as accession no./country or city/year/strain code using the reference or Web data in GenBank (1, 2, 5, 12, 15, 25).

The nucleotide sequences of E11 and E13 isolates were phylogenetically compared. For E13, the nucleotide divergence was less than 1.3% among isolates from the three rivers in the partial VP1 region (703 bp). The nucleotide sequence divergence was 0.9 to 1.3% between 12 environmental isolates and 5 clinical isolates, and at most, one amino acid substitution was found. Therefore, environmental isolates were closely related to clinical isolates in Toyama Prefecture (Fig. 3a). Compared to 19 other isolates from Japan during 2001 to 2002 found in GenBank, the divergence was 1.2 to 1.6%, with, at most, one amino acid substitution. Phylogenetic analysis showed that all E13 isolates in Japan belonged to the same cluster (Fig. 3a).

Moreover, isolates in Toyama from both environmental sources and patients were compared with the other E13 sequences available from GenBank, including the above-mentioned 19 strains from Japan. Phylogenetic analysis showed that the Toyama isolates belonged to the major genomic group labeled as the 2000–2002 cluster in Fig. 3b, which was described previously by other studies (1, 2, 5, 12, 15). Divergence of nucleotide sequences between Toyama isolates and others in this group was 1.9 to 2.5% (amino acid divergence, 0.3 to 0.7%), indicating that these Toyama isolates belonged to the major genomic group circulating worldwide.

On the other hand, nucleotide sequences of E11 were phylogenetically analyzed and antigenically categorized into two major strains: strain Gregory as the prototype and strain Silva as prime type (Fig. 4). There were several kinds of subgenotypes within these two major strains (6, 25). Although E11 was not isolated from patients in Toyama during the period of this

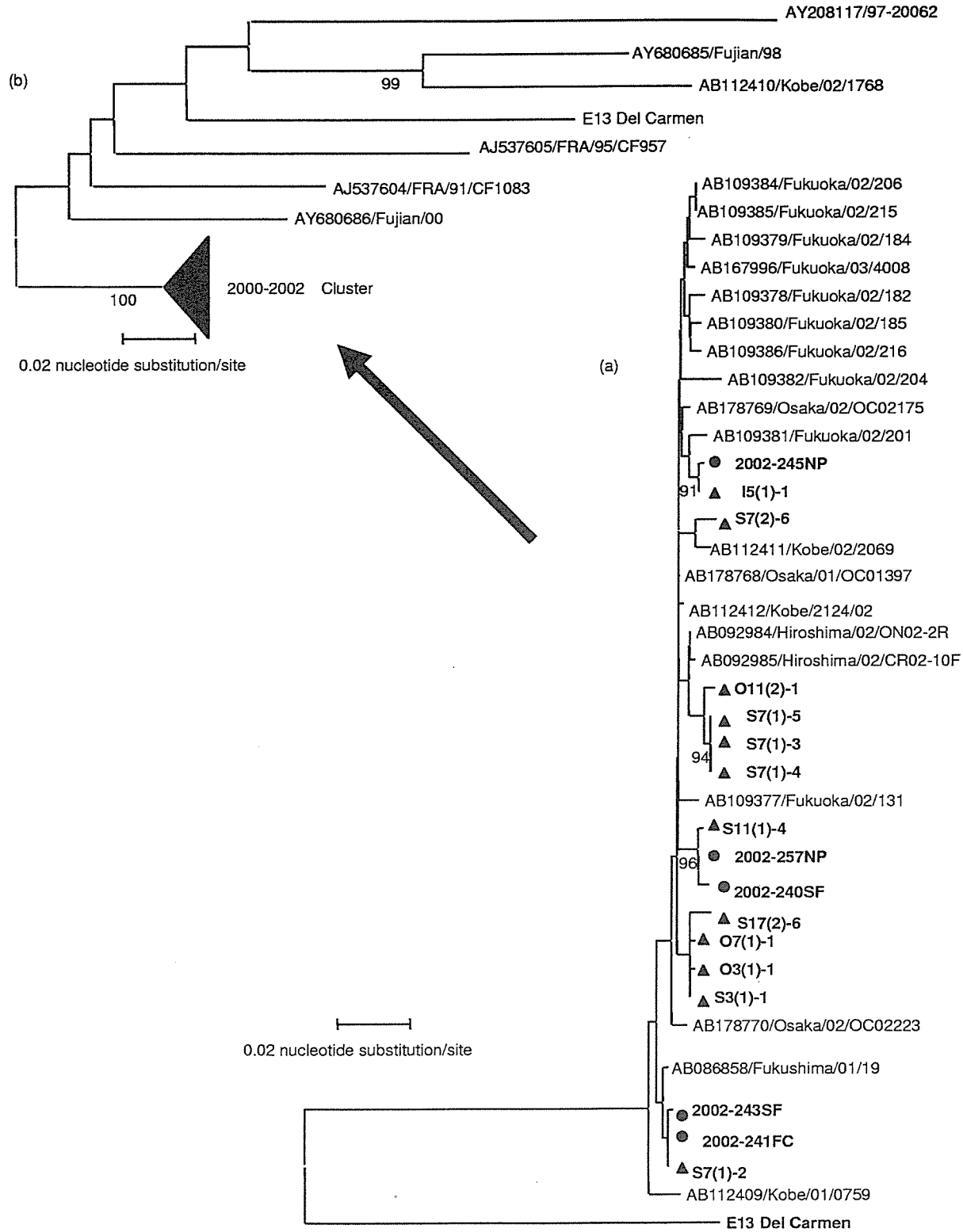

FIG. 3. (a) Phylogenetic relationships between the Japanese isolates. Phylogenetic trees for E13 using the partial VP1 region (703 bp) were generated by the neighbor-joining method with 36 strains: 17 Toyama isolates (12 from river samples and 5 from patients) and 19 other Japanese isolates (GenBank accession no. AB086858, AB092984, AB092985, AB109377 to AB109382, AB109384 to AB109386, AB112409, AB112411, AB112412, AB167996, and AB178768 to AB178770). Bootstrap values (in percentages) for 1,000 replicated trees are indicated. Circles and triangles specify patient isolates and environmental isolates, respectively. S, O, and I indicate Sembo, Oyabe, and Itachi, respectively. (b) Phylogenetic trees were reconstructed using 61 strains (accession no. AB112410, AJ537604 to AJ537609, AY227334 to AY227347, AY268561, AY268563 to AY268569, AY268571 to AY268580, AY680685, AY680686, and AJ241427 from GenBank and including the 19 other Japanese isolates). Bootstrap values (in percentages) for 1,000 replicated trees are indicated. The major genomic group isolated during 2000 to 2002 was compressed within the same cluster (2000–2002 cluster). Arrows from tree a to tree b indicate that Japanese isolates in tree a are included in the compressed cluster. The strains are represented as accession no./country or city/year/strain code using the reference or Web data in GenBank.

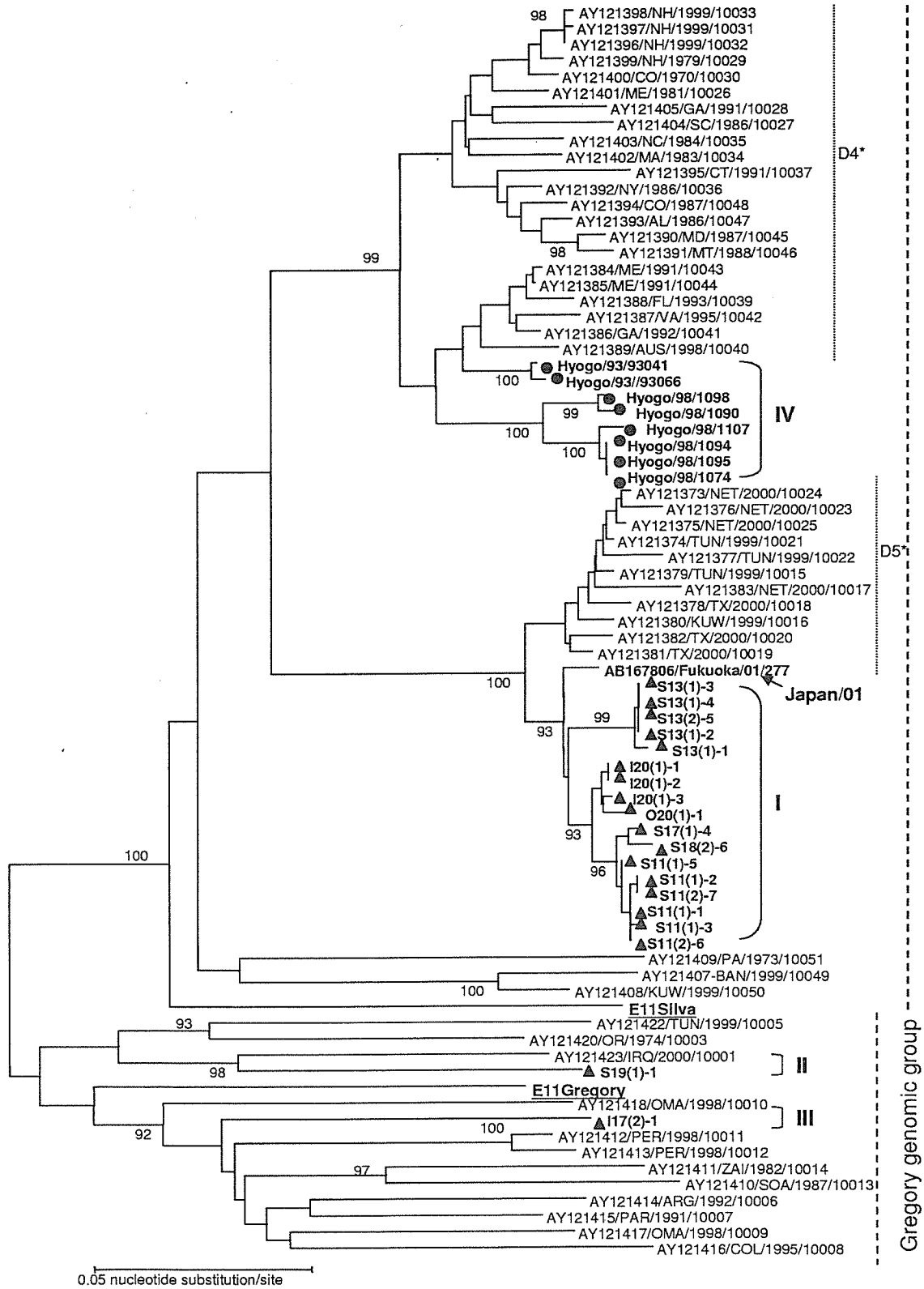

FIG. 4. Phylogenetic tree for E11 using the partial VP1 region (561 bp) generated by the neighbor-joining method with 78 strains: 19 Toyama River isolates, 8 patient isolates from Hyogo, and 51 other isolates (accession no. AY121373 to AY121405, AY121407 to AY121418, AY121420, AY121422, AY121423, AB167806, AF295498, and AF081326 from GenBank). Circles and triangles indicate viruses isolated from patients and the environment, respectively. Bootstrap values (in percentages) for 1,000 replicated trees are indicated. Subgenomic groups I to IV in this study are shown, with D4 and D5 clusters being named as suggested previously by Oberste et al. (25). The strains are represented as accession no./country or city/year/strain code using the reference or Web data in GenBank. Isolate identifiers consist of a three-letter country abbreviation (ARG, Argentina; AUS, Australia; BAN, Bangladesh; COL, Colombia; DOR, Dominican Republic; ZAI, Democratic Republic of Congo; IRQ, Iraq; KUW, Kuwait; NET, The Netherlands; OMA, Oman; PAR, Paraguay; PER, Peru; SOA, South Africa; TUN, Tunisia; Tur, Turkey; or USA, United States), with two-letter abbreviations for U.S. states. The viral isolate from Fukuoka City (accession no. AB167806) is indicated by arrows (Japan/01).

study, 19 E11 samples were isolated from the three rivers. Eight clinical isolates from Hyogo Prefecture between 1993 and 1998 were also used in the analysis together with the 51 E11 sequences available in GenBank.

Environmental isolates were divided into three genomic groups, groups I, II, and III (Fig. 4). Seventeen isolates fell into major genomic group I, with 1.5 to 2.1% nucleotide divergence within the partial VP1 region (561 bp). Oberste et al. previously described that the genomic group of Silva consisted of five subgroups, subgroups D1 to D5 (25). Toyama isolates had a mean of 18.3% divergence in nucleotides compared to the E11 Silva strain and only 3.9 to 5.1% divergence compared to the 1999–2000 D5 subgroup. Therefore, we classified Toyama isolates as belonging to subgroup D5. Moreover, nucleotide sequences of these isolates were very similar to aseptic meningitis isolates from Fukoaka City in 2001 (nucleotide divergence, 1.8 to 2.3%). Clinical isolates from Hyogo in 1993 and 1998 (group IV) were categorized as belonging to subgenomic group D4 from 1970 to 2001 (Fig. 4). I17(2)-1 (group III) and S19(1)-1 (group II) isolates were classified as belonging the Gregory genomic group and had nucleotide divergences from the genomic group of 21.2 to 24.0% and 18.9 to 21.5%, respectively.

Concluding remarks. There was a large outbreak of aseptic meningitis caused by E13 in the summer of 2002 in Japan, which coincided with a small outbreak caused by E11 (9). The aim of this study was to assess the performance of an environmental surveillance of river water isolates compared with isolates from clinical samples. These viruses were also isolated from the rivers in Toyama Prefecture by environmental surveillance. E13 was detected not only in Toyama Prefecture but also in other places in Japan simultaneously. The phylogenetic analysis of E13 showed that the isolates from both river waters and patients belonged to the same genomic cluster, one of the major genotypes circulating worldwide since 2000.

E11 was recently detected during the autumn/winter from river water in Toyama and was compared to isolates from elsewhere in Japan. Although there was no outbreak of aseptic meningitis in Toyama, E11 might have been silently circulating in the human population. Phylogenetic analysis showed that the isolates were divided into three genomic groups: isolates in group I belonging to the Silva genomic group, which seemed to be circulating mainly in Toyama, and minor E11 isolates of I17(2)-1 or S19(1)-1 belonging to the Gregory genomic group, which also seemed to be circulating in other areas of Japan. Thus, this environmental survey was capable of detecting enteroviruses of different genomic groups with high sensitivity and was capable of tracing minor circulating viruses.

In addition, many reoviruses were isolated during the surveillance, most frequently between December 2002 and February 2003. Matsuura et al. suggested in a previous study (19) that stool from not only humans but also animals might have contaminated the waters.

Since there were only a few reports of E11 and E13 isolation in Japan between 2003 and 2005 (11) according to the Infectious Agents Surveillance Report, these viruses appeared to temporarily cease circulation or to circulate silently. This report shows that the combination of conventional virus isolation from environmental sources together with phylogenetic analysis of clinical isolates is a useful approach in understanding enterovirus circulation and transmission. Therefore, environmental surveillance should be considered a complementary assessment tool to trace prevalent and minor enteroviruses circulating in the human population.

Nucleotide sequence accession numbers. The sequence data in this study were deposited in GenBank under accession no. AB239081 to AB239124.

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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

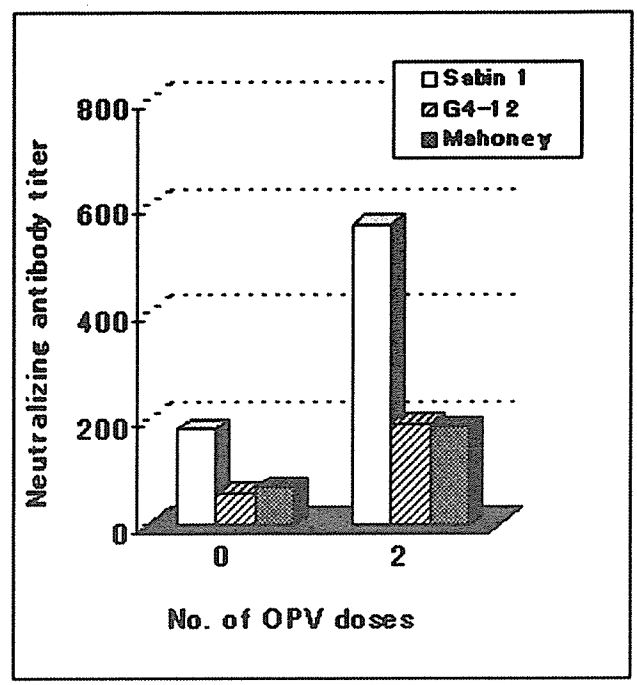

Fig. 1

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

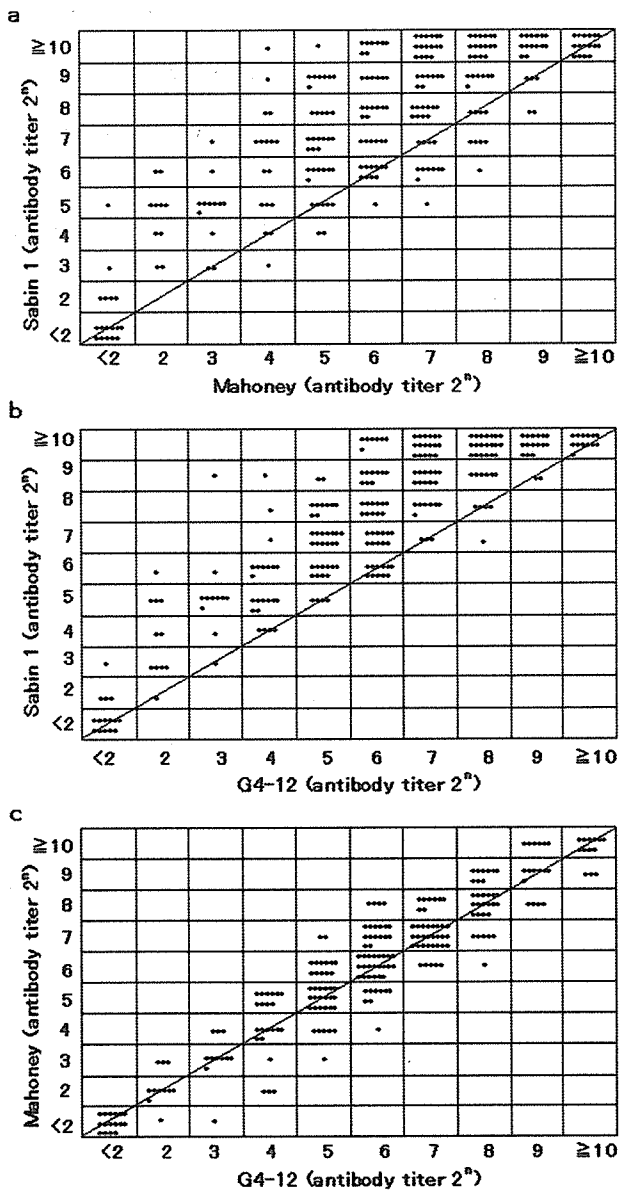

Fig. 2

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 pre-central 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

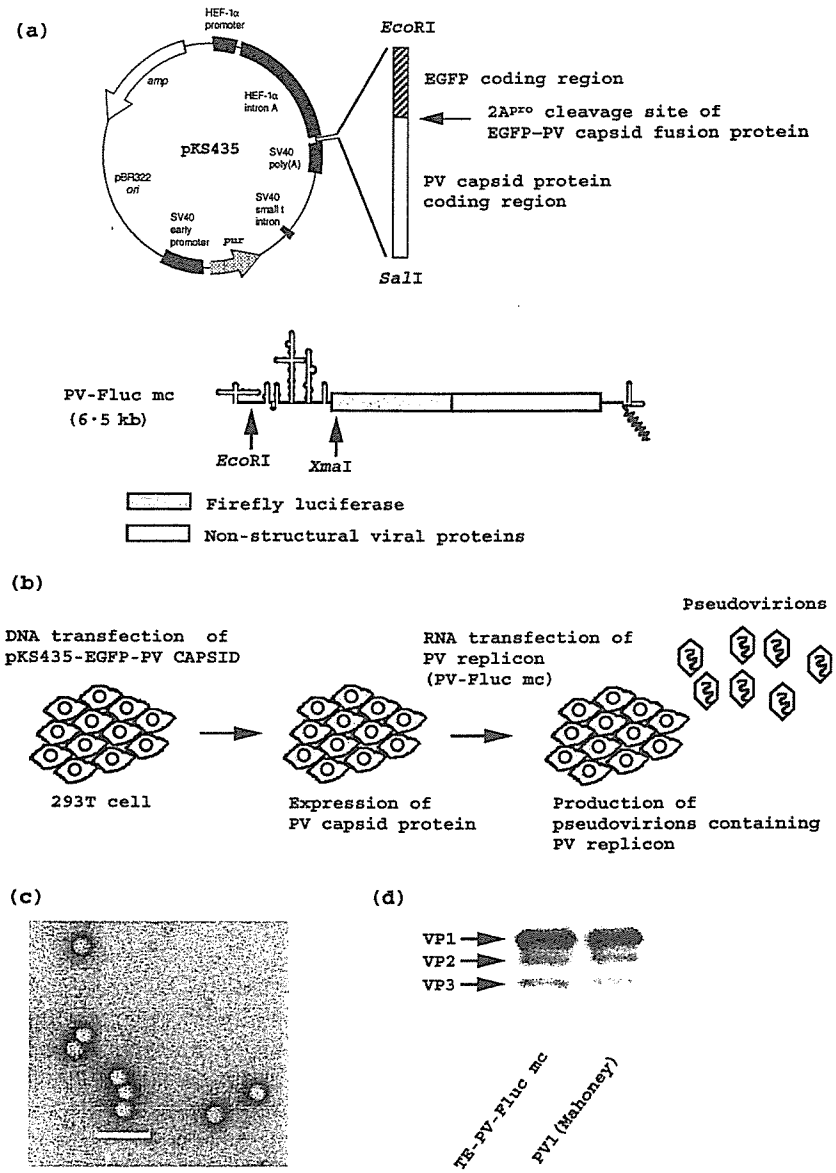

Fig. 1. *trans*-Encapsidation of a PV replicon encoding firefly luciferase. (a) Schematic view of expression vector pKS435 (upper panel) and PV replicon (lower panel) used for the *trans*-encapsidation. PV capsid was expressed as a fusion protein with EGFP in the N terminus, with a $2A^{pro}$ cleavage 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

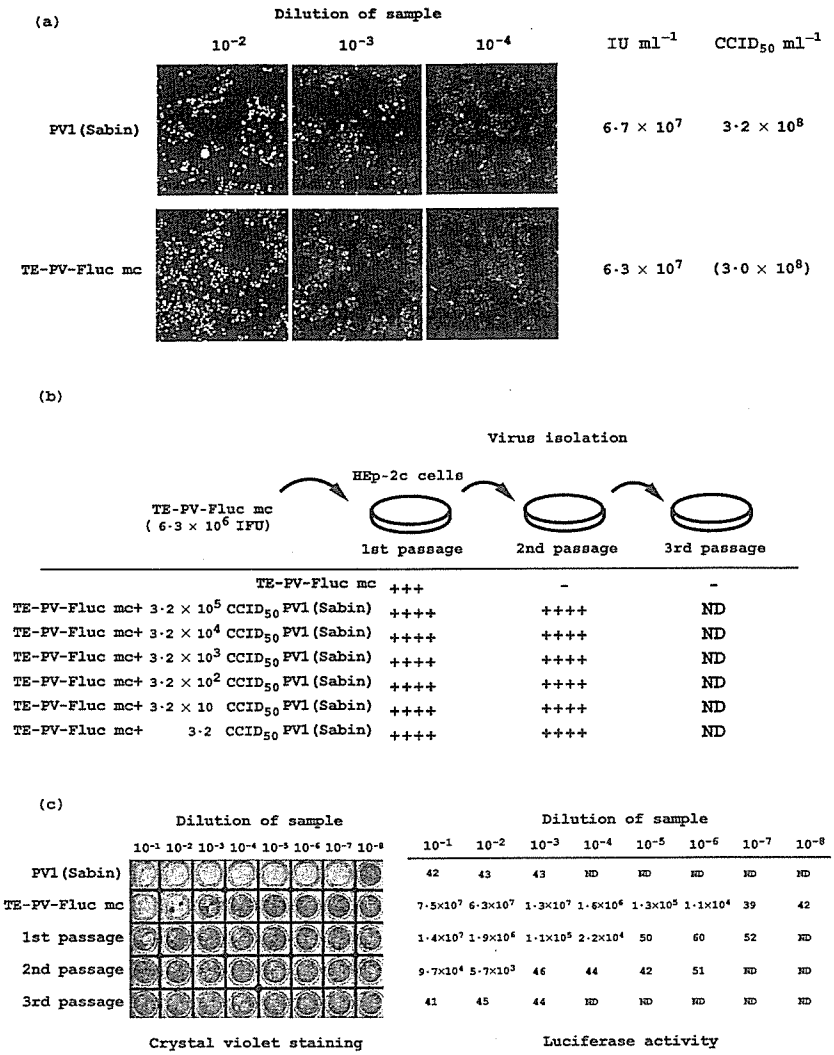

Fig. 2. Characterization of the *trans*-encapsidated PV replicon. (a) Titration of TE-PV-Fluc mc. The corresponding $CCID_{50}$ value of TE-PV-Fluc mc was estimated from that of PV1 (Sabin). (b) Isolation of viable viruses from the preparation of TE-PV-Fluc mc. TE-PV-Fluc mc alone or mixed with fixed 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

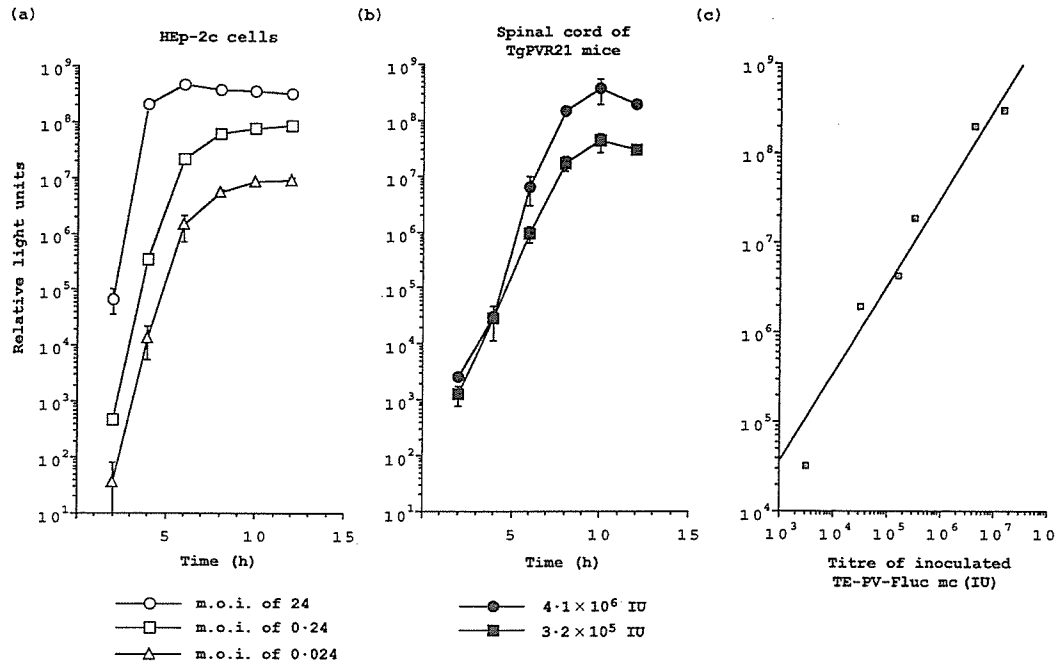

Fig. 3. *In vitro* and *in vivo* replication kinetics of TE-PV-Fluc mc. (a) Replication kinetics in HEp-2c cells. HEp-2c cells ($2{\cdot}6 \times 10^4$) were infected with $6{\cdot}3 \times 10^5$, $6{\cdot}3 \times 10^3$ or $6{\cdot}3 \times 10^2$ IU TE-PV-Fluc mc. (b) Replication kinetics in the spinal 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

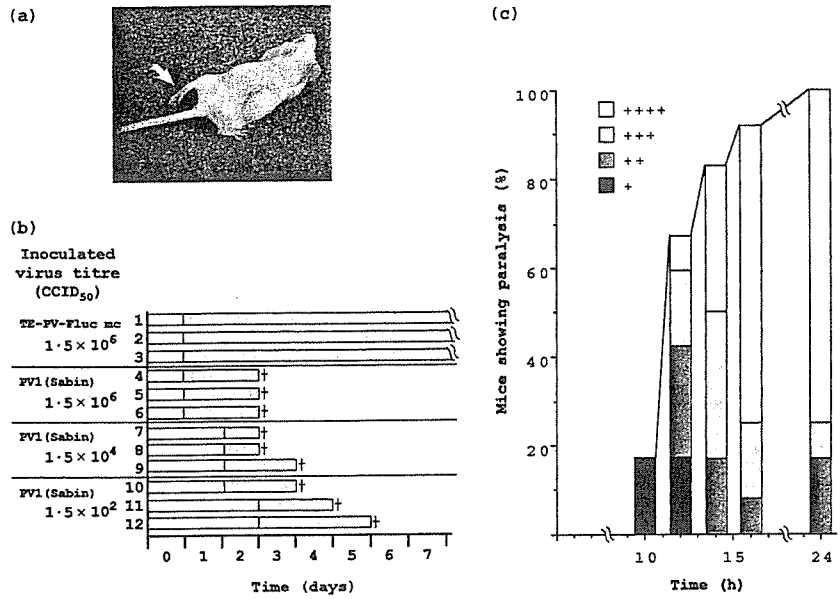

Fig. 4. Characterization of the paralysis of TgPVR21 mice inoculated with TE-PV-Fluc mc. (a) Paralysis of a TgPVR21 mouse inoculated with TE-PV-Fluc mc ($3{\cdot}2\times10^{5}$ IU) via the intraspinal route at 24 h p.i. Arrow indicates a paralysed left hindlimb. (b) Time course of the appearance of paralysis of TgPVR21 mice inoculated with TE-PV-Fluc mc or PV1 (Sabin). TgPVR21 mice were inoculated with $3{\cdot}2\times10^{5}$ IU ($1{\cdot}5\times10^{6}$ estimated $CCID_{50}$) TE-PV-Fluc mc or with $1{\cdot}5\times10^{2}$ to $1{\cdot}5\times10^{6}$ $CCID_{50}$ 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 $3{\cdot}2\times10^{6}$ IU ($1{\cdot}5\times10^{7}$ 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 $1{\cdot}9\times10^{2}$ IU (or $8{\cdot}9\times10^{2}$ $CCID_{50}$) 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,

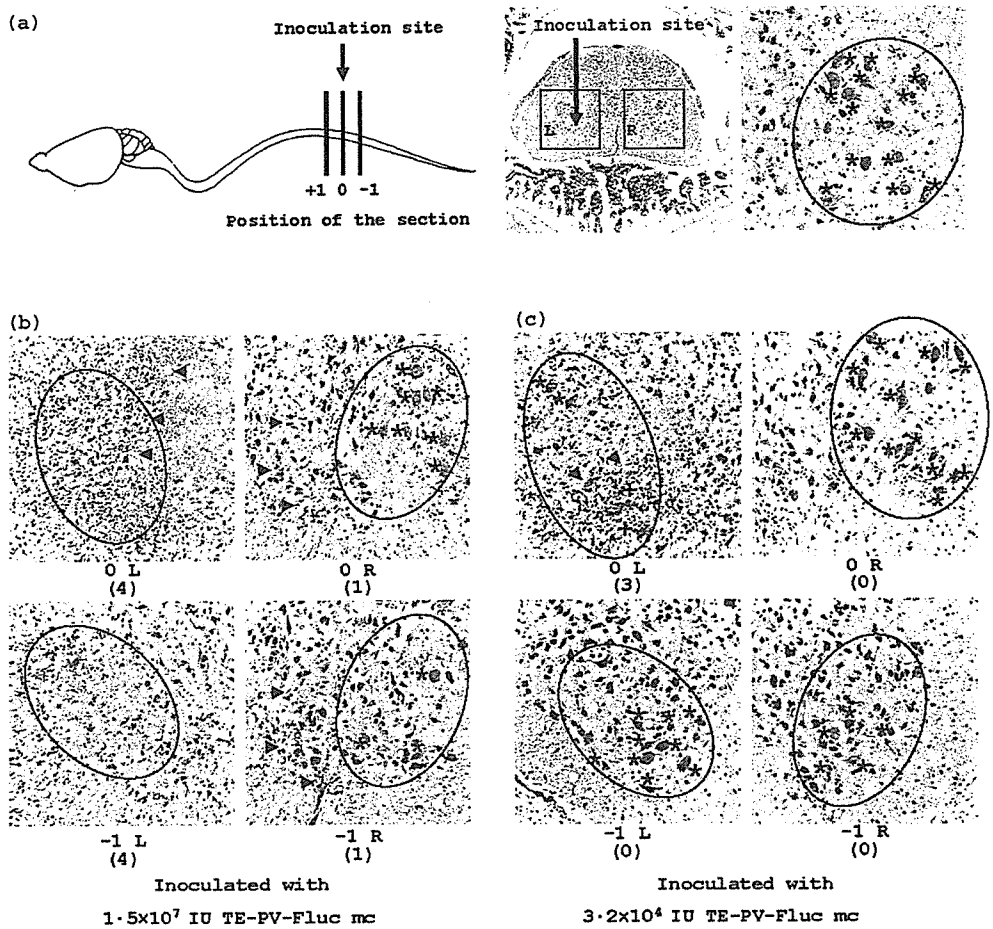

Fig. 5. Histological analysis of spinal cords in TgPVR21 mice inoculated with TE-PV-Fluc mc. TgPVR21 mice inoculated with TE-PV-Fluc mc via the intraspinal route were subjected to histological analysis at day 3 p.i. In each section, the areas of the anterior horn are encircled by ellipses, the cell bodies 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.