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# Transgenic Mice Carrying the Human Poliovirus Receptor: New Animal Model for Study of Poliovirus Neurovirulence

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**Recombinant viruses between the virulent Mahoney and attenuated Sabin 1 strains of poliovirus type 1 were subjected to neurovirulence tests using a transgenic (Tg) mouse line, ICR-PVRTg1, that carried the human poliovirus receptor gene. The Tg mice were inoculated intracerebrally with these recombinant viruses and observed for clinical signs, histopathological lesions, and viral antigens as parameters of neurovirulence of the viruses. These parameters observed in the Tg mice were different for different inoculated viruses. Dose-dependent incidences of paralysis and of death were observed in the Tg mice inoculated with any viruses used. This indicates that values of 50% lethal dose are useful to score a wide range of neurovirulence of poliovirus. The neurovirulence of individual viruses estimated by the Tg mouse model had a strong correlation with those estimated by monkey model. Consequently, the mouse tests identified the neurovirulence determinants on the genome of poliovirus that had been identified by monkey tests. In addition, the mouse tests revealed new neurovirulence determinants, that is, different nucleotides between the two strains at positions 189 and 21 and/or 935 in the 5′-proximal 1,122 nucleotides. The Tg mice used in this study may be suitable for replacing monkeys for investigating poliovirus neurovirulence.**

Poliovirus, known to be the causative agent of poliomyelitis, is a human enterovirus that belongs to the family *Picornaviridae* and is classified into three stable serotypes, 1, 2, and 3. Most poliovirus strains, with a few exceptions, infect only primates. Severe paralytic or, less frequently, fatal disease occurs as a result of lytic replication of poliovirus in neurons in the anterior horn of the spinal cord, motor cortex, and brain stem. To control poliomyelitis, attenuated poliovirus strains that cannot replicate well in the central nervous system (CNS) have been developed and effectively used as oral live vaccines, that is, Sabin 1 (type 1), Sabin 2 (type 2), and Sabin 3 (type 3) (29). However, the molecular mechanisms of poliovirus attenuation are not fully understood.

The difference in potential for causing the disease between the virulent and attenuated poliovirus strains must be due to the differences in the genome structures. The genome of poliovirus is an approximately 7.5-kb, single-stranded RNA with positive polarity. To date, cDNA copies of both the virulent and attenuated strains of all three poliovirus serotypes have been sequenced and cloned in *Escherichia coli* (6, 14, 20, 24, 25, 32–34). Comparative sequence studies performed on the genomes of attenuated Sabin 1 and its parental virulent Mahoney strains revealed 57 nucleotide substitutions within the 7,441 total heteropolymeric bases (20). The nucleotide changes were found to be scattered over the entire genome and resulted in 21 amino acid replacements within the viral polyprotein (20). Similarly, 10 nucleotide and three amino acid substitutions were found in the genomes of Sabin 3 and its parental virulent Leon strain (32, 33).

To identify neurovirulence determinants on the genomes of poliovirus, monkeys have been used as an experimental animal model. Neurovirulent variants of the vaccine strain and recombinant viruses between the virulent and attenuated strains were tested for their monkey neurovirulence (1, 2, 5, 8, 15, 16, 21, 35). The results have revealed that the loci influencing the attenuation phenotype are spread over several areas of the viral genome and that strong neurovirulence determinants reside in the 5′ noncoding regions of the genomes of all three serotypes (2, 8, 15, 21, 35). Particularly, nucleotide positions 480, 481, and 472 are identified to be the important determinants for poliovirus types 1, 2, and 3 (2, 5, 15). In contrast to a great contribution of position 472 of type 3 poliovirus to the attenuation phenotype, position 480 of type 1 poliovirus only partially influenced the phenotype (5). It is almost impossible, however, to identify other determinants in the 5′ noncoding region and weak determinants in other genome regions of type 1 poliovirus as long as experiments involve monkeys because of the difficulty in obtaining statistically reliable results (5). It is therefore desirable to establish a new experimental animal model which overcome disadvantages associated with monkey model.

Recent success in molecular cloning of the genomic and complementary DNAs for human poliovirus receptor made it possible to give permissiveness for poliovirus infection to cells of nonprimate origin (10, 19). Furthermore, the transgenic (Tg) mice carrying the human poliovirus receptor gene were shown to be susceptible to all three poliovirus serotypes. These Tg mice inoculated with poliovirus showed a paralytic disease which was clinically and pathologically similar to human poliomyelitis (11, 26, 28). The virulent and attenuated pheno-

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types of poliovirus strains appeared to be preserved in the Tg mice, since much larger doses of virus were required for the attenuated strain to cause paralysis compared with those of the virulent strain and since clinical behaviors caused by the virulent and attenuated strains are different from each other (11). These observations suggest that the Tg mice become a new animal model to study poliovirus neurovirulence.

This study was performed for the following two purposes: (i) establishment of neurovirulence tests using the Tg mouse model, including methods and criteria for evaluating neurovirulence levels of polioviruses, based on the comparison of the results obtained by mouse tests with those by monkey tests, and (ii) identification of neurovirulence determinants of type 1 poliovirus by using the Tg mouse model. Mouse neurovirulence tests were carried out with a number of recombinant viruses between the virulent Mahoney and the attenuated Sabin 1 strains, most of which had already been tested for their neurovirulence in monkeys (5, 8, 21). The Tg mice intracerebrally inoculated with viruses were observed for clinical signs, histopathological lesions, and viral antigens. By comparison of the data with those of monkey tests, the mouse neurovirulence tests are established. Furthermore, our results suggest that the Tg mice are suitable for replacing monkeys for investigating poliovirus neurovirulence.

## MATERIALS AND METHODS

**Strain of the Tg mice.** Tg mouse line ICR-PVRTg1 (Tg1), which is susceptible to poliovirus, was used for the experiments. Since the Tg1 mice are maintained with hemizygotes, PCR was employed to select the mice that carried the transgene (11). Tg1 mice 6 to 7 weeks old were used in this study. All mice used were free from specific pathogens.

**Construction of infectious cDNA clones of recombinant viruses.** To construct infectious cDNA clones, pVSM(1)IC-7b(T), pVSM(1)IC-17a(T), and pVSM(1)IC-18b(T), the cDNA segments of the corresponding nucleotide positions from 1 to 1813 of the Mahoney and Sabin 1 strains were subcloned into vector plasmid pML2 and designated pEP(M) and pEP(Sab), respectively (5, 12). Allele replacement experiments were carried out on these two plasmids with the restriction enzymes *Kpn*I, *Nco*I, *Fok*I, and *Ban*II. The resulting plasmids were designated pEP(SM)7b, pEP(SM)17a, and pEP(SM)18b. The shorter *Aat*II fragments of pVS(1)IC-0(T) (9) were replaced by the shorter *Aat*II fragments of these plasmids. As a result, we constructed three new recombinant cDNA clones that were infectious in mammalian cells.

**Cells and viruses.** African green monkey kidney (AGMK) cells were maintained in Dulbecco modified Eagle medium supplemented with 5% newborn calf serum. AGMK cells were transfected with 10 μg of the closed circular forms of the recombinant cDNA clones per 60-mm-diameter plastic dish by the modified calcium phosphate method described previously (9). The viruses recovered from the cells transfected with pVSM(1)IC-7b(T), pVSM(1)IC-17a(T), and pVSM(1)IC-18b(T) were designated PV1(SM)IC-7b, PV1(SM)IC-17a, and PV1(SM)IC-18b, respectively. These three recovered viruses were grown at 33.5°C in AGMK cells once and used as virus stocks. Other virus stocks were the same as viruses PV1(M)pDS306 and PV1(Sab)IC-0 reported by Kohara et al. (9); viruses PV1(SM)IC-2a, PV1(SM)IC-2b, PV1(SM)IC-4a, and PV1(SM)IC-4b reported by Omata et al. (21); viruses PV1(SM)IC-8a, PV1(SM)IC-8b, PV1(SM)IC-11a, and PV1(SM)IC-11b reported by Kohara et al. (8); and viruses PV1(SM)IC-5a, PV1(SM)IC-6a, PV1(SM)IC-9a, and PV1(SM)IC-10b reported by Kawamura et al. (5).

Nucleotide sequences of a 5′ portion of the genomes (positions 21 to 992) of PV1(Sab)IC-0, PV1(SM)IC-5a, PV1(SM)IC-6a, PV1(SM)IC-7b, PV1(SM)IC-9a, PV1(SM)IC-10b, PV1(SM)IC-17a, and PV1(SM)IC-18b were analyzed to confirm the modified nucleotide sequences by direct sequencing of the DNA fragments that had been amplified by PCR from cDNAs of the recovered virus genomes (4, 17). Nucleotide sequences were determined by the dideoxy chain termination method (30) using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp.).

**Clinical neurovirulence tests.** Into the brain of each Tg1 mouse, 30 μl of a virus suspension was inoculated. Ten Tg1 mice, five males and five females, were used for injection with each dose. For experiments with certain doses of viruses, 10 or 20 more mice were employed to obtain more reliable results. The Tg1 mice inoculated with viruses were observed for clinical symptoms, including paralysis, weakness, and death, at 12-h intervals up to 14 days. The 50% lethal doses ($LD_{50}$) and 50% paralytic doses and their standard errors were calculated according to the Dragstehdt-Behrens method, a modified Reed-Muench method (3), and the method of Pizzi (23), respectively.

**Examination of histopathological lesions and virus antigens.** The CNSs of the Tg1 mice that became moribund after inoculation of PV1(M)pDS306, PV1(Sab)IC-0, and PV1(SM)IC-4b were subjected to examination for histopathological changes and viral antigens. Mice inoculated with PV1(Sab)IC-0 sacrificed on the day of onset of paralysis were also subjected to histopathological examinations. All mice were sacrificed under anesthesia with ether, according to the guidelines for animal experiments in the Tokyo Metropolitan Institute of Medical Science.

The brains and spinal cords of the Tg mice inoculated with viruses were fixed in a solution of 10% buffered formalin and embedded in paraffin. The sections of the CNS were prepared, deparaffinized, and stained with hematoxylin-eosin and gallocyanin and by the method of Klüver and Barrera (KB) (7) to score the intensity of histopathological lesions. These parts of the CNS correspond to those of monkeys required for monkey neurovirulence tests of oral poliovirus vaccines (36).

Thin sections of the CNS, after deparaffinization, were reacted to rabbit hyperimmune sera against type 1 poliovirus at 4°C overnight, washed with phosphate-buffered saline, and treated with $H_2O_2$ to get rid of endogenous peroxidase activity. The thin sections were then treated with anti-rabbit immunoglobulin G conjugated with horseradish peroxidase by using the Vectastain ABC Anti-Rabbit Kit (Vector Laboratories). Poliovirus antigens were detected by incubation with diaminobentidine as an indicator. Methyl green-pyronine staining was also employed as the counterstain.

## RESULTS

**Clinical symptoms caused by polioviruses.** To find the mouse neurovirulence levels of recombinant viruses, the Tg1 mice were inoculated intracerebrally with the viruses. The genome structures of recombinant viruses are shown in Fig. 1. All viruses caused flaccid paralysis and death in infected mice. Incidences of paralysis and of death increased with doses of viruses inoculated (Table 1). The latency period was 3 to 10 days after inoculation in most paralytic cases, and the period also appeared to shorten with increasing doses of viruses (data not shown). However, the amount of virus required for causing disease was different from virus to virus. $LD_{50}$s and 50% paralytic doses calculated are shown in Table 1. Both values for PV1(M)pDS306 and PV1(Sab)IC-0 were the smallest and the

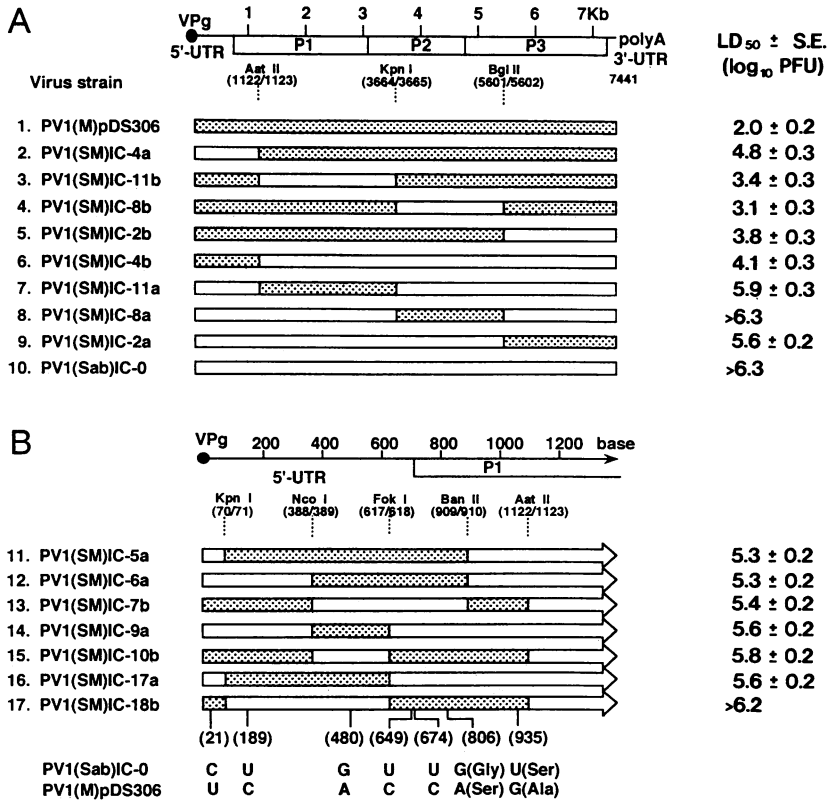


FIG. 1. Genome structures of recombinant type 1 polioviruses. The genome structures of the recombinant viruses are shown as a combination of the Mahoney (shadowed boxes) and the Sabin 1 (open boxes) sequences. The length of the genome of the type 1 poliovirus is shown at the top of the panels in kilobases (A) and bases (B) from the 5′ terminus. VPg is the genome-linked protein covalently attached to the 5′ terminus of the genome. P1 represents the protein region for viral capsid proteins. P2 and P3 represent protein regions for viral noncapsid proteins. UTR stands for untranslated region of the RNA. Restriction enzyme cleavage sites used for allele replacement experiments are shown by names of enzymes with nucleotide positions. Nucleotide and amino acid differences between the Mahoney [PV1(M)pDS306] and Sabin 1 [PV1(Sab)IC-0] strains are shown at the bottom of panel B. The nomenclatures for virus strains are indicated on the left. Logarithmic $LD_{50}$s and standard errors of each virus obtained by the mouse neurovirulence test are shown on the right.

largest, respectively. All other recombinant viruses showed intermediate values. Tg1 mice appear to be much more sensitive to poliovirus than monkeys are, since $10^6$ PFU of PV1(Sab)IC-0 paralyzed 3 of 20 mice, whereas no monkeys are paralyzed by inoculating $10^6$ PFU of the virus intracerebrally, and since more than $10^4$ PFU of PV1(M)pDS306 caused paralysis in all the mice although three of four monkeys are paralyzed by inoculating $10^6$ PFU of the virus intracerebrally (Table 1; see Fig. 3A) (21).

Development of the disease of infected mice appeared to reflect neurovirulence levels of individual viruses. In mice inoculated with PV1(M)pDS306, the disease developed very rapidly. All mice died within 24 h after the onset of paralysis in all limbs or, in some cases, only in the front limbs. These observations supported a strong neurovirulence phenotype of this virus in the Tg mice.

On the other hand, most Tg mice inoculated with PV1(Sab)IC-0, PV1(SM)IC-2a, PV1(SM)IC-5a, PV1(SM)IC-6a, PV1(SM)IC-7b, PV1(SM)IC-8a, PV1(SM)IC-9a, PV1(SM)IC-10b, PV1(SM)IC-11a, PV1(SM)IC-17a, and PV1(SM)IC-18b developed paralysis in the hind limbs and in the front limbs later on and then died 1 to 5 days after the clinical signs appeared. Some mice survived with paralysis in one or both hind limbs (Table 1). The results supported less neurovirulent phenotypes of this group of viruses in Tg1 mice.

About one-half of the Tg1 mice inoculated with PV1(SM)IC-2b, PV1(SM)IC-4a, PV1(SM)IC-4b, PV1(SM)IC-8b, and PV1(SM)IC-11b developed clinical signs similar to those of mice inoculated with PV1(M)pDS306, and the other half of the mice developed signs similar to those of mice inoculated with PV1(Sab)IC-0. In the latter case, however, most mice died 1 to 2 days after the onset of paralysis, and no mice survived. This group of viruses, therefore, can be classified into viruses with intermediate neurovirulence levels.

**Histopathological lesions and viral antigens in the CNS.** The CNSs of the Tg mice were examined for histopathological lesions caused by viruses PV1(M)pDS306, PV1(Sab)IC-0, and PV1(SM)IC-4b, which represented virus groups with strong, weak, and intermediate neurovirulence levels (Fig. 2 and 3). Histopathological changes such as degeneration of neurons and/or inflammatory cell infiltration were observed in the cerebral cortex, thalamus, midbrain, pons, medulla oblongata, and cervical, thoracic, and lumbar spinal cords of the Tg1 mice inoculated with any viruses used (Fig. 2B, C, E, and F and 3). Poliovirus antigens were detected in these areas.

Viruses with different neurovirulence levels induced different histopathological changes. In the mice paralyzed with PV1(M)pDS306, atrophic degeneration was observed in large numbers of neurons spread in the brain stem and spinal cord (Fig. 3). Poliovirus antigens were detected in damaged neurons

TABLE 1. Neurovirulence test on ICR-PVRTg1 mice

| Virus no. and strain | No. of animals dead/no. inoculated with ($log_{10}$ PFU/animal): | | | | | | $LD_{50}$ ± SE ($log_{10}$ PFU) | $PD_{50}$[a] ± SE ($log_{10}$ PFU) |
|---|---|---|---|---|---|---|---|---|
| | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 | | |
| 1. PV1(M)pDS306 | 2/20 | 10/20 | 18/20 | 10/10 | | | 2.0 ± 0.2 | 2.0 ± 0.2 |
| 2. PV1(SM)IC-4a | | 0/10 | 1/10 | 2/10 | 5/10 | 9/10 | 4.8 ± 0.3 | 4.8 ± 0.3 |
| 3. PV1(SM)IC-11b | 0/10 | 4/20 | 8/20 | 11/20 | 10/10 | | 3.4 ± 0.3 | 3.4 ± 0.3 |
| 4. PV1(SM)IC-8b | 0/10 | 2/10 | 10/20 | 15/20 | 20/20 | | 3.1 ± 0.3 | 3.1 ± 0.3 |
| 5. PV1(SM)IC-2b | 0/10 | 1/10 | 3/20 | 11/20 | 9/10 | | 3.8 ± 0.3 | 3.8 ± 0.3 |
| 6. PV1(SM)IC-4b | | 0/10 | 4/20 | 9/20 | 15/20 | 10/10 | 4.1 ± 0.3 | 4.1 ± 0.3 |
| 7. PV1(SM)IC-11a | | | | 0/10 | 3/10 | 4/10 (3)[b] | 5.9 ± 0.3 | 5.5 ± 0.3 |
| 8. PV1(SM)IC-8a | | | | 0/10 | 0/10 | 3/10 (2) | >6.3 | 6.0 ± 0.3 |
| 9. PV1(SM)IC-2a | | | | 0/10 | 1/10 | 8/10 | 5.6 ± 0.2 | 5.6 ± 0.2 |
| 10. PV1(Sab)IC-0 | | | 0/10 | 0/20 | 3/20 | 3/20 | >6.3 | >6.3 |
| 11. PV1(SM)IC-5a | | | 0/10 | 2/20 | 11/30 | 23/30 | 5.3 ± 0.2 | 5.3 ± 0.2 |
| 12. PV1(SM)IC-6a | | | 0/10 | 1/20 | 14/30 | 21/30 | 5.3 ± 0.2 | 5.3 ± 0.2 |
| 13. PV1(SM)IC-7b | | | | 0/20 | 8/30 (1) | 25/30 (3) | 5.4 ± 0.2 | 5.3 ± 0.2 |
| 14. PV1(SM)IC-9a | | | | 0/20 | 8/30 (1) | 18/30 (1) | 5.6 ± 0.2 | 5.6 ± 0.2 |
| 15. PV1(SM)IC-10b | | | | 0/20 | 8/30 (1) | 14/30 (4) | 5.8 ± 0.2 | 5.6 ± 0.2 |
| 16. PV1(SM)IC-17a | | | 0/10 | 3/30 | 7/30 | 17/30 | 5.6 ± 0.2 | 5.6 ± 0.2 |
| 17. PV1(SM)IC-18b | | | | 1/20 | 2/30 | 9/30 (4) | >6.2 | >6.0 |

[a] $PD_{50}$, 50% paralytic dose.
[b] Numbers in parentheses indicate the number of animals surviving with paralysis.

of these areas. Strong inflammatory cell infiltration was observed in the meninges of the brain stem and cerebral cortex and at the perivascular and perineuronal areas of the brain stem. In the spinal cord, severe neuronal atrophic degeneration was observed mainly in the ventral horns without apparent infiltration of mononuclear cells, including microglial cells in the gray matter (Fig. 2B and C). Poliovirus antigens were observed in most neurons in both the ventral and dorsal horns (Fig. 2A). Inflammatory cells were slightly seen in meninges (Fig. 2B).

In the mice inoculated with PV1(Sab)IC-0, the intensity of neuronal damage and inflammatory response in the brain appeared to be milder than in those inoculated with PV1(M)pDS306 (Fig. 3). Neuronal damage in the spinal cord was detected only in neurons in a limited area of the ventral horn (Fig. 2E and F). Virus antigens were also localized only in motor neurons in this area (Fig. 2D). The neuronal damages caused by PV1(Sab)IC-0 were accompanied by cellular infiltration, showing neuronophagia (Fig. 2E and F and 3). The inflammatory responses in the spinal cord were more severe than those caused by PV1(M)pDS306 infection, especially in meninges and perivascular areas. Viral antigens were usually detected only on the day of onset of paralysis (Fig. 3). This observation suggested that the viral antigens disappeared rapidly after degeneration of neurons. Intensities of histopathological changes were intermediate in the CNSs of the mice inoculated with PV1(SM)IC-4b (Fig. 3).

Neurovirulence levels of polioviruses were clearly differentiated by pathological studies of the infected Tg1 mice as described above. It should be noted that inflammatory responses induced in Tg 1 mice are less extensive than those in monkeys when the same virus is inoculated by the same route (data not shown).

**Genetic determinants influencing attenuation phenotype.** $LD_{50}$s of recombinant viruses in Tg1 mice are shown in Table 1. Allele replacement of the segment upstream of position 1122 of the virulent strain genome by the corresponding segment of the attenuated strain genome was most effective in increasing mouse $LD_{50}$s ($10^{2.0}$ to $10^{4.8}$ PFU), although every replacement of long segments of the genome resulted in changes of the values to a lesser extent (compare viruses 1 and 2 to 5 in Fig. 1A and Table 1). Inversely, replacement of this 5′-proximal segment of the attenuated strain genome by the corresponding segments of virulent strain genome was most effective in decreasing the mouse $LD_{50}$ among the allele replacements (compare viruses 10 and 6 to 9 in Fig. 1A and Table 1). These data indicate that the strong neurovirulence determinant(s) resides in the genome region of the 5′-proximal 1,122 nucleotides, which is compatible with the results obtained in monkey neurovirulence tests (8, 21).

The results of further mouse neurovirulence tests on recombinant viruses (viruses 11 to 17) with regard to the 5′-proximal portion of the genome are shown in Table 1. Every replacement involving position 480, an important nucleotide influencing monkey neurovirulence of type 1 poliovirus (5), resulted in considerable changes in mouse $LD_{50}$s (compare viruses 6 and 13, 15, and 17 in Fig. 1 and Table 1). Thus, mouse neurovirulence tests strongly suggested an important contribution of position 480 to the expression of neurovirulence or attenuation phenotype. However, one point substitution of the nucleotide (A to G) at position 480 was not sufficient for full expression of the attenuation phenotype encoded by this genome region (compare viruses 6, 10, and 15). Mouse neurovirulence tests suggested the contribution of positions 21 and/or 935 (compare viruses 6 and 11 in Table 1 and Fig. 1) and position 189 (compare viruses 15 and 17 in Table 1 and Fig. 1) to the phenotype. Two point mutations at positions 189 and 480 provided an attenuation phenotype close to that of PV1(Sab)IC-0 in Tg1 mice (virus 17 in Table 1 and Fig. 1). The contributions of nucleotide differences at positions 649, 674, and 806 were not evident in this experiment. These results strongly suggest that expression of the attenuation phenotype encoded by the genome region of the 5′-proximal 1,122 nucleotides of type 1 poliovirus is determined by substitutions of multiple nucleotides.

**Correlation between mouse $LD_{50}$s and monkey lesion scores.** Monkey neurovirulence tests of most viruses used in this study had already been carried out (5, 8, 21). It is therefore possible to compare the results obtained by monkey and mouse tests directly. Either of two conditions, intracerebral and intraspinal injection of virus, had been adopted to find the monkey neurovirulence of these viruses. The former is ade-

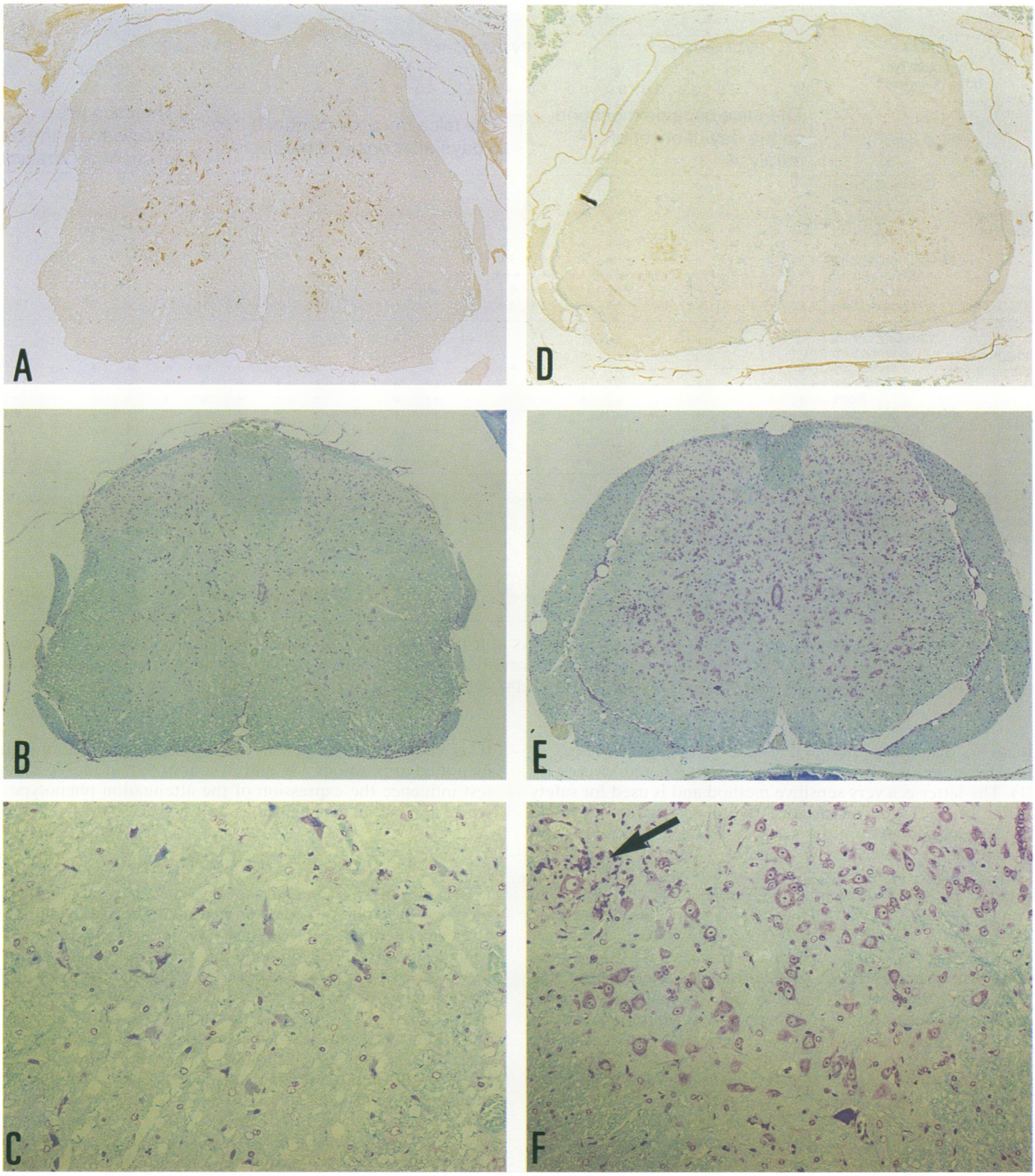


FIG. 2. Poliovirus antigens and histopathological lesions in the spinal cord of the Tg mice. (A) Poliovirus antigens in the lumbar spinal cord of a Tg1 mouse infected with PV1(M)pDS306 (mouse 5 in Fig. 3; ABC stain; original magnification, ×16). Viral antigens were seen in the neurons of the ventral and dorsal horns. (B) Lumbar spinal cord; serial section of panel A (KB stain; original magnification, ×16). Many neurons are lost (compare with panel E). Slight inflammatory cell infiltration was observed in the meninges. (C) Higher magnification of a part of the right ventral horn of panel B (KB stain; original magnification, ×66). Atrophic neurons and vacuolar degeneration are seen without cell infiltration. (D) Poliovirus antigens in the lumbar spinal cord of a Tg1 mouse inoculated with PV1(Sab)IC-0 on the day of onset of paralysis (mouse 21 in Fig. 3; ABC stain; original magnification, ×16). Poliovirus antigens are detected only in neurons of the ventral horns. (E) Lumbar cord of the mouse inoculated with PV1(Sab)IC-0 moribund on day 2 after onset of paralysis (mouse 14; KB stain; original magnification, ×16). Inflammatory cell infiltration is recognized in the meninges and ventral horns. Most neurons appear to be intact in both the ventral and dorsal horns. (F) Higher magnification of a part of the right ventral horn of panel E (original magnification, ×50). Most neurons of the ventral horn seem to be intact (compare with panel C). Glial and inflammatory cell infiltrations are observed around the damaged neurons of the ventral horn (arrow).

| viruses | | PV1(M)pDS306 | | | | | | PV1(SM)IC-4b | | | | | | PV1(Sab)IC-0 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tg mouse No. | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
| dose of viruses | | 2.0 | 2.0 | 2.0 | 3.0 | 2.0 | 2.0 | 5.0 | 5.0 | 4.0 | 6.0 | 6.0 | 5.0 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 | 6.5 |
| clinical signs | | The mice became moribund on the day of onset of paralysis | | | | | | The mice became moribund 2 to 6 days after onset of paralysis | | | | | | | | | | | The mice were sacrificed on the day of onset of paralysis | | | | |
| Neuronal damage | Cerebral cortex | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | Cerebellum | − | − | − | − | − | − | − | − | − | − | − | + | − | − | + | − | − | − | − | − | − | − |
| | Thalamus | + | − | ++ | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | Midbrain | + | + | + | + | − | ++ | + | + | + | − | − | − | − | + | − | + | − | − | − | − | − | − |
| | Pons | + | + | ++ | + | + | +++ | + | + | + | + | + | + | + | − | − | + | + | + | − | + | − | + |
| | Medulla oblongata | ++ | + | +++ | + | ++ | +++ | + | + | ++ | + | + | + | + | − | + | + | + | N.T. | + | + | + | + |
| | Cervical cord | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | + | + | ++ | + | + | + | ++ | ++ | + | + |
| | Thoracic cord | ++ | ++ | ++ | ++ | ++ | +++ | ++ | +++ | ++ | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | Lumbar cord | ++ | + | ++ | ++ | +++ | +++ | ++ | +++ | ++ | ++ | ++ | ++ | + | + | + | + | + | ++ | + | + | + | + |
| Inflammatory cell infiltration | Cerebral cortex | − | + | +++ | ++ | + | + | − | + | − | + | − | + | − | − | − | − | − | − | + | + | + | + |
| | Cerebellum | − | − | − | − | − | − | − | − | − | − | − | + | − | − | + | − | − | − | − | − | − | − |
| | Thalamus | ++ | + | +++ | ++ | ++ | + | + | + | ++ | + | + | + | + | + | + | + | ++ | + | + | + | + | + |
| | Midbrain | ++ | + | ++ | ++ | + | + | + | + | ++ | + | ++ | + | + | + | + | + | + | + | + | + | + | + |
| | Pons | ++ | + | ++ | ++ | + | + | + | + | + | + | ++ | + | + | + | + | + | + | + | + | + | + | + |
| | Medulla oblongata | + | + | ++ | ++ | ++ | + | + | + | ++ | + | ++ | + | + | + | + | + | ++ | N.T. | + | + | + | + |
| | Cervical cord | + | − | ++ | + | + | + | + | + | + | ++ | ++ | + | + | + | + | + | + | + | + | ++ | + | ++ |
| | Thoracic cord | + | − | + | + | + | + | ++ | ++ | + | + | + | + | + | + | + | + | + | + | + | − | + | ++ |
| | Lumbar cord | + | − | + | + | + | + | ++ | ++ | + | + | ++ | +++ | + | ++ | + | ++ | + | ++ | ++ | + | ++ | ++ |
| poliovirus antigens | Cerebral cortex | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | Cerebellum | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | Thalamus | ++ | + | − | − | − | − | − | − | + | − | − | − | − | − | − | − | − | − | + | + | + | − |
| | Midbrain | ++ | + | + | + | − | − | + | + | − | − | − | − | − | − | − | − | − | − | + | + | + | − |
| | Pons | +++ | ++ | ++ | + | + | +++ | ++ | ++ | + | − | − | − | − | − | − | − | − | + | − | − | + | − |
| | Medulla oblongata | +++ | ++ | ++ | + | + | +++ | ++ | ++ | + | + | − | − | − | − | − | − | − | + | + | + | + | − |
| | Cervical cord | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | + | + | − | − | − | − | − | − | − | + | + | + | + | + |
| | Thoracic cord | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + | + | − | − | − | − | − | − | − | + | + | + | + | − |
| | Lumbar cord | ++ | ++ | ++ | ++ | ++ | +++ | ++ | ++ | + | + | + | − | − | − | − | − | − | + | + | + | + | − |

FIG. 3. Histopathological lesions and poliovirus antigens in the CNS of ICR-PVRTg1 mice. Intensity of histopathological changes estimated by hematoxylin-eosin, KB, and gallocyanin staining and immunohistochemistry with antiviral antibodies: +++, strong; ++, intermediate; +, weak; −, not detected; N.T., not tested. Virus doses are given in $\log_{10}$ PFU per animal.

quate to distinguish between viruses with a wide range of neurovirulence, such as wild-type and attenuated viruses (8, 21). The latter is a very sensitive method and is used for safety testing of oral poliovaccine lots. Lesion scores for individual monkeys obtained from the tests involving intracerebral and intraspinal injections are shown in Fig. 4A and B, respectively.

$LD_{50}$s for Tg1 mice are plotted with monkey lesion scores obtained in the tests with intracerebral injection (Fig. 4A). The data suggest that the values have a good correlation with each other on a level of $P < 0.01$ (correlation coefficient: $r = -0.945$; $n = 10$). This result clearly indicates that the mouse neurovirulence tests detect the determinants that attenuate the viruses in primates. A similar result was obtained for the viruses with weak neurovirulence phenotypes by comparison of the $LD_{50}$s for mice with monkey lesion scores obtained by the tests with intraspinal injection, except for virus PV1(SM)IC-4b, at a level of $P < 0.05$ ($r = -0.946$; $n = 5$) (Fig. 4B). Significant correlation was not observed, taking data of PV1(SM)IC-4b into consideration (Fig. 4B), although the monkey neurovirulence level of PV1(SM)IC-4b obtained by the intracerebral test was well correlated with the mouse $LD_{50}$ of the virus (Fig. 4A). One possible explanation for this phenomenon is that the intraspinal injection method was too sensitive to assess the neurovirulence level of PV1(SM)IC-4b. Namely, the lesion score of virus PV1(SM)IC-9a already reached the plateau in this test (Fig. 4B). Thus, it is possible that the range of neurovirulence levels assessed by the mouse tests is wider than that assessed by monkey spinal tests. If that is the case, the neurovirulence levels of viruses PV1(SM)IC-4b, PV1(SM)IC-5a, PV1(SM)IC-6a, and PV1(SM)IC-9a might not be correctly estimated in monkey intraspinal tests in our previous work (5). It is thus possible that new neurovirulence determinants identified at positions 189 and 21 and/or 935 in the Tg mouse test influence the expression of the attenuation phenotype of type 1 poliovirus in primates.

## DISCUSSION

Because of characteristic species specificity of poliovirus, monkeys have been the only animal model for studying poliovirus neurovirulence. The monkey model, however, has disadvantages in (i) difficulty in handling, (ii) extremely high breeding costs, (iii) a high risk of zoonosis because of close evolutionary relatedness between humans and monkeys, (iv) the problem of species extinction in nature, (v) difficulties in controlling genetic background, and (vi) marked individual variations in the tests, as shown in Fig. 4. These situations made it difficult to obtain statistically reliable results from monkey neurovirulence tests. By introducing a human poliovirus receptor gene into the mouse genome, a new model that would overcome these problems was produced. As judged by the clinical and pathological analysis of infected Tg1 mice, this new model seems to be adequate for this purpose.

Our data strongly suggested that $LD_{50}$s obtained from mouse neurovirulence tests with intracerebral inoculation can be used to distinguish most polioviruses with a wide range of neurovirulence levels. Replication of the virus in vivo must involve a number of biological interactions between viral and host factors. The mouse model thus might have problems that would result in wrong conclusions because of species differences. Certain biological interactions between viral and mouse factors involved in development of the disease might not be

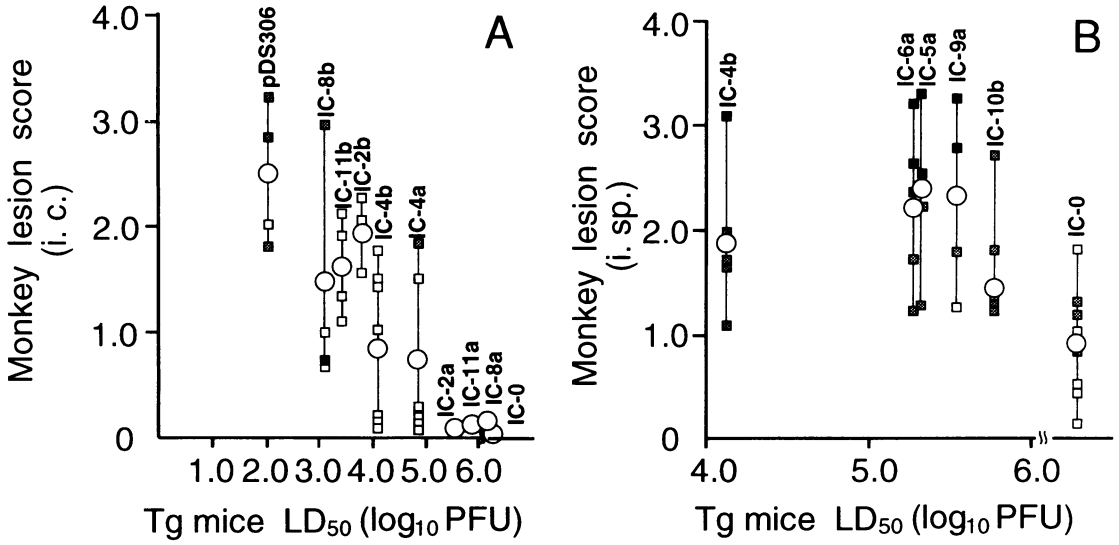


FIG. 4. Correlation of monkey lesion scores and Tg mouse $LD_{50}$. Lesion score values in monkey neurovirulence tests were plotted with logarithmic $LD_{50}$s in Tg mouse neurovirulence tests. Monkey neurovirulence tests were carried out by intracerebral (A) and intraspinal (B) injection. Lesion score values of individual monkeys are shown by squares. Closed and shaded squares indicate monkeys killed and paralyzed, respectively, during the experiments. The average lesion score for each virus is indicated by a circle. The nomenclature for virus strains was modified in that PV1(Sab), PV1(SM), and PV1(M) were omitted.

completely the same as those in primates. Species differences may generate different revertants in mice and primates during virus replication. It is possible that mouse neurovirulence does not always correlate with the neurovirulence levels in primates. Using the mouse-adapted virus system, however, Martin et al. (18), Ren et al. (27), and La Monica et al. (13) have identified the determinants in the 5′ noncoding region which attenuate the viruses in primates, suggesting that the interactions of the 5′ noncoding region with mouse factor(s) occur in a manner similar to that of those in primates. Our data presented here indicate that neurovirulence in Tg mice of recombinant viruses used in this study shows a good correlation with that in monkeys. These data suggest that interactions of neurovirulence determinants spread over the viral genome with mouse host factors and possible revertants generated in the CNS of Tg1 mice are not significantly influenced by the species differences. It is therefore possible that the neurovirulence levels of viruses in the Tg mouse model reflect those in primates. Ren et al. (27) reported an attenuation determinant in type 2 poliovirus using a Tg mouse line different from Tg1 mice. This determinant was proved to attenuate the virus also in monkeys (16).

As to the 5′-proximal 1,122 nucleotides of the genome of type 1 poliovirus, the mouse neurovirulence test described here revealed new determinants of nucleotide positions 189 and 21 and/or 935 in addition to position 480 that had been identified by monkey neurovirulence tests (5). According to models for possible secondary structures of the 5′ noncoding region of the type 1 poliovirus genome (22, 31), both nucleotide positions 189 and 480 participate in the formation of stem and loop structures around positions 200 and 500. The model suggests that substitutions of these nucleotides from virulent to attenuated virus types result in destabilization of the corresponding stem and loop structures, respectively. However, biological significances of these stem and loop structures have not been elucidated so far. Position 189 appeared not to function as a determinant when neurovirulence levels of PV1(SM)IC-5a and PV1(SM)IC-6a were compared with each other but appeared to function as a determinant when those of PV1(SM)IC-10b and PV1(SM)IC-18b were compared (Fig. 1). A possible explanation for this is that position 189 acts only in concert with position 480 in the expression of the attenuation phenotype. Interaction of these nucleotides in the expression of the attenuation phenotype of type 1 poliovirus, however, seems not to be simple and is not clear at present. Possible interaction between loci around positions 200 and 500 has been suggested to be important for the efficiency of certain steps in viral replication (12). It is therefore possible that determinants identified in the 5′ noncoding region of the genome influence formation of a highly ordered structure(s) required for a certain viral replication step(s).

The results described here strongly suggest that the Tg mouse model is very useful for the study of neurovirulence of polioviruses. In contrast to the monkey model, planned production with a much lower cost, greater control of genetic background, and greater control of pathogens can be done in the mouse system. Inbreeding of Tg1 mice is currently being undertaken. We believe therefore that the Tg mice are a better animal model for studying poliovirus neurovirulence than monkeys. It is possible to use the Tg mouse model for identification of minor neurovirulence determinants spread in the poliovirus genomes. Mouse neurovirulence tests presented here failed to distinguish polioviruses with very low neurovirulence such as oral live vaccine lots. However, we found that the intraspinal route of inoculation is more sensitive than the intracerebral route. With this route, development of new methods for safety testing is now in progress.

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