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Establishment of a Poliovirus Oral Infection System in Human Poliovirus Receptor-Expressing Transgenic Mice That Are Deficient in Alpha/Beta Interferon Receptor▽

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Poliovirus (PV) is easily transferred to humans orally; however, no rodent model for oral infections has been developed because of the alimentary tract's low sensitivity to the virus. Here we showed that PV is inactivated by the low pH of the gastric contents in mice. The addition of 3% $NaHCO_3$ to the viral inoculum increased the titer of virus reaching the small intestine through the stomach after intragastric inoculation of PV. Transgenic mice (Tg) carrying the human PV receptor (hPVR/CD155) gene and lacking the alpha/beta interferon receptor (IFNAR) gene (hPVR-Tg/*Ifnar*KO) were sensitive to the oral administration of PV with 3% $NaHCO_3$, whereas hPVR-Tg expressing IFNAR were much less sensitive. The virus was detected in the epithelia of the small intestine and proliferated in the alimentary tract of hPVR-Tg/*Ifnar*KO. By the ninth day after the administration of a virulent PV, the mice had died. These results suggest that IFNAR plays an important role in determining permissivity in the alimentary tract as well as the generation of virus-specific immune responses to PV via the oral route. Thus, hPVR-Tg/*Ifnar*KO are considered to be the first oral infection model for PV, although levels of anti-PV antibodies were not elevated dramatically in serum and intestinal secretions of surviving mice when hPVR-Tg/*Ifnar*KO were administered an attenuated PV.

Poliomyelitis is an acute disease of the central nervous system (CNS) caused by poliovirus (PV), a human enterovirus that belongs to the family *Picornaviridae*. In humans, an infection is initiated by oral ingestion of the virus, followed by multiplication in the alimentary mucosa (2, 38), from which the virus spreads through the bloodstream. Viremia is considered essential for leading to paralytic poliomyelitis in humans. By use of a PV-sensitive mouse model, previous studies (9, 44) demonstrated that after intravenous inoculation, circulating PV crosses the blood-brain barrier at a high rate, and a neural dissemination pathway from the skeletal muscle without injury is not the primary route by which the circulating virus disseminates to the CNS. Along with the blood-brain barrier pathway of dissemination, a neural pathway has been reported for humans (30), primates (11), and PV-sensitive transgenic mice (Tg) carrying the human PV receptor (hPVR/CD155) gene (31, 34); this pathway appears to be important in causing provocation poliomyelitis (9).

It has been proved that Tg carrying the hPVR gene (hPVR-Tg) are susceptible to all three PV serotypes, 1, 2, and 3 (22, 35), although mice without the hPVR gene are generally not susceptible to PV. This observation indicates that hPVR is the most important determinant of the host range of PV. After inoculation with PV by the intracerebral, intraspinal, intravenous, or intramuscular route (10, 20–22, 33–35), hPVR-Tg develop a flaccid paralysis in their limbs, which is clinically similar to human poliomyelitis. However, in contrast to its behavior in humans, PV does not replicate in the alimentary tracts of hPVR-Tg after oral administration, even in animals expressing high levels of hPVR in the intestinal epithelial cells (45). This result suggests that the expression of hPVR in the intestine is not solely responsible for the infection. It is also known that nonhuman primates are highly susceptible to PV by all routes except the oral route, yet the degree of oral susceptibility depends on the species (12). Thus, although oral infection is the most important route in humans, no adequate animal model has been established so far.

After an oral infection with PV, the virus must overcome at least three barriers before it can start to replicate efficiently in the first target cells in the small intestine: (i) the gastric acid solution, by which PV may be inactivated; (ii) inappropriate distribution of hPVR, by which PV may not be ushered to the correct target cells; and (iii) innate immunity, including interferon (IFN) signaling, by which the replication of PV may be hampered in the target cells (7). To know why orally administered PV hardly causes any paralysis in animals other than humans, we have to verify each step (see Fig. 7). In this report, barrier ii is defined as cell susceptibility and barrier iii is defined as cell permissivity.

To control poliomyelitis, attenuated PV strains of all three serotypes have been developed and used effectively as oral polio vaccines (37, 39). The attenuated Sabin strains can rep-

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licate well only in the alimentary tracts of humans without showing neuropathogenicity, enough to elicit neutralizing antibodies against PV after oral administration.

Picornaviruses are sensitive to IFNs (3, 5, 24, 28, 46). IFNs play an essential role in the innate immune antiviral response. Recently, Ida-Hosonuma et al. (13) found that deletion of the IFN-α/β receptor (IFNAR) gene in hPVR-Tg (hPVR-Tg/*Ifnar*KO) resulted in the disruption of IFN-α/β signaling (27), which is an important determinant of the tissue tropism and pathogenicity of PV. Similarly, it has been reported that IFN-α/β plays an important role in the pathogenicity and tissue tropism of some viruses, including coxsackievirus and Theiler's virus in the *Picornaviridae* (6, 8, 26, 36, 42). These results suggest that not only hPVR (cell susceptibility) but also IFN-α/β (cell permissivity) contributes to the pathogenicity and tissue tropism of PV.

In this paper, we have clarified the instability of the virus in the gastric environment, where the low pH of the gastric contents inactivates PV. Furthermore, using hPVR-Tg with or without IFNAR expression, we have shown that IFN-α/β plays a key role in preventing PV from replicating in the intestines of mice.

MATERIALS AND METHODS

Viruses and cells. The virulent Mahoney strain [PV1(M)OM] and the avirulent Sabin 1 strain [PV1(Sab)IC-0] of type 1 PV derived from infectious cDNA clones pOM1 (41) and pVS(1)IC-0(T) (19), respectively, were employed in this study. As other virulent strains, Lansing (type 2) and Leon (type 3) were used.

African green monkey kidney (AGMK) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum and were used for the preparation of viruses, transfection with infectious cDNA clones, and plaque assays.

Tg. The Tg strains used in this paper have been described previously (13). In brief, mice of a transgenic strain, ICR-PVRTg21 (21, 22), were backcrossed with C57BL/6 mice, and homozygotes with the C57BL/6 background (C57BL/6-PVRTg21) were produced. In this report, strain C57BL/6-PVRTg21 is referred to as PVRTg21. A129 mice, deficient in the *Ifnar* gene (27), were backcrossed with C57BL/6 mice and then further crossed with PVRTg21 or MPVRTg25-61 (MPVRTg25) (43). MPVRTg25 express hPVR under the control of the mouse PVR homolog (MPH) (25) regulatory gene. $PVR^{+/+}$ $Ifnar^{-/-}$ mice were obtained by intercrossing these PVRTg21/*Ifnar*KO (13) or MPVRTg25/*Ifnar*KO. All mice used were free of specific pathogens and were 7 to 10 weeks old. Mice were treated according to the guidelines for the Care and Use of Laboratory Animals of The University of Tokyo.

Assay of PV inactivation. PV1(M)OM (5 μl) was incubated at 37°C or 0°C for the periods indicated with 45 μl of each solution, and then the titer of virus was determined by a plaque assay. The pH 1 solution was 0.1 N HCl, the pH 2 solution was 0.01 N HCl, the pH 3 solution was 0.001 N HCl, and the pH 7 solution was saline. The pH of each solution was measured using pH test paper at the start and end points of the incubation.

Administration of viruses. For sampling of the gastric contents, mice were fasted overnight and then anesthetized with an intraperitoneal injection of 300 to 400 μl of ketamine (10 mg/ml) and xylazine (0.2 mg/ml) in saline. The stomach was exposed, and the pylorus was ligated with silk thread. The mice were inoculated with 200 μl of saline by using a gastric tube, and the gastric contents were collected. The gastric contents were centrifuged at 15,000 rpm for 10 min, and the supernatant was filtered. The filtrate was used for the experiments.

The viruses were administered orally using quantitative water bottles (Drinko-Measurer DM-G1; O'Hara & Co., Ltd., Japan). First, mice were fasted overnight, and then 2 ml of a viral solution containing 3% $NaHCO_3$ per mouse was administered within 24 h using the water bottles. The time point for starting the administration was taken as time zero. Twenty-four hours after the administration was started, the quantitative water bottles were replaced with conventional water bottles.

Recovery of viruses from tissues. For determination of the titer of virus in the tissues, the mice inoculated with the viruses were anesthetized and whole blood was recovered from the right ventricle. Immediately, the mice were perfused with saline through the left ventricle, and the tissues were excised. The tissues were homogenized in DMEM without serum to prepare a 10% emulsion. The homogenates were centrifuged to remove any debris, and the supernatant containing the virus was subjected to a plaque assay.

Labeling of PV. PV was purified by a protocol described previously (16). HeLa S3 cells were infected with PV1(M)OM at a multiplicity of infection of 10. The cells were harvested at 7 h postinfection, and the virus was purified from cytoplasmic extracts of the infected cells by using DEAE-Sepharose CL-6B (GE Healthcare Bio-Sciences KK) followed by centrifugation on a sucrose density gradient and CsCl equilibrium centrifugation. Purified virus was desalted by gel filtration on a PD-10 column (GE Healthcare Bio-Sciences KK) equilibrated with phosphate-buffered saline [PBS(−)] (per liter, 8.00 g NaCl, 1.15 g Na_2HPO_4, 0.20 g KCl, 0.10 g $MgCl_2 \cdot 6H_2O$, 0.20 g KH_2PO_4 [pH 7.4]). The concentration of poliovirions was determined by measuring the absorbance at 260 nm, where 1.0 optical density unit is regarded as equivalent to 9.4×10^{12} virions. The labeling of the virus is based on a protocol kindly provided by Lucas Pelkmans (32). PV (0.4 mg at 0.4 mg/ml) was labeled with 0.39 μl of Alexa Fluor 555-succinimidyl ester (10 mg/ml in dimethyl sulfoxide) according to the manufacturer's instructions (Invitrogen). These fluorophores react exclusively with free amines, resulting in a stable carboxamide bond. Labeled virus was repurified with NAP5 columns (GE Healthcare Bio-Sciences KK), dialyzed against PBS(−), and stored at −80°C. The labeling ratio was 14 mol of dye per mol of virus, and the specific infectivity of labeled virus was not reduced.

Detection of fluorescently labeled virus. After the MPVRTg25/*Ifnar*KO were anesthetized as described above, the intestines were exposed. The small intestine was ligated with silk thread at two points. Labeled virus was injected into the small intestine between the knots of the thread. One hour later, the injected portion of the small intestine was excised and washed with saline. The small intestine was directly observed under a confocal laser scanning microscope (LSM510; Carl Zeiss MicroImaging GmbH). For preparation of fixed tissue sections, the small intestine was immediately frozen in OCT compound (Sakura Fine Technical Co., Ltd.). Tissue sections were prepared by using a Jung CM3000 cryostat (Leica Instruments GmbH), mounted on 3-aminopropyltriethoxysilane-coated slides (Matsunami Glass Industries, Ltd.), and dried. All the staining procedures were performed at room temperature. First, the specimens were fixed in PBS(−) containing 2% paraformaldehyde for 1 min and washed four times in PBS(−). After treatment with 1.5% normal goat serum in PBS(−) for 20 min, 1 μg/ml of fluorescein isothiocyanate-labeled *Ulex europaeus* agglutinin-1 (UEA-1) was applied, and the specimens were incubated for 15 min and then washed with PBS(−). Nucleic acids were stained with 50 nM SYTO59 (Invitrogen). The sections were mounted with 80% (vol/vol) glycerol in PBS(−) and analyzed with a confocal laser scanning microscope.

Neutralizing assay. PVRTg21 and PVRTg21/*Ifnar*KO were orally administered 3×10^8 PFU of Sabin 1 along with 3% $NaHCO_3$ within 37 h by using quantitative water bottles. As a positive control, PVRTg21/*Ifnar*KO were intravenously injected with 1×10^5 PFU of Sabin 1. Twenty-one days after the administration, whole blood was collected from each mouse and serum was prepared after centrifugation. After incubation of the serum at 45°C for 1 h, 100 PFU of Sabin 1 in 100 μl was mixed with 100 μl of serially diluted serum and incubated for 1 h at 37°C. The virus-serum mixture was subjected to a plaque assay. Neutralizing activity is expressed as the maximum denominator for the dilution that can neutralize 100 PFU of Sabin 1.

RESULTS

Mouse gastric contents can inactivate PV. hPVR-Tg are much less susceptible to orally administered PV than humans. To explain this, we examined the stability of the virus during its passage through the mouse stomach after oral administration of PV. First, we examined whether a mouse gastric solution can inactivate PV. Under anesthesia, the pylorus was ligated and 200 μl of saline was inoculated using a gastric tube. Right after the inoculation, the gastric contents were collected, and the supernatant obtained by centrifugation was used as a gastric solution. As shown in Fig. 1A, 2×10^5 PFU of PV1(M)OM was incubated at 37°C or 0°C for the times indicated with or without the gastric solution from Tg or non-Tg. When PV was incubated at 37°C with the gastric solution from Tg or non-Tg, the titer of the virus was apparently reduced, whereas PV incubated with saline at 37°C for 4 h or PV just after mixing with the gastric solution showed no reduction. These results

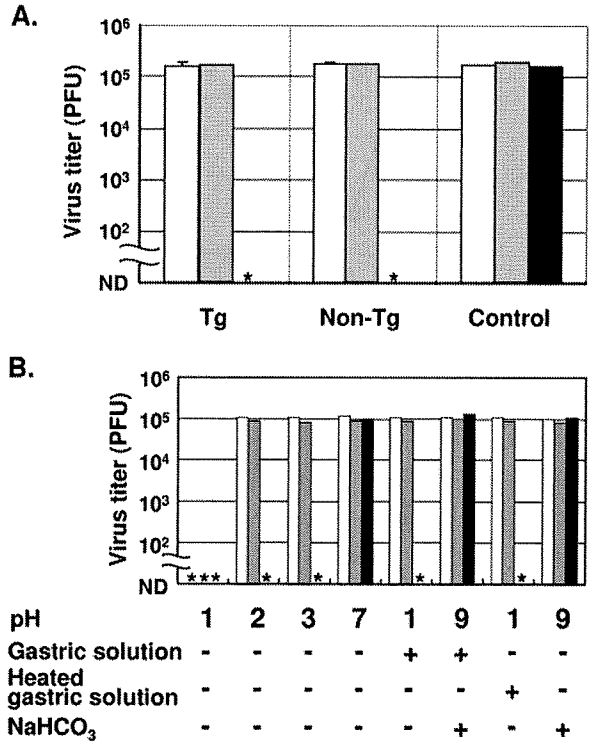

FIG. 1. Inactivation of PV with a mouse gastric solution. (A) PV1(M)OM at 2×10^5 PFU/5 μl was incubated at 37°C or 0°C for the periods indicated with or without 45 μl of a gastric solution from PVRTg21 (Tg) or C57BL/6 (non-Tg) mice, and then the titer of virus was determined by a plaque assay. As a control, PV was incubated with 45 μl of saline. Three animals were used per group. (B) PV1(M)OM at 1×10^5 PFU/5 μl was incubated at 37°C or 0°C for the periods indicated with 45 μl of each solution. After the incubation, the titer of virus was determined by a plaque assay. Open bars, samples just after mixing; solid bars, incubation at 37°C for 4 h; shaded bars, incubation at 0°C for 4 h. Asterisks, nondetectable titers. ND, not detected.

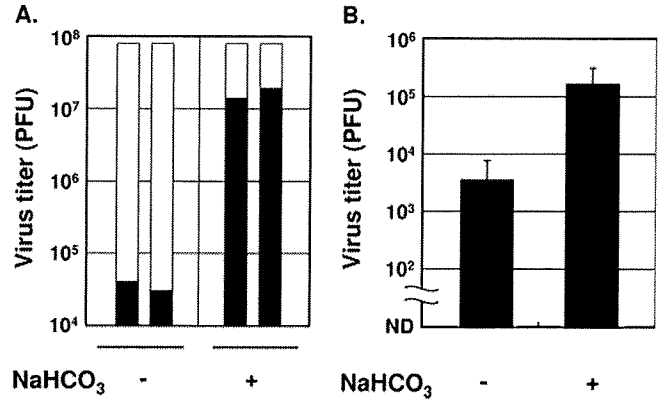

FIG. 2. Effect of passage through gastric contents on the virus titer after intragastric inoculation of mice with PV. (A) The effect of mouse gastric contents in the stomach on the retention of PV was examined. Under anesthesia, the pylorus was ligated and MPVRTg25 were intragastrically inoculated with 7.8×10^7 PFU of PV1(M)OM/500 μl with or without 3% $NaHCO_3$ by using a gastric tube. Five minutes after inoculation, the gastric contents were quickly recovered and the virus was detected by a plaque assay. Each bar represents an individual mouse. The black area indicates the titer detected in the gastric contents, and the white area indicates the decrease in the inoculated titer. (B) The rate of recovery of the virus from the small intestines of MPVRTg25 was examined after intragastric inoculation with 7.8×10^7 PFU of PV1(M)OM/500 μl with or without 3% $NaHCO_3$. Four hours after the inoculation, the contents of the small intestinal lumen were recovered and the virus was detected by a plaque assay. ND, not detected.

suggest that the gastric solution inactivated PV at 37°C independently of hPVR expression in mice.

Next, we investigated which factor influenced the inactivation. To examine the effect of low pH, 1×10^5 PFU of PV1(M)OM was incubated either with an HCl-containing solution at pH 1, pH 2, or pH 3, with saline (pH 7), or with the gastric solution, with or without $NaHCO_3$, either unheated or heated to inactivate the enzymatic activities. The gastric solution had a pH of ~1 without supplementation and a pH of 9 after it was mixed with $NaHCO_3$. As shown in Fig. 1B, PV was inactivated by the pH 2 and pH 3 solutions, as well as by the gastric solution after incubation at 37°C, but not by saline (pH 7). With the pH 1 solution, PV was inactivated without incubation at 37°C or 0°C. These results suggest that a low pH can inactivate PV. To confirm the effect of the low pH, the gastric solution with $NaHCO_3$ was incubated with PV. The pH 9 gastric solution did not inactivate PV even after incubation at 37°C. As a control, a saline solution with $NaHCO_3$ (pH 9) was examined; it had no effect on the viral titer. These results suggest that the low pH of the gastric solution leads to inactivation of the virus. When HEPES was used instead of $NaHCO_3$ to bring the viral solution to a pH of 9, the titer of virus was not decreased, either (data not shown). This result further supports the dependence of the PV-inactivating effect on a low pH. Finally, our experiment aimed to determine whether gastric enzymatic activities affect the infectivity of the virus. The enzymatic activities in the gastric solution were eliminated by heating at 95°C for 5 min. The heated gastric solution inactivated the virus after incubation at 37°C similarly to the unheated gastric solution without $NaHCO_3$. This result suggests that the enzymatic activities in the gastric solution do not affect the infectivity of PV under the conditions used.

Efficient delivery of PV to the intestine after intragastric inoculation with a pH neutralizer. To examine the survival rate of the virus at a low pH in the gastric environment in vivo, an assay of infectious PV was conducted after the virus was incubated in the stomach (Fig. 2A). Under anesthesia, the pylori were ligated and the mice were intragastrically inoculated with 7.8×10^7 PFU of PV/500 μl with or without 3% $NaHCO_3$ by using a gastric tube. Five minutes after inoculation, the gastric contents were quickly recovered and the titer of the virus was determined by a plaque assay. When the mice were inoculated with PV without 3% $NaHCO_3$, the titer was less than 0.1% of the original amount inoculated. On the other hand, when the mice were inoculated with PV together with 3% $NaHCO_3$, around 20% of the inoculated virus was recovered from the stomach. These results suggest that PV can be inactivated by gastric contents in vivo similarly to the inactivation in vitro (Fig. 1). As shown in Fig. 1B, the virus with the gastric solution containing 3% $NaHCO_3$ exhibited no loss of titer, whereas the virus inoculated intragastrically with 3% $NaHCO_3$ showed an 80% loss of titer in vivo (Fig. 2A). The gastric solution used for the in vitro experiments had been diluted with saline, which may have resulted in the minor effect on the inactivation of PV.

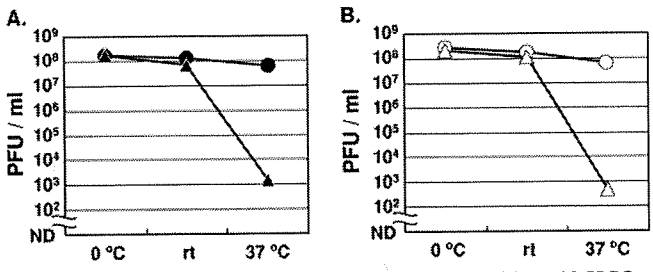

FIG. 3. PV titer after incubation for 24 h with or without $NaHCO_3$. A total of 1.5×10^8 PFU/ml of PV1(M)OM (A) or Sabin 1 (B) was incubated at the indicated temperatures for 24 h with (triangles) or without (circles) 3% $NaHCO_3$. The titer of virus after incubation was determined by a plaque assay. rt, room temperature. ND, not detected.

Next, we examined how much virus can reach the small intestine from the stomach after intragastric inoculation using a gastric tube. The rate of recovery of the virus from the small intestine was examined after intragastric inoculation of 7.8×10^7 PFU of PV with or without 3% $NaHCO_3$ by using a gastric tube. Four hours after inoculation, the contents of the entire small intestine were recovered and the titer of the virus was determined by a plaque assay (Fig. 2B). For mice inoculated with PV without 3% $NaHCO_3$, approximately 10^3 PFU/small intestinal lumen was recovered, whereas for mice inoculated with PV together with 3% $NaHCO_3$, about 10^5 PFU/small intestinal lumen was recovered. These results indicate that $NaHCO_3$ increased the recovery of the virus from the small intestinal lumen after intragastric inoculation with PV.

To know whether there is a reduction in the titer of the virus during the oral administration period (24 h), the stability of PV with 3% $NaHCO_3$ after 24 h was examined. The pH of a 3% $NaHCO_3$ solution was measured by pH test paper and determined to be 9. The titer was examined after incubation for 24 h at 0°C, room temperature, or 37°C (Fig. 3A and B). When PV1(M)OM was incubated with 3% $NaHCO_3$ or with H_2O, the titer did not decrease at 0°C or at room temperature. On the other hand, incubation of PV1(M)OM with 3% $NaHCO_3$ at 37°C reduced the titer by approximately 5 orders of magnitude, whereas incubation with H_2O at this temperature caused only about a 1-log-unit reduction (Fig. 3A). As for Sabin 1, when the virus was incubated at 0°C with 3% $NaHCO_3$ or with H_2O, no reduction in the titer was observed, whereas incubation at a higher temperature resulted in a reduction in the titer. Incubation of Sabin 1 with 3% $NaHCO_3$ or with H_2O at room temperature caused about a twofold reduction in the titer compared to that at 0°C. At 37°C, incubation of Sabin 1 with 3% $NaHCO_3$ reduced the titer by roughly 6 orders of magnitude, whereas incubation with H_2O caused only about a threefold reduction (Fig. 3B). These results suggest that incubation at room temperature for 24 h has only a minor effect on the titer and that incubation at 37°C for 24 h decreases the titer more severely, especially when the viral solution contains $NaHCO_3$. The relatively high pH of the 3% $NaHCO_3$ solution might have led to the instability of the viral RNA genome and virion particle. As for PV type 2 and 3 strains, incubation with 3% $NaHCO_3$ had only a minor effect on Leon, but Lansing showed a ~1-log-unit decrease in the titer after incubation with $NaHCO_3$ even at room temperature for 24 h (data not shown). These results suggest that the stability of PV with $NaHCO_3$ depends on the viral strain.

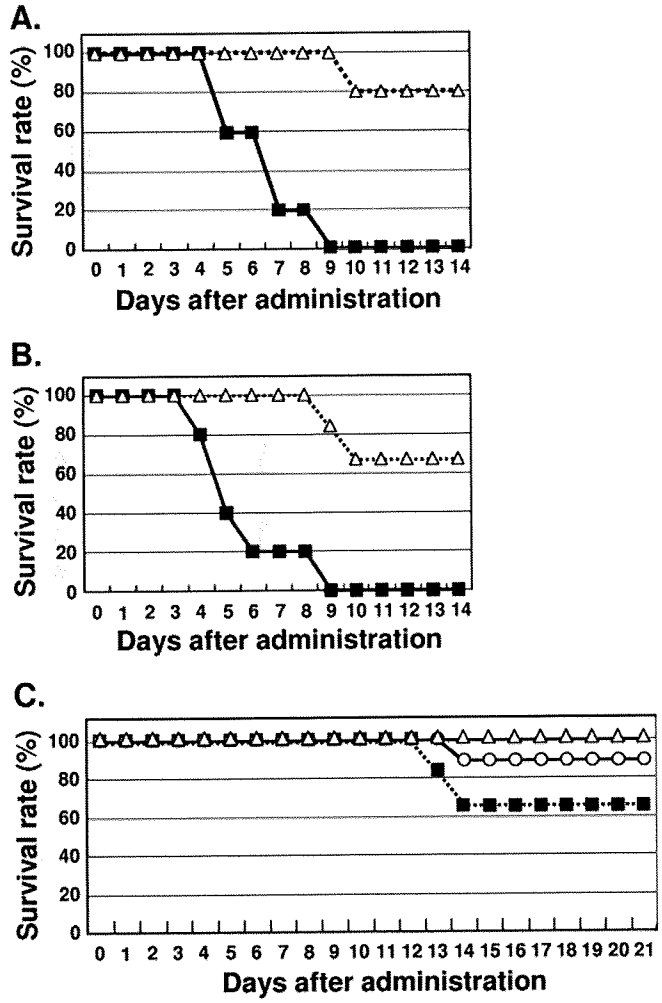

FIG. 4. Survival rates of mice after oral administration of PV. (A and C) PVRTg21/*Ifnar*KO (solid squares) or PVRTg21 (open triangles) were orally administered 3×10^8 PFU/2 ml of PV1(M)OM (A) or Sabin 1 (C), and the rate of survival was determined. Alternatively, PVRTg21/*Ifnar*KO were intravenously injected (open circles) with 1×10^5 PFU of Sabin 1/100 μl (C). Five (A), six (C) (oral administration), or nine (C) (intravenous injection) mice were observed for each group. (B) Similarly, MPVRTg25/*Ifnar*KO (solid squares) or MPVRTg25 (open triangles) were orally administered 3×10^8 PFU of PV1(M)OM/2 ml. Five or six mice were observed per group. The rate of survival was determined.

Effects of IFN-α/β signaling on the cell permissivity of orally ingested PV. To test whether PV orally ingested with 3% $NaHCO_3$ can cause paralysis in hPVR-Tg, PV with 3% $NaHCO_3$ was orally administered to PVRTg21 or to PVRTg21/*Ifnar*KO, which are deficient in *Ifnar*. To eliminate the possibility that the gastric tube might damage epithelia in the esophagus, 3×10^8 PFU of PV1(M)OM with 3% $NaHCO_3$ was orally administered without using a gastric tube. Eighty percent of PVRTg21 survived, whereas all the PVRTg21/*Ifnar*KO showed paralysis and died within 9 days of PV administration (Fig. 4A). These results suggest that PVRTg21/*Ifnar*KO are more susceptible to orally administered PV1(M)OM than PVRTg21. The susceptibility of

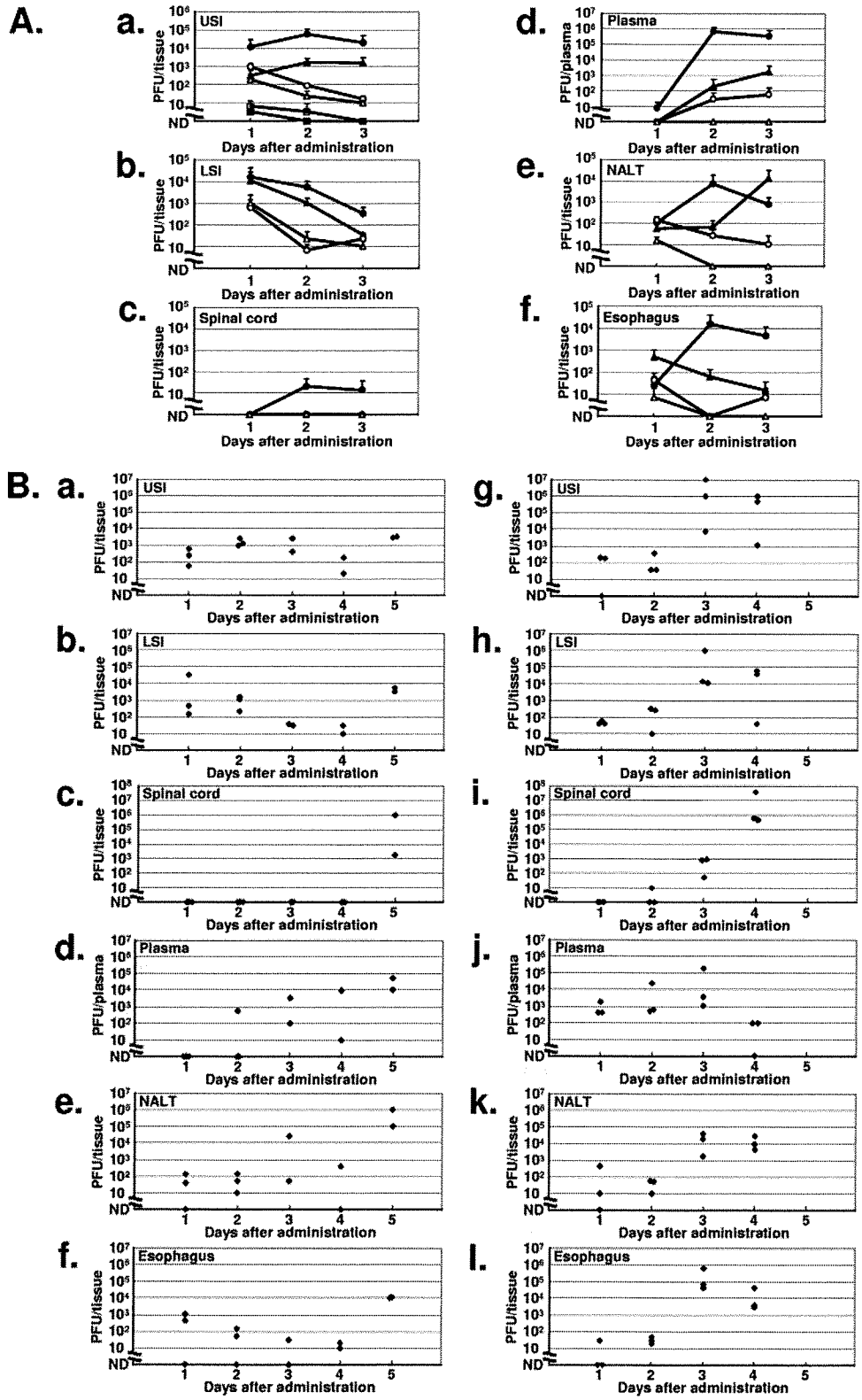

FIG. 5. Titers of PV recovered in tissues after oral administration of PV. (A) Virus was extracted from tissues of mice (PVRTg21 [open triangles], PVRTg21/*Ifnar*KO [solid triangles], MPVRTg25 [open circles], MPVRTg25/*Ifnar*KO [solid circles], C57BL/6 [open squares], and C57BL/6/*Ifnar*KO [solid squares]) 1, 2, and 3 days after the administration of 3×10^8 PFU of PV1(M)OM/2 ml. The vertical axis shows the amount (PFU) of PV detected in tissues by the plaque assay. USI, upper small intestine; LSI, lower small intestine. (B) Virus was extracted from each tissue of PVRTg21/*Ifnar*KO every 24 h after oral administration of 3×10^8 PFU of PV1(M)OM/2 ml (a to f) or intravenous injection of 1×10^5 PFU of PV1(M)OM/100 μl (g to l). Each point indicates one mouse. ND, not detected.

PVRTg21/*Ifnar*KO is dependent on the titer of virus (data not shown).

To confirm the effect of IFN signaling on the pathogenicity of orally administered PV in mice, another Tg strain (MPVRTg25) was examined, because hPVR is apparently detected in the small intestine and liver of MPVRTg25 by Western blotting (43). MPVRTg25 and MPVRTg25/*Ifnar*KO were orally administered PV with 3% $NaHCO_3$ without using a gastric tube, and the clinical symptoms were observed (Fig. 4B). In agreement with the results obtained with PVRTg21 and PVRTg21/*Ifnar*KO, all the MPVRTg25/*Ifnar*KO died whereas only 33% of MPVRTg25 died. These results suggest that MPVRTg25/*Ifnar*KO are more susceptible to oral administration of PV than MPVRTg25. These findings further support the notion that IFN signaling contributes to cell permissivity in mice orally administered PV. As for the clinical symptoms, MPVRTg25/*Ifnar*KO tend to show hepatocirrhosis rather than paralysis, and it is highly possible that these mice died of a hepatic disorder. Nevertheless, one can observe paralysis after intracerebral inoculation of MPVRTg25/*Ifnar*KO (data not shown). This result suggests that the virus can replicate in the CNS and cause paralysis even in MPVRTg25/*Ifnar*KO.

When PVRTg21/*Ifnar*KO or PVRTg21 were orally administered Sabin 1 with 3% $NaHCO_3$ without use of a gastric tube, more than 30% of PVRTg21/*Ifnar*KO showed paralysis and died, whereas all the PVRTg21 survived for 21 days (Fig. 4C). These results suggest that PVRTg21/*Ifnar*KO were more susceptible to oral administration of Sabin 1 than PVRTg21. PVRTg21/*Ifnar*KO also showed susceptibility to the type 2 strain Lansing or the type 3 strain Leon despite the fact that that Lansing was less pathogenic than PV1(M)OM or Leon (data not shown). Together with these results, our study strongly suggested that IFN signaling plays a key role in cell permissivity following oral administration of PV, although we cannot exclude the possibility that components of the innate immune system other than IFN-α/β affect the cell permissivity.

Time course of the replication of PV in tissues after oral administration. To assess the ability of PV to replicate in different tissues after oral administration, the titers of the virus in the small intestine, colon, nasopharynx-associated lymphoid tissue (NALT), esophagus, spinal cord, and plasma were determined at 1, 2, and 3 days after administration to mice. PV was orally administered to PVRTg21, PVRTg21/*Ifnar*KO, MPVRTg25, MPVRTg25/*Ifnar*KO, C57BL/6, and C57BL/6/*Ifnar*KO, and the tissues were excised each day after administration. The tissues were then homogenized, and the titers of the virus in the solutions were determined by a plaque assay (Fig. 5A). In all the tissues tested, titers were always higher in *Ifnar* knockout hPVR-Tg than in *Ifnar*-expressing hPVR-Tg. For instance, the titers were higher in PVRTg21/*Ifnar*KO than in PVRTg21, and similarly, they were higher in MPVRTg25/*Ifnar*KO than in MPVRTg25 (Fig. 5Aa to c, e, and f). As for C57BL/6/*Ifnar*KO and C57BL/6 mice, the titers in the upper small intestine were negligible (Fig. 5Aa). These results suggest that PV can replicate in all of the tissues examined more efficiently in *Ifnar* knockout mice than in *Ifnar*-expressing mice.

As for plasma, the virus caused viremia from the second day in PVRTg21/*Ifnar*KO, MPVRTg25, and MPVRTg25/*Ifnar*KO (except for one animal that showed slight viremia on the first day) (Fig. 5Ad). The results imply that the virus leaks into the blood after replicating in tissues, probably the alimentary tract, because little virus was detected in plasma on the first day.

We next investigated the chronological titer of the virus in different tissues after an oral or systemic challenge (Fig. 5B). When PVRTg21/*Ifnar*KO were intravenously inoculated with PV1(M)OM, the virus had started replicating extensively in all tissues examined on the third day (Fig. 5Bg to l). Almost all the mice injected intravenously with the virus developed paralysis and died on the fifth day (data not shown). Following oral administration of PV1(M)OM to PVRTg21/*Ifnar*KO, all the tissues examined showed a burst of proliferation of the virus on the fifth day (Fig. 5Ba to f), and the mice began to die on the fifth day (Fig. 4A). These findings indicate that the burst of replication after oral administration may be due to the virus that appeared on the second day in the bloodstream, because such a burst took 3 days after the intravenous injection. It seems feasible that the paralysis after oral administration of the virus is mainly due to the circulating virus that invaded from the alimentary tract.

Inoculated PV was incorporated into mouse small intestinal epithelia. To examine which kind of cells in the alimentary tract incorporate the virus, fluorescently labeled PV was injected into the ligated small intestine in MPVRTg25/*Ifnar*KO. One hour after the injection, the ligated tissue was subjected to confocal laser scanning microscopic analysis. As shown in Fig. 6Ab and c, the virus was detected inside the microvillus, whereas no fluorescence was detected in the small intestine without the injection (Fig. 6Ae and f). When the intestine was observed after frozen sectioning, the virus was detected in the cytoplasm of the epithelial cells in the microvilli (Fig. 6Bd and e). No fluorescence was detected in the small intestine without injection of the virus (Fig. 6Bi and j). These results suggest that the virus inside the cavity of the small intestine can be incorporated into the epithelial cells. It is highly possible that the incorporated virus starts to replicate in these epithelial cells. To further clarify which kinds of cells incorporate the virus from the intestinal cavity, fluorescently labeled UEA-1 (specific for α-L-fucose residues) was used, since UEA-1 has been shown to possess high affinity for selected intestinal epithelial cells, including mouse microfold (M) cells and goblet cells (4, 17). As shown in Fig. 6C, PV was not detected in the UEA-1-positive fraction of epithelial cells. On the other hand, the cells that contained PV were the UEA-1-negative fraction in the epithelia (Fig. 6B). It has been reported that some microorganisms, such as *Salmonella enterica* serovar Typhimurium and *Yersinia pseudotuberculosis*, can be efficiently incorporated into M cells under similar experimental conditions (15). These results suggest that in the IFN-α/β-free environment of the intestinal epithelium, PV is incorporated into the UEA-1-negative fraction of epithelial cells, although we cannot exclude the possibility that M cells are involved in the dissemination of the virus and the subsequent infection process.

Oral administration of attenuated PV did not effectively generate neutralizing antibodies in hPVR-Tg/*Ifnar*KO. Inasmuch as orally administered PV is incorporated into the intestinal epithelium and starts replicating in hPVR-Tg/*Ifnar*KO, it is important to examine whether oral administration of attenuated PV can induce the production of neutralizing antibodies in mice. To this end, attenuated PV was orally administered to

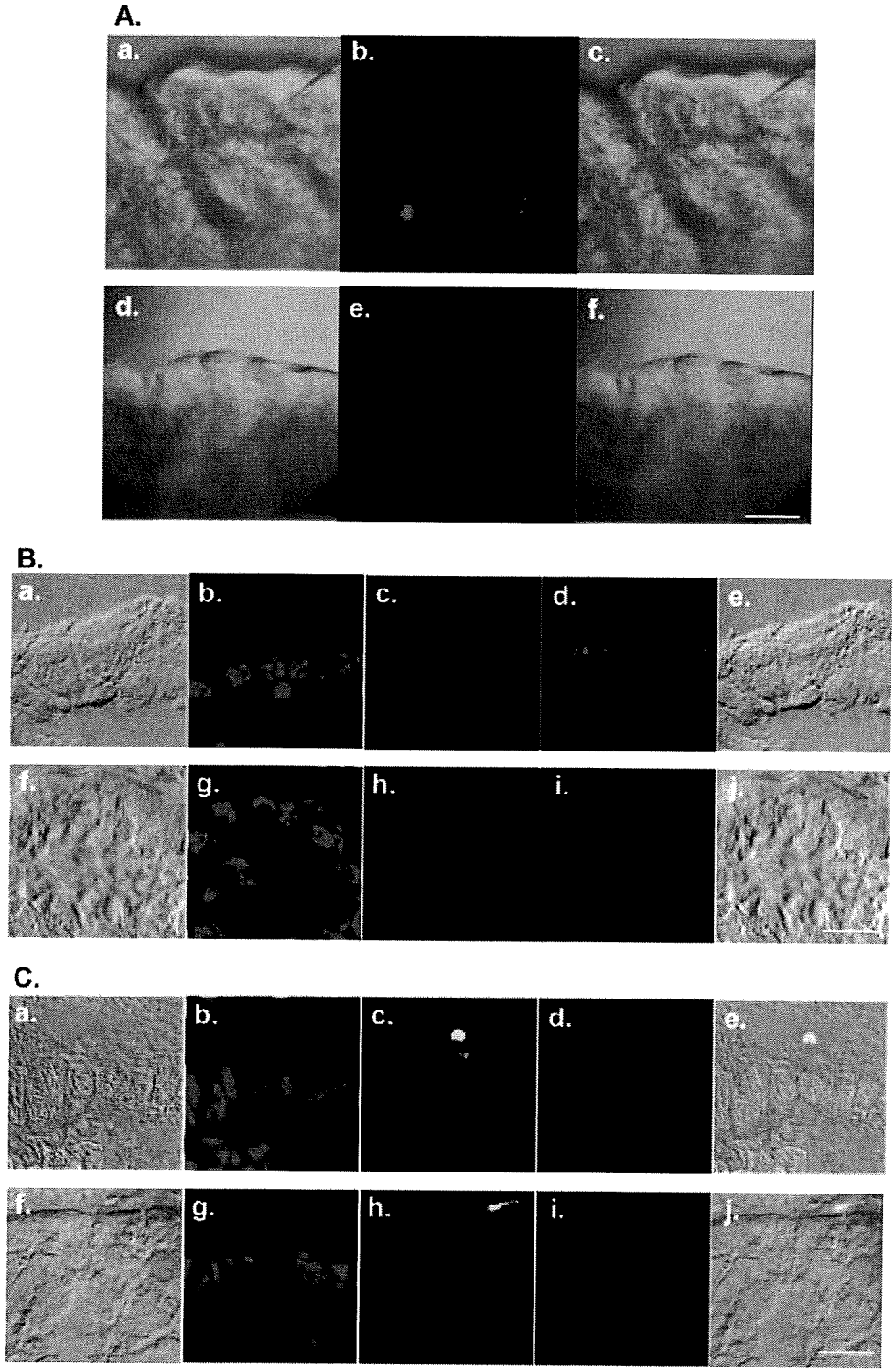

FIG. 6. Fluorescently labeled PV is detected in intestinal epithelia. Alexa Fluor 555-labeled PV1(M)OM (1.5×10^7 PFU/10 μl) was injected into the ligated small intestines of MPVRTg25/*Ifnar*KO (top row of images in each panel). PV-negative controls were also used (bottom row in each panel). One hour after injection, the ligated portion was excised and subjected to confocal laser scanning microscopy. Intestines were observed without (A) or with (B and C) fixation and frozen sectioning. (B) UEA-1-negative epithelia; (C) epithelia that contain UEA-1-positive cells. Red, Alexa Fluor 555-labeled PV1(M)OM (Ab, c, e, and f; Bd, e, i, and j; Cd, e, i, and j); blue, nuclei (Bb, e, g, and j; Cb, e, g, and j); green, fluorescein isothiocyanate-labeled UEA-1 (Bc, e, h, and j; Cc, e, h, and j). Aa and d, Ba and f, and Ca and f show bright-field images only. Ac and f, Be and j, and Ce and j show merged images of bright-field and fluorescence micrographs. Bars, 100 μm.

TABLE 1. PV-neutralizing activity in serum after oral administration of Sabin 1

Mouse strain	Substance	Route of administration	Amt of immunizing virus (PFU/mouse)	Neutralizing activity[a]
PVRTg21/*Ifnar*KO	DMEM	Oral	0	0/6
	Sabin 1	Oral	3×10^8	0/4
	Sabin 1	Intravenous	1×10^5	8/8
PVRTg21	Sabin 1	Oral	3×10^8	0/6

[a] Expressed as the number of mice whose serum showed neutralizing activity (≥16)/number of mice examined.

PVRTg21/*Ifnar*KO or PVRTg21. Serum was collected 21 days after virus administration, and the neutralizing activity in the serum was assayed. In the case of PVRTg21/*Ifnar*KO, all the mice showed neutralizing activity after intravenous inoculation of 10^5 PFU of Sabin 1 whereas no mouse showed neutralizing activity after oral administration of 3×10^8 PFU of the strain or DMEM (Table 1), although the virus was detected in the intestine until 4 days after oral administration of Sabin 1 (data not shown). As for PVRTg21, no mouse showed neutralizing activity after oral administration of 3×10^8 PFU of Sabin 1. Similar results were obtained for MPVRTg25/*Ifnar*KO after oral administration of the strain (data not shown). These results suggest that oral administration of Sabin 1 to PVRTg21/*Ifnar*KO or MPVRTg25/*Ifnar*KO was ineffective at raising the neutralizing activity.

DISCUSSION

Some of the recent outbreaks in areas certified as being clear of polio were caused by a circulating vaccine-derived PV that had mutated from the oral polio vaccine used to prevent polio (18). This suggests to us a need to develop new polio vaccines or antipolio drugs for the control of polio outbreaks. For that purpose, it is necessary to establish a useful animal model that mimics the natural infection route and subsequent disease development in humans in order to evaluate candidate vaccines or drugs.

A paper about the effects of pH on PV infectivity (40) indicates that PV can be easily inactivated at pH 3.0 or 9.0 at 30°C, but it depends on the buffers. This means that, at least at pH 3.0 or 9.0, the stability of viral infectivity is determined by the stability of the virions in particular buffers.

There is a discrepancy in the data obtained at pH 1 between the HCl solution and the gastric solution (Fig. 1B). The fact that the pH was measured using pH test paper and we cannot know the value precisely might explain the discrepancy, especially at the extremely low pH.

The present study has shown that IFNAR plays an important role in the infection and multiplication of orally administered PV in the small intestine of hPVR-Tg (Fig. 4 and 5A). The deletion of IFNAR resulted in successful infection by oral PV via the intestinal epithelium and the subsequent development of clinical symptoms. Viremia seems to be essential for the symptoms to appear (Fig. 5B), and a histopathology for similar symptoms caused by artificial viremia (intravenous inoculation) in hPVR-Tg/*Ifnar*KO has been reported (13). We thus established an oral administration system using hPVR-Tg/*Ifnar*KO, with which one can assess the 100% lethal doses of PV strains. This is the first in vivo system in which all the animals showed paralysis after oral administration of PV.

From the results presented in Fig. 5B, the orally administered virus disseminates mainly through the bloodstream in mice, although other, minor routes might be involved. It is possible, for example, that a neural pathway exists from the alimentary tract to the CNS through the vagus nerve or from the skeletal muscle to the CNS through the peripheral nerve. After PVRTg21/*Ifnar*KO were orally administered 3×10^8 PFU of Sabin 1, low titers (from 4×10^1 PFU/plasma to 8×10^2 PFU/plasma) of virus were detected in the plasma for 2 of 3 mice 3 days after administration and for 1 of 3 mice 4 days after administration. In spite of the ineffective serum conversion (Table 1), some lethal infection occurs after oral administration of Sabin 1 in PVRTg21/*Ifnar*KO (Fig. 4C). These results imply that the neural pathway from the alimentary tract to the CNS might contribute to death after oral administration of Sabin 1 in PVRTg21/*Ifnar*KO. After oral administration, the titer of virus in skeletal muscle did not rise until the virus started replicating efficiently in all the tissues examined (data not shown). This result implies that the neural pathway from skeletal muscle to the CNS is not essential, at least in this system. Nevertheless, we do not know which pathway has an essential role in causing paralysis and death.

It is possible that the virus was incorporated accidentally via the intranasal pathway after oral administration. In our experiments, when 1.5×10^8 PFU/ml of the Mahoney strain was orally administered to PVRTg21, the mice showed hardly any signs of paralysis. This concentration was higher than 10^6 PFU/20 μl, which was enough to cause death among 60% of intranasally inoculated PVRTg21 (29). Furthermore, the distribution of the virus after oral administration differs from that after intranasal inoculation. From these results, it is unlikely that orally administered virus enters the intranasal pathway.

We have shown previously that hPVR is expressed in the small intestines of MPVRTg25 but is not detected in those of PVRTg21 by Western blotting (43). As for immunohistochemistry, hPVR expression was barely observed in the intestinal epithelium and was not detected in germinal centers within Peyer's patches in PVRTg21 (14), and an assertive hPVR antigen was not detected in small intestinal epithelia in PVRTg21 or MPVRTg25 (M. Takano-Maruyama and H. Ohno, personal communication). These results suggest that hPVR is not expressed at high levels in the small intestines of PVRTg21, MPVRTg25, or the *Ifnar* knockout versions of these mice, although it is possible that the levels of hPVR expression on the intestinal epithelia differ among these mice. Incorporated fluorescently labeled virus was observed in the intestines of MPVRTg25/*Ifnar*KO but not PVRTg21/*Ifnar*KO or C57BL/6 mice (data not shown). This might be due to the level of hPVR expression on the apical side of the intestinal epithelia. Despite the fact that PVRTg21 express little if any hPVR in the small intestine, the titers of virus in the upper small intestines of PVRTg21 and PVRTg21/*Ifnar*KO 1 day after administration were ~10^2-fold higher than those in C57BL/6 mice and C57BL/6/*Ifnar*KO, respectively (Fig. 5A), and PVRTg21/*Ifnar*KO showed susceptibility to oral administration of PV (Fig. 4A and C and 5). These results suggest that hPVR ex-

pressed in the small intestines of PVRTg21/*Ifnar*KO contributes to cell susceptibility to orally administered PV.

We observed hepatocirrhosis in MPVRTg25/*Ifnar*KO after oral administration of PV1(M)OM despite the fact that MPVRTg25 do not show hepatocirrhosis. These results suggest that MPVRTg25/*Ifnar*KO show irregular tissue tropism of the virus compared to MPVRTg25, which have a native immune system. A previous paper has reported that viral antigen-positive cells were detected in the liver 1 day after intravenous inoculation of 2×10^7 PFU of PV1(M)OM into MPVRTg25/*Ifnar*KO (13). Moreover, a high titer of the virus was recovered 2 days after oral administration of 3×10^8 PFU of PV1(M)OM to MPVRTg25/*Ifnar*KO (data not shown). It seems that this occurred because hPVR in the liver causes the virus to replicate in MPVRTg25/*Ifnar*KO mice. Indeed, we have reported previously that the liver in MPVRTg25 expresses hPVR (43). Moreover, the virus in the bloodstream can easily access the liver (1). Considering these results, it is feasible that the viremia led to a liver infection that then caused a secondary viremia, leading to invasion of the CNS in MPVRTg25/*Ifnar*KO. As for PVRTg21/*Ifnar*KO, it has been reported that the disruption of IFNAR enables the virus to replicate in nonneuronal tissues, although both PVRTg21 and PVRTg21/*Ifnar*KO developed paralysis by similar points in time after intravenous inoculation (13). All together, the oral infection system using hPVR-Tg/*Ifnar*KO does not serve as an adequate animal model for analyzing virus tissue tropism in the whole body, although the clinical symptoms seen in PVRTg21/*Ifnar*KO were similar to those in PVRTg21. This notion is also supported by the fact that healthy humans are highly susceptible to poliovirus infection in spite of a robust innate immune response. Nevertheless, this oral administration system might be applicable to the study of initial infection events in vivo. It is also worth elucidating the role of the IFNAR-related signaling cascade in the infection of intestinal epithelial cells.

We succeeded in detecting the fluorescently labeled virus in the small intestine 1 h after its injection (Fig. 6) but failed to detect the viral antigen 2 days after oral administration of the virus using immunohistochemistry (data not shown). It is difficult to detect antigens in the intestine because of high background levels and the quick turnover of infected cells. It is possible that the virus does not replicate prominently in the intestinal epithelia or that the infected cells tend to drop out easily from the epithelial layer. Although the titers of virus in other alimentary tissues were relatively low after oral administration of the virus (Fig. 5A) and the viral antigen was not detected in the NALT, esophagus, or colon 2 days after oral administration (data not shown), we cannot exclude the possibility that PV replicates in the alimentary tract early in the course of infection. Alternatively, the fluorescence observed could have derived from inactive PV taken up by cells during a nonproductive infection.

The mechanism for oral infection with PV in humans has not been made clear, though it is possible that PV can replicate in and/or permeate M cells or lymphatic tissues in humans, because attenuated PV vaccine strains can readily generate neutralizing antibodies. In the present study, oral administration of Sabin 1 did not lead to the generation of neutralizing activity in the serum of PVRTg21/*Ifnar*KO (Table 1) and MPVRTg25/*Ifnar*KO (data not shown) or the binding activity of immunoglobulin A's in feces (data not shown), despite the fact that the virus proliferated in the upper small intestine (data not shown). Our results also showed that PV was not preferentially detected in the UEA-1 fraction of epithelial cells, which contains M cells, in hPVR-Tg/*Ifnar*KO (Fig. 6C), and the virus did not proliferate efficiently in NALT, a lymphatic tissue (Fig. 5Ae and Be). These results may correlate with the difficulty of raising the neutralizing activity after oral administration of the virus in hPVR-Tg/*Ifnar*KO. However, it should be emphasized that a lack of IFNAR hampered enhancement of the antibody-evoked response (23). Thus, the phenomena we observed might be due to the lack of IFNAR. Although we still could not reconcile the discrepancy between humans and the murine model in the exact site of PV invasion in the intestinal tract, the present study offers a new opportunity to address the issue, since hPVR-Tg/*Ifnar*KO are susceptible to orally administered PV for the initiation of infection and subsequent development of disease symptoms. And it is probable that the expression of hPVR in different intestinal cells is important in determining the heightened permissiveness of oral PV administration in humans compared to rodents. PV is highly successful at infection of the alimentary tracts of humans, and intestinal secretion of the virus can persist for months, even in healthy individuals, regardless of age. This means that the virus infects and replicates in some cells or tissues in the human gastrointestinal tract in spite of a possible early innate immune response. However, it is also possible that the innate immune response of humans is weaker than that of rodents.

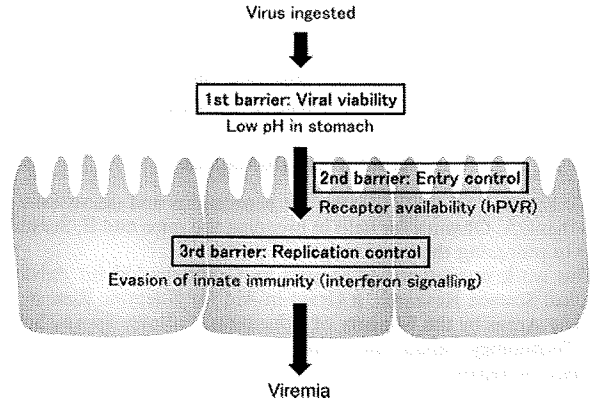

FIG. 7. Presumed PV dissemination routes and characteristics. The presumed barriers to orally ingested PV are shown. First, the ingested virus enters the stomach and suffers a low-pH environment (the first barrier). Second, the virus has to find the appropriate receptor in order to enter the intestinal cells (the second barrier, cell susceptibility). Third, the virus can start replicating in the cells depending on the lack of an IFN system (the third barrier, cell permissivity). Finally, the virus probably invades the bloodstream.

The presumed barriers to orally ingested PV are shown in Fig. 7. First, the ingested virus enters the stomach and experiences a low-pH environment (the first barrier). If the virus overcomes this, it has to find the appropriate receptor in order to enter the intestinal cells (the second barrier, cell susceptibility). Then the virus can start replicating in the cells depending on the lack of an IFN system (the third barrier, cell permissivity). Finally, the virus probably invades the bloodstream. In this report, we clarified that the low pH of the gastric

environment inactivates PV and decreases the titer of virus that reaches the intestine, and the lack of an IFN system allows the virus to replicate in the intestine more efficiently. These three barriers may serve to protect individuals from viremia.

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ORIGINAL ARTICLE

Evaluation of a two-dose administration of live oral poliovirus vaccine for wild and virulent vaccine-derived poliovirus type 1, 2, 3 strains in Japan

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Abstract

We evaluated the efficacy of Japan's vaccination policy, a 2-dose administration of live oral poliovirus vaccine (OPV) against wild and virulent vaccine-derived poliovirus (VDPV) type 1, 2, 3 strains, by investigating the neutralizing antibody titers of residents in Toyama Prefecture, Japan. Seropositivities against the virulent type 1 and 2 strains were more than 90%, but the values against the virulent type 3 strains were approximately 60%. Also, while geometric mean antibody titers against virulent type 1 and 2 strains were more than 180, those against the virulent type 3 strains were 58–59, and 9–12, in particular, at 10 to 19 y of age. A booster dose of the vaccine for the type 3 virus is recommended for adolescents. However, high herd immunity against type 1, 2 and 3 viruses has been maintained for these 22 y, although the seropositivity against type 3 virus was always lower than other types. Our results suggest that Japan's vaccination policy might be enough to prevent an epidemic of poliomyelitis caused by wild and virulent VDPV type 1, 2, 3 strains, even though the titers against type 3 viruses were the lowest.

Introduction

The trivalent live oral poliovirus vaccine (OPV) is constituted of attenuated Sabin strains of each of the 3 serotypes. It is regarded as one of the most effective and safest vaccines in current use, and it has been used as a major tool for the global poliomyelitis eradication program of the World Health Organization (WHO). Two additional merits of the OPV are extreme simplicity of administration and low cost of manufacture. In Japan, following the introduction of the 2-dose administration of OPV, which was imported from Canada and the Soviet Union, to children of 3 months to 12 y of age in 1961–1963, the number of patients decreased markedly, while 1000 to 5000 paralytic cases of poliomyelitis were reported annually before the introduction of the OPV [1,2]. A 2-dose administration of domestic OPV to infants 3–48 months of age (between 1964–1994) and 3–90 months (since 1995 until now) of age at intervals longer than 6 weeks has been performed routinely since 1964. A wild poliovirus was isolated from 1 patient with poliomyelitis in 1980, and from 2 patients with non-acute flaccid paralysis in 1984 and 1993, respectively, but since then no wild poliovirus has been isolated from patients with poliomyelitis in Japan [3].

OPV is usually given to an individual 3 times or more, which confers a high seropositivity against 3 types of polioviruses [4–7]. Moreover, it is in Japan that the epidemic of poliomyelitis has been prevented with only a 2-dose administration of OPV. A large-scale investigation was carried out to evaluate antibody titers that neutralize virulent wild polioviruses by the vaccination of imported monovalent OPV

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(prepared by British Pfizer Co.) in 1961 before the introduction of mass vaccination [8,9]. However, both the virus titer of each type of poliovirus and the administration method that inoculated each type of monovalent vaccine virus, respectively, in the investigation were different from Japan's current trivalent OPV policy. Furthermore, it was suggested that natural infections of poliovirus influenced the results because of continually occurring epidemics of poliomyelitis caused by poor public health conditions at that time in Japan. Therefore, a regular measurement program of the neutralizing antibody titers against Sabin attenuated strains has been performed by the Ministry of Health and Welfare in Japan since 1973, but few investigations on antibody titers against virulent wild polioviruses have been carried out.

The global poliomyelitis eradication program of the WHO is close to the final stage, at which the virulent wild-type poliovirus is replaced with the vaccine strains from OPV [10]. Recently, epidemics of poliomyelitis by vaccine-derived polioviruses (VDPVs) [11,12] and imported wild polioviruses [13] have been reported worldwide. We have surveyed polioviruses regularly since 1979 in Toyama Prefecture. Although the wild poliovirus was not isolated, a total of 78 VDPV strains (type 1: 16 strains, type 2: 31 strains, type 3: 31 strains) were isolated from the river water and wastewater from sewage disposal plants between October 1993 and September 1995 [14]. Furthermore, using the method of 'mutant analysis by PCR and restriction enzyme cleavage (MAPREC)' to estimate the ratio of polioviruses containing genomes of a virulent nature in a vaccine-virus population [15,16], we have found that some strains of type 1 [17,18], type 2 [19] and type 3 [20] VDPV possess virulent genotype.

We have been considering why an epidemic of poliomyelitis caused by virulent polioviruses like virulent VDPV has not occurred in Japan, and have studied this using virulent poliovirus type 1 strains [21]. In this study, we attempt to assess retrospectively the efficacy of Japan's current OPV vaccination policy in preventing epidemics of poliomyelitis caused by wild and virulent VDPV type 1, 2, 3 strains.

Material and methods

Serum samples of residents used for this study were obtained with the agreement of the residents or their guardians in October, 1998.

Viruses

Type 1, 2 and 3 strains of Sabin and wild-type Mahoney, Lansing and Saukett were obtained from the National Institute of Infectious Diseases, Tokyo, Japan. Type 1, 2 and 3 VDPV G4-12, G18-2 and G5-1 strains were originally isolated from a sewage disposal plant located downstream of the Oyabe River, Toyama Prefecture, Japan [14]. They were estimated to be strong neurovirulent strains by the molecular analysis method (MAPREC method) that quantifies genomic changes [17,19,20]. Differences in both nucleotide and amino acid sequences in the VP1 capsid protein region and 5' non-coding region between the genomes of Sabin strains and the isolated VDPVs are described in Table I. The VDPVs that had strong neurovirulence and the most abundant nucleotide mutations in the VP1 region were used in this research. According to WHO criteria, a VDPV is defined as a strain provided there is 1–15% drift in the VP1 region at the nucleotide level [12]. Although the G4-12 and G5-1 strains do not belong to the category of VDPV since their nucleotide divergences

Table I. Differences in nucleotide sequences between the genomes of Sabin strains and VDPVs.

		Change from Sabin to VDPV [a]			
		VP1 region		5' non-coding region	
	VDPV	Nucleotide	Amino acid	Nucleotide	Ratio of reversion [b]
Type 1	G4-12	C→U (2743)		G→A (480)	
		A→G (2795)	T→A (106)	U→C (525)	92.1%
Type 2	G18-2	T→C (2537)	V→A (19)	A→G (481)	83.2%
		G→A (2566)	D→N (29)		
		C→T (2568)			
Type 3	G5-1	G→A (2536)	A→T (54)	U→C (472)	88.6%
		C→T (2713)			
		T→C (2790)	M→T (105)		
		A→G (2815)			

[a] Numbers in parentheses are positions.
[b] Contents of 480-A + 525-C for type 1, 481-G for type 2 and 472-C for type 3 by using the MAPREC method.

were less than 1% in the VP1 region, we included them as VDPVs in this research.

Sera

In this research, the serum that remained in the regular measurement program between 1984 and 2005 was used. The selection of serum was decided based on the following criteria: 1) serum which had been collected at the time in 1993 to 1995 close to when the virulent VDPVs were isolated; 2) sufficient amount of serum was residual for measurement of neutralizing antibody titer. Consequently, a total of 191 individual serum samples from residents of Toyama Prefecture, Japan, of 0–76 y of age, collected in 1998, were used for this study. Of these serum samples, 85 that received the standard 2 doses of OPV were measured for neutralizing antibody titers against wild and virulent VDPV strains. The vaccination history of individual groups of residents is shown in Table II. In addition, 67–295 serum samples every y between 1984 and 2005 (total 4050 samples) which contain non-vaccinated, 1- or 2-dose OPV-vaccinated individuals from residents of Toyama Prefecture were measured for neutralizing antibody titers against Sabin type 1, 2 and 3 strains.

Measurement of neutralizing antibody titers

The neutralizing test was performed in 96-well microtiter plates using Vero cells according to the standard method recommended by the WHO [22]. The original virus titers and the challenge virus titers used for the neutralization test were as follows: Sabin 1: $10^{6.75}$, $10^{2.08}$, Sabin 2: $10^{6.75}$, $10^{2.06}$, Sabin 3: $10^{6.75}$, $10^{2.36}$, Mahoney: $10^{6.38}$, $10^{2.32}$, Lansing: $10^{6.50}$, $10^{2.11}$, Saukett: $10^{6.25}$, $10^{2.25}$, G4-12: $10^{6.75}$, $10^{2.25}$, G18-2: $10^{5.50}$, $10^{2.06}$ and G5-1: $10^{6.25}$, $10^{2.17}$ $TCID_{50}$/0.05 ml, respectively. The cytopathic effect was scored 7 d after infection. A serum sample with a neutralizing antibody titer equal to or more than 8 was regarded as seropositive.

Results

Seropositivity and neutralizing antibody titers against Sabin type 1, 2, 3 vaccine strains

The serum samples were measured for neutralizing antibody titers against each type of Sabin vaccine strain using a microneutralization assay. In individuals who were born after the enforcement of domestic OPV vaccination and received the standard 2 doses of OPV, the seropositivities against the Sabin type 1, 2 and 3 strains were 98.8%, 100% and 68.2%, respectively (Table II). The rates were higher than those of either non-vaccinated individuals (corresponding rates were 40.0%, 80.0% and 20.0%, respectively), or 1-dose OPV-vaccinated individuals (75.0%, 87.5% and 37.5%, respectively). Similarly, the geometric means of neutralizing antibody titers of the 2-dose vaccinated individuals against each type of Sabin strain was obviously higher (569, 426 and 72.1, respectively) compared to those of the non-vaccinated individuals (corresponding titers were 79.2, 180 and 6.4, respectively). Although both the seropositivity and antibody titers against the Sabin type 3 strain were the lowest among 3 types of Sabin strains, the efficacy of 2-dose administration of OPV against all types

Table II. Vaccine history, seropositivity and neutralizing antibody titers against Sabin type 1, 2 and 3 strains of residents in Toyama Prefecture.

			Seropositivity (%) [c]/ neutralizing antibody titer		
	Vaccine history [a] (no. of OPV doses)	No. of serum samples [b]	Sabin 1	Sabin 2	Sabin 3
Residents born after 1964 [d] (after introduction of domestic OPV)	Non-vaccinated	5	40.0/79.2	80.0/180	20.0/6.4
	Vaccinated (1)	8	75.0/649	87.5/489	37.5/69.5
	Vaccinated (2)	85	98.8/569	100/426	68.2/72.1
	Unknown	24	87.5/323	95.8/365	66.7/41.3
Residents born before 1964 [e] (before introduction of domestic OPV)	0	69	92.8/158	94.2/212	85.5/90.9

[a] The OPV, prepared by the Japan Poliomyelitis Research Institute, Tokyo, Japan, contained $10^{6.0\pm0.5}$, $10^{5.0\pm0.5}$, $10^{5.5\pm0.5}$ $TCID_{50}$ of Sabin type 1, 2 and 3 strains, respectively, in 1 dose.
[b] Serum specimens were collected in October, 1998.
[c] A titer equal to or more than 8 was regarded as seropositive.
[d] <35 y of age in 1998.
[e] Equal to or more than 35 y of age in 1998.

of Sabin strains seems to be valid compared to those of non-vaccinated individuals.

High seropositivity (92.8%, 94.2% and 85.5%, respectively) and high geometric mean antibody titers (158, 212 and 90.9, respectively) were observed in individuals who were born before the enforcement of domestic OPV vaccination and, as a rule, were not vaccinated (Table II). This may be explained by natural infection, as there were continually occurring epidemics of poliomyelitis because of poor public health conditions at that time in Japan.

Seropositivity and neutralizing antibody titers against virulent type 1, 2, 3 strains

Efficacy of the 2-dose administration of OPV against virulent strains was analysed. Seropositivity and geometric mean antibody titers against type 1, 2 and 3 virulent wild strains of Mahoney, Lansing and Saukett, and virulent VDPVs G4-12, G18-2 and G5-1 are shown, in Figure 1, in the residents who received the standard 2-dose administration of domestic OPV. The seropositivities against virulent type 1 and 2 strains were more than 90%, but the values against virulent type 3 strains were 62.4% (Figure 1A). Differences in seropositivity between the Sabin vaccine strains and virulent strains in all types were hardly observed.

The geometric mean antibody titers against type 1 strains of Sabin, Mahoney and G4-12 were 569, 186 and 190, respectively (Figure 1B). Similarly, the titers against type 2 strains of Sabin, Lansing and G18-2 were 426, 260 and 325, respectively. Although high antibody titers were observed against virulent type 1 and 2 strains, those were obviously lower than those of the Sabin attenuated strains. In contrast, the titers against type 3 strains of Sabin, Saukett and G5-1 showed lower values of 72, 58 and 59, respectively, compared with those of the other 2 types.

Comparison of neutralizing antibody titers among age group

The geometric mean neutralizing antibody titers among age group of the residents who received the standard 2-dose administration of OPV were investigated, although the number of serum samples used in the investigation was unsatisfactory (85 samples) (Figure 2). Sufficient titers against virulent type 1 and 2 strains were observed in all generations (Figure 2A, B), while the titers against the virulent strains of Mahoney and G4-12 were 2 to 4 times lower than those against the Sabin strain in the case of type 1 viruses. Such large differences were not

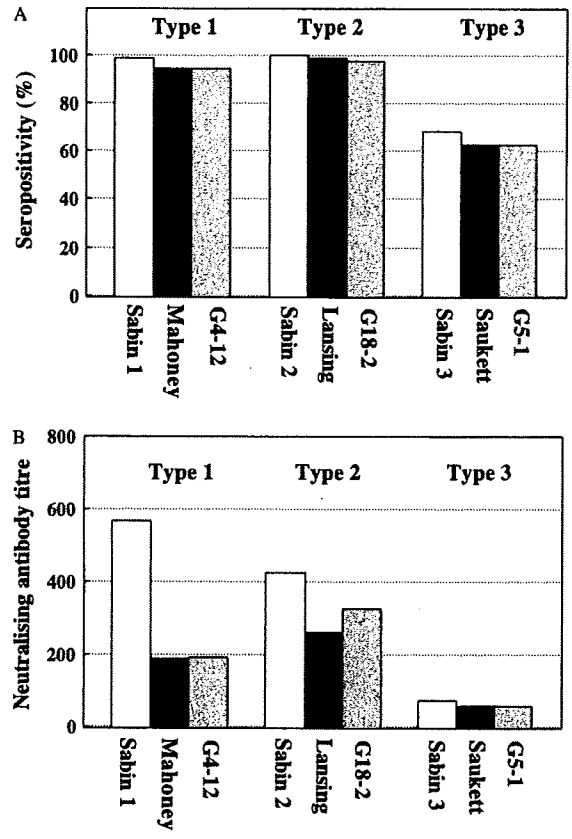

Figure 1. Seropositivity (A) and geometric mean neutralizing antibody titers (B) of residents who were vaccinated with the standard 2-dose administration of OPV against strains of type 1 Sabin, Mahoney and G4-12, type 2 Sabin, Lansing and G18-2, and type 3 Sabin, Saukett and G5-1.

observed in type 2 and 3 viruses (Figure 2A, B, C). These data are consistent with the results shown in Figure 1B. The geometric mean antibody titers against type 3 strains of Sabin, Saukett and G5-1 were extremely low at the ages of 10 to 13 y (corresponding titers were 19.1, 12.0 and 11.4, respectively) and 14 to 19 y (10.0, 9.0 and 9.0, respectively) compared with other age groups. The corresponding seropositivities in both generations also showed low values of 38.5%, 30.8%, 30.8% and 50.0%, 25.0%, 25.0%, respectively (data not shown).

Furthermore, the seropositivity of total 4050 serum samples from residents of Toyama Prefecture against Sabin type 1, 2 and 3 strains for these 22 y is shown in Figure 3. The vaccine coverage was 90–97% in each y except for the individuals with unknown vaccination history (data not shown). Large difference of the vaccine coverage was not observed compared with the general situation in Japan (90–94%) [23]. Since the seropositivity against type 3 strain was always lower than other

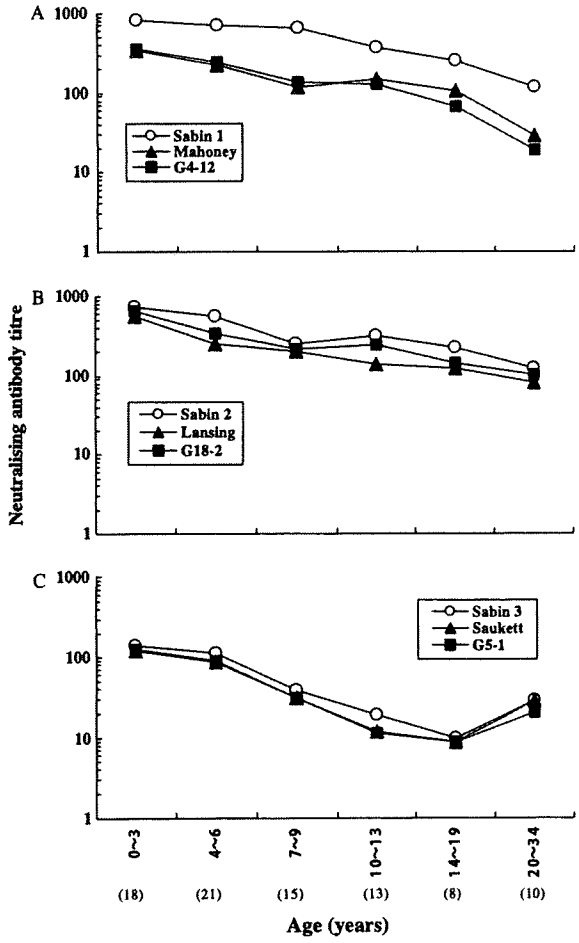

Figure 2. Geometric mean neutralizing antibody titers according to age group of the residents who were vaccinated with the standard 2-dose administration of OPV against strains of type 1 Sabin, Mahoney and G4-12 (A), type 2 Sabin, Lansing and G18-2 (B), and type 3 Sabin, Saukett and G5-1 (C) in each generation. The number of serum samples used in the investigation is shown in parentheses.

types, the antibody titer against type 3 virus would be postulated to be lower than the other types as well. However, high herd immunity against type 1, 2 and 3 viruses has been maintained long term.

Discussion

A 2-dose administration, in spring and autumn, respectively, of OPV is routinely carried out in infants in Japan. The effect of this vaccination protocol has been a concern, because it is only in Japan (of the whole world) that the vaccination is performed just twice. Although there are some reports of a high seropositivity using the 2-dose vaccination of OPV against all types of polioviruses [24], the continuation of the effect is unclear. Therefore, a large-scale surveillance of neutralizing antibody titers against virulent wild polioviruses administrated by imported monovalent OPV was performed before the introduction of mass vaccination [8,9]. However, the virus titers and inoculation method were different from Japan's current trivalent OPV vaccination policy. Furthermore, there is also the possibility that natural infections of poliovirus influenced the results because of continually occurring epidemics of poliomyelitis caused by poor public health conditions at that time in Japan. Nishio et al. [25] reported that the neutralizing antibody titers, particularly against the type 3 virus, decreased gradually for a period of 5 y after 2-dose vaccination of OPV, and thereafter some of them increased due to natural infection.

To assess the effect of the vaccination under current public health conditions with few natural infections, we assayed here the neutralizing antibody titers against type 1, 2 and 3 strains of Sabin, wild, and virulent VDPV of residents in an area of Toyama

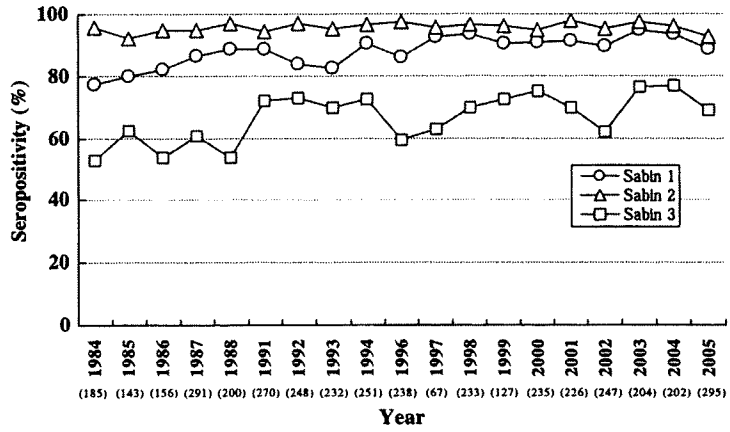

Figure 3. Seropositivity against type 1, 2 and 3 strains of Sabin (1984–2005). The number of serum samples used in the investigation is shown in parentheses.

Prefecture where the virulent VDPVs were isolated. We have already reported that the current vaccination policy gave a sufficient effect on virulent type 1 viruses [21]. In this study, >90% of the individuals were seropositive, and high neutralizing antibody titers of >180 were observed in virulent type 1 and 2 polioviruses in residents who were vaccinated with the standard 2-dose administration of OPV (Figure 1A, B). Geometric neutralizing antibody titers against isolated VDPV strains were lower than those against Sabin strains, especially in type 1 strains. Challenge virus titers of Sabin 1 and G4-12 strains for neutralizing test were almost the same (see Material and methods). It is therefore considered that the amino acid divergence in the VP1 region takes part in the difference of neutralizing antibody titre. In contrast, remarkably low seropositivity and neutralizing antibody titers were revealed in type 3 viruses. Large differences in antibody titers were also observed among the age groups of type 3 viruses especially at 10 to 19 y of age (Figure 2C). It is unclear why the antibody titers were low in these age groups, although a 2-dose administration of OPV had been received as well as other age groups. This phenomenon was similarly observed not only in 1998, but also in other y (Table III), i.e. a tendency that the antibody titer and seropositivity of 10–19 y age group were lower than those of other age groups was shown between 1996 and 2005. A booster dose of the vaccine in adolescents to ensure personal and herd immunity is recommended.

Lago et al. [4] reported similar results, showing that while the seropositivities of poliovirus type 1 and 2 strains were >90%, that of the type 3 virus was 45.9%, after a 2-dose administration of OPV in Cuba. It has been also reported that the seropositivity of the type 3 virus after a 2-dose vaccination of OPV was approximately 40% in Brazil, which value increased after the booster vaccination [6]. Thus, it should be considered that solid immunity will be acquired by performing the booster vaccination for adolescents in Japan, even though Japan's current OPV vaccination policy has been effective in preventing poliomyelitis caused not only by the wild poliovirus, but also by virulent VDPV in types 1, 2 and 3. Although the objective was an increase in type 1 antibody titer, the notification of the booster vaccination of OPV was initiated by the Ministry of Health and Welfare in Japan in 1996 for individuals who were born in 1975 to 1977, and subsequently the vaccination was performed. The efficacy of the booster vaccination in Japan will be clarified by analysing the effects of this.

Recently, epidemics of poliomyelitis caused by VDPVs have been reported worldwide [11,12]. Also,

Table III. Seropositivity and geometric mean neutralizing antibody titers in each age group of residents who were vaccinated with the standard 2-dose administration of OPV against Sabin type 3 strain in recent y.

	Seropositivity (%)/neutralizing antibody titer against Sabin type 3 strain.								
Age group	1996	1997	1998	1999	2001	2002	2003	2004	2005
0 ~ 3	76.9/172 (26) [a]	83.3/106 (6)	61.1/142 (18)	79.2/88.2 (24)	83.3/49.1 (18)	73.3/30.9 (15)	73.3/131 (15)	71.4/182 (14)	83.3/176 (24)
4 ~ 6	85.0/63.8 (20)	71.4/102 (7)	76.2/114 (21)	84.0/26.2 (25)	75.0/39.0 (8)	58.8/22.8 (17)	53.8/23.4 (13)	85.7/159 (14)	94.1/93.4(17)
7 ~ 9	87.5/28.0 (16)	75.0/45.0 (8)	86.7/38.4 (15)	93.8/87.8 (16)	87.5/76.5 (16)	69.2/21.5 (13)	75.0/28.3 (12)	66.7/52.7 (12)	71.4/30.9 (7)
10 ~ 13	53.8/9.2 (13)	– (0)	38.5/19.1 (13)	71.4/32.0 (14)	44.8/16.3 (29)	45.5/9.8 (22)	50.0/21.2 (10)	69.2/45.8 (26)	66.7/28.5 (33)
14 ~ 19	42.4/16.4 (33)	66.7/14.7 (3)	50.0/10.0 (8)	42.9/20.6 (7)	63.6/39.3 (11)	46.2/14.5 (13)	68.0/24.0 (25)	71.4/48.0 (7)	57.1/19.4 (14)
20 ~ 34	0/4.0 (5)	88.9/61.8 (9)	90.0/28.4 (10)	63.6/23.3 (11)	60.0/17.6 (5)	50.0/20.0 (8)	75.0/9.0 (4)	–(0)	40.0/11.2 (10)

[a] The number of serum samples used in the investigation is shown in parentheses.

imported cases of wild polioviruses have been reported in polio-free countries [13]. Furthermore, virulent VDPVs were easily isolated from river and sewage water in Japan [14,19]. Poliomyelitis cases caused by VDPVs or imported wild viruses have not been reported in Japan, since it is considered that high herd immunity has been maintained for a long time. Our results suggest that Japan's vaccination policy might be enough to prevent an epidemic of poliomyelitis caused by wild and virulent VDPV type 1, 2, 3 strains, even though the titers against type 3 viruses were the lowest.

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An Attenuated Strain of Enterovirus 71 Belonging to Genotype A Showed a Broad Spectrum of Antigenicity with Attenuated Neurovirulence in Cynomolgus Monkeys▽

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Enterovirus 71 (EV71) is a causative agent of hand, foot, and mouth disease and is also sometimes associated with serious neurological disorders. In this study, we characterized the antigenicity and tissue specificity of an attenuated strain of EV71 [EV71(S1-3′)], which belongs to genotype A, in a monkey infection model. Three cynomolgus monkeys were inoculated with EV71(S1-3′), followed by lethal challenge with the parental virulent strain EV71(BrCr-TR) via an intravenous route on day 45 postinoculation of EV71(S1-3′). Monkeys inoculated with EV71(S1-3′) showed a mild neurological symptom (tremor) but survived lethal challenge by virulent EV71(BrCr-TR) without exacerbation of the symptom. The immunized monkey sera showed a broad spectrum of neutralizing activity against different genotypes of EV71, including genotypes A, B1, B4, C2, and C4. For the strains examined, the sera showed the highest neutralization activity against the homotype (genotype A) and the lowest neutralization activity against genotype C2. The order of decreasing neutralization activity of sera was as follows: A > B1 > C4 > B4 > C2. To examine the tissue specificity of EV71(S1-3′), two monkeys were intravenously inoculated with EV71(S1-3′), followed by examination of virus distribution in the central nervous system (CNS) and extraneural tissues. In the CNS, EV71(S1-3′) was isolated only from the spinal cord. These results indicate that EV71(S1-3′) acts as an effective antigen, although this attenuated strain was still neurotropic when inoculated via the intravenous route.

Enterovirus 71 (EV71) is a small nonenveloped virus with a genome of single-strand positive RNA of about 7,500 nucleotides and belongs to the genus *Enterovirus* of the family *Picornaviridae* (8, 48). EV71 is classified as *Human enterovirus species A* along with some coxsackie A (CA) viruses, such as CA10 and CA16 (8, 44). CA10, CA16, and EV71 cause hand, foot, and mouth disease (HFMD) and herpangina. EV71 infection is also sometimes associated with severe neurological diseases, such as brain stem encephalitis and poliomyelitis-like paralysis, mainly in infants and young children (11, 34, 56). The neuropathogenic features of EV71 were first emphasized during an outbreak in Bulgaria in 1975 in which poliomyelitis-like paralysis was the major symptom (21.1% of patients), with a high fatality rate (29.5%) among the paralytic cases (51). In recent large-scale outbreaks of HFMD in Malaysia (1997) (1, 50) and Taiwan (1998 and 2000) (20, 28, 29, 55), several fatal encephalitis cases were reported. In the EV71 outbreak in Taiwan in 1998, out of 129,106 cases of HFMD or herpangina, there were 405 severe cases, including 78 fatal cases (20); thus, the severity rate of this outbreak was <0.3%. These findings underscore the high neuropathogenicity of EV71 as well as poliovirus (PV), which causes poliomyelitis in 0.1 to 1.0% of infected individuals (reviewed in reference 36).

Most of the fatal EV71 cases in Taiwan were in young children (age, ≤5 years) and involved pulmonary edema and/or pulmonary hemorrhage (20). These symptoms were of neurogenic origin, and brain stem involvement (direct destruction of the vasomotor and respiratory centers) was critical (10, 21, 25, 31, 57). Disseminated infection of EV71 in the central nervous system (CNS) might in part explain the EV71-specific neuropathogenesis (40).

Molecular epidemiological studies indicate that EV71 consists of at least 10 genotypes (A, B1 to -5, and C1 to -4) (7, 26, 33, 37, 49). The prototype BrCr strain is the sole member of genotype A (8). In the HFMD outbreak in Malaysia in 1997, the predominant genotype was B3, but in the outbreak in Taiwan in 1998, the predominant genotype was C2. Isolates from the outbreak in Bulgaria in 1975 and the outbreak in Hungary in 1978 belong to genotype B1. In more recent EV71 outbreaks in Asia, genotype C4 was predominant (26, 27, 37). These findings indicate that, in general, the genotype is not the sole determinant of the observed pathogenesis of EV71 (14, 50). The relationship between EV71 genotypes and cross-neutralizing reactivity remains unclear.

Recently, we generated an attenuated strain of EV71 [EV71(S1-3′)] derived from the prototype BrCr strain by defined genetic manipulation (5). The manipulation was based on the temperature-sensitive determinants of the type 1 PV vaccine strain (Sabin 1) (45), some of which are located in the

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conserved regions of the enterovirus genome, i.e., the 5′ nontranslated region (NTR), the $3D^{pol}$, and the 3′ NTR (24, 43, 53). Monkeys inoculated with EV71(S1-3′) exhibited a mild, nonlethal neurological disorder with limited spread in the CNS on day 10 postinoculation (p.i.).

In the present study, we characterized the antigenicity and tissue specificity of EV71(S1-3′) in cynomolgus monkeys. We examined the tissue specificity of EV71(S1-3′) in the CNS and extraneural tissues. We examined the humoral immune response of monkeys inoculated with EV71(S1-3′) and the antigenicity of EV71(S1-3′) against EV71 strains belonging to genotypes A, B1, B4, C2, and C4.

MATERIALS AND METHODS

Cells and viruses. Vero cells (derived from African green monkey kidney cells) and RD cells (derived from human rhabdomyosarcoma) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Vero cells were used for preparation of stocks of the viruses EV71(S1-3′) and EV71(BrCr-TR), titration of those viruses, and isolation of viruses from monkey tissues. RD cells were used for preparation of stocks of other virus strains, titration of those viruses, and neutralization assays. The following EV71 strains were used: EV71(BrCr-TR), a genotype A temperature-resistant variant of the prototype BrCr strain; EV71(S1-3′), a genotype A temperature-sensitive mutant of the prototype BrCr strain; Nagoya (genotype B1) (16); C7-Osaka (genotype B4); 1095 (genotype C2) (49); 75-Yamagata-2003 (genotype C4); 2399-Yamagata-2003 (genotype C4); and 1530-Yamagata-2003 (genotype C4) (26, 27, 37). In cynomolgus monkeys, EV71(BrCr-TR) has the neurovirulent phenotype of the BrCr strain and EV71(S1-3′) has an attenuated phenotype (5, 17, 39, 40). The virus stocks of EV71(BrCr-TR) and EV71(S1-3′) were prepared in Vero cells by RNA transfection of the transcripts derived from corresponding infectious clones, as described elsewhere (5). The virus stocks of other strains were prepared in RD cells by RNA transfection of the transcripts derived from corresponding infectious clones [EV71(Nagoya) and EV71 (Nagoya-HIS)] or by direct amplification from the original virus stocks. For the enzyme-linked immunosorbent assay (ELISA), EV71(Nagoya-HIS) was propagated from the virus stock in RD cells cultured in serum-free medium (VP-SFM; Gibco).

General methods of molecular cloning. *Escherichia coli* strain XL10gold (Stratagene) was used for the preparation of plasmids. Ligation of DNA fragments was performed using a Quick Ligation kit (New England BioLabs). Site-directed mutagenesis (SDM) was performed using KOD plus DNA polymerase (Toyobo) (47).

RNA extraction, RT-PCR, and sequencing. Viral genomic RNA was extracted from the culture fluid of infected cells using a High-pure viral RNA purification kit (Roche). Reverse transcription-PCR (RT-PCR) was performed using a ReverTra-Plus kit (TOYOBO). PCR products were purified using a PCR purification kit (QIAGEN). Direct sequence analysis of full-length genomic sequences of EV71(Nagoya) was performed using DNA fragments amplified by RT-PCR as the templates of the sequencing. The 5′ end of the viral genome was sequenced using the 5′RACE system, version 2.0 (Invitrogen), according to the manufacturer's instructions. The 3′ end of viral genomes was sequenced using an RT-PCR product obtained using the primers 7200F+ and EcoRI-3END (Table 1). DNA sequencing was performed using a BigDye Terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems), and the results were analyzed using an ABI PRIZM 3130 genetic analyzer (Applied Biosystems).

RNA transfection. RNA transcripts were obtained using a RiboMAX large-scale RNA production system T7 kit (Promega), using AvrII-linearized DNA of the infectious clones of EV71(Nagoya) or EV71(Nagoya-HIS) as the template. The in vitro-synthesized RNA transcripts were transfected onto monolayers of RD cells in six-well plates (Falcon), using the DEAE-dextran method, followed by incubation at 37°C in 10% FCS–DMEM (2 ml per well) (30). The cells were harvested when all the cells exhibited the cytopathic effect (CPE) and were then stored at −70°C. The titers of recovered viruses were about 10^7 50% cell culture infectious doses ($CCID_{50}$) per ml.

Virus titration. The virus titer was determined by measuring the $CCID_{50}$ in a microtitration assay using RD cells, as described elsewhere (40). Briefly, inoculated RD cells were cultured at 37°C for 7 days and were then observed for CPE. The $CCID_{50}$ was calculated using the Behrens-Kärber method (23).

TABLE 1. Primers used for construction of infectious clones of EV71(Nagoya) and EV71 (Nagoya-HIS)

Primer	Sequence[a]
7200F+	AACACTCAAGATCACGTGCGCTCCC
EcoRI-3END-	ACTGGAATTCTTTTTTTTTTTTTTT TTTTTTTTV
SnaBI-T7-Nagoya+	TTAATACGTATTAATACGACTCACT ATAGGTTAAAACAGCCTGTGGGT TGTTCC
Nagoya2200+	CTACGTGGTTCCAATTGGGGCGCC
Nagoya3800-	CTAGCTTCCACATATATGAGGCTGG
A2MluI-	AAAAACGCGTTTTTTTTTTTTTTTT TTTTTTTTGCTATTCTGG
NagoyaVP1-HIS+	CACCATCATCACCATCACACTAACC CAAATGGTTATGCTAACTG
NagoyaVP1-HIS-	GTGTGATGGTGATGATGGTGAGTA CCCTCAAGAGGGAGGTCTAT CTCC

[a] The variable sequence position in the primer is expressed according to the IUPAC system. Sequences read from the 5′ position on the left.

Titration of neutralization activity of monkey serum. The neutralization titer of monkey serum was determined using two different methods: (i) observation of CPE in infected cells (CPE method), and (ii) counting the number of infected cells by indirect immunofluorescence (IF method) (2).

For the CPE method, twofold dilutions of each monkey serum were prepared using 10% FCS–DMEM. Then, in 96-well plates (Falcon; two wells per dilution), 50 μl of each dilution was mixed with 50 μl of 10% FCS–DMEM containing 20 $CCID_{50}$ of an EV71 strain, followed by incubation at 37°C for 3 h. Then, 100 μl of RD cell suspension (containing 2.0×10^5 cells) was added to each well, followed by culturing at 37°C for 7 days for observation of CPE. The reciprocal of the highest dilution of serum that protected cells from infection in at least one of the two inoculated wells (per dilution) was recorded as the neutralization titer.

For the IF method, we used the following infectious doses of EV71 in 50 μl of 10% FCS–DMEM, which each yielded about 100 infected cells, as detected by indirect immunofluorescence: EV71(BrCr-TR), 3.8×10^2 $CCID_{50}$; EV71 (Nagoya), 1.9×10^2 $CCID_{50}$; EV71(C7-Osaka), 3.3×10^2 $CCID_{50}$; EV71 (1095), 1.1×10^2 $CCID_{50}$; EV71(75-Yamagata-2003), 2.1×10^3 $CCID_{50}$; EV71(2399-Yamagata-2003), 5.6×10^4 $CCID_{50}$; EV71(1530-Yamagata-2003), 2.3×10^2 $CCID_{50}$. These doses were incubated with 10-fold dilutions of monkey sera in 50 μl of 10% FCS–DMEM, or were incubated with 50 μl of 10% FCS–DMEM without monkey sera (control), in 96-well plates (Falcon) at 37°C for 3 h. After incubation, 100 μl of RD cell suspension (2.0×10^5cells) was added, followed by culturing at 37°C for 16 h, except for cells inoculated with 75-Yamagata-2003, which were cultured at 37°C for 8 h because 75-Yamagata-2003 spreads faster than other strains in RD cells. Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline [PBS(-); 10 mM phosphate buffer, pH 7.0, 137 mM NaCl, and 2.6 mM KCl] at room temperature for 10 min and were then incubated with rabbit anti-EV71(C7-Osaka) hyperimmune serum [1:100 dilution in 0.1% Triton X-100–PBS(-)] at 37°C for 1 h. After washing three times with PBS(-), goat anti-rabbit immunoglobulin G (IgG; H+L) conjugated with fluorescein isothiocyanate [1:300 dilution in 0.1% Triton X-100/PBS(-); Zymed] was added to the cells, followed by incubation at 37°C for 20 min. The cells were observed with a Biozero fluorescence microscopy system (KEYENCE). The number of infected cells obtained in the control wells (without serum) was designated as 100%, and the percentage of neutralization was calculated for each dilution. The 50% neutralization units (NU_{50}) for 50 μl of monkey serum was calculated according to the Behrens-Kärber method (23).

Construction of infectious cDNA clones of EV71(Nagoya) and EV71(Nagoya-HIS). A DNA fragment, containing 4 kb of the 5′ region of the viral genome of EV71(Nagoya), was amplified by RT-PCR using the primers SnaBI-T7-Nagoya+ and Nagoya3800- (Table 1). The resultant cDNA fragment was cloned into plasmid pEV71(BrCr-TR), which contains the infectious cDNA of strain BrCr-TR (5), after digestion by SnaBI and AgeI. Next, the 3′-end sequence of EV71(Nagoya) was amplified by RT-PCR using the primers Nagoya2200+ and A2MluI- and was then cloned into the above construct after digestion by AgeI and MluI. The resultant construct was sequenced and then differences in nucleotides between this construct and the parental Nagoya strain were corrected by SDM using appropriate primers. This infectious clone of EV71(Nagoya) was designated as pEV71(Nagoya).