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REVIEW

Japanese vaccinations and practices, with particular attention to polio and pertussis

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Summary This article introduces Japanese vaccinations and practices, focusing on polio and pertussis.

Japan is one of the few industrialized countries still using live attenuated oral poliovirus vaccine (OPV). Current status of vaccine-associated paralytic poliomyelitis in Japan is discussed. This review is intended to encourage early conversion of OPV to inactivated poliovirus vaccine (IPV) for the routine vaccination as soon as possible.

The other topic pertains to the results of a study designed to evaluate the safety and immunogenicity of the Japanese DPT vaccine in adults when administered at the dose of 0.2 ml (2/5th of the ordinary dose). In Japan, there is no system for providing advice to adults on vaccination once the childhood schedule is completed. The author, however, wishes to propose here that if the currently approved DPT vaccine can be better utilized as Tdap, we may improve the means for disease prophylaxis.

Introduction

It has been realized that Japan is behind other countries in terms of vaccine issues, specifically the vaccination system, availablity of vaccine products, licensure procedure delay, and others. The term "vaccine gap" is frequently used when referring to Japan. However, in Japan the vaccine to prevent Japanese encephalitis (a viral infection of the central nervous system prevalent in Asia) was first developed. A cellular pertussis vaccine, less likely to induce adverse reactions than the whole-cell pertussis vaccine, was also developed first in Japan. At that time, Japan was not behind other countries in terms of vaccination. Despite these past achievements, the current situation relating to vaccination in Japan is rather regretful. Introspection is now needed in many respects by both the administrative authorities and the medical professionals in Japan. Instead of accepting the current situation, concrete measures to improve the situation must be devised. In view of this situation, this paper will deal with two topics, vaccine-associated paralytic poliomyelitis in Japan and DPT vaccination with an adjusted dose for younger adults in Japan.

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Vaccine-associated paralytic poliomyelitis in Japan

Background

Poliovirus belongs to the genus *Enteroviridae*. The most characteristic clinical symptom of poliomyelitis caused by this virus is "paralysis." OPV, used currently for children's routine vaccination program in Japan, is a very effective vaccine, but is known to induce paralysis occasionally by itself. This adverse reaction is due to acquisition of neurovirulence of the attenuated strain, although the incidence is very low. The present study was undertaken to investigate the features of vaccine-associated paralytic poliomyelitis (VAPP) through analysis of the clinical course, risk factors for disease onset, and virological data collected from recently reported VAPP cases in Japan.

Subjects and methods

Data of cases suspected of having VAPP, reported in the literature or at medical/scientific society meetings since 2000 in Japan, were analyzed and compared.

Results

Tables 1–4 show a list of the cases reported during 10 years.[1–10] An overwhelming majority of the VAPP cases were boys presenting after the first dose of OPV. In 2 of the 10 cases, perianal abscess was found to be present at the time of administration of the OPV (Table 1).

In many cases, fever developed 2–3 weeks after administration of the OPV, followed several days to 5 days later by paralysis. Examination of the cerebrospinal fluid revealed a moderately increased cell count and a slightly higher protein level than normal. Diagnostic imaging revealed the characteristic finding of a high signal intensity in the anterior horn of spinal cord, but not in all cases. In the neurophysiological test, some cases showed reduced motor nerve action potentials or conduction rate in the affected area. Sequelae were seen in all cases. Among the cases checked for immunological abnormalities, no evident sign of immunodeficiency was noted in any case (Tables 2 and 3).

The number of cases from which the poliovirus vaccine strain could be isolated was small; however, sampling at the appropriate time-point (immediately after onset of paralysis) was not carried out in all cases. In 5 of the 10 cases, fecal samples tested positive for the virus, and type 2 or 3 virus was isolated in these cases. There were no cases in which poliovirus was isolated from the cerebrospinal fluid (Table 4).

Discussion

For many years, VAPP has been known to occur at a high incidence in boys after the first dose of OPV.[11] A similar result was obtained in the present study. Of the 10 VAPP cases reported during the latest 10-year period, 2 were children with perianal abscess. Another overseas report has also pointed out perianal abscess as a risk factor for the onset of VAPP.[12] Further study is therefore needed on this association. According to the current package insert for OPV in Japan, the risk factors for the onset of VAPP are a history of tooth extraction, tonsillectomy, non-emergency surgery or frequent intramuscular injections within one month of administration of OPV, and avoidance of these risk factors is recommended. However, the package insert does not still refer to perianal abscess (Table 5).

It is said that immunodeficiency (e.g., abnormal humoral immunity) increases the risk of development of VAPP as an adverse reaction to OPV.[11] Therefore, it would be desirable to examine cases of VAPP in detail for the presence/absence of immunological abnormalities, in an attempt to elucidate clearly the involvement of host factors in VAPP.

Among the cases developing paralysis after intake of OPV in whom VAPP was suspected, virological investigations were not performed consistently in samples collected at an appropriate point of time (i.e., soon after the onset of paralysis). In view of the data from cases reported previously, it is considerably difficult to isolate the responsible virus from the cerebrospinal fluid. We therefore cannot over-emphasize the importance of checking for the virus in samples of feces, in which virus isolation seems to be possible over longer periods of time.

Table 1 List of reported VAPP cases in Japan.

No.	Year of VAPP onset	Age (months)	Gender	OPV	Underlying disease, etc.	Prefecture	Reference
1	2008	7	Male	1st dose	Perianal abscess	Mie	1
2	2008	11	Male	1st dose	None	Osaka	2
3	2008[a]	10	Male	1st dose	None	Fukushima	3
4	2008[a]	4	No description	1st dose	None	Yamaguchi	4
5	2008[a]	6	Male	1st dose	HBs carrier	Hokkaido	5
6	2007	9	Male	1st dose	None	Nara	6
7	2007[a]	6	Male	1st dose	Perianal abscess	Hokkaido	7
8	2006[a]	18	Male	None	None	Tokyo	8
9	2004[a]	8	Male	1st dose	None	Yamaguchi	9
10	2000	13	Male	2nd dose	None	Saga	10

[a] The year when the case was reported is described for cases 3, 4, 5, 7, 8, and 9, because the time of VAPP onset was not mentioned in the literature.

Table 2 List of reported VAPP cases in Japan.[2]

No.	OPV – fever (days)	OPV – paralysis (days)	Paralyzed site	Spinal MRI finding	Neuroconduction rate	Sequelae
1	19	23	Right lower leg	No abnormality	No abnormality	Present
2	20	26	Right lower leg	Abnormality on T2-weighted image	Reduced conduction rate	Present
3	15	18	Left lower leg	No abnormality	Reduced action potential	Present
4	18	21	Left lower leg	No description	Reduced action potential	Present
5	17	21	Both lower legs	High signal intensity at the anterior horn of the spinal cord on T2-weighted images	No abnormality	Present
6	Orally taken in October, fever in November (within 1 month)	Nuchal rigidity 5 days after fever	Left lower leg	High signal intensity on T2-weighted images (axial and sagittal view on Day 8 of paralysis)	No description	Present
7	15	21	Both lower legs	Abnormal signal intensity in the anterior horn of spinal cord	No description	Present
8	–	–	Right lower leg	Not performed	Not performed	Present
9	14	18	Both lower legs	No description	Reduced action potential	Present
10	22	22	Right lower leg	High signal intensity at the anterior horn of the spinal cord on T2-weighted images (axial and sagittal views on Day 13 of paralysis)	Reduction in both action potential and conduction rate	Present

Diagnostic imaging by MRI or neurophysiological testing may sometimes be useful for the diagnosis, because both yield characteristic findings. However, positive findings are not always obtained in cases of VAPP. The ability of these methods to yield useful findings diagnostically may depend on the stage or severity of the disease, the timing of the test, the testing technique, and the imaged section in case of MRI.

All of the cases suspected of having VAPP who were analyzed in the present study had some sequelae, such as motor dysfunction, deformed atrophy, etc. The prognosis is poor in cases of paralysis caused by VAPP, just as in the case of paralysis caused by wild strains of poliovirus. In Japan, where an epidemic of wild-type poliovirus infection has not occurred for many years, it is now essential to reduce the incidence of VAPP, which occurs at a low incidence but can cause sequelae, to zero as soon as possible. In this respect, it would be desirable to switch, at the earliest possible, from OPV to IPV for routine vaccination.

VAPP and poliovirus vaccine in Japan

VAPP can be diagnosed on the basis of a history of intake of OPV, the characteristic clinical symptoms, laboratory data, and virological results. To facilitate virologically confirmed diagnosis, it is essential to collect fecal samples soon after the onset of paralysis.

Several factors, including male gender, first OPV intake, immunodeficiency, surgery/tissue damage/frequent intramuscular injections within one month of intake of OPV, have been pointed out as risk factors for the onset of VAPP. In addition to paying close attention to these factors, there is also the necessity to check for the presence of perianal abscess as a risk factor for the onset of VAPP.

However, even when appropriate measures are taken against avoidable risk factors, VAPP can still develop following the intake of OPV. The incidence of VAPP is one out of several million doses of OPV, as suggested by the survey data from Japan and around the world.[11] In Japan, about 1.1 million babies are born annually. According to the current schedule for routine vaccination in Japan, OPV is administered twice. If the incidence of VAPP is calculated from these figures, about one case of VAPP develops inevitably each year, and this estimation is endorsed by the results of our review of previously reported cases. VAPP is a serious adverse reaction which can cause sequelae. If the current situation of Japan is judged from a general viewpoint, it would be desirable, as early as possible, to switch from OPV to IPV. The rationale for this judgment is described below.

In Japan, the last case of paralysis caused by wild-type poliovirus was reported in 1980. Also in periodic virus surveillances, wild-type poliovirus has not been detected since it was last isolated in 1993 (isolated from a 13-year-old boy with acute upper respiratory infection, but no paralysis). Nevertheless, Japan has been continuing to use OPV, resulting in the onset of VAPP in several ten cases over the

Table 3 List of reported VAPP cases in Japan.[3]

No.	WBC	CRP	CRP cerebrospinal fluid cell count (/mm^3)	Cerebrospinal fluid protein (mg/dl)	Immunological test
1	14400	1	15	54	No abnormality in IgG, lymphocyte subset, PHA and ConA blastgenesis
2	10700	0.01	5	17	No description
3	Normal	Normal	19	58	No abnormality in immunoglobulins
4	No description	No description	No description	No description	No description
5	Slight increase	No description	Increase	No description	No abnormality
6	9600	0.04	156	50	No abnormalities in IgG, and lymphocyte subsets
7	No description	No description	No description	No description	No description
8	No description	No description	51	38	No description
9	No description	No description	25	No description	No description
10	10500	0.14	6	30	No abnormality in IgG, and lymphocyte subsets

past three decades, during which period not even one case of wild-type poliovirus infection has been reported to occur. Around the world, there are many countries which cannot afford to use IPV owing to budget considerations. Japan, however, is not one of such countries. The vaccination system in Japan is now said to be many years behind the vaccination systems in other countries. This is a serious problem which can affect adversely the future of children in Japan. Immediate countermeasures are essential to deal with this situation.

OPV is simple to use (via oral route) and is inexpensive. In addition, it enables local immunity of intestine. Because of these features, the use of OPV is expected to bring herd immunity. In practice, only OPV has been successful in controlling the poliomyelitis epidemic in various districts of the world over relatively very short periods of time. To date, it has not been demonstrated whether IPV might also be as powerful. Therefore, while recommending early switching from OPV to IPV for childhood routine vaccination in Japan, we would also like to underline the necessity of keeping an adequate stock of OPV in view of the current situation where eradication of poliovirus appears to be unlikely to be achieved in the very near future.

DPT vaccination with an adjusted dose for younger adults in Japan

Background

It has recently been pointed out that the prevalence of pertussis among adults and older children is increasing in Europe, USA and Japan.[13,14] The clinical symptoms of pertussis in adults and older children are often atypical, making an early precise diagnosis of the disease frequently difficult. If pertussis is transmitted to neonates and infants, in whom this disease follows a severe course, a serious problem may be posed.

Table 4 List of reported VAPP cases in Japan.[4]

No.	Virus in feces	Virus in cerebrospinal fluid
1	Sabin type 3-positive	Virus isolation and PCR negative
2	No description	No description
3	Sabin type 2 and 3-positive	Virus isolation and PCR negative (Enterovirus PCR also negative: Days 8 and 15 after disease onset)
4	No description	No description
5	Sabin-positive (checked at NIID[a])	Negative
6	Sabin-positive (checked at NIID[a])	Negative
7	Sabin type 3-positive	Virus isolation negative
8	Not performed: type 2 serum neutralizing antibody titer elevated	Not performed
9	No description	Enterovirus PCR negative
10	Virus isolation and PCR negative	Virus isolation and PCR negative

[a] NIID: National Institute of Infectious Diseases, Japan.

Table 5 Package insert for oral poliovirus vaccine produced in Japan (6th version, revised in September 2009; excerpts).

Adverse reactions and significant adverse reactions
Onset of flaccid paralysis after vaccination has been reported, although it is very rare. It is difficult to attribute this event definitely to the vaccine alone.
The incidence of VAPP seen among Japanese individuals with a history of immunization with OPV was 1 out of about 4.86 million vaccinations in individuals without immunological abnormalities during the period between 1981 and 2006. The incidence of this event in individuals with a history of contact with the vaccinated individuals is 1 out of about 7.89 million vaccinations
Precautions
As a rule, it is advisable to avoid non-emergency surgery (tooth extraction, tonsillectomy, hernia surgery, etc.) for about one month after the vaccination
Other precautions
In view of the report that the frequent intramuscular injections (of antibiotics, etc.) during the approximately one-month period after vaccination can elevate the incidence of VAPP, it is advisable to avoid intramuscular injections during this period. According to the same report, this kind of paralysis is not attributable to the drug administered intramuscularly, but to the strong stimulus to the intramuscular injection and invasion by the virus in the vaccine of the central nervous system

In several countries, additional booster vaccination using the Tdap vaccine containing the pertussis antigen has been implemented to consolidate immunity around the nation and to control pertussis in older children and adults. In Japan, the Tdap vaccine is not yet approved, and it is expected to take high expenses and a long time until this vaccine is adopted through clinical trials for clinical use. However, the currently used mixed diphtheria/pertussis/tetanus (DPT) vaccine can have a composition similar to that of the Tdap vaccine used in Western countries if the dose of inoculation of this vaccine is adjusted.

The present study[14] was undertaken to evaluate the safety and immunogenicity of the DPT vaccine prepared in Japan in adults, at an adjusted inoculation dose. This study was originally aimed at devising a countermeasure against pertussis in older children and adults. However, since the results of this study also seemed to be useful for discussing vaccination of Japanese adults for whom no recommendation on vaccination after childhood is available (e.g., useful in reinforcing immunization for overseas travelers), the results are presented below.

Subjects and methods

The DPT vaccine "Trivic®" (Lot. 3E07A), manufactured by the Research Foundation for Microbial Diseases of Osaka University (BIKEN), was administered subcutaneously to 164 adults at a single dose of 0.2 ml in the left upper arm, after obtaining their written informed consent. The mean age of the subjects was 27.3 ± 6.0 years (range, 22–50 years). The median age was 24 years. There were 83 males and 81 females.

To ensure safe use of the vaccine, the health condition of each subject was checked using a health observation diary carrying information on systemic symptoms (fever, headache, malaise, etc.) and local reactions (redness, pain, itching sensation, etc.) for a 4-week period after the vaccination.

For evaluation of the immunogenicity, blood samples were collected immediately before and 4 weeks after the vaccination to check for the IgG antibody titers to pertussis toxin (PT) and filamentous hemagglutinin (FHA) using ELISA. Antibody to diphtheria toxin was measured by the colorimetric method and that to tetanus toxin was assayed by KPA. All of these measurements were carried out at the Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University (Kanonji, BIKEN). The tests for anti-PT antibody and anti-FHA antibody were judged as positive if the respective antibody titers were 10 ELISA unit (EU)/ml or more. The test for anti-diphtheria toxin antibody was rated as positive if the titer was 0.1 IU/ml or more. The test for anti-tetanus toxin antibody was rated as positive if the titer was 0.01 IU/ml or more. In the evaluation of the immunogenicity, a judgment of significant elevation of the antibody titer was made in the following instances: (1) if the antibody test rated as negative before the vaccination became positive after the vaccination, and (2) if the antibody test rated as positive before the vaccination was followed by a two-fold or greater rise of the antibody titer after the vaccination.

The history of DPT vaccination in individual subjects was checked on the basis of the records in the Maternal and Child Health Handbook carried by individual subjects. Analysis of the history of DPT vaccination revealed that there were 58 subjects (35%) who had received the vaccination (3 times in 10 subjects and 4 times in 48 subjects), 8 subjects (5%) who had not received the vaccination, and 98 subjects (60%) for whom no clear records were available (loss of the Maternal and Child Health Handbook, etc.).

Results

Analysis of the adverse events following the vaccination revealed the development of systemic symptoms in 10 subjects (6.1%), fever (37.5 °C or more) in 2 subjects (1.2%), headache in 6 subjects (3.6%), and malaise in 4 subjects (2.4%). The duration of fever was 1 day in 1 of the subjects and 2 days in the others, with a maximum body temperature of 37.8 °C (recorded on Day 1) and 37.5 °C (Day 7), respectively. Injection-site reactions were noted in 32 subjects (19.5%), including pain (23 subjects [14.0%]), itching sensation (14 subjects [8.5%]), redness (11 subjects [6.7%]), swelling (10 subjects [6.1%]), and heat sensation (9 subjects [5.5%]). Two subjects (1.2%) developed both redness (5 cm or more) and swelling. None of these adverse events required any particular intervention, and there were no serious adverse reactions.

Table 6 Amount of antigens in several vaccine products.

DPT/DT/Tdap	Pertussis -PT (μg)	Pertussis -FHA (μg)	Diphtheria toxoid (Lf)	Tetanus toxoid (Lf)
Trivic® 0.2 ml (BIKEN, Japan)	9.4	9.4	6 or less	1 or less
DTVic® 0.1 ml (BIKEN, Japan)	–	–	5 or less	1 or less
Adacel® 0.5 ml (Sanofi Pasteur)	2.5	5	2	5
Boostrix® 0.5 ml (GlaxoSmithKline)	8	8	2.5	5

In the evaluation of the pertussis antibody titer prior to the vaccination, the test for anti-PT antibody was positive in 87 subjects (53%) and negative in 76 subjects (47%). When the anti-PT antibody-positive cases were analyzed by age, the positivity rate was the highest in the 20–29 years age group and decreased with advancing age, being 59% in the 20–29 years age group, 43% in the 30–39 years age group, and 22% in the 40–49 years age group. The test for anti-FHA prior to the vaccination was positive in 139 subjects (85%) and negative in 24 subjects (15%). When the anti-FHA antibody-positive cases were analyzed by age, the positivity rate was the highest in the 20–29 years age group and decreased with advancing age, being 88% in the 20–29 years age group, 80% in the 30–39 years age group, and 67% in the 40–49 years age group. These results suggest that the titers of antibodies for host defense against *Bordetella pertussis* infection gradually decrease over time after vaccination.

Also in the 58 subjects for whom clear records of the history of 3 or more vaccinations (with vaccine containing the pertussis antigen) were available, the pre-vaccination test for anti-PT antibody was negative in 21 subjects (36%) and that for anti-FHA antibody was negative in 9 subjects (16%).

In the 76 subjects in whom the test for anti-PT antibody was negative before vaccination, the geometric mean (GMT) of the anti-PT antibody titer was $10^{0.71\pm0.22}$ EU/ml (GMT, 5.13; range, 3.09–8.51) before vaccination and $10^{1.94\pm0.47}$ EU/ml (GMT, 87.10; range, 29.01–257.04) after vaccination. Thus, the test for the antibody became positive after vaccination in all of these subjects, except two. The two subjects in whom the antibody test remained negative had not received any vaccine containing the pertussis antigen during childhood. In the 87 subjects in whom the test for anti-PT antibody was positive before the vaccination, the geometric mean of the anti-PT antibody titer was $10^{1.45\pm0.35}$ EU/ml (GMT, 28.18; range, 12.60–63.10) before the vaccination and $10^{2.36\pm0.52}$ EU/ml (GMT, 229.09; range, 69.18–758.58) after the vaccination. In 77 subjects (89%), the post-vaccination anti-PT antibody titer rose by two-fold or greater as compared with the pre-vaccination titer in terms of the EU.

In the 24 subjects in whom the test for anti-FHA antibody was negative before the vaccination, the geometric mean of the anti-FHA antibody titer was $10^{0.83\pm0.67}$ EU/ml (GMT, 6.76; range, 1.45–31.62) before the vaccination and $10^{2.10\pm0.38}$ EU/ml (GMT, 125.89; range, 52.48–302.00) after the vaccination. Thus, the test for anti-FHA antibody became positive after vaccination in all of these subjects. In the 139 subjects in whom the test for anti-FHA antibody was positive before the vaccination, the geometric mean of the anti-FHA antibody titer was $10^{1.58\pm0.42}$ EU/ml (GMT, 38.02; range, 16.98–100.00) before the vaccination and $10^{2.43\pm0.47}$ EU/ml (GMT, 269.15; range, 91.20–794.33) after the vaccination. In 122 subjects (88%), the post-vaccination titer of anti-FHA antibody rose by two-fold or greater as compared with the pre-vaccination titer in terms of the EU.

Prior to the vaccination, the test for anti-tetanus toxin antibody was positive in more than 90% of the subjects aged below 39 years, but in only 25% of the subjects aged 40 years or older. This is probably because the generation aged 40 years and older had not acquired basic immunity through vaccine containing tetanus toxoid during infancy and childhood under the Japanese routine vaccination program not covering tetanus in those days. Routine vaccination against tetanus for infants in Japan was started only in 1968. Detection of the anti-tetanus antibody in some of these subjects can probably be explained by the DT vaccination received by them at the age of 11 years.

In the analysis of the time-course of changes in the anti-tetanus toxin antibody titers following vaccination in the present study, 94% of the subjects judged to have had a positive antibody test prior to the vaccination showed a two-fold or greater rise in the titer after the vaccination. Of the subjects in whom the antibody test was negative before the vaccination, 2 subjects (aged 39 and 46, respectively) failed to become antibody-positive after the vaccination. The 46-year-old subject belonged to the generation prior to the introduction of the routine anti-tetanus vaccination program and did not give any history of vaccination against tetanus. The 39-year-old subject also had low antibody titers against diphtheria and pertussis and did not give any history of vaccination against these diseases.

The pre-vaccination anti-diphtheria toxin antibody positivity rate tended to be lower than the pre-vaccination anti-tetanus toxin antibody positivity rate. It was about 50% in the subjects aged 30 years and older. Of the subjects in whom this antibody test was positive before the vaccination, 99% showed a two-fold or greater rise in the titer after the vaccination. There were 5 subjects in whom the rise in the antibody titer after the vaccination was inadequate. Of these 5, 2 (aged 20–29) had no history of vaccination and the history of vaccination was indeterminate in the other 3 (aged 35, 46, and 50 years old). Because routine vaccination with diphtheria toxoid in Japan was started only in 1958, individuals aged 50 years and older are unlikely to have received this vaccination.

Discussion

Trivic®, DPT vaccine available in Japan, contains 9.4 μg of PT antibody and 9.4 μg of FHA antigen per 0.2 ml, equivalent to or higher than the amount of these antigens contained in Adacel® (a Tdap vaccine, containing PT 2.5 μg, FHA 5 μg) and Boostrix® (containing PT 8 μg, FHA 8 μg). The amount of diphtheria toxoid and tetanus toxoid contained in 0.2 ml of Trivic® is 6 Lf or less and 1 Lf or less, respectively, larger than the amount of diphtheria toxoid and smaller than the amount

of tetanus toxoid contained in Adacel® (2 Lf, 5 Lf) and Boostrix® (2.5 Lf, 5 Lf). The DT vaccine DT Vic® (a product of Research Foundation for Microbial Diseases of Osaka University (BIKEN)), used for additional booster vaccination against diphtheria and tetanus in individuals aged 10 years and older in Japan, contains diphtheria toxoid and tetanus toxoid in amounts of 5 Lf or less and 1 Lf or less, respectively, per 0.1 ml, equivalent to the amounts contained in 0.2 ml of Trivic® produced by the same manufacturer. These results indicate that use of the antigen composition of Trivic® 0.2 ml was rational for additional booster vaccination of the adults covered by this study (Table 6).

Local reactions to the vaccine seen during this study period did not seem to be as serious as those observed following DTP vaccination in infants or Tdap vaccination overseas. No significant systemic reactions were noted either in this study. Thus, overall, we may say that DPT vaccine (0.2 ml) can be used safely in adults.

The results of the evaluation of immunogenicity through analysis of the time-course of changes of the antibody titers after vaccination in adults suggest that acquisition of basic immunity during infancy improves the effectiveness of additional vaccination against all of pertussis, tetanus, and diphtheria. To protect the health of overseas travelers, both basic immunity acquisition during infancy and additional immunization during adulthood appear to be important.

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Conflict of interest

The author has no conflict of interest to declare.

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The TLR3/TICAM-1 Pathway Is Mandatory for Innate Immune Responses to Poliovirus Infection

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Cytoplasmic and endosomal RNA sensors recognize RNA virus infection and signals to protect host cells by inducing type I IFN. The cytoplasmic RNA sensors, retinoic acid inducible gene I/melanoma differentiation-associated gene 5, actually play pivotal roles in sensing virus replication. IFN-β promoter stimulator-1 (IPS-1) is their common adaptor for IFN-inducing signaling. Toll/IL-1R homology domain-containing adaptor molecule 1 (TICAM-1), also known as TRIF, is the adaptor for TLR3 that recognizes viral dsRNA in the early endosome in dendritic cells and macrophages. Poliovirus (PV) belongs to the Picornaviridae, and melanoma differentiation-associated gene 5 reportedly detects replication of picornaviruses, leading to the induction of type I IFN. In this study, we present evidence that the TLR3/TICAM-1 pathway governs IFN induction and host protection against PV infection. Using human PVR transgenic (PVRtg) mice, as well as IPS-1$^{-/-}$ and TICAM-1$^{-/-}$ mice, we found that TICAM-1 is essential for antiviral responses that suppress PV infection. TICAM-1$^{-/-}$ mice in the PVRtg background became markedly susceptible to PV, and their survival rates were decreased compared with wild-type or IPS-1$^{-/-}$ mice. Similarly, serum and organ IFN levels were markedly reduced in TICAM-1$^{-/-}$/PVRtg mice, particularly in the spleen and spinal cord. The sources of type I IFN were CD8α^{+}/CD11c^{+} splenic dendritic cells and macrophages, where the TICAM-1 pathway was more crucial for PV-derived IFN induction than was the IPS-1 pathway in ex vivo and in vitro analyses. These data indicate that the TLR3/TICAM-1 pathway functions are dominant in host protection and innate immune responses against PV infection. *The Journal of Immunology*, **2011, 187: 5320–5327.**

When RNA viruses infect mammalian cells, type I IFN is generated to suppress viral infection. IFN-inducing pathways evoked by viral dsRNA have been identified in humans and mice, and the possible involvement of these pathways in protection against viruses has been examined using gene-disrupted mice and various virus species (1). The sensing of dsRNA by the innate immune system is accomplished either by TLR3 or by cytoplasmic sensors such as dsRNA-dependent protein kinase (so-called PKR), retinoic acid inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) (2). In virus-infected cells, RIG-I and MDA5 mainly participate in type I IFN induction in conjunction with the adaptor molecule IFN-β promoter stimulator-1 (IPS-1; also known as MAVS, Cardif, or VISA) (1). The role of these molecules in host cell protection has been clearly delineated in RNA virus infection.

Toll/IL-1R homology domain-containing adaptor molecule 1 (TICAM-1; also called TRIF) is the adaptor of TLR3 (3–5). When TLR3 senses dsRNA on the endosomal membrane, it induces type I IFN (6, 7). The adaptor TICAM-1 plays a pivotal role in TLR3-mediated IFN-α/β induction. Once dsRNA stimulates TLR3, TICAM-1 transiently couples with TLR3 and forms a multimer, translocating to a distinct region of the cytoplasm (8). In its multimeric form, TICAM-1 recruits the kinase complex to activate IFN regulatory factors (IRF)-3 and -7, which induce type I IFN production (7, 9). Historically, this IFN-inducing pathway was identified earlier than the cytoplasmic RIG-I/MDA5 pathway (10, 11). Many reports have mentioned the possibility that the TLR3/TICAM-1 pathway is involved in the anti-viral IFN response (12), but no definitive evidence of the anti-viral properties of this pathway has been obtained using TICAM-1$^{-/-}$ mice (13). Only a DNA virus, mouse CMV (MCMV), has been shown to infect TICAM-1$^{-/-}$ mice, and thus mouse cells are partly protected from MCMV by the TICAM-1 pathway (5, 14).

Poliovirus (PV) is a positive strand ssRNA virus that produces dsRNA intermediates during viral replication (15), modified with 5′ terminal Vpg protein (16), a characteristic feature of picornaviruses. It is generally accepted that picornaviruses are recognized by MDA5 but not RIG-I in infected cells, presumably due to the generation of this unusual dsRNA. This concept was confirmed by the finding that MDA5$^{-/-}$ mice fail to induce type I IFN in response to encephalomyocarditis virus (EMCV) and permit severe EMCV infection (13, 17). However, another picornavirus, coxsackie B virus (CBV) serotype 3, is recognized by TLR3 in infected cells and induces IFN-γ as an effector for suppressing CBV infection

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Abbreviations used in this article: BM, bone marrow; BM-DC, bone marrow-derived dendritic cell; BM-Mf, bone marrow-derived macrophage; CBV, coxsackie B virus; DC, dendritic cell; EMCV, encephalomyocarditis virus; HCV, hepatitis C virus; IFIT-1, IFN-induced protein with tetratripeptide repeats 1; IP-10, IFN-γ–induced protein 10; IPS-1, IFN-β promoter stimulator-1; IRF, IFN regulatory factor; KO, knockout; MCMV, mouse cytomegalovirus; MDA5, melanoma differentiation-associated gene 5; MEF, mouse embryonic fibroblast; Mf, macrophage; MOI, multiplicity of infection; PV, poliovirus; PVRtg, poliovirus receptor transgenic; RIG-I, retinoic acid inducible gene I; RT-qPCR, real-time quantitative PCR; TICAM-1, Toll/IL-1R homology domain-containing adaptor molecule 1; WNV, West Nile virus; WT, wild-type.

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(18). In this study, we analyzed in vivo infection of a popular picornavirus, PV, using PVR transgenic (PVRtg) mice, which show a neurotropic phenotype during PV infection similar to humans (19, 20). Using this mouse model, in combination with TICAM-1$^{-/-}$ or IPS-1$^{-/-}$ mice, we present evidence that the host TICAM-1 pathway, particularly in macrophages (Mf), serves as a source of type I IFN induction and protects host PVRtg mice from PV infection and paralytic death. Thus, the strategy for host protection against picornaviruses is not simply based on the MDA5-dependent dsRNA recognition, but is variable depending on picornavirus species.

Materials and Methods

Mice

All mice were backcrossed with C57BL/6 mice more than seven times before use. TICAM-1$^{-/-}$ (21) and IPS-1$^{-/-}$ mice (this study) were generated in our laboratory. TLR3$^{-/-}$ (4), IRF-3$^{-/-}$, and IRF-7$^{-/-}$ mice (22) were provided by Drs. S. Akira (Osaka University, Osaka, Japan) and T. Taniguchi (University of Tokyo, Tokyo, Japan). PVRtg mice were provided as reported previously (20). All mice were maintained under specific pathogen-free conditions in the Animal Facility at Hokkaido University Graduate School of Medicine (Sapporo, Japan). Animal experiments were performed according to the guidelines set by the Animal Safety Center, Japan.

Generation of IPS-1–deficient mice

The *IPS-1* gene was amplified by PCR using genomic DNA extracted from embryonic stem cells. The targeting vector was constructed by replacing the second and third exons with a neomycin-resistance gene cassette (Neo), and an HSV thymidine kinase driven by the PGK promoter was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into 129/Sv mice-derived embryonic stem cells, G418 and ganciclovir doubly resistant colonies were selected and screened by PCR. The targeted cell line was injected into C57BL/6 blastocysts, resulting in the birth of male chimeric mice. These mice were then backcrossed with C57BL/6 mice. The disruption of the *IPS-1* gene was confirmed by PCR for the long and short arms. The abolishment of IPS-1 mRNA expression was confirmed by real-time quantitative PCR (RT-qPCR).

Cells, viruses, and reagents

Wild-type (WT) and TICAM-1$^{-/-}$ mouse embryonic fibroblasts (MEF) were prepared from 12.5- to 13.5-d-old embryos. PV, strain Mahoney, was amplified in Vero cells, and the viral titer was determined by a plaque assay. Bone marrow (BM) cells were prepared from the femur and tibia. The cells were cultured in RPMI 1640 (Invitrogen, New York, NY) supplemented with 10% FCS, 100 μM 2-ME, and 10 ng/ml murine GM-CSF or the culture supernatant of NIH3T3 cells expressing M-CSF. After 6 d, cells were collected and used as bone marrow-derived dendritic cells (BM-DC) or BM-derived macrophages (BM-Mf). For the preparation of BM-DC and BM-Mf, the medium was changed every 2 d. Splenic DC and NK cells were isolated using the MACS system (Miltenyi Biotec, Auburn, CA).

Experimental infection of mice

Five- to 8-wk-old C57BL/6 female mice were used throughout this study. Mice of different genotypes were i.p. or i.v. infected with PV at the doses indicated. The viability of the infected mice was monitored for 2 wk. We collected sera from the mice at different time points to measure viral titers by a plaque assay and cytokine levels by an ELISA. To determine the tissue viral titer, mice were euthanized and organs were aseptically removed and frozen by liquid nitrogen. Because the organs were not perfused before organs were removed, virus titers were determined including blood. Specimens were homogenized in 2 ml PBS on ice, and titers were determined by a plaque assay.

ELISA

Culture supernatants of cells (10^5) seeded on 24-well plates or sera were collected and analyzed for cytokine levels with ELISA. ELISA kits for IFN-α and IFN-β were purchased from PBL Biomedical Laboratories. ELISA was performed according to the manufacturer's instructions.

qPCR

For qPCR, total RNA was extracted with TRIzol (Invitrogen), and 0.2–0.5 μg RNA was reverse-transcribed using a high-capacity cDNA transcription kit (Applied Biosystems, Piscataway, NJ) with random primers according to the manufacturer's instructions. qPCR was performed using a Step One real-time PCR system (Applied Biosystems).

In vivo blocking of NK activity

Mice (PVRtg and PVRtg/TICAM-1$^{-/-}$) were i.p. injected with 250 μg anti-NK1.1 Ab, asialoGM1 Ab, or control PBS as described previously (21). One day later, the mice were i.p. inoculated with 10^4 PFU PV. One to 7 d after PV injection, depletion of peripheral NK1.1$^+$ cells was confirmed by flow cytometry. Then, the mortality of the mice was monitored. In some experiments, the spleen cells were harvested and NK cells (DX5$^+$ cells) were positively isolated using the MACS system (Miltenyi Biotec). The DX5$^+$ NK cells were suspended in RPMI 1640 containing 10% FCS and mixed with ^{51}Cr-labeled B16D8 cells at the indicated E:T ratios. After 4 h, the supernatants were harvested and [^{51}Cr] release was measured.

Statistical analysis

Statistical significance of differences between groups was determined by the Student *t* test, and survival curves were analyzed by the log-rank test using Prism 4 for Macintosh software (GraphPad Software). Student *t* tests and χ^2 goodness-of-fit tests were performed using Microsoft Excel software and a χ^2 distribution table.

Results

TICAM-1 is essential for protection of PVRtg mice against PV infection

Mice lacking the *mda-5* gene abrogate the production of type I IFN in response to EMCV infection and are more susceptible to infection with EMCV (13, 17). Because EMCV is a picornavirus, it has been proposed that MDA5 is critical for sensing picornavirus infection. In infected cells, picornaviruses efficiently generate long dsRNA, which is recognized by the cytoplasmic dsRNA sensor MDA5 (23). The 5′ end of the PV genomic RNA is linked to a VPg protein (16), not to a 5′-triphosphate, a major ligand for another cytoplasmic RNA sensor, RIG-I (24, 25). Thus, we first tested, using the PVRtg mouse model (20), whether the mortality of PV-infected mice is affected by disruption of IPS-1 (Fig. 1*A*). Approximately 70% of WT (PVRtg) mice and ~40% of IPS-1$^{-/-}$ mice survived >10 d postinoculation at an i.p. dose of 2×10^4 PFU. No statistical significance between these two groups was detected (Fig. 1*A*). In the same experiments, TICAM-1$^{-/-}$ mice died within 5 d by paralysis (Fig. 1*B*).

We next investigated the effect of the route of PV infection on mortality in this mouse model. PV (2×10^3 PFU) was injected i.p. or i.v. into WT and TICAM-1 mice and their mortality was examined (Fig. 1*C*, 1*D*). All TICAM-1$^{-/-}$ mice died by paralysis within 7.5 d irrespective of the injection route. The significance of this early mortality rate of PV-infected TICAM-1$^{-/-}$ mice was supported by statistical analysis. The mortality rates were slightly high in WT mice compared with IPS-1$^{-/-}$ mice when PV loads in mice were not very high (Supplemental Fig. 1*A*). This tendency seemingly diminished by early death of IPS-1$^{-/-}$ mice with high doses of PV input. These data suggested that TICAM-1, rather than IPS-1 (or the sensors RIG-I and MDA5), is a critical factor in protecting mice from PV-mediated paralytic death. This conclusion was confirmed using RIG-I$^{-/-}$ and MDA5$^{-/-}$ mice with a PVRtg background (S. Abe, K. Fujii, and S. Koike, submitted for publication).

These results showed a discrepancy with previous indications that MDA5 is critical in picornavirus protection (13). We therefore tested the dose dependence of PV in the survival of WT versus TICAM-1$^{-/-}$ mice. Surprisingly, high doses of PV (2×10^5 and 2×10^6 PFU) induced paralytic death in all WT as well as TICAM-1$^{-/-}$ mice within 6 d (Fig. 2*A*, 2*B*). Thus, high doses of PV (>2×10^5 PFU) appear to overpower the TICAM-1 PV-protective activity in vivo, which confirmed previous findings using other picornaviruses (13). TICAM-1 was most effective in

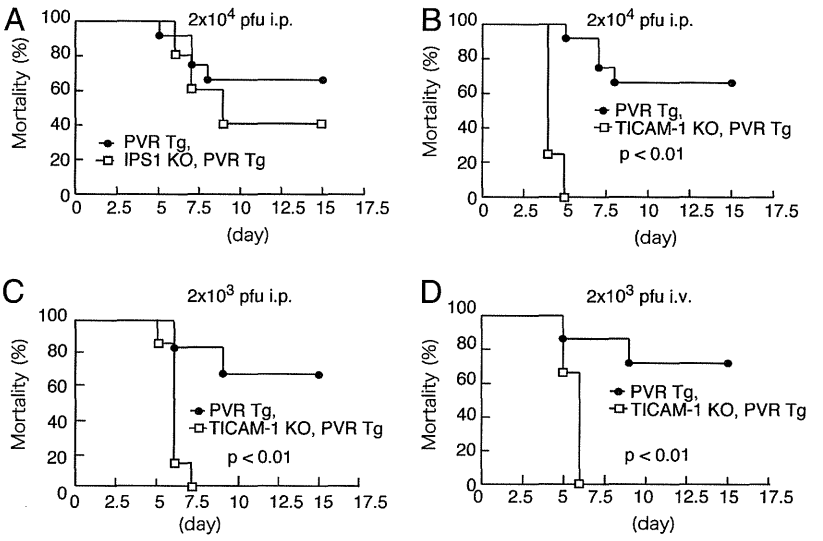

FIGURE 1. Survival of WT, TICAM-1 KO, and IPS-1 KO mice following i.p. or i.v. PV infection. *A* and *B*, PV (2×10^4 PFU) was infected via the i.p. route into WT and IPS-1 (*A*) or TICAM-1 (*B*) KO mice ($n \geq 5$), and survival was monitored for 14 d. *C* and *D*, PV (2×10^3 PFU) was infected via the i.p. (*C*) or i.v. (*D*) route into WT and TICAM-1 KO mice ($n \geq 5$), and survival was monitored for 14 d.

the survival against PV infection at low dose ($<2 \times 10^4$ PFU) (Figs. 1*B*, 1*C*, 2*C*). Similar results were obtained with the PV infection study (S. Abe, K. Fujii, and S. Koike, submitted for publication) when TICAM-1$^{-/-}$ mice were substituted with TLR3$^{-/-}$ or IRF-3/7 double-knockout (KO) mice. Results were confirmed using IRF-3$^{-/-}$ and IRF-7$^{-/-}$ mice (26). These results are essentially consistent with previous reports using a PVRtg/IFNAR$^{-/-}$ mouse model (27), in which type I IFN is critical for PV permissiveness, particularly in the intestine of PVRtg mice.

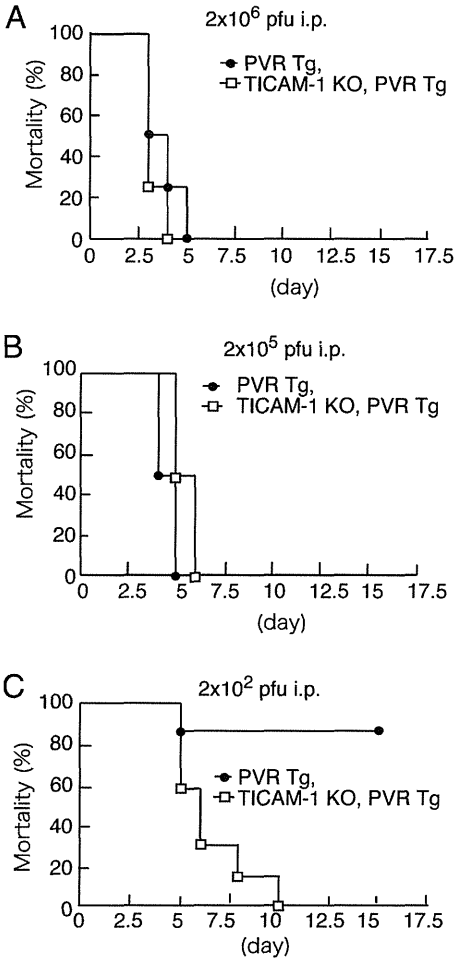

FIGURE 2. High doses of PV disable the protective effect of TICAM-1. WT and TICAM-1 KO mice ($n \geq 6$) were i.p. infected with 2×10^6 (*A*), 2×10^5 (*B*), or 2×10^2 PFU (*C*) PV and survival was monitored for 14 d.

TICAM-1–dependent type I IFN induction in PVRtg mice

PV titers in various organs were measured with WT and TICAM-1$^{-/-}$ mice i.p. injected with 2×10^4 PFU PV. In most organs, PV titers were higher in TICAM-1$^{-/-}$ mice than in WT mice at day 3 post-infection (Fig. 3*A*). The PV titer ratio in TICAM-1$^{-/-}$ versus WT mice was also high in the lung (Fig. 3*A*). In most organs except for the large intestine, high PV titers were harvested in TICAM-1$^{-/-}$ mice compared with WT mice. The difference in local PV titers between WT and TICAM-1$^{-/-}$ mice was culminated in the lung and spinal cord (Fig. 3*A*). Serum PV titers were increased within 48 h

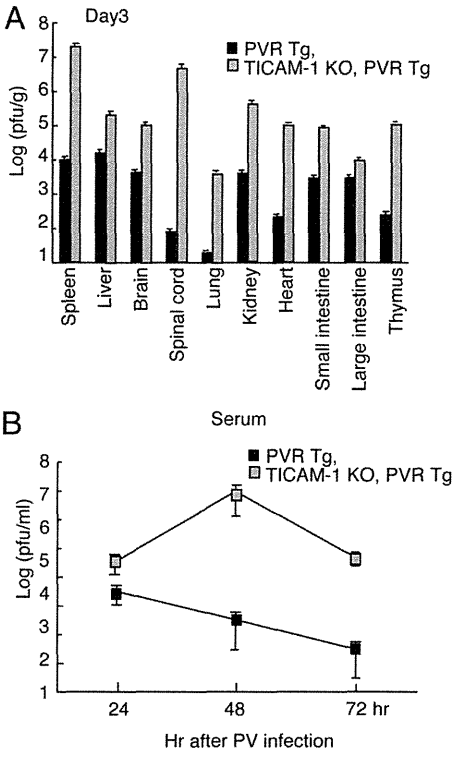

FIGURE 3. Viral titers in organs and serum following PV infection. WT and TICAM-1 KO mice were infected i.p. with 2×10^4 PFU PV. The viral titers in each organ (*A*) and sera (*B*) were measured by a plaque assay. Data are shown as means ± SD of three independent samples.

after PV i.p. injection in TICAM-1$^{-/-}$ mice compared with WT mice (Fig. 3*B*).

IFN-α/β levels were measured with sera from WT, IPS-1$^{-/-}$, and TICAM-1$^{-/-}$ mice, but they were barely detected in these PV-infected mice (Supplemental Fig. 1*B*). Only i.v. injection of high PV titers (an example shows >4 × 10^6 PFU) allowed WT mice to release type I IFN within 12 h (Supplemental Fig. 1*B*). No IFN was detected in blood in TICAM-1$^{-/-}$ and IPS-1$^{-/-}$ mice even in this high-dose setting. However, IFN-α production was reproduced in a cell type level (peritoneal Mf) in vitro (Supplemental Fig. 1*C*). PV infection-mediated cell death (28) and degradation of MDA5 protein (29) may be major causes for this undetectable type I IFN production during in vivo PV infection.

TICAM-1 pathway contributes to IFN-β induction in WT mice with low PV titers

We next determined the mRNA levels of type I IFN in each organ extracted from PV (2 × 10^4 PFU)-infected WT and TICAM-1$^{-/-}$ mice. IFN-β mRNA was upregulated in all of the organs tested in WT mice within 12 h in response to PV injection (i.p.) (Fig. 4*A*). In contrast, only a low increase in IFN-β mRNA was detected in the organs of TICAM-1$^{-/-}$ mice (Fig. 4*A*). IFN-α2 mRNA was upregulated in the organs of TICAM-1$^{-/-}$ and WT mice to similar extents in response to PV injection (2 × 10^4 PFU, i.p.) (Fig. 4*B*). Notable decreases in IFN-α2 mRNA were observed in the TICAM-1$^{-/-}$ spleen and spinal cord compared with WT controls (Fig. 4*B*). The mRNA levels of genes associated with type I IFN induction were evaluated by qPCR, and no unique differences were observed between the splenocytes from PV-injected TICAM-1$^{-/-}$ and IPS-1$^{-/-}$ mice (Supplemental Fig. 1*D*). Hence, type I IFN mRNA is generally upregulated via TICAM-1 in the local organs of PVRtg WT mice during PV infection.

The mRNA levels of IFN-inducible genes and other cytokines were determined in spleen cells after PV infection. IFN-λ and IFN-γ–induced protein 10 (IP-10) mRNA were upregulated in the spleen cells of WT, but not TICAM-1$^{-/-}$ mice, after PV infection (multiplicity of infection [MOI] of 1) (Fig. 4*C*), with profiles similar to that of IFN-β mRNA (Fig. 4*C*). A sensor for 5′-triphosphorylated RNA, IFN-induced protein with tetratripeptide repeats 1 (IFIT-1), was also upregulated through PV infection (Fig. 4*C*). TNF-α, IL-10, IL-12p40, and IFN-γ, which may be associated with infectious cell death, were barely upregulated in spleen cells in response to PV infection (Supplemental Fig. 1*E*).

TICAM-1–dependent type I IFN induction by PV depends on Mf in PVRtg mice

The types of cells that participate in type I IFN induction in the spleen were examined by sorting spleen cells. IFN-β and IFN-α2 were found to be induced in WT CD11c$^+$ DC (Fig. 5*A*), whereas CD11c$^-$ cells barely induced type I IFN. Furthermore, IFN-β and IFN-α2 were barely induced in TICAM-1$^{-/-}$ CD11c$^+$ cells (Fig. 5*B*). Participation of IPS-1 in type I IFN induction in CD11c$^+$ myeloid cells is less compared with that of TICAM-1 (Fig. 5*B*).

Splenic CD8α$^+$CD11c$^+$ and CD4$^+$CD11c$^+$ cells were separated by MACS beads and their response to PV (MOI of 1) was analyzed by determining the mRNA levels of type I IFN (Fig. 5*C*). CD8α$^+$CD11c$^+$ cells, but not the CD4$^+$CD11c$^+$ cells, of WT mice were responsible for type I IFN induction by PV. There was a CD4$^-$CD8α$^-$ population of DC in the spleen and this type of cells did not induce type I IFN in response to PV (Supplemental Fig. 2). The generation of the mRNA of type I IFN and IFIT-1 by PV infection was abrogated in the TICAM-1$^{-/-}$ CD8α$^+$CD11c$^+$ splenic DC (Fig. 5*D*). Also, CD4/8α double-negative DC failed to express type I IFNs (Supplemental Fig. 2). Thus, CD8α$^+$CD11c$^+$ DC, which reportedly express TLR3 (30), are the source of type I IFN in PV-infected PVRtg mice.

We finally confirmed that type I IFN is locally induced in TLR3$^+$ myeloid cells during PV infection. BM-Mf and BM-DC were prepared from mouse BM and challenged with PV (MOI of 1). These cells express TLR3 in the endosome as previously reported about mouse BM-DC (30) and human monocyte-derived DC (31). BM-Mf showed similar profiles of type I IFN mRNA to those of PV-infected splenocytes (Figs. 4*C*, 6*A*). However, IFN-λ and IP-10 mRNA were not detectable in PV-infected BM-Mf, the reason for which remains unclear (Fig. 6*A*). IL-12p40, a representative TICAM-1–dependent gene, was transiently upregulated

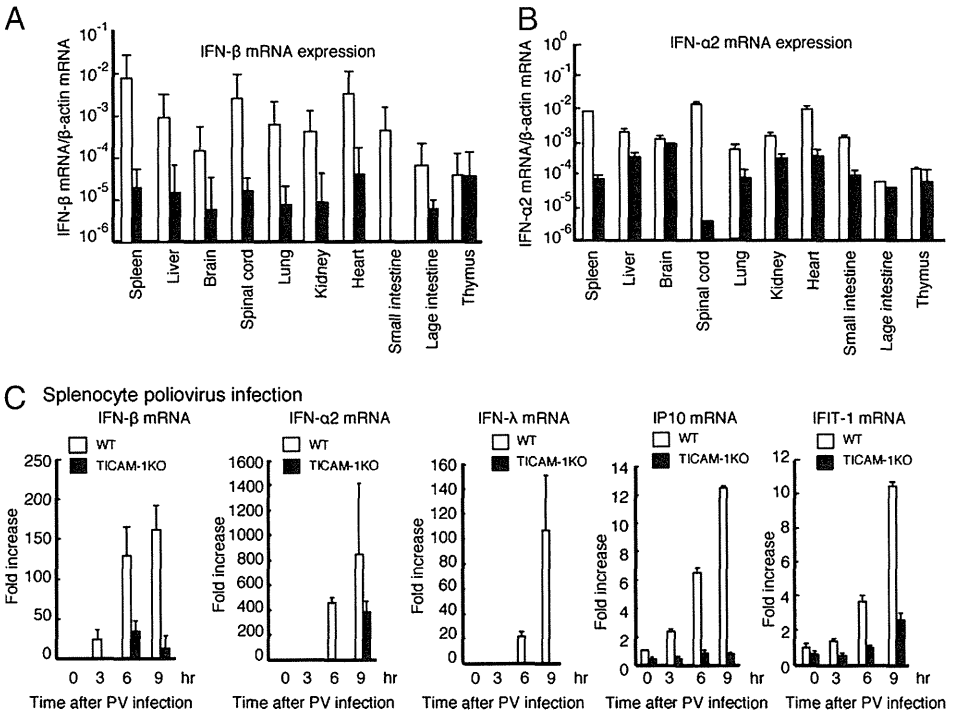

FIGURE 4. The expression of type I IFN following PV infection. *A* and *B*, WT and TICAM-1 KO mice were infected i.p. with 2 × 10^4 PFU PV. Three days postinfection, the mRNA expression levels of IFN-β (*A*) and IFN-α (*B*, *C*) were determined by RT-qPCR. *C*, Splenocytes (5 × 10^5) were infected with PV (MOI of 1) and the mRNA expression levels of IFN-β, IFN-α2, IFN-λ, IP-10, and IFIT-1 were measured by RT-qPCR. Data are shown as means ± SD and are representative of three independent experiments.

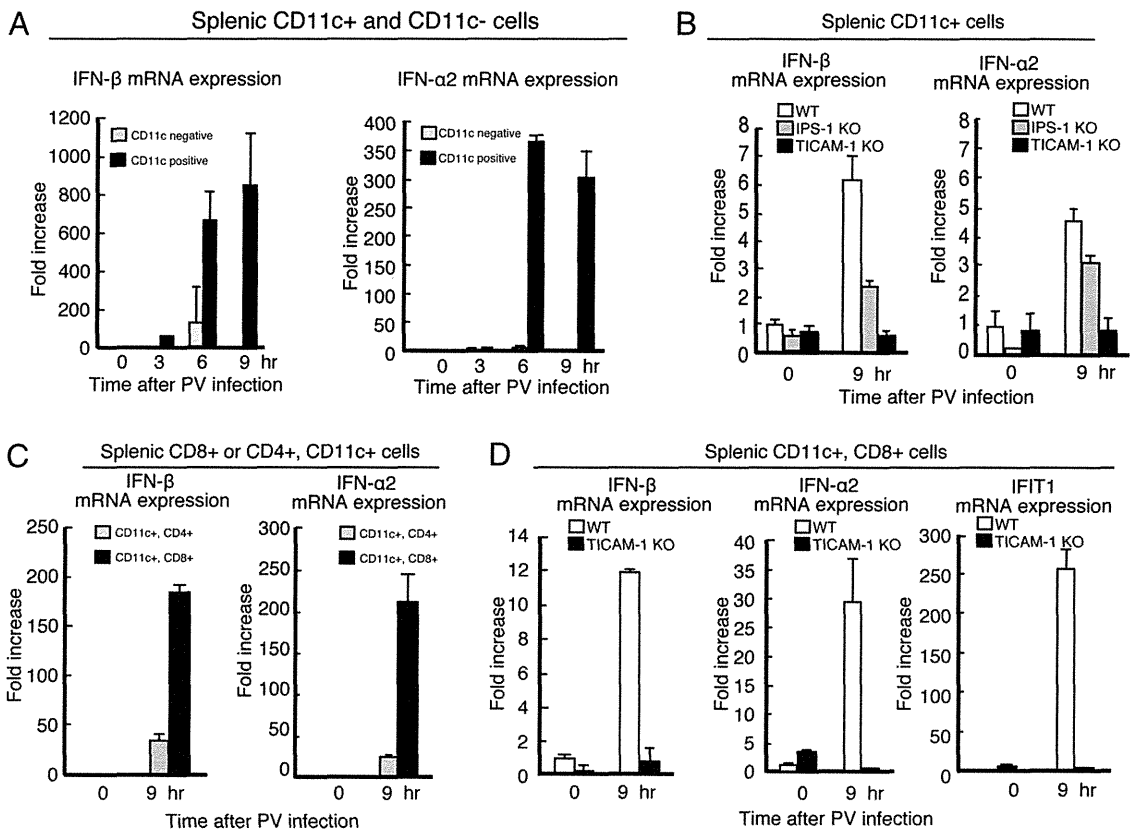

FIGURE 5. The expression of type I IFN in splenic DC. *A*, were isolated from WT spleens using the MACS system. $CD11c^+$ or $CD11c^-$ cells (5×10^5) were infected with PV (MOI of 1), and the mRNA expression of type I IFNs was measured by RT-qPCR. *B*, WT, TICAM-1, and IPS-1 knockout splenic $CD11c^+$ cells were infected with PV, and the expression of type I IFNs was measured by RT-qPCR. *C*, $CD8\alpha^+CD11c^+$ cells and $CD4^+CD11c^+$ cells were isolated from WT spleens and infected with PV (MOI of 1). The expression of type I IFNs was measured by RT-qPCR. *D*, $CD8\alpha^+CD11c^+$ splenic cells were isolated from WT and TICAM-1 KO mice and infected with PV (MOI of 1). The expressions of type I IFNs and IFIT-1 were measured by RT-qPCR. Data are shown as means ± SD and are representative of three independent experiments.

in BM-Mf ~4 h after PV infection (Supplemental Fig. 3). Similarly, but less prominently, the profiles of type I IFN and IL-12p40 were observed in BM-DC (Fig. 6*A*, Supplemental Fig. 3) and $CD11c^+CD8^+$ splenic DC (Fig. 5*D*). Therefore, taken together, these results indicate that IL-12 and IFN-α/β are only minimally upregulated in splenic DC in a PV-dependent manner.

The production of IFN-α was determined by ELISA in the supernatant of PV-infected BM-Mf and BM-DC (Fig. 6*C*). BM-Mf prepared from WT mice generated higher amounts of IFN-α than did those from TICAM-$1^{-/-}$ mice. Although similar results were obtained with BM-DC, the effect of TICAM-1 depletion was not statistically significant (Fig. 6*C*).

NK cells and MEF do not play major roles in protection against PV infection

Using NK1.1-depleted mice, we tested the possible participation of NK cells in the protection of PVRtg mice from PV infection (Fig. 7). $NK1.1^+$ cells were depleted from mouse blood 1 d after injection (i.p.) of NK1.1 Ab into WT (Fig. 7*A*) and TICAM-$1^{-/-}$ mice. After PV challenge, WT mice inoculated with control saline and NK1.1 Ab survived similarly, whereas TICAM-$1^{-/-}$ mice were all killed by PV within 7.5 d irrespective of NK1.1 pretreatment (Fig. 7*B*). Hence, NK cell activation does not affect PV-derived death. The lack of TICAM-1 was also found to have no effect on the NK cell-mediated rescue of PV-infected mice.

Mouse fibroblasts are known to be a potential source of type I IFN (13). We therefore checked whether MEF induce type I IFN and protection against PV (Supplemental Fig. 4). MEF from WT PVRtg mice were susceptible to PV, with cell death being observed at an MOI of 1. MEF from TICAM-$1^{-/-}$ PVRtg mice were 1 log more susceptible to PV, with cell death occurring at an MOI of 0.1 (Supplemental Fig. 4*A*). IFN-β was upregulated in PV-infected MEF to only a slightly higher level in PVRtg MEF than in TICAM-$1^{-/-}$ PVRtg MEF (Supplemental Fig. 4*B*). These results suggested that the large difference in the PV survival rate between WT and TICAM-$1^{-/-}$ mice is not caused by NK cells or type I IFN induction by fibroblasts. The TICAM-1 pathway plays a key role for producing IFN-α/β in Mf/DC, but not in fibroblasts, during PV infection in PVRtg mice.

Discussion

In this study, we demonstrated that PV infection is exacerbated in TICAM-$1^{-/-}$ PVRtg mice. There are a number of RNA-sensing molecules that serve as anti-virus agents and function in a cell type-specific manner. Based on trials using gene-disrupted mice and human viruses, RIG-I has been reported to be essential for sensing infection by rhabdoviruses, influenza viruses, paramyxoviruses, and flaviviruses, whereas MDA5 is important for sensing picornavirus infection (13, 33). In previous studies on picornaviruses, however, only EMCV and several species of picornaviruses have been employed for the KO mice analyses (13). The essential role of type I IFN in PV tropism has been well characterized in PVRtg mice (34). To our knowledge, this study is the first to investigate the sensor that detects PV infection in PVRtg PV-sensitive mice. Because RIG-I and MDA5 use the adaptor IPS-1, we constructed an IPS-$1^{-/-}$ mouse strain for this

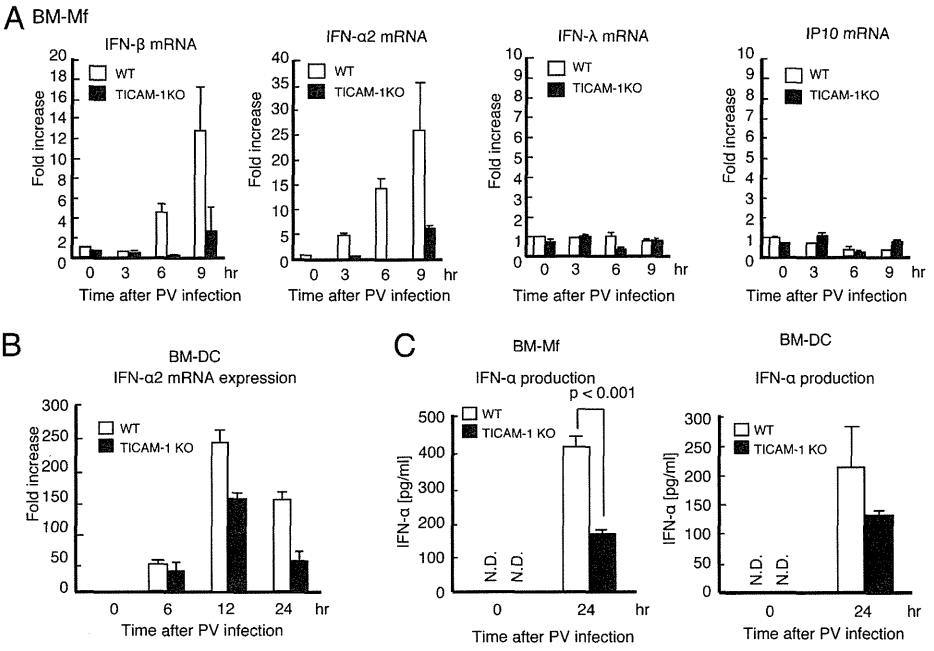

FIGURE 6. Production of type I IFN from BM-Mf and BM-DC. BM-Mf (*A*) and BM-DC (*B*) were prepared from BM cells with M-CSF and GM-CSF, respectively (32). The cells were infected with PV (MOI of 1), and the expression levels of IFN-β, IFN-α2, IFN-λ, and IP-10 were determined by RT-qPCR. *C*, IFN-α produced by PV-infected BM-Mf and BM-DC was measured by ELISA. BM-Mf and BM-DC were prepared from BM cells of WT and TICAM-1$^{-/-}$ mice as in *A* and *B*. Data are shown as means ± SD and are representative of three independent experiments.

study. Unexpectedly, however, IPS-1 was dispensable for protection against PV infection in vivo. This study, taken together with other reports (33, 35, 36), suggests that each virus species has its own strategy to evade host immune attack. This is true even in picornavirus subspecies. Although the IPS-1 pathway involving RIG-I and MDA5 is important for sensing and preventing cytoplasmic virus replication, other steps also participate in critical regulation of virus replication. PV infection is the case where MDA5 is not absolutely critical, but TICAM-1 is essential, for virus protection.

The TICAM-1 pathway participates in driving NK/CTL activation in DC/Mf (21, 37). This pathway is involved in type I IFN induction, as in the IPS-1 pathway, but cells expressing TLR3 are limited. The TLR3 distribution profile by flow cytometry confirms its expression in myeloid cells in mice (30). The TICAM-1 pathway converges with the IPS-1 pathway via the molecular complex of IRF-3–activating kinases (38), and therefore activation of the TICAM-1 pathway induces type I IFN and other IFN-inducible genes (39). Nevertheless, gene induction profiles differ between the TICAM-1 and IPS-1 pathways (40), which may explain the functional distinction between the sensor that is triggered in the virus-infected cells (MDA5/IPS-1) and the sensor that is required for DC/Mf to mount immune responses. Studying these gene functions will be an important issue for functional discrimination between the intrinsic versus extrinsic sensors.

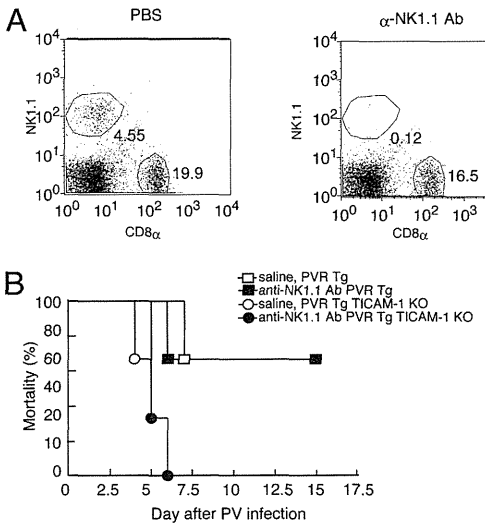

FIGURE 7. Effect of NK cells on mortality of PV-infected TICAM-1$^{-/-}$ PVRtg mice. *A*, To block the NK cell activity in mice, NK1.1 Ab or PBS (control) was i.p. injected into WT mice ($n \geq 6$). After 24 h, spleen cells were isolated from the mice and the fraction of NK1.1$^+$ cells was measured by FACS analysis. *B*, NK1.1 Ab or PBS was i.p. injected into WT and TICAM-1 KO mice. After 24 h, the mice were infected i.p. with PV, and survival was monitored for 15 d.

RIG-1/MDA5 are distributed over almost all organs, including Mf/DC. An interesting point concerns what the function is of the IPS-1 pathway in Mf/DC. Without conditional KO mice, we have an experimental limit to discriminate between their intrinsic function that is triggered in PV-infected cells and the extrinsic function leading Mf/DC to driving the innate immune response. Because the TLR3/TICAM-1 pathway is conserved in Mf/DC, the CNS, fibroblasts, and epithelical cells, it is reasonable that their functions are rather specified in Mf/DC and the neuronal system in PV infection.

However, except several examples such as rhabdovirus (41) and hepatitis C virus (HCV) (32), no definitive evidence has been reported supporting the role of TLR3/TICAM-1 in anti-RNA virus function using KO mice, unlike IPS-1 (35, 36). In previous studies, we used RNA viruses and their mouse models of measles virus, respiratory syncitial virus, vesicular stomatitis virus, influenza virus, and rotavirus infection (12), but we were unable to demonstrate solid antiviral function of the TLR3/TICAM-1 pathway in these models (12). Accordingly, which type I IFN, IFN-inducible gene, NK cell, or CTL is an effector for antagonizing viral replication still remains uncharacterized. To our knowledge, the results of our present study first demonstrated that the TLR3/TICAM-1 pathway is indispensable for induction of the type I IFN effector, but not NK cell activation, which is a critical event in the elimination of virus-infected cells and host protection against PV. IL-12 and IFN-γ are not upregulated in splenic DC in a PV-dependent manner. Furthermore, CTL are unlikely to be involved in our present model, since they would not function within the time scale of several days after initial infection (42).

How PV circumvents host-inducible type I IFN is an intriguing point. Three lines of evidence have supported the presence of unique mechanisms by which PV infection abrogates MDA5-mediated type I IFN production by infected cells and accelerates TLR3-mediated DC maturation through phagocytosis of PV-infected cell debris. First, proteases encoded in the PV genome process the PV polyprotein to produce functional viral proteins (43). PV 2A and 3C proteases also contribute to the degradation of eIF4G (44) and TATA-binding protein (45), respectively, the cleavage of which induces the translational and transcriptional "shutoff" of host protein synthesis (28). Thus, blocking the synthesis of host cell proteins by PV involves stopping IFN production. Second, MDA5 is degraded in PV-infected cells in a proteasome- and caspase-dependent manner, resulting in the lack of type I IFN production (29). Third, PV-mediated apoptosis occurs in a caspase-dependent manner to disable infected cells from inducing an IFN response (46), with the MDA5-dependent innate response to PV infection becoming minimal within 3 h postinfection. Additionally, RIG-I is also cleaved by the viral protease 3C (47), and additional RIG-I functions are subsequently disrupted. Hence, the RIG-I/MDA5 functional time frames should be narrow and ineffective in PV-infected cells.

The hijacked cells release virions and die irrespective of blocking of the IPS-1 pathway. These infected cells are degrading into apoptotic debris containing virus dsRNA when RIG-I/MDA5 is ineffective at inducing IFN (48). Phagocytic internalization of this infected debris containing viral dsRNA into endosomes in Mf/DC is a critical event for TLR3 stimulation (37). If this is the case in PV-infected PVRtg mice, dsRNA-containing debris produced by apoptosis of PV-infected cells may play a major role in the activation of the TICAM-1 pathway in myeloid cells, as is the case for another positive-stranded RNA virus, HCV (32). In HCV studies, dead cells act as carriers of viral dsRNA to the endosomes of DC (32). HCV induces cellular immunity including NK activation driven by the DC TICAM-1 pathway. PV, however, barely induces NK cell activation.

The results of the present study were obtained using the PVRtg mouse model for human PV infection. Possible limitations of this model may include the fact that PV natural infection in humans occurs postinfection of the intestine by a low dose of PV and the PV mouse model is unable to reproduce this infectious route (27). The difference in PV infection between human and the PVRtg mouse might reflect the difference of the IFN-inducing system in humans and mice. However, the response to neurovirulence and death by PV infection occurs similarly in mice and humans. PVRtg mice are susceptible to neuronal infection and the IFNAR$^{-/-}$ phenotype further enhances systemic PV infection (27, 34). The G (Sabin vaccine) and A forms (WT) of PV, which harbor G or A residues in their stem-loop V structures, respectively, show different levels of toxicity or neurovirulence (49). The lower toxicity of the vaccine strain is due to suppression of PTB-mediated protein synthesis in the G form. These results are essentially reproducible in the PVRtg mouse model (50). Our findings further indicate the essential role of the TICAM-1 pathway in the PVRtg model system for the PV-mediated induction of type I IFN in vivo. How this finding is associated with PV-mediated paralytic death and aberrance in the neuronal system is an open question for further understanding the PV neurovirulence and host defense.

In studies on virus infection in neurons, there was no difference between TLR3$^{-/-}$ and WT mice in the brain of reovirus infection (51). TLR3$^{-/-}$ mice have less severe neuroinvasiveness and survive longer than do WT mice in rabies virus infection (41). Further extensive studies have been performed with West Nile virus (WNV). TLR3$^{-/-}$ or TICAM-1$^{-/-}$ mice became more resistant to WNV infection than did WT mice (52). Compared to these earlier results, a recent report showed that lack of TLR3 enhances WNV mortality and increases viral burden in the brain (53). TNF-α and IL-6 are induced for inflammation, and high IL-10 production causes an increase of mortality in WNV-infected mice (54). TICAM-1 signaling is undoubtedly involved in the modulation of these cytokine productions and WNV replication in the nervous system (53, 54). In patients with herpes simplex encephalitis, functional deficiency of TLR3 or TICAM-1 is a critical factor for disease progression (55). The TLR3 responses in the CNS may differ from those in the immune system we examined (54, 56). How PV infection modulates IFN/cytokine-inducing signaling in the nervous system is an interesting issue. The possibility remains that cytokines, such as TNF-α, IL-10, IL-12p40, and IFN-γ, might be associated with the removal of infectious cells as in other virus infections, and the antiviral function of TLR3 ligands in PV-infected mice requires further elucidation.

A picornavirus CBV activates the TLR3/TICAM-1–IFN-γ axis in host-infected cells to induce type II IFN (18). It is possible that CBV promotes TLR3-dependent IFN-γ induction in lymphocytes rather than the type I IFN-inducing pathway. In the model of PV infection, however, the TICAM-1 pathway does not contribute to type II IFN induction. These findings indicate that picornaviruses, that is, EMCV, CBV and PV, have independently evolved to adapt to the host innate immune system and cope with the IFN-inducing system. If this is the case, host responses against picornaviruses may not be unimodally raised by MDA5 but may provide differentially adapted strategies. EMCV tropism reported previously (13) is clearly distinct from those of other picornaviruses. In this article, we present evidence that PV infection is protected by the TICAM-1 pathway that extrinsically induces type I IFN. Virus-produced dsRNA may differentially act on host cells depending on each virus species and accomplish circumvention from host innate sensing systems, maintaining virus tropism.

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Disclosures

The authors have no financial conflicts of interest.

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Endemic Transmission of Echovirus 30 in Toyama, Japan in 2010 Is Verified by Environmental Surveillance

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Although human enteroviruses are the most commonly identified causes of aseptic meningitis in infants and children and are known to circulate worldwide, infection with these viruses is mostly asymptomatic.

Echovirus 30 (E30) was a major causative agent of aseptic meningitis in Japan in 2007–2008 (1), although in 2010 relatively few E30 isolates were reported in Japan (1). In contrast, outbreaks of aseptic meningitis caused by E30 were reported in European countries such as Latvia and Serbia (2,3). In Toyama, E30 was isolated from 2 children aged 1 and 4 years with gastroenteritis and upper respiratory illness diagnosed in pediatric clinics in April and July 2010, respectively. E30 was isolated from fecal specimens and a throat swab, respectively, using RD-18S cells and identified by a neutralization test with rabbit antisera against enteroviruses (25U; Denka-Seiken, Tokyo, Japan and National Institute of Infectious Diseases of Japan). We reported previously that environmental surveillance is a sensitive method for detecting silently circulating viruses in the community as environmental water, such as raw sewage, could contain enteric viruses shed by infected individuals (4–7). During the same survey period from September 2007 to August 2010, 89 E30 isolates were obtained from raw sewage samples in Toyama.

To identify the origin and lineage of the E30 isolates obtained from the above-mentioned patients, nucleotide sequences in a partial VP1 region (746 bases) of the virus were determined and phylogenetically compared with those of randomly selected E30 isolates (21 of 89 strains) obtained from raw sewage (environmental isolates) in Toyama between 2007 and 2010, and with E30 strains available in GenBank (8–15). Viral RNA was extracted from 140 μL of the culture fluid of cells that appeared to be cytopathic using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized for 15 min at 37°C using an RT reagent kit (Takara, Otsu, Japan) with a random hexamer, according to the manufacturer's procedures, then submitted to polymerase chain reaction (PCR). PCR was performed using the primers 187 (sense; 5′-ACI GCI GYI GAR ACI GGN CA-3′) and 011 (antisense; 5′-GCI CCI GAY TGI TGI CCR AA-3′), which amplify an 809-bp DNA fragment corresponding to nucleotides 2553–3361 of E30, Bastianni (GenBank accession no. AF311938), as described by Oberste et al. (16). The PCR products were used directly for sequence analysis using an ABI Prism BigDye Terminator v3.1 cycle sequencing kit and an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, Calif., USA). The genetic relationship between the isolates and reference strains was analyzed using the MEGA 3.1 software (17) for the partial VP1 regions (746 bases of nucleotides 2573–3318).

Figure 1 shows the phylogenetic relationships of the partial VP1 region for the E30 isolates. The clinical isolates obtained in Toyama in 2010 were found to be closely related to the environmental isolates obtained in the period January 2008 to August 2010. The identities in the nucleotide sequences of the clinical isolates compared with the environmental isolates in 2008 and 2009–2010 were 96.4–97.2% and 98.0–99.6%, respectively. The nucleotide sequences of these isolates were also similar to those of the E30 strains isolated in Kobe, Japan, Korea, and Malaysia (93–96%) in 2003–2004. On the other hand, the similarity with the nucleotide sequences for the strains isolated in the United Kingdom in 2007–2008, Korea in 2008, and Russia in 2008–2009 was low (approximately 80%). The E30 isolates obtained in Toyama in 2010 were therefore not related to those from outbreaks in Europe. The clinical isolates obtained in Toyama in 2010 were genetically similar to environmental isolates obtained in the period 2008–2010 and the strains isolated in Kobe in 2003, thus suggesting that they were derived from viruses that had been circulating among individuals in Japan for several years. As enteric viruses from raw sewage are thought to reflect mostly asymptomatic infection in communities (4–6), E30 may have been transmitted asymptomatically in Toyama since 2008.

The nucleotide sequences determined in this study were deposited in GenBank under accession no. AB600148 to AB600170.

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Conflict of interest None to declare.

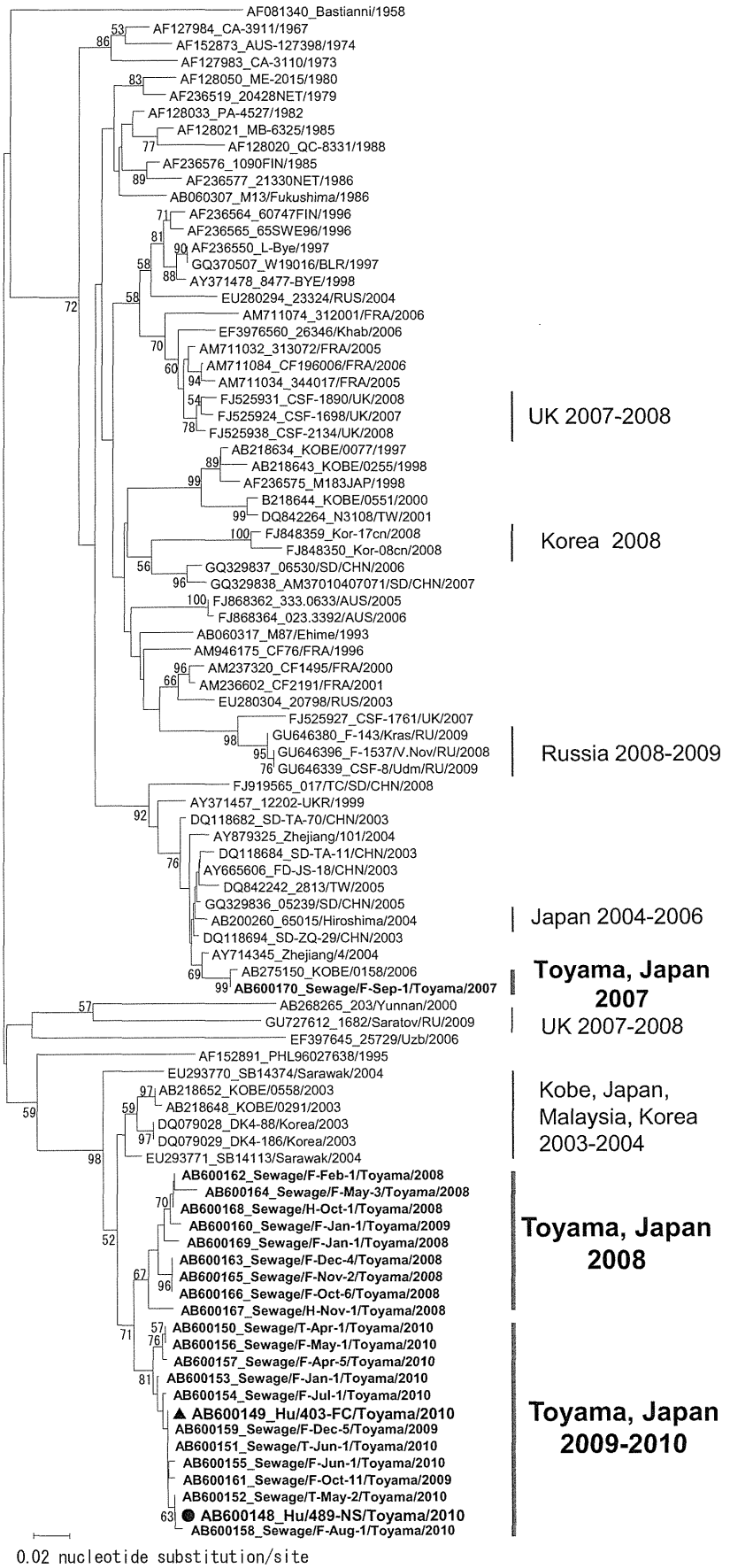

Fig. 1. Phylogenetic relationships among E30 isolates. Phylogenetic tree of E30 using the partial VP1 region (746 bases) was generated by the neighbor-joining method. Bold, triangle (▲), and circle (●) specify environmental and clinical isolates with gastroenteritis and upper respiratory illness, respectively. The strains are presented as accession number_strain name/year. Bootstrap values (in percentages) for 1,000 replicated trees are indicated.

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NOTES

Human Parechovirus Infection in Children Hospitalized with Acute Gastroenteritis in Sri Lanka[▽]

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Of 362 fecal specimens collected from infants and children hospitalized with acute gastroenteritis in Sri Lanka from September 2005 to August 2006, 30 (8.3%) were positive for human parechovirus (HPeV). Six different HPeV genotypes, including HPeV1, -3, -4, -5, -10, and -11, were identified, of these, HPeV11 was reported for the first time.

Human parechoviruses (HPeVs) are classified in the *Parechovirus* genus of the large family *Picornaviridae*, which is a highly diverse family of important pathogens of humans and animals (19). The previous findings reveal the genetic variability of HPeVs, and the number of newly identified HPeV genotypes has been on the increase. Based on VP1 sequence comparisons, the HPeVs have been divided into 14 genotypes (HPeV1 to -14) (http://www.picornaviridae.com/parechovirus/hpev/hpev.htm). Of these, 10 HPeV genotypes (HPeV1 to -8, -10, and -14) have been published to date (1, 3–8, 10–14, 20).

Little is known about the spectrum of viral agents causing acute gastroenteritis in Sri Lanka, except for recent studies mainly focused on rotavirus infection among infants and children less than 5 years old (2, 15). The present study aimed to screen stool samples collected from Sri Lankan children hospitalized with acute gastroenteritis due to infection with HPeV, one of the less-explored viral pathogens that has been reported to be associated with diarrhea recently, and to characterize the molecular properties of the HPeV strains detected.

Three hundred sixty-two fecal specimens collected from infants and children hospitalized with acute gastroenteritis in Kandy, Sri Lanka, from September 2005 to August 2006 were screened for HPeV. First, the viral genome was extracted from 140 μl of a 10% fecal suspension by using the QIAamp viral RNA Minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription (RT)-PCR and sequence analysis were then performed as described in our earlier report (18). All fecal samples which were positive for HPeV were tested further for common viral pathogens that cause diarrhea, including rotavirus, adenovirus, norovirus, sapovirus, and astrovirus, by RT-PCR (22, 23) to see if there were coinfections with HPeV and these viruses.

Of the 362 samples tested, 30 were positive for HPeV and the HPeV detection rate was 8.3%. HPeV was detected nearly year round but not in May, with the peak incidence in October and November (data not shown). Among the 30 positive samples, 20 were coinfected with other viruses, such as rotavirus (14 samples, 46.7%), adenovirus (3 samples, 10%), norovirus GII (2 samples, 6.7%), and norovirus GII and adenovirus (1 sample, 3.3%), and 10 (33.3%) were infected with HPeV alone.

For genotyping, the VP1 region of 27 of 30 HPeV-positive samples was successfully amplified and sequenced. Phylogenetic analysis of the VP1 segments of reference HPeV strains and the strains studied showed that the strains studied could be identified as HPeV1 (11 strains), HPeV3 (1 strain), HPeV4 (5 strains), HPeV5 (3 strains), or HPeV10 (5 strains). Notably, the two remaining strains (LK-73 and LK-223) showed VP1 sequences that clustered together with none of the 10 known HPeV genotypes (HPeV1 to -8, -10, and -14) (Fig. 1).

Identical matrix analysis of the VP1 nucleotide sequences of these two strains and global reference strains of the 10 known genotypes available in the GenBank databases was then performed. The results showed that the highest mean nucleotide and amino acid similarities of these two study strains with HPeV10 were only 64.7 and 73.6%, respectively, while their lowest mean nucleotide and amino acid similarities with HPeV6 were 54.8 and 68.1% (Table 1). Therefore, these strains were expected to be classified into a new or previously unpublished HPeV genotype (HPeV9 and -11 to -13) on the basis of previous proposed criteria for the assignment of HPeV genotypes (16).

The VP1 sequences of the two strains studied were submitted to the International Committee on Taxonomy of Viruses

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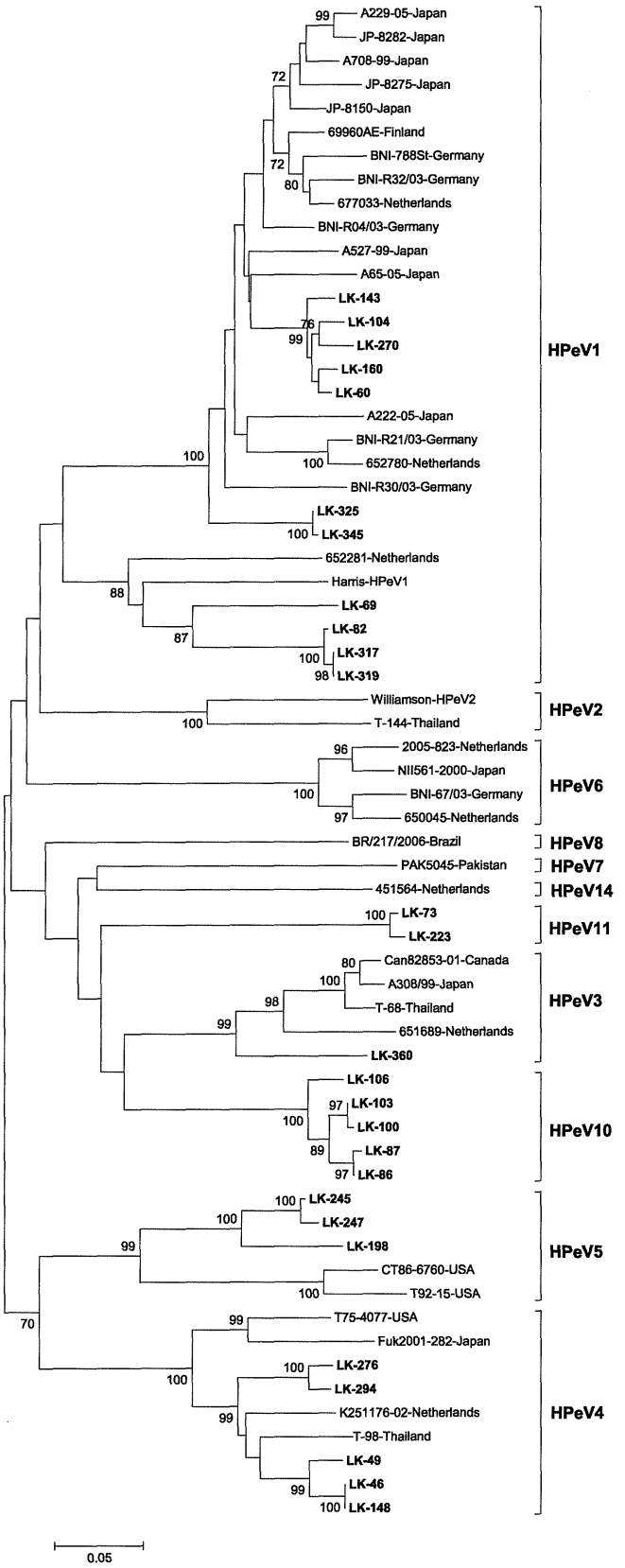

FIG. 1. Phylogenetic tree constructed from nucleotide sequences of the VP1 segments of the strains studied and reference HPeV strains with 500 bootstrap repetitions. Bootstrap values of >70% are shown at the branch nodes. The HPeV strains studied are in boldface type.

TABLE 1. Mean nucleotide similarities between HPeV genotypes

Genotype	Mean % nucleotide identity										
	1	2	3	4	5	6	7	8	10	**11**	14
1											
2	61.9										
3	54.6	55.3									
4	61.1	61.9	56.7								
5	58.4	57.9	48.9	62.4							
6	61.8	57.3	57.8	58.4	56.7						
7	53.8	55.9	60.8	53.5	51.4	49.9					
8	58.0	57.6	58.2	58.1	53.2	55.1	58.9				
10	56.9	51.5	67.6	56.8	49.2	51.3	63.7	60.5			
11	**57.3**	**58.6**	**62.7**	**61.3**	**57.4**	**54.8**	**63.4**	**63.4**	**64.7**		
14	56.8	53.9	63.3	55.0	51.8	52.4	63.4	59.6	67.0	59.9	

Picornavirus Study Group in order to identify their genotype (http://www.picornastudygroup.com/types/index.html). These two strains received the designation HPeV11, with nucleotide and amino acid sequence identities to the prototype HPeV11 strain of 90.5 and 99.1% (strain LK-73) and 90 and 98.6% (strain LK-223) (unpublished data).

The medical records of the 10 patients with samples positive for HPeV alone were reviewed. All 10 patients were children 2 to 26 months old. Of these, five patients (50%) were less than 12 months of age. Besides diarrhea, fever and vomiting were found in 30 and 40% of the patients, respectively. Respiratory tract symptoms such as wheezing, coughing, and coryza were present in 30% of the patients. According to the WHO guidelines for assessing dehydration, the majority of the patients (9 [90%] out of 10) suffered from dehydration and 1 of these patients experienced severe dehydration (21). Ten episodes of diarrhea per day along with vomiting were noted in both cases of HPeV11 infection.

This study is the first to evaluate HPeV infection among hospitalized infants and children with acute gastroenteritis in Sri Lanka. The percentage of HPeV-positive specimens (8.3%) was similar to that found in other studies of HPeV infection (4, 5). In addition, coinfections with HPeV and various types of common diarrheal viruses, such as rotavirus, norovirus, and adenovirus, were noted for the first time. The rate of coinfection with HPeV and these diarrheal viruses accounted for as many as 66.7% (20/30) of the HPeV-positive cases.

In this study, six different HPeV genotypes, HPeV1, -3, -4, -5, -10, and -11, were present among Sri Lankan infants and children with acute gastroenteritis. Obviously, the circulation of various HPeV genotypes was noted among Sri Lankan infants and children. Regarding HPeV1 infection, the finding was in good agreement with previous studies which reported that HPeV1 was predominant over other HPeVs found in patients with acute gastroenteritis (4, 5, 7, 11, 17, 18).

The alignment of deduced amino acid sequences of the strains studied and global HPeV reference strains of HPeV1 to -8, -10, and -14 revealed that the arginine-glycine-aspartic acid (RGD) motif, which is considered to be critical for HPeV1 entry (9), was not present in the two HPeV11 strains studied (data not shown). The lack of the RGD motif in HPeV11 may mean that HPeV11 has an RGD-independent entry pathway.

In conclusion, this is the first report of the circulation of HPeV in infants and children with acute gastroenteritis in Sri