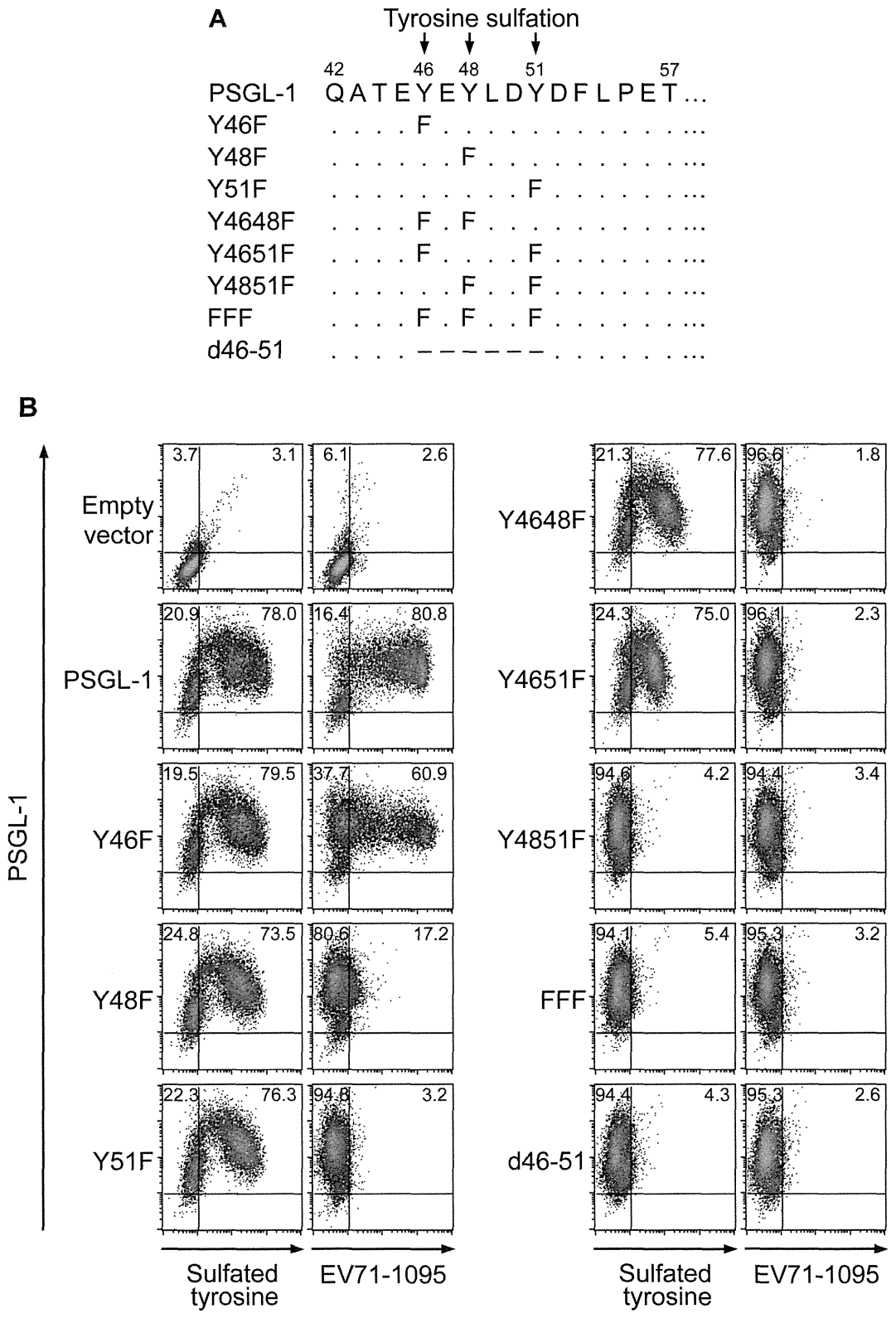

Figure 4. Sulfated tyrosines in the N-terminal region of PSGL-1 are important for binding to EV71-1095. (A) Putative sites of tyrosine sulfation (Y46, Y48, and Y51) in the N-terminus (aa 42–57) of PSGL-1 and the series of PSGL-1 mutants constructed. Identical and deleted amino acids are indicated by dots (.) and dashes (–), respectively. (B) EV71-1095 binding to PSGL-1 mutants. The percentage of cells expressing tyrosine sulfated proteins or bound to EV71-1095 is indicated in the upper right quadrant. The data are representative of three independent experiments.
doi:10.1371/journal.ppat.1001174.g004

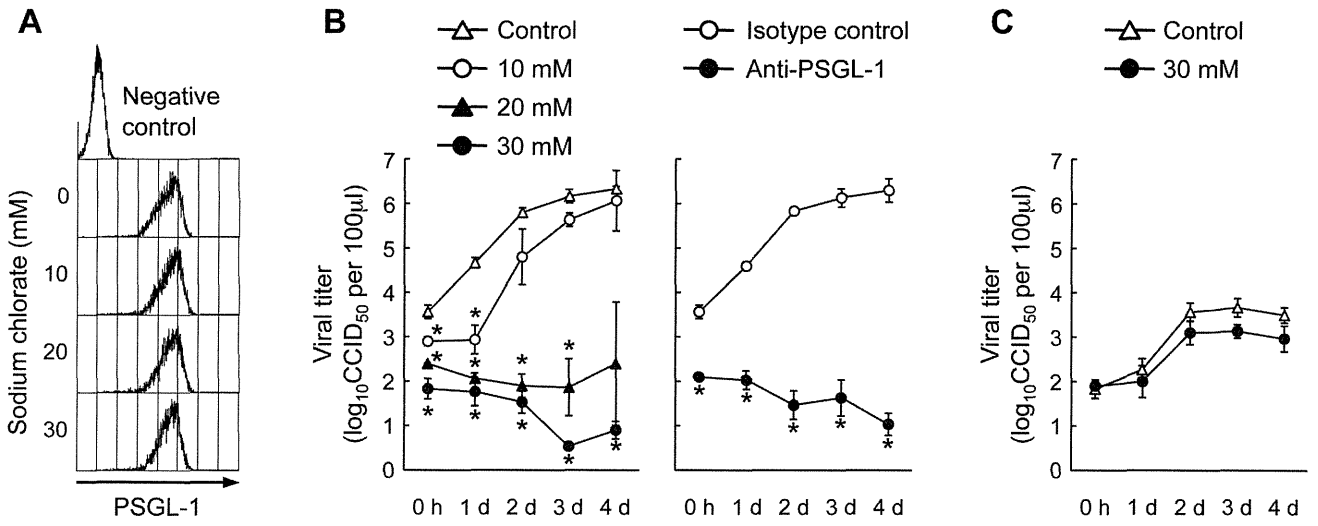

Figure 5. Effect of sodium chlorate on EV71-1095 replication in Jurkat T cells. (A) PSGL-1 expression on the Jurkat T cell surface, as measured by flow cytometry. As a negative control, Jurkat T cells maintained in the absence of sodium chlorate were stained with an isotype control antibody. (B) EV71-1095 growth kinetics in Jurkat T cells in the presence of sodium chlorate. Viral titers were determined at 0 h, 1 day, 2 days, 3 days, and 4 days after EV71-1095 inoculation in Jurkat T cells. As a control for inhibition of EV71 replication, EV71-1095 growth kinetics in Jurkat T cells in the presence of anti-PSGL-1 (KPL1) and control antibodies are shown. (C) EV71-02363 (EV71-non-PB) growth kinetics in Jurkat T cells in the presence of sodium chlorate. Viral titers are indicated as the mean ± S.D. of triplicate analyses. Asterisks indicate $P<0.01$ compared to those of the controls.
doi:10.1371/journal.ppat.1001174.g005

1095 and examined viral titers at 24 h posttransfection in the presence or absence of 30 mM sodium chlorate. Although infectious viruses were recovered in the presence of sodium chlorate, the mean viral titer in the presence of sodium chlorate was over 10 times lower than that of the control experiments (data not shown). Although sodium chlorate inhibited EV71-PB-binding to PSGL-1 expressing cells (Figs. 3C and 5B (0 h postinfection)), we could not rule out the possible involvement of the sodium chlorate treatment during the later stages of viral replication. Further studies are needed to elucidate the inhibitory mechanism of action of sodium chlorate in a receptor dependent or independent manner during different stages of viral replication of EV71.

Replication of the G-10 strain of coxsackievirus A16, which may use another unidentified receptor(s) to infect Jurkat T cells [5,24], was significantly inhibited by sodium chlorate (Fig. 6). This result suggests that some sulfated molecules other than PSGL-1 might be involved in the replication of coxsackievirus A16 in Jurkat T cells in a PSGL-1-independent manner.

Discussion

We have shown that tyrosine sulfation, but not *O*-glycosylation, of the N-terminal region of PSGL-1 is critical for EV71-PB binding to PSGL-1 and for virus entry and subsequent replication

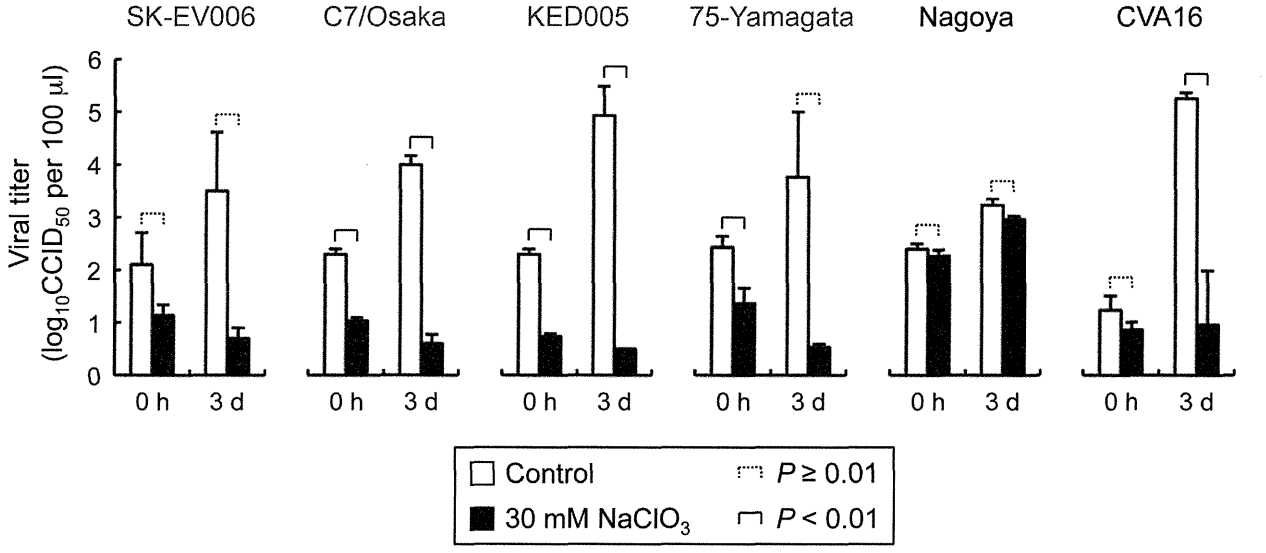

Figure 6. Replication of five EV71 strains and coxsackievirus A16 (CVA16) in Jurkat T cells in the presence of sodium chlorate. Viral replication was determined in Jurkat T cells incubated with 30 mM sodium chlorate. EV71-PB strains are indicated in red. Titers are expressed as the mean, and error bars indicate the S.D. of triplicate or quintuplicate (CVA16) analyses.
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of EV71-PB in Jurkat T cells. First, unlike P-selectin-Fc, EV71-PB bound to a PSGL-1 mutant with an alanine substitution at the potential *O*-glycosylation site (T57) in a calcium-independent manner (Figs. 1 and S1). Second, removal of sialyl Lewis x by sialidase did not reduce PSGL-1 binding to EV71 (Fig. 2). Third, a sulfation inhibitor, sodium chlorate, significantly impaired EV71-PB binding to PSGL-1 in a dose-dependent manner (Figs. 3 and S1). Fourth, EV71-PB binding to PSGL-1 was inhibited when phenylalanine substitutions were made at one or more potential tyrosine sulfation sites in the N-terminal region of PSGL-1 (Figs. 4 and S1). Finally, PSGL-1-dependent viral replication of EV71-PB strains in Jurkat T cells, but not EV71-non-PB strains, was inhibited by sodium chlorate (Figs. 5 and 6).

Human PSGL-1 is one of the most characterized tyrosine sulfated proteins at the molecular level [11]. The involvement of *O*-glycans and sulfated tyrosines in the structural and functional basis of PSGL-1 binding to its natural ligands has been extensively studied, and distinct requirements for tyrosine sulfation for PSGL-1 binding to selectins have been elucidated. Among the three potential sulfated tyrosines of human PSGL-1, Y46 and Y51, but not Y48, are important for PSGL-1 binding to L-selectin along with a core-2 based *O*-glycan with sialyl Lewis x at T57 [22]. On the other hand, the crystal structure of the lectin and EGF domains of P-selectin co-complexed with the N-terminal domain of PSGL-1 revealed a critical involvement of sulfated tyrosines at Y48 and Y51 for direct molecular contact with P-selectin [11]. The corresponding interactions via sulfated tyrosines are not formed in E-selectin binding in the crystal structure of the PSGL-1–E-selectin complex [11]. Thus, tyrosine sulfation is critical for PSGL-1 binding to L- and P-selectins, but not to E-selectin [14]. In our study, we have shown that sulfated tyrosines at Y48 and Y51 play a critical role in PSGL-1 binding to EV71-PB. However, *O*-glycosylation at T57 and sialyl Lewis x moieties on the potential *O*-glycans of PSGL-1 were not required for the PSGL-1–EV71 interaction, suggesting distinct structural requirements between EV71 and P-selectin for PSGL-1 binding. To elucidate the structural basis of the PSGL-1–EV71 interaction, further studies will be needed to identify genetic determinants in EV71 capsid proteins required for PSGL-1 binding using both EV71-PB and non-PB strains.

Yang et al. [21] have recently reported that EV71 may use sialylated glycans as receptors for infection in intestinal DLD-1 cells. In our current study, we showed that potential *O*-glycans at T57 and sialic acids are not critical for binding to EV71-PB (Figs. 1 and 2). However, our study does not exclude possible contributions of sialic acids and other proteins with or without *O*-glycans on the cell surface of various cells during the course of EV71 replication in a PSGL-1-dependent or -independent manner [21,24,25].

In contrast to the structural requirements of *O*-glycans for PSGL-1 binding to selectins, all three sulfated tyrosines, but not *O*-glycans at T57, are required for PSGL-1 binding with the skin-associated chemokine, CCL27 [9]. PSGL-1 facilitates P-selectin-mediated T cell migration in the inflamed skin [26,27] and interacts with the chemokine CCL27 to regulate skin-homing T cells [9]. HFMD pathogenesis due to EV71 can be characterized as acute skin inflammation. Therefore, it is possible that binding of EV71-PB with PSGL-1-positive skin-homing T cells and/or Langerhans cells, and subsequent viral replication in those cells, may participate in HFMD pathogenesis and progression. The status of tyrosine sulfation of PSGL-1 on those cells may modulate cell migration and PSGL-1-dependent replication of EV71-PB in the inflamed skin.

An important role for tyrosine sulfation of a specific cellular receptor in viral entry and replication has been demonstrated for the first time in a co-receptor for HIV-1, CCR5 [18]. CCR5 is a functional receptor for macrophage inflammatory protein (MIP)-1α and MIP-1β, and is expressed on memory/effector T cells, macrophages, and immature dendritic cells [28]. The N-terminal region of CCR5 is highly modified by tyrosine sulfation and *O*-glycosylation, and sulfated tyrosines play critical roles in CCR5 interactions with chemokines [18]. Site-directed mutagenesis and treatment with sodium chlorate revealed that sulfation of tyrosine residues in the N-terminal region of CCR5 is required for efficient CCR5 binding to MIP-1α and MIP-1β, and to HIV-1 gp120-CD4 complexes, without affecting the expression of CCR5 [18]. Likewise, the efficacy of HIV-1 entry was significantly reduced in cells expressing CCR5 mutants with one or more phenylalanine substitutions at four potential tyrosine sulfated residues compared to that in cells expressing native CCR5 [18]. Tyrosine sulfation may be a common phenomenon in chemokine receptors expressed on immune cells such as leukocytes, platelets, and dendritic cells [16]. Therefore, tyrosine sulfation seems to regulate not only the migration of immune cells but also the infectivity of viruses.

Although the occurrence of severe EV71 infection with a number of fatal cases mainly in children continues to be a major public health threat in the Asia Pacific region, no vaccines or antiviral agents are currently available for EV71 [29]. Our data suggest that the virus-receptor interaction may be a promising target for potential antiviral agents. Thus, soluble PSGL-1 as one such agent may have an inhibitory effect on EV71-PB replication [5]. In our current study, we have demonstrated the possible involvement of tyrosine sulfation of PSGL-1 on EV71 entry into target cells, and accordingly, we showed the inhibitory effect of a tyrosine sulfation inhibitor on viral replication of EV71-PB strains in Jurkat T cells. Thus, the elucidation of the structural and functional basis of virus-receptor interactions will provide novel and unique antiviral approaches for the treatment of severe EV71-associated diseases.

Materials and Methods

Cells

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Wako) supplemented with 10% fetal calf serum (FCS). Jurkat T cells were maintained in RPMI medium (Sigma) supplemented with 10% FCS.

Viruses

All EV71 strains (Table 1) and the coxsackievirus A16 prototype strain (G-10) were propagated in RD or Vero cells. Because some of the strains produced diffuse plaques on RD cells, the viral titers were determined by a microtitration assay using 96-well plates and

Table 1. EV71 strains.

Strain (Subgenogroup)	PSGL-1 binding phenotype[1)]	Accession No.	Reference
SK-EV006 (B3)	PB	AB059819	[33]
C7/Osaka (B4)	PB	AB059818	[33]
KED005[2)] (C1)	PB		[33]
1095 (C2)	PB	AB059817	[30,34]
75-Yamagata (C4)	PB	AB177813	[35]
Nagoya (B1)	Non-PB	AB059813	[36]
02363 (C1)	Non-PB	AB115495	[34]

[1)]PB: PSGL-1–binding, Non-PB: PSGL-1–non-binding [5].
[2)]The VP1 nucleotide sequence of KED005 is identical to that of the 03784-MAA-97 strain (accession no. AY207612) isolated in Malaysia [37].
doi:10.1371/journal.ppat.1001174.t001

RD cells, as previously described [30]. Briefly, 10 wells were used for each viral dilution, and the viral titers were expressed as 50% cell culture infectious dose ($CCID_{50}$). For flow cytometry, we used concentrated viruses unless otherwise stated. To prepare virus concentrations, viruses were ultracentrifuged, and the amount of EV71 virions was measured.

Antibodies and recombinant proteins

The anti-EV71 monoclonal antibody (mAb) MA105 (mouse IgG_{2b}) was generated from mice immunized with EV71-1095 (Y. Tano et al., unpublished data) Immunization to mice, fusion, selection of hybridomas, and propagation of hybridomas in the ascite fluid of the mice, were outsourced to Nippon Biotest Laboratories Inc., Tokyo, Japan. The anti-human PSGL-1 mAb KPL1 and anti-sialyl Lewis x mAb CSLEX1 were purchased from BD Biosciences. Anti-human PSGL-1 mAb PL2 was purchased from Beckman–Coulter. Anti-sulfotyrosine mAb Sulfo-1C-A2 [31] was purchased from Millipore. For the negative control, mouse IgG_1 (MOPC-21) and IgG_{2a} (G155–178) were purchased from BioLegend and BD Biosciences, respectively. Recombinant P-selectin-Fc was purchased from R&D Systems.

Plasmids and mutagenesis

For directional cloning using a *Cpo*I recognition site [32], we introduced a *Cpo*I recognition-compatible (*San*DI) site into the pcDNA3.1(+) plasmid (Invitrogen). The *Bam*HI-*Eco*RI fragment of pcDNA3.1(+) was replaced with 5′-ggatccgggtcccggtaagaattc-3′ (*Bam*HI+*San*DI+gg+Stop+*Eco*RI) to produce pcDNA3.1SS. Human *FUT7* cDNA was amplified from Jurkat T cell cDNA with the primers FUT7-F1 (5′-atacggtccggccatgaataatgctgggcacggc-3′) and FUT7-R1 (5′-tgacggaccgtcaggcctgaaaccaaccct-3′). The *FUT7* ORF was sub-cloned into a *San*DI site in pcDNA3.1SS to produce pcDNA-FUT7. The sequence of the cloned *FUT7* ORF was identical to that of *FUT7* (NM_004479).

The primers used for mutagenesis/deletion are provided in Table S1. Briefly, cDNA of human *SELPLG* was cloned into pEF6-Flag-3S [5] to produce pEF-PSGL-1 [5]. Mutations and deletions were introduced into the N-terminal region of human PSGL-1 with PCR, and the mutated *SELPLG* cDNA was cloned into pEF6-Flag-3S.

Transfection of 293T cells

293T cells were transfected with expression plasmids using Lipofectamine 2000 (Invitrogen), and DMEM medium was replaced with fresh medium 4 h after transfection. The cells were collected 24 h after transfection by pipetting, and were used for flow cytometry. For inhibition of tyrosine sulfation of PSGL-1, 293T cells were treated with 10–50 mM sodium chlorate in DMEM for 1 day. Four hours after transfection with pEF-PSGL-1, the medium was replaced with medium containing sodium chlorate, and the cells were further incubated for 20 h.

Flow cytometry

The cells were washed once with flow cytometry buffer (FC buffer; PBS(−) supplemented with 2 mM EDTA, 2% FCS, and 0.1% NaN_3) and incubated with the indicated mAb on ice for 30 min. After washing with FC buffer, the cells were incubated with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen). To detect sialyl Lewis x, the cells were incubated with secondary antibodies conjugated with R-phycoerythrin (SouthernBiotech). To detect PSGL-1 by two-color flow cytometry, PL2 was labeled with a Zenon mouse IgG_1 R-phycoerythrin labeling kit (Invitrogen). To detect P-selectin-Fc binding, PBS(−) supplemented with 2 mM $CaCl_2$, 2% FCS, and 0.1% NaN_3 was used instead of FC buffer. The cells were washed and analyzed with FACSCalibur (Becton Dickenson).

EV71-binding assay by flow cytometry

293T cells (5×10^5) transfected with the indicated expression plasmid were washed once with FC buffer and incubated with the EV71-1095 preparation (1×10^7 $CCID_{50}$) supplemented with 0.1% NaN_3, or concentrated viruses (containing 0.5 μg of VP1 protein) per 50 μl FC buffer. The cells were washed and stained for 30 min on ice with Alexa Fluor 488-conjugated MA105.

Sialidase treatment of cells

Cells were processed as in the EV71-binding assay and flow cytometry described above. Prior to the addition of EV71, P-selectin-Fc, or mAb, cells (2.5×10^6) were incubated with 50 mU/ml of *Vibrio cholerae* sialidase (Roche) in 500 μl of DMEM supplemented with 2% FCS for 1 h at 37°C and then washed once.

Viral infection assays

Jurkat T cells (4×10^4 cells) were inoculated with viruses at 1 $CCID_{50}$/cell for 1 h on ice, washed, and incubated in medium (200 μl in a 48-well plate) at 34°C. For inhibition of tyrosine sulfation of PSGL-1, the cells were pretreated with 10–30 mM sodium chlorate in medium for more than 3 days, inoculated with viruses, washed, and maintained in medium supplemented with sodium chlorate. For mAb inhibition, the cells were pretreated with 10 μg/ml mAb for 1 h, washed, and maintained in medium with 10 μg/ml mAb. At the indicated time, the infected cells and supernatants were freeze-thawed, and viral titers were determined by $CCID_{50}$ titration in RD cells. All infection assays were carried out in triplicate unless otherwise stated, and the mean viral titers were compared using Student's *t*-test (two-tailed). P values<0.01 were considered statistically significant.

Supporting Information

Table S1 Substitution/deletion mutant primers.
Found at: doi:10.1371/journal.ppat.1001174.s001 (0.04 MB DOC)

Figure S1 Binding of four EV71-PB strains to 293T cells expressing PSGL-1. 293T cells were transfected with the indicated expression plasmids (wild-type PSGL-1, T57A, or FFF) and cultured in the absence (PSGL-1, T57A, and FFF) or presence (PSGL-1+$NaClO_3$) of 50 mM sodium chlorate. The transfectants were incubated with concentrated EV71 and used for the EV71 binding assay using flow cytometry. As a negative control, cells were incubated with concentrated supernatant from the RD cell culture (RD sup.). The percentage of cells bound to EV71 is indicated in the upper right quadrant.
Found at: doi:10.1371/journal.ppat.1001174.s002 (2.14 MB TIF)

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Author Contributions

Conceived and designed the experiments: YN. Performed the experiments: YN. Analyzed the data: YN TW HS. Contributed reagents/materials/analysis tools: YN HS. Wrote the paper: YN TW HS.

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Development of a Particle Agglutination Method with Soluble Virus Receptor for Identification of Poliovirus[▽]

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In the Global Polio Eradication Initiative, laboratory diagnosis plays a critical role by isolating and identifying poliovirus (PV) from the stool samples of patients with acute flaccid paralysis (AFP). In this study, we developed a particle agglutination (PA) method with a soluble human PV receptor (hPVR) in the form of an immunoadhesin (PVR-IgG2a) for the simple and rapid identification of PV. Sensitized gelatin particles with PVR-IgG2a showed specific agglutination with the culture fluid of PV-infected cells within 2 h of reaction in a one-step procedure. Detection limits for type 1, 2, and 3 PV(Sabin) strains were 1.5×10^6 50% cell culture infectious doses ($CCID_{50}$), 5.3×10^5 $CCID_{50}$, and 9.1×10^5 $CCID_{50}$, respectively. Wild-type PVs and PV isolates from acute flaccid paralysis cases examined were identified correctly with this PA method, except for some samples with a mixture of different serotypes of PVs, where a minor population of PV failed to be detected. These results suggest that this PA method is useful for the simple and rapid identification of PV, although the sensitivity was not high enough to detect a minor population of PV (<1/10 of the major population) among mixed PVs.

In the Global Polio Eradication Initiative, laboratory diagnosis plays a critical role by isolating and identifying poliovirus (PV) from stool samples of patients with acute flaccid paralysis (AFP). In the World Health Organization (WHO) Global Polio Laboratory Network, PV isolation and identification are performed at WHO national polio laboratories by use of a cell culture system with a human rhabdomyosarcoma cell line (RD cells) and a mouse L cell line expressing the human PV receptor (hPVR) (L20B cells) (17, 18), followed by differentiation of the isolated strains into oral PV vaccine (OPV)-related PVs, vaccine-derived PVs, and wild-type PVs at WHO regional reference laboratories by several methods, including enzyme-linked immunosorbent assay (ELISA), probe hybridization, and reverse transcription-PCR (RT-PCR) (7, 18).

For the identification of PV, a neutralization test with anti-PV antibodies has been performed with the cell culture system and takes 3 to 5 days for results (18). The latest procedure for the laboratory diagnosis of PV (called New Algorithm) gives priority to minimizing the time to report. Accordingly, PV identification by neutralization testing at the national laboratories is no longer necessary, in principle, before the differentiation, except for samples with a mixture of different serotypes of PVs. In this procedure, RT-PCR systems have a critical role in identification and differentiation to minimize the overall time for analysis (on the order of hours) (7, 16). However, identification by RT-PCR, based on the mobility of PCR products, has 4 steps (i.e., viral RNA preparation, RT reaction, PCR, and gel electrophoresis) and might be laborious for laboratories with high workloads. Real-time RT-PCR requires additional equipment for the measurement and quality control system. In the posteradication era of PV, the availability of simple and rapid identification procedures at the national laboratories would be helpful for rapid confirmation of polio cases.

In the present study, we developed a novel particle agglutination (PA) method with a soluble hPVR for the simple and rapid identification of PV. With this PA method, PV strains, including Sabin vaccine strains, wild-type PVs, and PV isolates from AFP cases, were identified within 2 h of reaction in a one-step procedure.

MATERIALS AND METHODS

Cells, viruses, and antibodies. RD cells (human rhabdomyosarcoma cell line), HEp-2c cells (human larynx epidermoid carcinoma cell line), and GP2-293 cells (Clontech) were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). HEK293 cells were cultured as monolayers in Opti-Pro SFM medium (Gibco) supplemented with 2% FCS. RD cells were used for the titration of PV, coxsackievirus B (CVB), echoviruses, and enterovirus 71 (EV71). HEp-2c cells were used for the titration of coxsackievirus A (CVA). GP2-293 cells were used for the production of a recombinant retrovirus for the expression of PVR-IgG2a. HEK293 cells were used for the stable expression of PVR-IgG2a. RD cells, HEp-2c cells, and L20B cells infected with viruses were collected after freezing and thawing and then used as virus samples. For characterization of the PA method for the identification of PV, we examined vaccine strains [PV1(Sabin), PV2(Sabin), and PV3(Sabin)], wild-type strains [PV1(Mahoney), PV1(Brunhilde), PV2(MEF-1), PV3(Leon), PV3(Saukett), and PV3(Suwa-3)], and isolates from AFP cases. Nonpoliovirus enteroviruses examined to show the specificity of the PA method were as follows: echovirus 11 (strain Gregory; 6.8×10^5 50% cell culture infectious doses [$CCID_{50}$]/μl), echovirus 25 (strain JV-4; 1.0×10^6 $CCID_{50}$/μl), CVB3 (strain Nancy; 5.6×10^4 $CCID_{50}$/μl), CVB4 (strain JVB; 3.2×10^4 $CCID_{50}$/μl), EV71 (strains BrCr-TR, Nagoya, C7-Osaka, 1095, and 75-Yamagata-2003 [1.8×10^4 $CCID_{50}$/μl, 6.3×10^4 $CCID_{50}$/μl, 3.3×10^5 $CCID_{50}$/μl, 1.2×10^5 $CCID_{50}$/μl, and 6.3×10^5 $CCID_{50}$/μl, respectively]), CVA17 (CAM2163 isolate; 1.8×10^4 $CCID_{50}$/μl), and CVA20 (CAM1976 isolate; 3.2×10^4 $CCID_{50}$/μl). Virus titers were determined by measuring the $CCID_{50}$ at 35°C by a microtitration assay (13). Type-specific anti-PV antibody (RIVM PV typing antiserum) was reconstituted with 0.5 ml of distilled water and then diluted by adding 4.5 ml of 2% FCS–DMEM. Anti-PV1+PV2+PV3 (anti-P1+P2+P3), -P1+P2, -P2+P3, and

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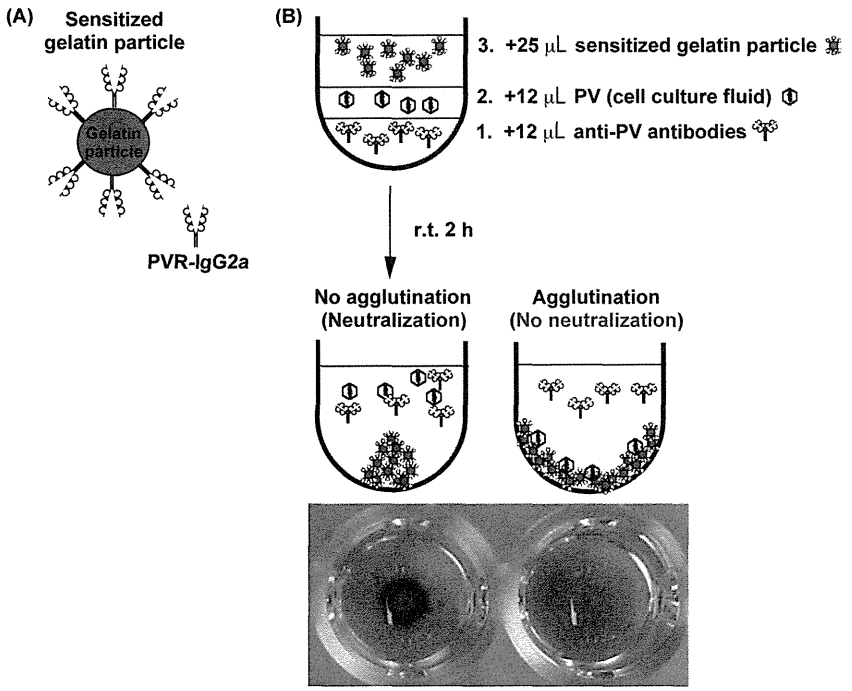

FIG. 1. PA method for the identification of PV. (A) Schematic view of a sensitized gelatin particle with a soluble PVR (PVR-IgG2a). (B) Procedure of the PA method for the identification of PV and the appearance of agglutination by PV. The order of sample addition to the reaction plate is as follows: 1, anti-PV antibodies; 2, PV solution; and 3, sensitized gelatin particle solution. r.t., room temperature.

-P3+P1 antibody pools were prepared by mixing equal volumes of each type-specific antibody and by adding a 1/5 volume of FCS (final FCS concentration, 18.2%).

Purification of PVR-IgG2a. PVR-IgG2a, which is an immunoadhesin molecule consisting of the extracellular domains of hPVR and the Fc domains of mouse IgG2a (1), was expressed in HEK293 cells by use of a retrovirus expression system. A DNA fragment of the coding region of PVR-IgG2a was obtained by a PCR using primers 5′CCATAGATCTACCATGGCCCGAGCCATGGC3′ and 5′ATGAATCGATTCATTTACCCGGAGTCCGGG3′ (BglII and ClaI sites in the primers are underlined), with a baculovirus expression vector for PVR-IgG2a as the template (1). The DNA fragment was digested with BglII and ClaI and then was cloned into the corresponding sites of plasmid pLEGFP-N1 (BD Biosciences Clontech). The resultant plasmid was designated pPVR-IgG2a. GP2-293 cells were cotransfected with pPVR-IgG2a and pVSV-G (Clontech). The cell culture supernatant of the transfected cells was collected at 96 h posttransfection. HEK293 cells were inoculated with the collected supernatant and then used for the expression of PVR-IgG2a. For the purification of PVR-IgG2a, the cell culture supernatant of PVR-IgG2a-expressing HEK293 cells was collected on day 5 after passage. PVR-IgG2a was purified from 100 ml of the supernatant by use of protein A Sepharose Fast Flow (GE Healthcare) in 4 ml of elution buffer at a concentration of 0.03 mg/ml, as described previously (1).

Quantification of PVR-IgG2a. The amount of PVR-IgG2a was quantified by a dot blot analysis, with purified mouse IgG2a (BD Pharmingen) as a standard sample. Purified PVR-IgG2a and purified mouse IgG2a (0.05, 0.025, and 0.0125 mg/ml) in a 5-μl volume were adsorbed to an Immobilon-P transfer membrane (Millipore) by use of an SRC 96 D Minifold I dot blotter (Schleicher & Schuell). The filter was blocked in phosphate-buffered saline (PBS) (10 mM phosphate buffer [pH 7.0], 135 mM NaCl, and 2.6 mM KCl) containing 5% nonfat dry milk and then incubated at room temperature for 20 min. The filter was washed with PBS containing 0.1% Tween 20 three times for 5 min each and then subjected to detection by use of a SuperSignal West Femto maximum sensitivity substrate kit (Pierce). The filter was incubated with goat anti-mouse IgG antibodies conjugated with horseradish peroxidase (1:1,000 dilution in PBS containing 0.1% Tween 20 and 0.5% nonfat dry milk) at room temperature for 1 h. The filter was washed with PBS containing 0.1% Tween 20 three times for 5 min each and then treated with substrate solution for detection of the signal by use of an LAS-3000 imaging system (Fujifilm). The concentration of PVR-IgG2a was estimated from that of mouse IgG2a and presented as the corresponding concentration of mouse IgG2a.

Sensitization of gelatin particles with PVR-IgG2a. Gelatin particles which had been activated with tannic acid were mixed with 5.8 to 12.6 μg/ml of PVR-IgG2a in phosphate buffer solution (pH 6.0) and incubated at 37°C for 1 h. After being washed, the gelatin particles were suspended in phosphate buffer solution (pH 6.0) supplemented with 2% normal rabbit serum and lyophilized.

PA procedure. For the PA procedure, 12 μl of anti-PV antibody solution or 10% FCS–DMEM without anti-PV antibodies (positive control for PA) was added to the reaction plates (Fastec U-bottomed microplate; Fujirebio Inc.), and then 12 μl of PV solution (cell culture fluid obtained after freezing and thawing of the infected cells) was added to the anti-PV antibody solutions. Finally, 25 μl of reconstituted sensitized gelatin particle solution was added to the plate. The plates were mixed in a plate mixer (Micro Mixer P; Taitec) for 30 s and then incubated at room temperature for 2 h. Agglutination of the sensitized gelatin particles was judged by visual observation.

RESULTS

Development of a PA method for the identification of PV. To detect all the serotypes of PV by a PA method, we used soluble hPVR in the form of an immunoadhesin (PVR-IgG2a), consisting of extracellular domains of hPVR and the hinge and Fc portions of mouse IgG2a (1, 3), for the sensitization of gelatin particles (Fig. 1A). In the PA method, anti-PV antibody solutions or 10% FCS–DMEM without anti-PV antibodies (a positive control for PA) was added to the reaction plates, and then PV solutions (cell culture fluid obtained after freezing and thawing of infected cells) were added to the anti-PV antibody solutions. Finally, the reconstituted sensitized gelatin particle suspension was added to the plates, and then the plates were mixed and incubated at room temperature for 2 h to observe

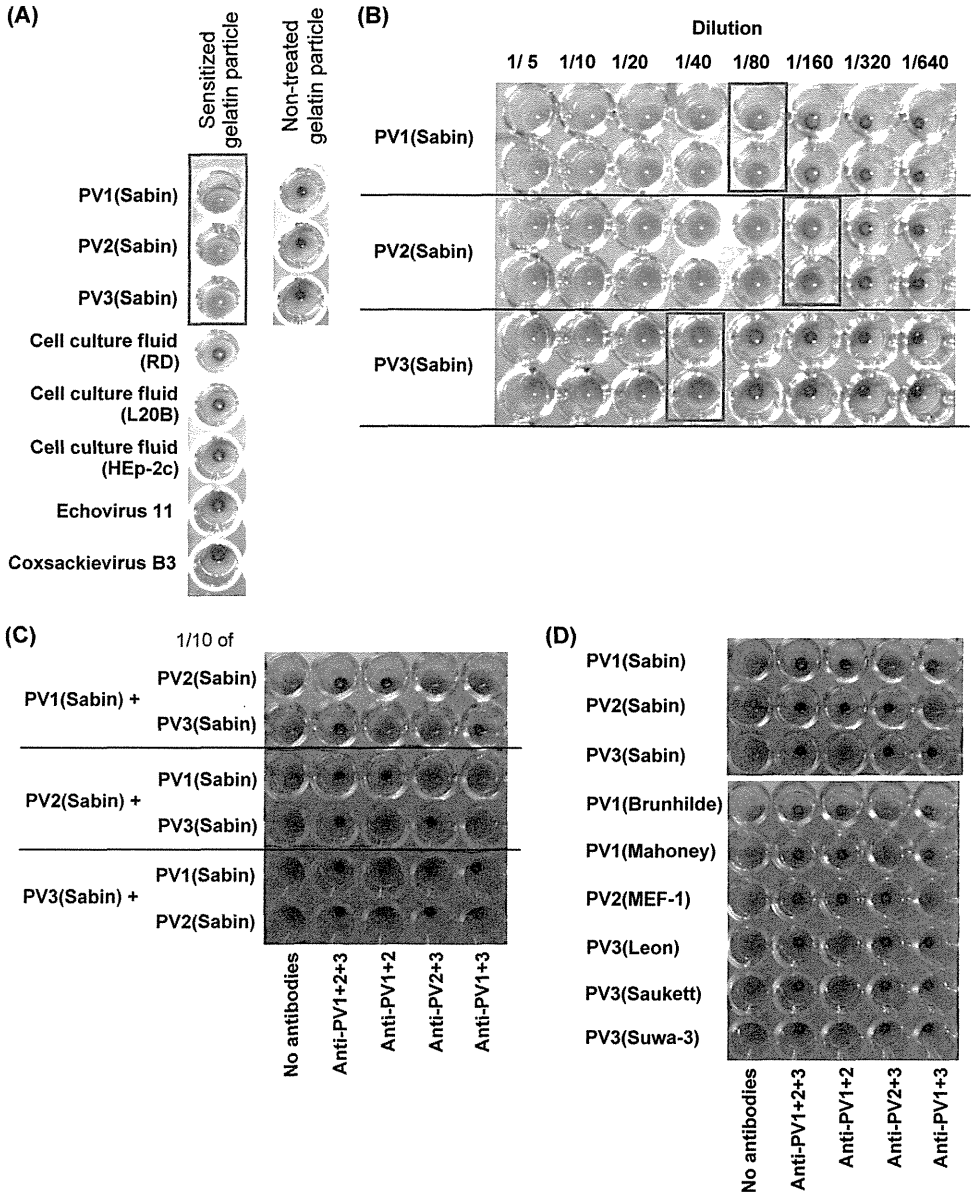

FIG. 2. Characterization of PA method and identification of PV strains. (A) Specificity of sensitized gelatin particles for PV. The agglutination activity of gelatin particles was examined with nonpoliovirus enteroviruses (echovirus 11 and CVB3) and nontreated gelatin particles. Wells that showed agglutination are shown in a box. (B) Sensitivity of the PA method for PV(Sabin) strains. Virus solutions of PV1(Sabin), PV2(Sabin), and PV3(Sabin) (virus titers of 9.5×10^6 $CCID_{50}/\mu l$ [1.1×10^8 $CCID_{50}$ in 12 μl], 6.9×10^6 $CCID_{50}/\mu l$ [8.2×10^7 $CCID_{50}$ in 12 μl], and 2.9×10^6 $CCID_{50}/\mu l$ [3.5×10^7 $CCID_{50}$ in 12 μl], respectively) were diluted 1/5 to 1/640 and then examined by the PA method. The detection limit of the PA method for each sample observed on the reaction plate is shown with a box. (C) Identification of mixed PV(Sabin) strains by the PA method. Virus solutions of PV1(Sabin), PV2(Sabin), and PV3(Sabin) (virus titers of 9.5×10^6 $CCID_{50}/\mu l$ [1.1×10^8 $CCID_{50}$ in 12 μl], 6.9×10^6 $CCID_{50}/\mu l$ [8.2×10^7 $CCID_{50}$ in 12 μl], and 2.9×10^6 $CCID_{50}/\mu l$ [3.5×10^7 $CCID_{50}$ in 12 μl], respectively) were mixed with a 1/10 volume (1.2 μl) of each PV(Sabin) strain and then subjected to the PA method for identification. (D) Identification of wild-type PV strains by the PA method. Virus titers of wild-type strains examined [PV1(Brunhilde), PV1(Mahoney), PV2(MEF-1), PV3(Leon), PV3(Saukett), and PV3(Suwa-3)] were 3.2×10^6 $CCID_{50}/\mu l$, 1.8×10^6 $CCID_{50}/\mu l$, 1.0×10^7 $CCID_{50}/\mu l$, 1.0×10^6 $CCID_{50}/\mu l$, 1.0×10^6 $CCID_{50}/\mu l$, and 5.6×10^5 $CCID_{50}/\mu l$, respectively.

the agglutination and inhibition of agglutination by anti-PV antibodies (Fig. 1B).

We examined the specificity of this PA method and the detection limit for PV(Sabin) strains. Agglutination was observed for PV(Sabin) strains but not for cell culture fluids of mock-infected RD cells, L20B cells, HEp-2c cells, and RD cells infected with other enteroviruses, including echoviruses 11 and 25, CVB3 and -4, EV71, and CVA17 and -20) (Fig. 2A; data not shown). Nontreated gelatin particles did not show any apparent agglutination. Detection limits of this PA method for type 1, 2, and 3 PV(Sabin) strains were 1.5×10^6 $CCID_{50}$, 5.3×10^5 $CCID_{50}$, and 9.1×10^5 $CCID_{50}$, respectively (Fig. 2B). These results suggested that the observed agglutination was caused by the specific interaction of PV with its soluble receptor.

Identification of PV strains by the PA method. Next, we examined PV samples consisting of mixed Sabin strains. A minor

TABLE 1. PV identification by cell culture and PA method

Sample no.	PV isolate[a]	Virus titer[b] (log_{10} $CCID_{50}$/50 μl)	Virus identification by cell culture[c]	Virus identification by PA method
1	CAM1967-R1L1	6.25	P3	P3
2	CAM1967-R2	7.25	P3	P3
3	CAM2057-L2	6.5	P3	P3
4	CAM2057-R2	6.75	P3	P3
5	CAM2057-R1L1	NA	P3	P3
6	CAM2058-L2	6.25	P3 + NPV	P3
7	CAM2058-R2	7.75	P3 + NPV	P3
8	CAM2058-R1L1	NA	P3 + NPV	P3
9	CAM2294-L1	NA	P3	P3
10	CAM2294-R2	7.5	P3	P3
11	CAM2553-L1	6.5	P2	P2
12	CAM2554-L1	7.0	P1 + P2	P1 + P2
13	CAM2554-R1	7.5	P1 + P2	P2
14	CAM2840-L2	7.25	P2	P2
15	CAM2906-L3	7.25	P3	P3
16	CAM2907-L3	7.0	P3	P3
17	CAM2936-L2	7.0	P3 + NPV	P3
18	CAM2936-R2	7.25	P3 + NPV	P3
19	CAM2937-L2	6.25	P3 + NPV	P3
20	CAM2937-R2	7.25	P3 + NPV	P3
21	CAM2970-L2	6.25	P1 + P3	P1
22	CAM2995-L2	7.0	P3 + NPV	P3
23	CAM2995-R2	NA	P3 + NPV	P3
24	CAM3017-L2	8.0	P2	P2
25	CAM3017-R2	NA	P2	P2
26	CAM3017-R2L1	NA	P2	P2

[a] Passage histories of the isolates are shown in the sample names. R, recovered from RD cells; L, recovered from L20B cells. Numbers after R and L represent the numbers of passage of the virus in the cells (e.g., R1L1 means one passage in RD cells followed by one passage in L20B cells).
[b] NA, not available.
[c] NPV, non-PV.

population of different serotypes of Sabin strains (1/10 of the major strains) was detected and correctly identified (Fig. 2C). We examined wild-type PVs and also PV isolates from AFP cases. All of the wild-type PVs examined were identified correctly (Fig. 2D). PV isolates with single PV strains showed consistent results with those obtained by neutralization test in cell culture, irrespective of the cell line (RD cells or L20B cells) used for the isolation (Table 1). However, for some isolates with a mixture of different serotypes of PV (CAM2554-R1 and CAM2970-L2), minor PV strains were not detected by the PA method. These results suggest that the PA method is useful for the identification of PV, although minor strains might not be detected in samples with a mixture of different serotypes of PV.

DISCUSSION

In this study, we developed a novel PA method for the identification of PV, using a soluble hPVR and gelatin particles. We used a soluble hPVR in the form of an immunoadhesin (PVR-IgG2a) (1) rather than anti-PV antibodies to sensitize gelatin particles, because hPVR binds all serotypes of PV with its specific interaction and uniform affinity (4, 8, 10, 11). For PVR-IgG2a, some avidity to PV would be expected that could not be attained by monomeric forms of soluble hPVR, which have relatively weak affinities for a single binding site on the virion (K_d [dissociation constant] of 6.7×10^{-7} to 4.5×10^{-8} M) (2, 9). Gelatin particles have little or no nonspecific reaction caused by their own epitopes, and their utility has been well established for the diagnosis of a number of infectious diseases, including strongyloidiasis (infection with *Strongyloides stercoralis*) (15), leprosy (infection with *Mycobacterium leprae*) (5), human immunodeficiency virus infection (19), human T-cell leukemia virus infection (6), infection with *Mycoplasma pneumoniae* (Fujirebio Inc.), and exposure to diphtheria, pertussis, and tetanus toxins (12), by detection of antibodies against infectious agents in the sera of suspected patients. In accordance with the given properties of hPVR and gelatin particles, sensitized gelatin particles formed specific agglutination with all serotypes of PV, with comparable sensitivities (Fig. 2).

The advantages of the PA method developed in this study over currently available PV identification procedures are as follows: (i) it is simple (one-step procedure, mixing PV isolates, anti-PV antibodies, and sensitized particles), (ii) it is rapid (2 h of incubation of the sample at room temperature, which is comparable to the time for analysis by RT-PCR systems and faster than a cell culture-based neutralization test [3 to 5 days]), and (iii) it allows easy evaluation of the results (visual observation without equipment). Disadvantages of the PA method compared to neutralization testing with a cell culture system are as follows: (i) it has a lower sensitivity (10^5 $CCID_{50}$ versus about 1.5 $CCID_{50}$) and (ii) it cannot isolate single serotypes of PV in a mixture of different serotypes of PVs. Actually, PV isolates examined in this study showed virus titers of $10^{6.25}$ to $10^{8.0}$ $CCID_{50}$/50 μl, and all samples were correctly identified by the PA method, except for some mixtures (Table 1). For the identification of major PV strains in the samples, isolates from both L20B cells and RD cells are detectable with the PA method. For the isolation of minor strains, isolates from RD cells might be more suitable than those from L20B cells because of their higher titers (14). However, one isolate from L20B cells (CAM2554-L1), but not other isolates, from L20B cells and from RD cells (CAM2970-L2 and CAM2554-R1), was correctly identified by the PA method. In the current procedure for laboratory diagnosis of PV, identification in a cell culture system is indispensable for isolation of each PV strain for differentiation (18). Utilization of type-specific monoclonal antibodies in the PA method might allow differentiation of PVs along with genetic differentiation by RT-PCR systems (7, 16). The PA method is not an alternative to identification by the cell culture system or RT-PCR systems in the current procedure but could provide supportive information as a rapid and simple confirmation test for PV at national polio laboratories without facilities for molecular diagnosis. The PA method would be useful in cases with a limited time for reporting (within a day) and where reliable specificity is required, e.g., for identification at the national laboratories in the posteradication era of PV.

In summary, we have developed a PA method for the identification of PV. This PA method is useful for the simple and rapid identification of PV.

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Isolation of a recombinant type 3/type 2 poliovirus with a chimeric capsid VP1 from sewage in Shandong, China

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ABSTRACT

The genetic and phenotypic characterization of poliovirus strain P3/Jinan/1/09, isolated from sewage sample in Jinan city, Shandong province, China, was described. The strain had a Sabin type 3/type 2/type 3 recombinant genome, with the first crossover site located in capsid VP1 coding region between nucleotide positions 3293 and 3294 (numbering according to Sabin 3), and the second crossover site in 3D region between positions 6374 and 6378. The recombinant had introduced six Sabin 2-derived amino acids into the carboxyl terminus of Sabin 3 VP1 capsid protein. The complete genome of the isolate revealed eight nucleotide substitutions in Sabin 3 region with two substitutions resulting in amino acid alteration, and two missense substitutions in the Sabin 2 region. An estimation based on the evolution rate of the P1 coding region of Sabin 3 background suggested that evolution time of strain P3/Jinan/1/09 might be 76–80 days. The person who excreted the recombinant was not known and no evidence was obtained for its circulation in population via acute flaccid paralysis surveillance. The virus showed Sabin 3 serological characterization in neutralization test, and it did not lose temperature sensitivity phenotype at 40 °C. The significance of environmental surveillance and the presence of natural capsid recombinant poliovirus strain in the context of the global polio eradication initiative are discussed.

1. Introduction

Poliovirus is a member of *Enterovirus* genus in the *Picornaviridae* family (Stanway et al., 2005). It possesses a positive-sense single-stranded RNA genome which contains an open reading frame (ORF) flanked by a 5′-untranslated region (UTR) and a 3′-UTR. The ORF is translated into a polyprotein and is then cleaved into capsid proteins (VP1 to VP4) and nonstructural proteins. The polioviruses have three serotypes and the antigenic characterization is determined by the capsid epitopes.

Recombination is a common event in poliovirus evolution. The oral poliovirus vaccine (OPV) consists of three serotypes of attenuated polioviruses and provides the opportunity of intertypic recombination in gut cells infected by vaccine strains of more than one serotypes. Most recombinants were found among vaccine strains of serotypes 2 and 3 (Guillot et al., 2000; Cuervo et al., 2001), and a small portion were Sabin 1 recombinant or wild-vaccine recombinant (Furione et al., 1993). The crossover sites have almost invariably been found in genomic regions coding for nonstructural proteins, and natural intertypic capsid recombinant between vaccine strains was very rare. To the best of our knowledge, four recombinant strains with an intertypic capsid protein were reported to be isolated from clinical specimens (Martin et al., 2002; Blomqvist et al., 2003; Dedepsidis et al., 2008; Zhang et al., 2009), and three of them possessed a chimeric type 3/type 2 VP1 with the junction site located at the 3′-end of VP1 region (Martin et al., 2002; Blomqvist et al., 2003; Dedepsidis et al., 2008). Besides, three PV3/PV2 capsid recombinants were recently reported to be isolated from environmental samples in Argentina (Mueller et al., 2009).

Although the last poliomyelitis case caused by wild-type poliovirus in Shandong province of China occurred in 1991, routine OPV program for infants and young children was still implemented with a high level of vaccination coverage. The World Health Organization (WHO) strategy for monitoring the wild type and reverted vaccine poliovirus is to identify acute flaccid paralysis (AFP) cases and their contacts. But because of the high rate of inapparent

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infection for poliovirus, AFP surveillance is not sufficient enough (Birmingham et al., 1997), and environmental surveillance offers an alternative method. Wild-type poliovirus, Sabin strains, vaccine-derived polioviruses (VDPVs), and capsid recombinant polioviruses were reported to be isolated from environmental sample previously (Minor et al., 1999; Yoshida et al., 2002; Blomqvist et al., 2004; Mueller et al., 2009). However, no capsid recombinant polioviruses isolated from sewage in China or other Asian countries have been reported yet.

Surveillance for enteroviruses in environmental samples has been implemented in our laboratory for more than 1 year. Poliovirus strain P3/Jinan/1/09 was isolated from a sewage sample collected on May 28, 2009 in Jinan city, Shandong province of China. Preliminary sequencing of VP1 region revealed a type 3/type 2 recombination event at the 3′-end of the VP1 region. This finding led us to further explore the complete genome characterization of this virus.

2. Materials and methods

2.1. Sampling

Sewage sample was collected in the No. 2 waste water treatment plant in Jinan city, the capital of Shandong province, China. On May 26, 2009, a gauze-wrapped cotton pad was placed in the inlet collector canal. After 2 days, the soaked cotton pad was picked up and transferred to the laboratory and kept at 4 °C until they were treated.

2.2. Concentration of sewage water

Sewage samples were concentrated by method described by Iwai et al. (2006). Briefly, the cotton pad containing sewage sample was squeezed vigorously and the fluid (about 500 ml) was collected into a sterile beaker, and then centrifuged at 3000 rpm for 30 min at 4 °C. 2.5 mM $MgCl_2$ was added to the supernatant to a final concentration of 0.05 mM. The pH value was adjusted to 3.5. Then the solution was filtered through a 0.45 μm mixed cellulose ester membrane filter (ADVANTEC, A045A142C, Tokyo, Japan) by positive pressure pump. Absorbents on the filter were then eluted with 10 ml 3% beef extract solution by ultrasonication for three times (1 min for each time), and the solution was centrifuged at 12 000 rpm for 30 min. Subsequently the supernatant was filtered through a 0.22 μm filter and was ready for cell inoculation.

2.3. Virus isolation

L20B, RD and HEp-2 cell lines were used for virus isolation. 200 μl of treated solution was added to each of the cell culture tube (18 tubes for each cell line). After absorption at 36 °C for 2 h, 1 ml of Eagle's minimal essential medium with 2% fetal calf serum was added and the tubes were kept in 36 °C incubator for 7 days and were examined every day. After 7 days, the tubes were freezed and thawed and re-passaged in L20B, RD and HEp-2 cell lines, and another 7-day examination was performed.

2.4. Serotyping

According to standard protocols recommended by the WHO (2004), the microneutralization assays were carried out in 96-well tissue culture plates using poliovirus type-specific rabbit polyclonal antisera (National Institute for Public Health and the Environment, RIVM, the Netherlands). The antisera–virus mixtures were incubated for 1 h at 36 °C. Subsequently, suspension fluid of L20B cell was added to the plate which was subsequently examined daily for the presence of CPE. The antiserum that prevented the development of CPE indicated the serotype of the poliovirus.

2.5. RT-PCR and sequencing

Total RNA was extracted from 140 μl of the infected L20B cell culture using QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommended procedure. RT-PCR was performed using Access RT-PCR System (Promega, USA). Primers UG1 and UC11 (Balanant et al., 1991) were used to amplify the entire VP1 coding region, and primers for amplifying the complete genome were designed by "primer-walking" strategy. The protocols were as followed. 3 μl of viral RNA was added to RT-PCR mixtures (total volume 50 μl) containing 0.2 mM each of dNTP, 1 mM $MgCl_2$, 5 U AMV reverse transcriptase, 5 U Tfl DNA polymerase, 5 U RNasin ribonuclease inhibitor (Promega, USA), and 1 μM each of the primers. The mixtures were incubated at 45 °C for 45 min and then 94 °C for 2 min, followed by 35 cycles of amplification (94 °C for 20 s, 45 °C for 30 s and 72 °C for 1 min). The products were analyzed by agarose gel electrophoresis, and positive products were sequenced at BGI Sequencing Company (Beijing, China).

2.6. Sequence analysis

The sequence of the strain P3/Jinan/1/09 was aligned with the Sabin strains (AY184219, AY184220 and AY184221 for Sabin 1, Sabin 2 and Sabin 3, respectively) using the BioEdit 7.0.5.3 software (Hall, 1999). The crossover sites were identified as being located between the last nucleotide, differentiating the environmental sequence from the 3′-partner reference sequences, and the first nucleotide, differentiating the environmental sequence from the 5′-partner reference sequence (Zhang et al., 2009).

The time from the date of OPV administration to the date of sampling of the recombinant strain was estimated via the calculation of *Ks* (synonymous substitutions per synonymous site) and *Kt* (all the substitutions per site) in the capsid coding region. The *Ks* and *Kt* of evolving polioviruses have a linear character with time according to previous studies (Kew et al., 1998; Martin et al., 2000). In P1 region, the *Ks* parameter is 3.2% per year and the *Kt* parameter is 1.1% per year (Jorba et al., 2008).

2.7. Temperature sensitivity

Temperature sensitivity of isolate P3/Jinan/1/09 was performed on monolayer RD cells in 6-well plates as described before (Blomqvist et al., 2003). 200 μl of Virus stocks were added onto the cells. After absorption at 36 or 40 °C for 1 h, the supernatant was removed, and 2.5 ml of maintenance medium was added and incubated at 36 or 40 °C, separately. After 8, 24, and 48 h, the plates were harvested, and the $CCID_{50}$ were calculated in 96-well plates. More than 2 logarithms reduction of the titers at different temperatures was considered to be temperature sensitive. In order to avoid experiment error, the assay was repeated three times.

2.8. Nucleotide sequence accession number

The complete genomic sequence of isolate P3/Jinan/1/09 described in this study was deposited in the GenBank database under the accession number GU256222.

3. Results

3.1. Preliminary characterization of the isolate

Strain P3/Jinan/1/09 was isolated from sewage sample via L20B cell line. Neutralization test using poliovirus type-specific rabbit polyclonal antisera showed that it was a type 3 poliovirus. Full-

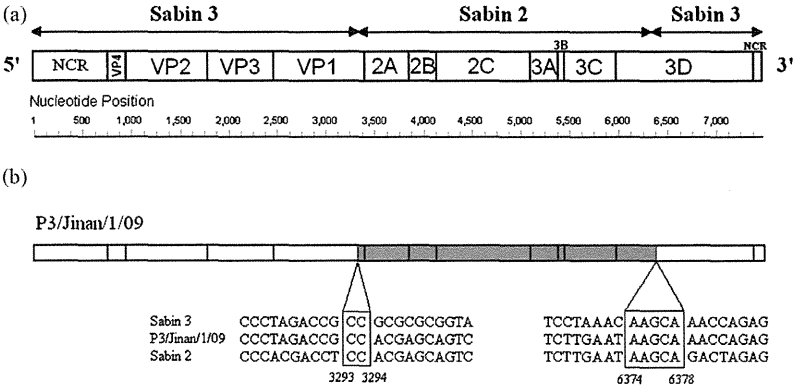

Fig. 1. Scheme of genomic structure of strain P3/Jinan/1/09. The Sabin 3/2/3 genomic structure (a) and the proposed crossover sites (b) are shown in detail.

Table 1
Nucleotide and amino acid substitutions of P3/Jinan/1/09 compared with Sabin strains.

Origin	Region	Nucleotide			Amino acid		
		Position	Sabin	P3/Jinan/1/09	Position	Sabin	P3/Jinan/1/09
Sabin 3	5′-NCR	336	U	C			
	5′-NCR	472	U	C			
	VP4	853	A	G			
	VP3	1938	G	A	399	S	N
	VP3	2140	U	A			
	VP1	2493	C	U	584	T	I
	VP1	2503	U	A			
	VP1	3214	U	C			
Sabin 2	3D	6114*	U	C	1888	R	Q
	3D	6291*	C	U	1893	T	A

Positions with an asterisk are according to Sabin 2 (AY184220), and others refer to Sabin 3 (AY184221).

length VP1 region amplification and sequencing revealed a Sabin type 3/type 2 recombinant with a crossover site at the 3′-end of VP1 region. Totally three nucleotide substitutions were found in the 900-nucleotide VP1 coding region, and the homology with the relative Sabin 3 strain was 99.7%.

3.2. Analysis of the genome

The genome of P3/Jinan/1/09 was Sabin type 3/type 2/type 3 recombinant with two crossover sites (Fig. 1). The first crossover site located in the 3′-end of VP1 region between the nucleotide positions 3293 and 3294 (numbering according to Sabin 3), which resulted in the insertion of a 84-nucleotide Sabin 2 sequence in a Sabin 3 genomic background, effectively resulting in six amino acid substitutions at positions 279, 286, 287, 288, 290, and 293 in VP1 sequence. The second crossover site was in 3D region between the nucleotide positions 6374 and 6378.

The complete genome of strain P3/Jinan/1/09 contains 7432 nucleotides. Compared with Sabin strains, there were totally ten nucleotide substitutions in the whole genome (Table 1). Eight of them located in the Sabin 3 background before the first crossover site, and the other two located in Sabin 2 donor between the two crossover sites. No substitutions were found in the Sabin 3 region after the second crossover site. Two substitutions associated with increased neurovirulence of Sabin 3 (Rezapkin et al., 1995) were both found in the genome of P3/Jinan/1/09. One was the U-to-C reversion at nucleotide position 472 in the 5′-non-coding region, and the other was the C-to-U reversion at nucleotide position 2493 in VP1 coding region. Totally four substitutions resulted in amino acid substitutions. One was in VP3 coding region, another was in VP1 coding region, and the two left were in 3D region.

In comparison with other Sabin 3/Sabin 2 capsid recombinants, Substitutions at nucleotide position 472 could also be found in previously reported capsid recombinant strains 31043, PV3/NOR/018, and K/2002. Substitution at position 1938 was also common in strains 31043 and PV3/NOR/018, and substitution at position 2493 was seen in strain K/2002.

3.3. Estimation of the evolution time of P3/Jinan/1/09

The approximate evolution time of the recombinant strain was estimated in the P1 coding region. The *Ks* and *Kt* parameters for capsid coding region was 0.67% and 0.24% respectively. Compared with the reported evolution rate of 3.2% (*Ks*) and 1.1% (*Kt*) in P1 region per year (Jorba et al., 2008), we estimated the evolution time of the virus was 76 days (based on *Ks* estimate) or 80 days (based on *Kt* estimate). Because poliovirus can only replicate in vivo, the evolution time estimated did not include the duration in environment.

3.4. Temperature sensitivity

The assay for temperature sensitivity showed Sabin 3 was temperature sensitive with titer reduction of more than 2 logarithms at different centigrade. The property of temperature sensitivity of strain P3/Jinan/1/09 reduced slightly. The reduction between titers at 36 and at 40 °C 8 h after infected was less than 2 logarithms, but the reductions at 24 or 48 h post-infection were more than 2 logarithms, just slightly lower than the reductions of Sabin 3 (Table 2). The three times of repeat showed the similar results.

4. Discussion

4.1. General

We have described the isolation and genetic and phenotypic characterization of a Sabin type 3/type 2/type 3 poliovirus recom-

Table 2
Temperature sensitivity of P3/Jinan/1/09.

Virus	Titers at 37 °C			Titers at 40 °C			Log titer reduction		
	8 h p.i.	24 h p.i.	48 h p.i.	8 h p.i	24 h p.i.	48 h p.i.	8 h p.i	24 h p.i.	48 h p.i.
Sabin 3	7.625	7.000	6.500	4.375	4.375	3.375	2.250	2.625	3.125
P3/Jinan/1/09	7.375	7.500	6.875	5.625	5.125	4.125	1.750	2.375	2.750

binant. Strain P3/Jinan/1/09 was isolated from sewage samples collected on May 28, 2009 in sewage disposal facility of Jinan city, the capital city of Shandong province, China.

4.2. Sequence analysis

In comparison with other type 3/type 2 capsid recombinants reported previously, the sequence of strain P3/Jinan/1/09 was the most conservative with only ten nucleotide substitutions in the complete genomic sequence. The substitutions at the attenuation sites (positions 472 and 2493) for Sabin 3 (Chumakov et al., 1992) might suggest the partial reversion of poliovirus neurovirulent phenotype.

Among all the Sabin 3/Sabin 2 capsid recombinants, the first recombination junctions located exclusively in the 3′-end of the VP1 coding region, and the second junctions, if existed, located in the 3D region without exception. However, the exact crossover sites were not constant. Among all such recombinant strains, no identical crossover sites were observed in the 3D region, and only two crossover sites were identical in the VP1 coding region. This phenomenon of similar recombination junction with variable crossover sites suggested although VP1 and 3D region were hot spot regions for recombination, the exact nucleotide sequences involved in recombination were not exclusive.

Based on the calculation of *Ks* and *Kt* parameters of P1 coding region, the evolution time of this recombinant was estimated to be 76–80 days. This period was the duration in replication, and the time in environment was not included. Taking into consideration the several months of surviving in sewage for poliovirus, the age of strain P3/Jinan/1/09 could not be estimated exactly.

4.3. Environmental surveillance

The WHO program for global poliomyelitis eradication requires the intensive immunization of OPV and the surveillance of polio cases, which includes epidemiological and virological investigation of AFP cases, supplemented in some countries by analysis of poliovirus circulation in populations through surveillance in sewage contaminated by human fecal. After the declaration of polio-free in West Pacific Region of WHO, the environmental surveillance is of great importance in investigating the circulation of wild-type poliovirus (El Bassioni et al., 2003) or in detection the existence of VDPV (Shulman et al., 2000; Yoshida et al., 2002; Blomqvist et al., 2004). In this study, a recombinant type 3/type 2 poliovirus with a chimeric VP1 protein was isolated from sewage, and to the best of our knowledge, this is the first report of such recombinant isolated from environmental surveillance in Asian countries.

The origin of the recombinant virus was unknown. The person who excreted the recombinant was not known via AFP surveillance, and no evidence was obtained for its circulation in human populations. It is not clear whether the poliovirus capsid recombinant poliovirus can generate potentially dangerous VDPVs, but the risk is supposed not to be ruled out. The existence of this kind of recombinant vaccine virus highlights the importance of the environmental surveillance of polioviruses before stopping the usage of OPV.

4.4. Temperature sensitivity

Strain P3/Jinan/1/09 did not lose the temperature-sensitive phenotype of parent Sabin strain. This was not identical with three previously reported capsid recombinants which had almost entirely lost the temperature sensitivity. Compared with them, number of substitutions of strain P3/Jinan/1/09 was the least with only 4 amino acid mutations. It had been reported altogether twelve amino acids substitutions have been suggested to be related to temperature sensitivity (Blomqvist et al., 2003), and no was found in strain P3/Jinan/1/09. This might explain the preservation of temperature-sensitive phenotype of virus. But the titer reductions of the strain P3/Jinan/1/09 at 24 or 48 h post-infection were slightly less than those of Sabin 3 strain, and the reduction at 8 h post-infection was less than 2 logarithms. This result suggested that the temperature sensitivity of strain P3/Jinan/1/09 was slightly weakened, and whether the phenomenon came from recombination needed further investigation.

4.5. Conclusions

In this study, we described the isolation and characterization of a recombinant Sabin type 3/type 2/type 3 poliovirus isolated from sewage sample. The results presented here confirmed the importance of environmental surveillance.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2010.02.014.

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特集 第57回日本ウイルス学会シンポジウム特集

1. 世界ポリオ根絶の失われた10年とポリオ根絶計画のこれから

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世界ポリオ根絶計画は，当初，2000年までの根絶達成を目標としていた．しかし，目標から10年が経過した2010年においても，近い将来のポリオ根絶の目途は立っていない．2010年現在，ポリオ常在国であるアフガニスタン，パキスタン，インド，ナイジェリア4カ国以外の国・地域では，地域固有の野生株ポリオウイルス伝播は認められていない．単価経口生ポリオワクチンの積極的使用にも関わらず，ポリオ常在国4カ国では，いまだ1型および3型野生株ポリオウイルス伝播が継続しており，ナイジェリアとインドに由来する野生株ポリオウイルスが，いったんポリオフリーを達成したアフリカ，アジア，ヨーロッパの国々へ，頻繁かつ広範な伝播を引き起こしている．さらに，ナイジェリアやインドのポリオ流行地では，2型ワクチン由来ポリオウイルス伝播が発生しており，一部地域における2型ポリオウイルスに対する集団免疫の低下が危惧されている．その一方，2009年には，北部ナイジェリアにおけるポリオ根絶活動指標の改善が報告されており，実際，2009-2010年にかけて，ナイジェリアの1型および3型野生株ポリオウイルス伝播は顕著に減少ししつつある．さらに，ハイリスク地域における追加接種活動の質を改善し，より簡便化するための効果的な手段として，Sabin 1型と3型を含む二価経口生ポリオワクチンが2010年に導入された．2000-2009年の10年間，ポリオ症例数の上では顕著な減少は認められていないが，「世界ポリオ根絶の失われた10年」の間に得られた経験を十分に踏まえることが，世界ポリオ根絶計画を最終段階に進めるために必要とされる．

はじめに

本稿では，世界保健機関（World Health Organization; WHO）を中心に進められている世界ポリオ根絶計画の現状と問題点について概説する．本誌前号で，宮村達男国立感染症研究所前所長が，ポリオ根絶計画の原理原則と現在の諸課題について，明快かつ詳細にまとめられているので[1]，本稿では，この10年間のポリオ根絶計画停滞の原因に論点をしぼり，世界ポリオ根絶計画の今後の展望について，私見を交え解説する．

世界ポリオ根絶計画の基本戦略は，経口生ポリオワクチン（oral poliovirus vaccine; OPV）の集団接種によって，野生株ポリオウイルス伝播を遮断することにある．WHOが世界ポリオ根絶計画を開始した1988年当時，毎年125カ国余において35万人程度のポリオ症例が発生していたと推定されているが，2009年の野生株ポリオウイルスによるポリオ確定症例数は，世界全体で1606症例と報告されている（2010年5月4日現在）．現在，地域固有の野生株ポリオウイルス伝播がいまだに継続しているポリオ常在国は，パキスタン，アフガニスタン，インド，ナイジェリアの4ヶ国となっている[2]．ポリオ確定症例数とポリオ流行地域の推移でみる限り，ポリオ根絶計画の成果は2000年までは顕著であるが，その後の10年間は，多くの資金と公衆衛生リソースの投入にも関わらず，計画は一進一退を繰り返している（**図1**，右上図）．とくに最近5年間は，1型および3型野生株ポリオウイルスが交互に流行を繰り返すとともに，ワクチン由来ポリオウイルス（vaccine-derived poliovirus; VDPV）によるポリオ症例も増加傾向にある（**図2**）．WHOが当初ポリオ根絶の目標とした2000年以降の10年を「ポリオ根絶計画の失われた10年」と表現すると言い過ぎであろうか．しかし，「ポリオ根絶計画の失われた10年」から

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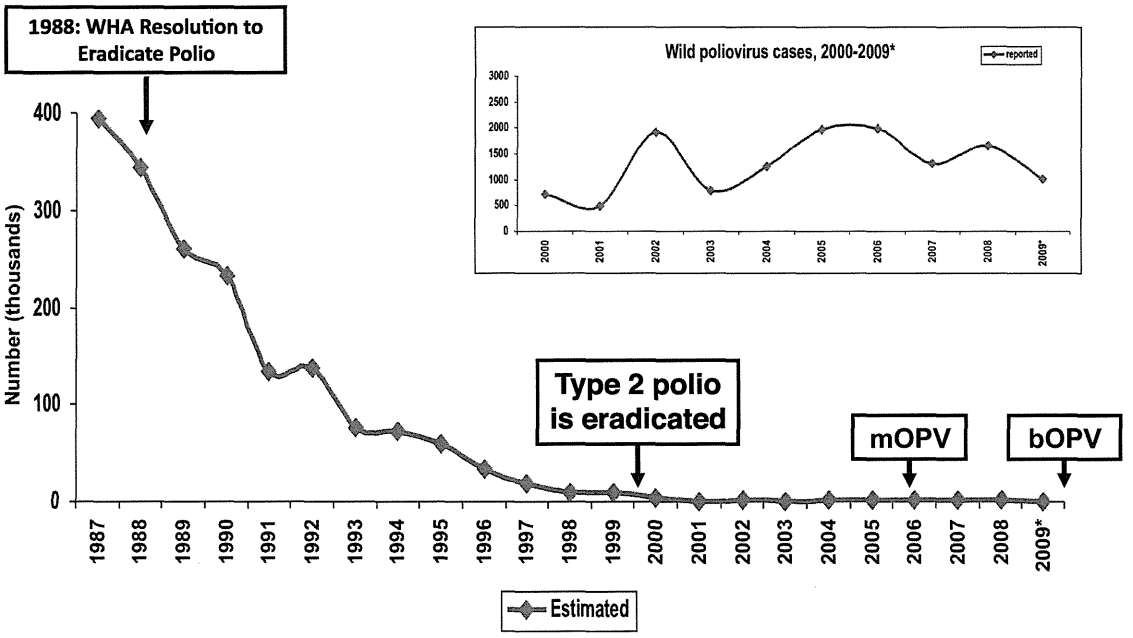

図1 全世界のポリオ症例数の推移
1987～2009年における世界的なポリオ症例数の推移．2000年以降10年間のポリオ症例数については，別スケールの図を右上に示した(WHO提供資料を一部改変)．

虚心に学ぶこと以外に，世界ポリオ根絶を達成する道筋は見えてこないと筆者は考える．

ポリオ常在国における野生株ポリオウイルス伝播の継続

この10年の世界ポリオ根絶計画停滞のもっとも大きな要因は，残されたポリオ流行地における野生株ポリオウイルス伝播の継続にある．世界中の他の地域において成功したtrivalent OPV（tOPV）接種の徹底による野生株ポリオウイルス伝播の遮断という，ある意味単純明快な予防接種戦略が，現在残されている野生株ポリオ常在国では，結果的には功を奏しなかった．その原因は単純ではなく，地域ごとに異なる要因が複合的に関与している．

1）インド

2009年時点において，インドの野生株ポリオウイルス伝播地域は，北部の限られた地域，Uttar Pradesh州西部とBihar州中部に，ほぼ限局されている（**図3**）．これらの地域では，tOPVによる頻回のワクチン接種キャンペーンの実施にも関わらず，1型および3型野生株ポリオウイルス伝播が継続し，tOPV頻回接種では結果的にウイルス伝播停止につながる十分な集団免疫が誘導されないことが臨床疫学研究により明らかされている[3,4]．Uttar Pradeshにおける症例対象研究により，1型monovalent OPV（mOPV）とtOPVの有効性を比較したところ，1型mOPVは5回接種により78%の感染防御効果を示したが，同程度の防御効果を示すためには14回のtOPV接種を必要とした[4]．また，mOPVはtOPVと比較してウイルス排泄抑制効果が高く，ポリオウイルス伝播を効率よく制御することが示唆された[5]．これらの臨床疫学研究の結果は，野生株ポリオ常在地域おける現行のポリオワクチン戦略に応用され，mOPV導入に際しての理論的裏付けとされた．インドの野生株ポリオ流行地では，2005年の1型mOPV導入により，1型野生株ポリオ症例が顕著に減少したものの根絶には至らず，2009年後半まで，1型野生株ポリオウイルスの伝播が継続している．その一方，同地域では2008～2009年にかけて，3型野生株によるポリオ症例が大幅に増加した(**図2**)．主として1型mOPVを用いたSupplementaly Immunization Activity（SIA）により，結果的に3型ポリオウイルスに対する集団免疫が低下したことが，3型ポリオ症例の増加につながった可能性が高い．また，2009～2010年にかけて，ナイジェリア北部同様，インド北部でも2型VDPVの伝播が確認されており[6]（**表1**），tOPVによる定期予防接種率がもともと低い同地域における，2型VDPVの長期的伝播が危惧されている．インド北部の特定の地域で，なぜOPVによる免疫誘導が他の地域と比較して不十分なのかについては，いまだ多くの議論があり，その要因は科学的に解明されていない[3,4,6-8]．緻密化の一方で過度に複雑化したワクチン戦略以前に，ワクチン接種キャンペーンの質やワクチン接種回数のモニタリング等，流行現場の実情に即した問題点を指摘する意見も依然根強い[6,7]．インド北部におけ

表1 ワクチン由来ポリオウイルス(VDPV)によるポリオ流行 (2000-2010年)

国 (地域)	発生年	ポリオ症例数	血清型	ゲノム遺伝子組換え*	推定伝播期間(年)
ハイチ・ドミニカ共和国 (ヒスパニオーラ島)	2000-2001	21	1型	有	2.5
フィリピン	2001	3	1型	有	2.5
マダガスカル	2001-2002	5	2型	有	2.5
中国 (貴州省)	2004	2	1型	無	1.0
マダガスカル	2005	3	2型	有	1.5
インドネシア	2005	46	1型	有	1.5
カンボジア	2005-2006	2	3型	有	2.0
ミャンマー	2006-2007	5	1型	有	2.0
エチオピア	2008-2009	4	2型	?	1.0
コンゴ民主共和国	2008-2009	16	2型	?	4.0?
インド(西部UP**)	2009-2010	12	2型	有	1.0
ナイジェリア	2005-2010	307	2型	有	5.0

*非ポリオエンテロウイルスとのゲノム遺伝子組換えの有無

** Uttar Pradesh 州

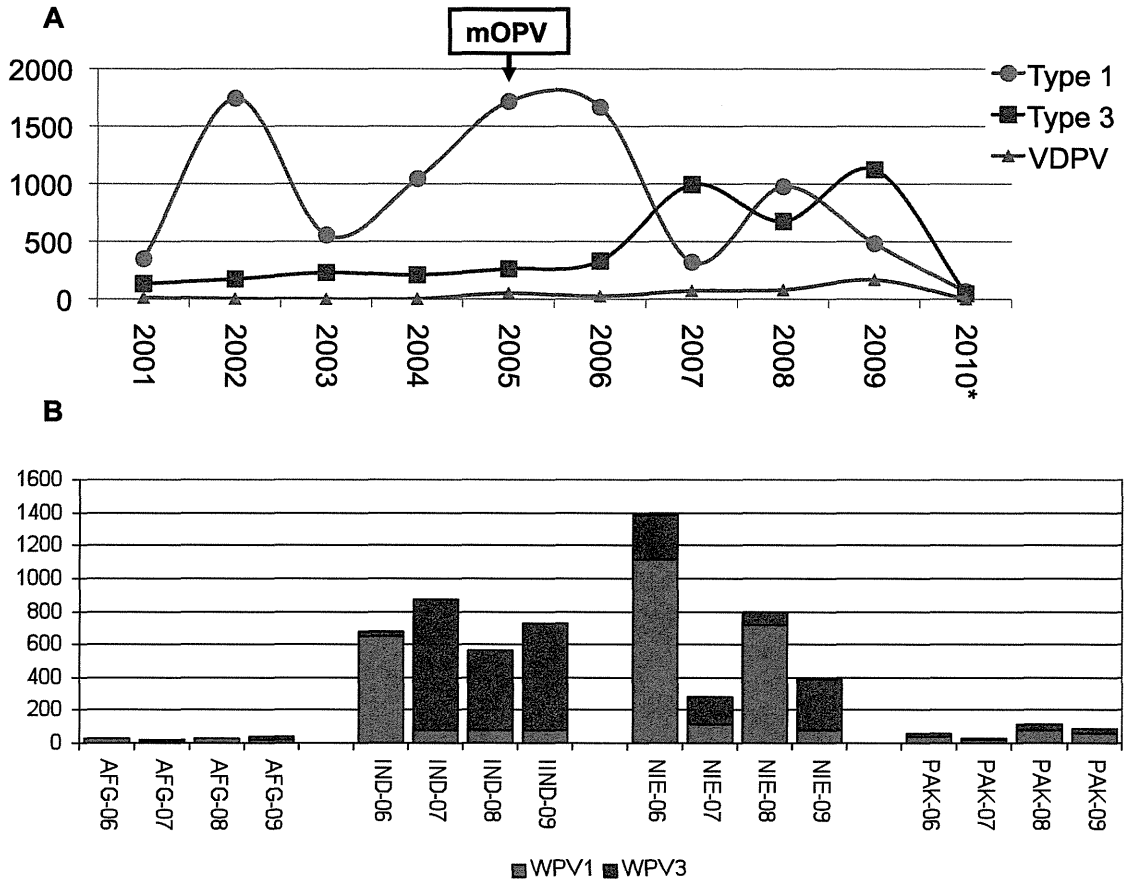

図2 1型，3型野生株，およびVDPVによるポリオ症例数

(A) 2001 ～ 2010年におけるポリオ症例数を，1型および3型野生株，VDPVに分けて示した．(B)野生株ポリオ常在国4カ国(アフガニスタン(AFG)，インド(IND)，ナイジェリア(NIE)，パキスタン(PAK))における1型および3型野生株によるポリオ症例数の推移(2006 ～ 2009年)(WHO提供資料を一部改変)．

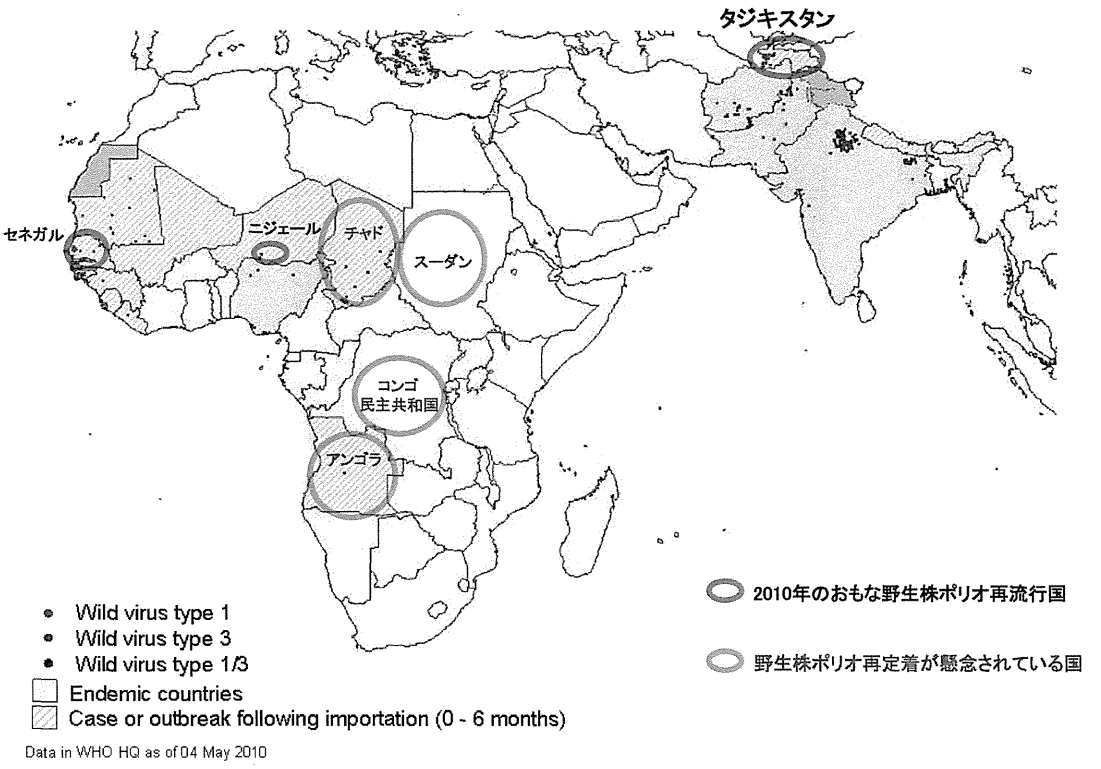

図3 確定ポリオ症例の分布

2009年11月5日～2010年5月4日の半年間における1型野生株(赤)および3型野生株(青)由来ポリオ症例の分布．ひとつのドットが1症例を示す（WHO提供資料を一部改変）．

る1型および3型野生株ポリオウイルス伝播の継続と2型VDPV流行の発生は，1型および3型mOPVを用いたSIAスケジュール至適化の困難さを改めて示した．そのため，インド北部では，2010年から1型および3型弱毒化株を含むbivalent OPV（bOPV）が導入された．1型および3型弱毒株を含むbOPVは，tOPVと比較すると，1型および3型ポリオウイルスに対して効果的に免疫を誘導し，mOPVとほぼ同等の有効性を示すとされている（**図4**）．bOPV導入による，よりシンプルなSIA戦略により，1型および3型野生株ポリオウイルス伝播を同時にコントロールすることが期待されている．

2）ナイジェリア

ナイジェリアは，2005年以降，アフリカ唯一の野生株ポリオ常在国であるとともに，周辺国を介して多くのアフリカ・アジア諸国へ，断続的に野生株ポリオウイルスを輸出し続けていることが問題視されている．インドとは異なり，ナイジェリア北部における野生株ポリオウイルス伝播継続のおもな要因は，もともと脆弱な公衆衛生基盤による低い定期予防接種率とポリオ根絶計画へのコミットメントの低さによる不完全なSIAキャンペーンにあるとされてきた．ナイジェリア北部では，2006-2007年のmOPV導入後も1型および3型野生株ポリオウイルス伝播が継続しており，2005年以降，2型VDPVの長期伝播によるポリオ流行が，野生株流行地域とほぼ同じ地域で継続している（**表1**）[9]．2005年と比較すると顕著な改善が認められるが，2007年時点での北部および北西部における4回以上OPV接種率は32%以下と推定されている[10]．

とくに地方政府レベルでのポリオ根絶計画へのコミットメント強化のために行われた2009年2月の“Abuja Commitments to Polio”を含む様々な取り組みにより，ナイジェリア北部でのワクチン接種指標に明らかな改善が認められている（**図5**）．その結果を反映し，2009年後半以降，ナイジェリア北部の野生株ポリオ流行は沈静化しつつある．2010年のナイジェリアのポリオ確定症例は，いまのところ3型野生株による2例のみであり，1型野生株症例は2009年10月以来，半年以上報告されていない（**図5**）．2型VDPVによるポリオ症例も，2009年の153症例と比較して2010年は1例のみとなっている．ニジェール，チャド等，ナイジェリアの近隣国では，2010年になって1型あるいは3型野生株による新たなポリオ症例発生が報告されており（**図3**），にわかに楽観視は出来ないが，アフリカにおけるポリオ根絶の進展にとって，ナイジェリア国内のポリオ症例数の顕著な減少には大きな期待が持たれる．

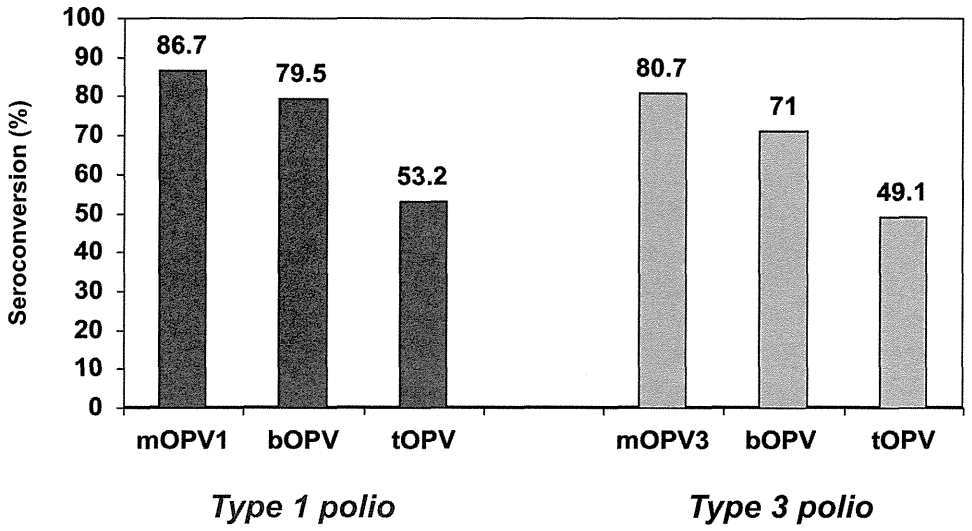

図4　ポリオ流行地における bOPV の有効性の検討
インドのポリオ流行地における1型および3型ポリオウイルスに対する OPV の有効性の比較．mOPV, bOPV あるいは tOPV を2回接種した後の，1型あるいは3型ポリオウイルスに対する中和抗体陽性率(%)，(WHO 提供資料を一部改変)．

3）パキスタンおよびアフガニスタン

国境を接するパキスタンおよびアフガニスタンでは，共通のウイルス遺伝子型の1型および3型野生株ポリオウイルス伝播が継続しており，パキスタン/アフガニスタンは，インドおよびアフリカとは独立した大きなひとつの野生株ポリオ流行地域となっている．2006年以降，パキスタン/アフガニスタンではポリオ症例数の顕著な減少は認められず，とくに2008年以降，症例数および伝播地域の増加傾向が認められる[11]．この地域における野生株ポリオウイルス伝播継続の大きな要因は，以前より指摘されている通り，不安定な政情と悪化する治安により，一部ハイリスク地域において効果的な SIA が実施できないことにある．また，定期予防接種率の低さ，接種現場へのワクチン供給システムの不備，不十分な SIA のモニタリング，国際的なプロジェクトに対して非協力的な一部勢力の存在等，様々な問題点が指摘されている[12]．一部ポリオ流行地域では，インド北部同様，OPV 頻回接種にも関わらずポリオを発症する症例が報告されており，パキスタンでも OPV の有効性についての検証が必要とされている．

4）野生株ポリオ常在国に由来するポリオ再流行

2000年以降，いったんポリオフリーを達成した多くの国々で，野生株ポリオ流行国に由来するポリオ再流行が頻繁に発生している．2009年に限っても，西アフリカを中心とした19カ国において，1型あるいは3型野生株ポリオウイルスよるポリオ再流行が発生しており，1型野生株ポリオウイルスは，2010年にはこれまで10年間ポリオフリーを維持していたアフリカ最西端のセネガルまで達した（**図3**）．度重なるポリオ再流行の発生と広範な地域への野生株ポリオウイルスの伝播は，ポリオ再流行発生と outbreak response のモグラたたきのような繰り返しという点で，アフリカにおけるポリオ根絶計画にとって大きな負担となっている[1,9]．アンゴラ，チャド，スーダン，コンゴ民主共和国等，公衆衛生基盤の脆弱な一部のアフリカ諸国では，輸入野生株ポリオウイルスが長期間定着する傾向が認められ，再定着国から周辺国への野生株ポリオウイルス伝播が報告されている．2009年後半以降のナイジェリア北部における野生株ポリオ症例数の減少は，この10年続いたナイジェリアからの野生株ポリオ輸出の大元を断つ意味で期待が持たれるが，アフリカ全体のポリオ根絶達成のためには，サーベイランスおよび outbreak response 体制の再構築を含めた野生株ポリオ再定着国に対する対策が，今後より重要となると考えられている．

ワクチン由来ポリオウイルスによるポリオ流行

2000-2001年にかけて，1型 VDPV 伝播による大規模なポリオ流行が，ヒスパニオーラ島で初めて報告されて以来，VDPV によるポリオ流行の発生は，様々な地域で毎年のように報告されており，野生株ポリオ根絶前後におけるリスク要因として重要視されるに至った[13-18]．この10年間に世界各地で発生したポリオ流行事例に由来する VDPV 分離株のウイルス学的解析から得られた知見は数多い．なぜなら，ポリオウイルスワクチン株（Sabin 株）はすでに全塩