nature medicine

Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71

Yorihiro Nishimura[1], Masayuki Shimojima[2], Yoshio Tano[1,3], Tatsuo Miyamura[1], Takaji Wakita[1] & Hiroyuki Shimizu[1]

Enterovirus 71 (EV71) is a major causative agent of hand, foot and mouth disease (HFMD), a common febrile disease occurring mainly in young children. Although clinical manifestations of HFMD are usually mild and self limiting, a severe EV71 outbreak can lead to a diverse array of neurological diseases. Identification of the specific cellular receptors is crucial for elucidating the mechanism of early virus-host interactions and the pathogenesis of enteroviruses[1]. Here we identify human P-selectin glycoprotein ligand-1 (PSGL-1; CD162), a sialomucin membrane protein expressed on leukocytes that has a major role in early stages of inflammation[2–4], as a functional receptor for EV71 using an expression cloning method by panning[5]. The N-terminal region of PSGL-1 binds specifically to EV71. Stable PSGL-1 expression allowed EV71 entry and replication, and development of cytopathic effects in nonsusceptible mouse L929 cells. Five out of eight EV71 strains bound soluble PSGL-1 and used intact PSGL-1 as the primary receptor for infection of Jurkat T cells. Three other EV71 strains did not use PSGL-1, suggesting the presence of strain-specific replication of EV71 in leukocytes. EV71 replicated in nonleukocyte cell lines in a PSGL-1–independent manner, indicating the presence of alternative receptor(s) for EV71. The identification of PSGL-1 as a receptor for EV71 sheds new light on a role for PSGL-1–positive leukocytes in cell tropism and pathogenesis during the course of HFMD and other EV71-mediated diseases.

EV71 and coxsackievirus A16 (CVA16) belong to the human enterovirus species A of the genus *Enterovirus*[6] and are the major causative agents of HFMD. EV71 may cause various neurological diseases, such as aseptic meningitis, acute flaccid paralysis and fatal neurogenic pulmonary edema. Severe EV71 outbreaks have been reported periodically throughout the world, representing a major public health concern, particularly in the Asia-Pacific region[7–9]. During large outbreaks of EV71, individuals with severe EV71-associated encephalitis and neurogenic pulmonary edema showed a marked depletion of T cells and high levels of proinflammatory cytokines[10,11]. Because of this T cell involvement, we generated a retroviral complementary DNA (cDNA) library from Jurkat T cells that are susceptible to EV71 infection[12] and used it for expression cloning[5] to identify a receptor that specifically binds EV71 virions (EV71-1095 strain, **Supplementary Table 1**). Transduction of mouse myeloma P3X63Ag8U.1 (P3U1) cells with the Jurkat cDNA library resulted in the formation of four colonies that bound EV71-1095–coated dishes, all of which encoded human PSGL-1 (*SELPLG*) (**Fig. 1a** and **Supplementary Fig. 1**).

To confirm the specific binding of PSGL-1 to EV71-1095, we used 293T cells, which express undetectable amounts of endogenous PSGL-1. Transient expression of human PSGL-1 on 293T cells markedly increased the binding of EV71-1095 to 293T cells (**Fig. 1b**); however, expression of control ligands (sialomucin proteins CD34 and CD43) and mouse Psgl-1 did not (**Supplementary Fig. 2**). To identify the region of human PSGL-1 that is important for EV71 binding, we first constructed a chimeric PSGL-1 (hmPSGL-1) containing amino acids 1–61 of the human PSGL-1 followed by amino acids 63–397 of mouse Psgl-1 (**Supplementary Fig. 3a,b**). EV71-1095 bound hmPSGL-1 expressed on 293T cells (**Supplementary Fig. 3c**). To confirm this finding, we examined whether monoclonal antibodies (mAbs) recognizing PSGL-1 (KPL1 (refs. 13,14) and PL2 (ref. 15); **Fig. 1a**) block the PSGL-1–EV71 interaction. KPL1, which blocks P-selectin binding to PSGL-1 (ref. 13), inhibited EV71-1095 binding to 293T cells transiently expressing PSGL-1 in a dose-dependent manner; however, PL2, which does not block binding to P-selectin, did not (**Fig. 1c**). These findings suggest that the N-terminal region (amino acids 42–61) of human PSGL-1 is crucial for interactions with EV71-1095.

To test whether PSGL-1 is involved in the later steps of viral entry after binding to cells, we used mouse cells that do not support EV71 infection. Mouse myeloma P3U1 cells expressing human PSGL-1 did not support efficient EV71 replication (data not shown). Therefore, we obtained an L929 cell clone (L-PSGL-1.1) that stably expresses high amounts of human PSGL-1 (**Fig. 2a**). L-PSGL-1.1 cells showed a cytopathic effect 4 d after infection, and we detected EV71 antigen by immunofluorescence in the infected L-PSGL-1.1 cells (**Fig. 2b**). The development of cytopathic effect and detection of viral antigens induced by EV71-1095 infection was markedly inhibited by KPL1 (**Fig. 2b**), but not by PL2 (data not shown), as were the replication kinetics of EV71-1095 in infected L-PSGL-1.1 cells (**Fig. 2c,d**),

[1]Department of Virology II, National Institute of Infectious Diseases, Musashimurayama-shi, Tokyo, Japan. [2]Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan. [3]Present address: Japan Poliomyelitis Research Institute, Higashimurayama-shi, Tokyo, Japan. Correspondence should be addressed to H.S. (hshimizu@nih.go.jp).

Received 2 December 2008; accepted 6 April 2009; published online 21 June 2009; doi:10.1038/nm.1961

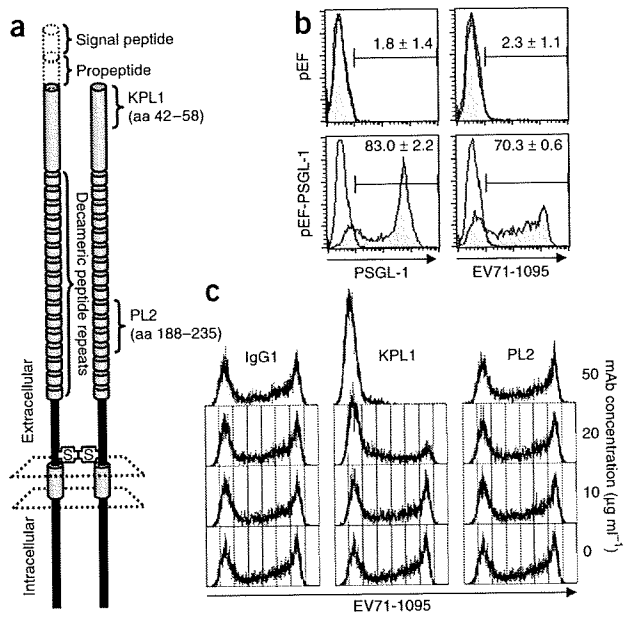

Figure 1 EV71-1095 binds human PSGL-1 expressed on 293T cells. (**a**) Schematic structure of human PSGL-1. The binding sites of PSGL-1–specific mAbs (KPL1 (ref. 14) and PL2 (ref. 15)) are indicated. aa, amino acids. (**b**) Flow cytometric analysis of 293T cells transfected with pEF-PSGL-1 or pEF (a control plasmid). Left, shaded and open areas represent staining with KPL1 mAb and isotype control, respectively. Right, shaded and open areas represent EV71-1095 binding assay and binding control with the mock-infected culture supernatant, respectively. The percentage of PSGL-1– or EV71-positive cells is indicated (mean ± s.d., $n = 3$). (**c**) EV71-1095 binding inhibition assay by flow cytometry. Dose-dependent inhibition of EV71-1095 binding to 293T cells transiently expressing PSGL-1 by mAb.

indicating PSGL-1–dependent EV71 replication in these cells. Furthermore, pretreatment of EV71-1095 with a soluble form of recombinant PSGL-1 (PSGL-1–Fc) impaired viral replication 2–4 d after viral inoculation of L-PSGL-1.1 cells (**Fig. 2e**) and RD cells (**Supplementary Table 2**) expressing undetectable PSGL-1 (data not shown), suggesting that the inhibitory effect of PSGL-1–Fc is due to either direct binding to EV71 virions or virion uncoating induced by the PSGL-1–Fc–EV71 interaction.

To elucidate the biological role of PSGL-1–dependent cell tropism of EV71-1095, we investigated the relationship between PSGL-1 expression on the cell surface (**Fig. 3a**) and replication of EV71 (**Fig. 3b**) in various cell lines (**Supplementary Table 2**). Among the four leukocyte cell lines examined, we found PSGL-1–dependent viral replication, as indicated by the reduction of viral titers in the presence of KPL1 in Jurkat T cells and U937 monocytic cells, which express large amounts of PSGL-1 on the cell surface. EV71-1095 induced a faint cytopathic effect in Jurkat T cells but no apparent cytopathic effect in the other leukocyte cell lines (data not shown). EV71-1095 replication was not affected by KPL1 in MT-2 cells and four nonleukocyte cell lines (RD, HEp-2c, SK-N-MC and Vero) that have little or no PSGL-1 expression, suggesting PSGL-1–independent replication through unidentified receptors in MT-2 and nonleukocyte cells. Taken together, these results suggest that EV71-1095 can use PSGL-1 as a functional cellular receptor in PSGL-1–positive leukocytes (**Supplementary Fig. 1a**), but it may use alternative mechanism(s) for replication in cells that have little or no PSGL-1 expression (**Supplementary Fig. 1b**).

Although the association of a specific genogroup with severe neurological diseases has not been identified through molecular epidemiological analyses of previous and recent EV71 isolates (genogroups A, B and C)[9,16], *in vitro* and *in vivo* phenotypes may be associated with certain amino acid determinants of EV71. Using eight representative EV71 strains (**Supplementary Table 1**), we investigated the strain specificity of EV71 and PSGL-1 use. We first examined the direct biochemical interaction between EV71 strains and PSGL-1–Fc by co-immunoprecipitation. The VP1 proteins of the SK-EV006, C7/Osaka, KED005, 1095 and 75-Yamagata strains of EV71 immunoprecipitated with PSGL-1–Fc (**Fig. 4a**). In contrast, the VP1 proteins of the BrCr, Nagoya and 02363 strains of EV71 did not immunoprecipitate with PSGL-1–Fc (**Fig. 4a**). Thus, these EV71 strains can be classified into two distinct phenotypes according to their PSGL-1–binding capability, regardless of genogroup: five PSGL-1–binding strains (EV71-PB; SK-EV006 (genogroup B3), C7/Osaka (B4), KED005 (C1), 1095 (C2) and 75-Yamagata (C4)) and three PSGL-1–nonbinding strains (EV71–non-PB; BrCr (A), Nagoya (B1) and 02363 (C1)). We then tested the PSGL-1–dependent replication competence of EV71-PB and EV71–non-PB strains in Jurkat T cells

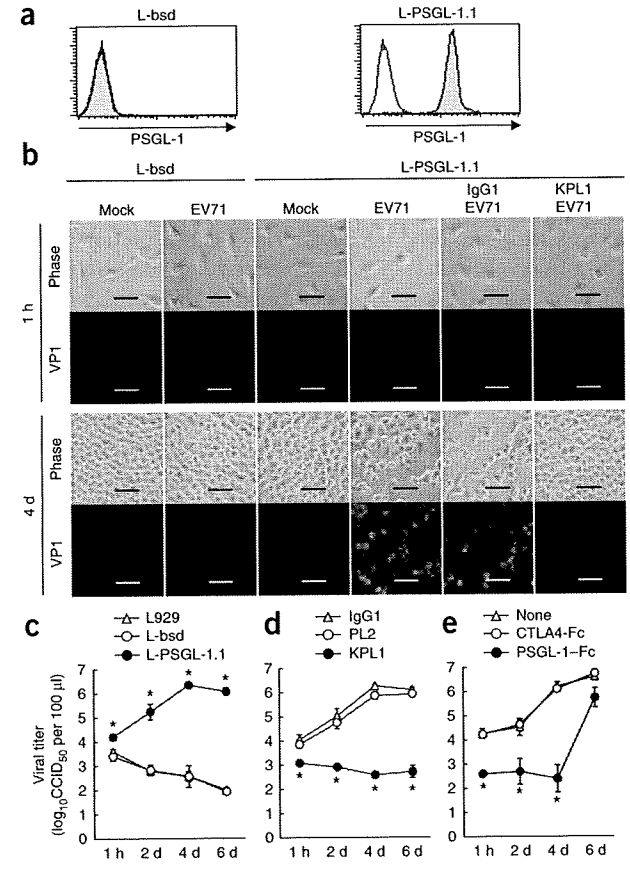

Figure 2 Stable expression of human PSGL-1 on mouse L929 cells permits infection by EV71-1095. (**a**) PSGL-1 expression on L-PSGL-1.1 and L-bsd (PSGL-1 negative control) cells, as measured by flow cytometry. (**b**) Development of cytopathic effect (Phase) and EV71 antigens by immunofluorescence (VP1). Scale bars, 100 μm. (**c**) Replication kinetics of EV71-1095 in L-PSGL-1.1 and PSGL-1–negative cells. (**d**) Replication kinetics of EV71-1095 in L-PSGL-1.1 cells in the presence of PSGL-1–specific (KPL1 and PL2) and control mAbs. (**e**) Replication kinetics of EV71-1095 in L-PSGL-1.1 cells after treatment with soluble PSGL-1. Viral titers and error bars are indicated as the means ± s.d. of triplicate analyses. Asterisks indicate $P < 0.01$ compared to the two controls.

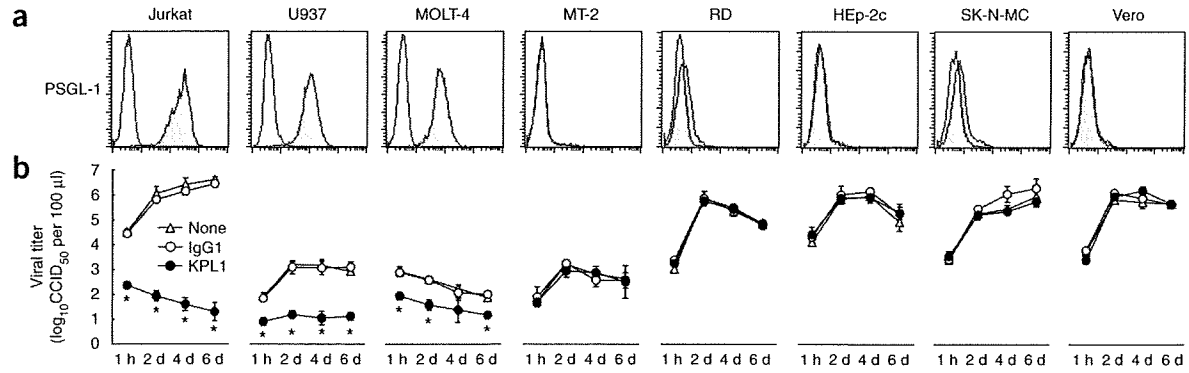

Figure 3 PSGL-1 expression and EV71-1095 replication kinetics in leukocyte and nonleukocyte cell lines. (**a**) PSGL-1 expression on the cell surface, as measured by flow cytometry. The shaded and open areas represent staining with PSGL-1–specific mAb (KPL1) and isotype control, respectively. (**b**) EV71-1095 replication kinetics in the presence of KPL1 and control mAbs. The titers and error bars are the means ± s.d. of triplicate analyses. Asterisks indicate $P < 0.01$ compared to the two controls.

(**Fig. 4b**). All five EV71-PB strains replicated in Jurkat T cells in a PSGL-1–dependent manner, as indicated by the reduction of viral titers in the presence of KPL1 at 4 d after inoculation (**Fig. 4b**). Among the three EV71–non-PB strains, two (Nagoya and 02363) replicated in Jurkat T cells in a PSGL-1–independent manner, and the BrCr strain replicated poorly (**Fig. 4b**). These data indicate that EV71-PB strains use PSGL-1 as the primary and functional receptor for infection of Jurkat T cells (**Supplementary Fig. 1a**), whereas some of the EV71–non-PB strains may use unidentified receptors for viral replication even in PSGL-1–positive T lymphocytes (**Supplementary Fig. 1c**). Although the KED005 and 02363 strains (genogroup C1) show distinct differences in their PSGL-1–binding phenotypes, only four amino acids (Ile55 of VP3 and Lys98, Glu145 and Ile262 of VP1 for the EV71-02363 strain) are different in the proteins comprising the entire capsid region of these two strains, suggesting that a few amino acid determinants in the capsid proteins are fully responsible for the phenotype (**Fig. 4**). Among the four amino acid differences, Glu145 of VP1 may be exposed on a surface-exposed loop in the capsid VP1 protein, and this has been identified as a major virulence determinant in mouse models[17,18].

We next investigated PSGL-1–dependent replication of the prototype CVA16-G10 strain. This strain replicated in L-PSGL-1.1 cells but not in L-bsd cells (a PSGL-1–negative control), and virus replication in L-PSGL-1.1 cells was inhibited by KPL1 (**Supplementary Fig. 4a,b**). The CVA16-G10 strain used PSGL-1 to infect L-PSGL-1.1 cells but not Jurkat T cells (**Supplementary Fig. 4c**), suggesting that CVA16-G10 represents a strain that uses an alternative entry mechanism via unidentified functional receptors for infection of Jurkat T cells.

PSGL-1 expressed on leukocytes has a crucial role in the tethering and rolling of leukocytes during recruitment of cells from blood vessels to the sites of acute inflammation upon stimulation by infection[2–4]. The tissue expression of PSGL-1 is primarily restricted to hematopoietic myeloid, lymphoid and dendritic lineages and to platelets. However, PSGL-1 is also expressed on the dendritic cells of lymph nodes and on macrophages in the intestinal mucosa[2], which could be the primary site of EV71 replication after viral ingestion. During the viremic phase of infection, a variety of circulating leukocytes expressing PSGL-1 may be responsible for the *in vivo* replication of EV71 (ref. 19) and subsequent EV71-induced apoptosis in the infected cells[12,20], possibly resulting in the T cell depletion and changes in cytokine levels observed in severe

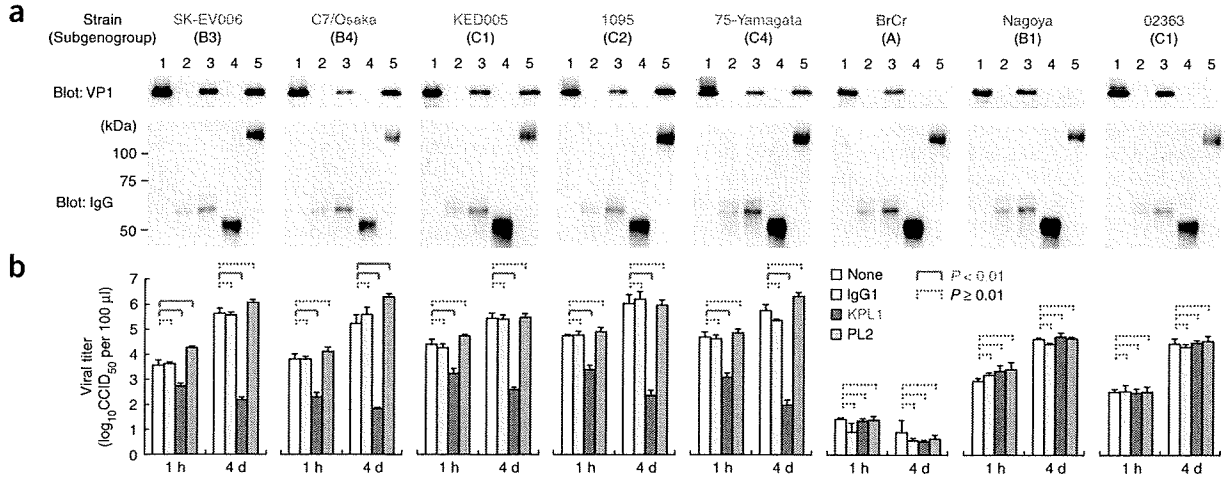

Figure 4 EV71 strain–specific binding to PSGL-1 and replication in Jurkat T cells. The strains that are EV71-PB and EV71–non-PB are indicated in red and blue, respectively. (**a**) Western blot showing the EV71 VP1 protein immunoprecipitated with PSGL-1–Fc. Concentrated viruses (lane 1) were incubated with isotype control mAb (lane 2), EV71-VP1–specific mAb (MA105, lane 3), negative control recombinant protein (CTLA4-Fc, lane 4) or PSGL-1–Fc (lane 5) and immunoprecipitated. (**b**) Viral replication in Jurkat T cells incubated with PSGL-1–specific (KPL1 and PL2) and control mAbs. Titers are expressed as the mean, and error bars indicate s.d. of triplicate analyses.

encephalitis cases with pulmonary edema[10,11]. In addition, the distribution and recruitment of PSGL-1–positive Langerhans cells and lymphocytes in inflamed skin[2] are consistent with the apparent HFMD pathogenesis associated with EV71 and CVA16 infection, which is characterized by acute skin inflammation. In this regard, our findings suggest the involvement of PSGL-1–positive inflammatory cells during the course of EV71 and CVA16 infection.

Although EV71 infects neurons and causes acute brainstem encephalitis, paralysis or both in humans[21,22] and experimental animals[17,23–25], PSGL-1 is apparently not expressed in the adult human brain[2]. Consistent with this observation, we have shown that EV71-1095 may also use PSGL-1–independent mechanism(s) for replication in nonleukocyte cells, including SK-N-MC neuroblastoma cells. Furthermore, CVA16, another causative agent of HFMD, can also use PSGL-1 as a functional receptor in L-PSGL-1.1 cells. Thus, PSGL-1–dependent viral replication is unlikely to be directly responsible for the neuronal cell apoptosis induced by EV71 (refs. 26,27). However, we cannot exclude the possible involvement of PSGL-1–positive inflammatory cells in the pathogenesis of HFMD and a variety of EV71 diseases with or without neurological manifestations.

We have shown that human PSGL-1 is a functional cellular receptor for EV71 infection. The occurrence of severe EV71 infection with a number of fatal encephalitis cases continues to be a major public health threat even now[28], but, currently, no vaccines or specific antiviral agents are available for EV71. Because soluble PSGL-1 inhibits EV71 replication, it may be a potential inhibitor of EV71-PB infection. The identification of PSGL-1 as a functional EV71 receptor on leukocytes is the first major step in elucidating EV71 pathogenesis at the molecular level. However, other EV71 and human enterovirus species A receptors on leukocyte and nonleukocyte cells may also have key implications in EV71 tropism and pathogenesis, particularly for severe central nervous system diseases. Further structural and functional analyses of interactions of EV71 with PSGL-1 and other unidentified receptors may provide new therapeutic approaches for the treatment of severe EV71 diseases.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank N. Takeda, S. Morikawa, Y. Matsuura, K. Moriishi, S. Koike, S. Yamayoshi and Y. Izumiya for helpful discussions; Y. Ami for technical advice regarding FACS; and N. Nishimura for preparing figures. We also thank M. Sinniah, M. Yusof (Institute for Medical Research, Malaysia) for providing EV71-SK-EV006 and KED005, N. Onnimala, Y. Pongsuwanna (National Institute of Health, Thailand) for providing EV71-02363, Y. Okuno (Osaka Prefectural Institute of Public Health) for providing EV71-C7/Osaka, K. Mizuta (Yamagata Prefectural Institute of Public Health) for providing EV71-75-Yamagata, A. Makino, Y. Tohya, H. Akashi (University of Tokyo) for providing P3U1 cells, H. Sakata (National Institute of Infectious Diseases, Japan) for providing L929 cells, and H. Shirato (National Institute of Infectious Diseases, Japan) for providing MOLT-4 and MT-2 cells. We are grateful to J. Wada for technical assistance. This work was supported by a Grant-in-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Y.N.). Y.N. and H.S. were supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases and a Grant-in-Aid for the Promotion of Polio Eradication, from the Ministry of Health, Labour and Welfare, Japan.

AUTHOR CONTRIBUTIONS

Y.N. designed and performed experiments, analyzed data and wrote the paper; M.S. improved the expression cloning method; Y.T. prepared and characterized EV71-specific mAbs; and T.M. and T.W. analyzed data and wrote the paper. H.S. planned the project, designed experiments, analyzed data and wrote the paper. All authors discussed the results and commented on the manuscript.

Published online at http://www.nature.com/naturemedicine/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Rossmann, M.G., He, Y. & Kuhn, R.J. Picornavirus-receptor interactions. *Trends Microbiol.* **10**, 324–331 (2002).
2. Laszik, Z. *et al.* P-selectin glycoprotein ligand-1 is broadly expressed in cells of myeloid, lymphoid, and dendritic lineage and in some nonhematopoietic cells. *Blood* **88**, 3010–3021 (1996).
3. Sako, D. *et al.* Expression cloning of a functional glycoprotein ligand for P-selectin. *Cell* **75**, 1179–1186 (1993).
4. Somers, W.S., Tang, J., Shaw, G.D. & Camphausen, R.T. Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe^X and PSGL-1. *Cell* **103**, 467–479 (2000).
5. Shimojima, M. *et al.* Usage of myeloma and panning in retrovirus-mediated expression cloning. *Anal. Biochem.* **315**, 138–140 (2003).
6. Oberste, M.S., Maher, K., Kilpatrick, D.R. & Pallansch, M.A. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. *J. Virol.* **73**, 1941–1948 (1999).
7. Chan, L.G. *et al.* Deaths of children during an outbreak of hand, foot and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. *Clin. Infect. Dis.* **31**, 678–683 (2000).
8. Ho, M. *et al.* An epidemic of enterovirus 71 infection in Taiwan. *N. Engl. J. Med.* **341**, 929–935 (1999).
9. McMinn, P.C. An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS Microbiol. Rev.* **26**, 91–107 (2002).
10. Lin, T.Y., Hsia, S.H., Huang, Y.C., Wu, C.T. & Chang, L.Y. Proinflammatory cytokine reactions in enterovirus 71 infections of the central nervous system. *Clin. Infect. Dis.* **36**, 269–274 (2003).
11. Wang, S.M. *et al.* Pathogenesis of enterovirus 71 brainstem encephalitis in pediatric patients: roles of cytokines and cellular immune activation in patients with pulmonary edema. *J. Infect. Dis.* **188**, 564–570 (2003).
12. Chen, L.C. *et al.* Enterovirus 71 infection induces Fas ligand expression and apoptosis of Jurkat cells. *J. Med. Virol.* **78**, 780–786 (2006).
13. Snapp, K.R. *et al.* A novel P-selectin glycoprotein ligand-1 monoclonal antibody recognizes an epitope within the tyrosine sulfate motif of human PSGL-1 and blocks recognition of both P- and L-selectin. *Blood* **91**, 154–164 (1998).
14. Thatte, A. *et al.* Binding of function-blocking mAbs to mouse and human P-selectin glycoprotein ligand-1 peptides with and without tyrosine sulfation. *J. Leukoc. Biol.* **72**, 470–477 (2002).
15. Li, F. *et al.* Visualization of P-selectin glycoprotein ligand-1 as a highly extended molecule and mapping of protein epitopes for monoclonal antibodies. *J. Biol. Chem.* **271**, 6342–6348 (1996).
16. Brown, B.A., Oberste, M.S., Alexander, J.P. Jr., Kennett, M.L. & Pallansch, M.A. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. *J. Virol.* **73**, 9969–9975 (1999).
17. Arita, M., Ami, Y., Wakita, T. & Shimizu, H. Cooperative effect of the attenuation determinants derived from poliovirus Sabin 1 strain is essential for attenuation of enterovirus 71 in the NOD/SCID mouse infection model. *J. Virol.* **82**, 1787–1797 (2008).
18. Chua, B.H., Phuektes, P., Sanders, S.A., Nicholls, P.K. & McMinn, P.C. The molecular basis of mouse adaptation by human enterovirus 71. *J. Gen. Virol.* **89**, 1622–1632 (2008).
19. Kung, C.M. *et al.* Differences in replication capacity between enterovirus 71 isolates obtained from patients with encephalitis and those obtained from patients with herpangina in Taiwan. *J. Med. Virol.* **79**, 60–68 (2007).
20. Kuo, R.L., Kung, S.H., Hsu, Y.Y. & Liu, W.T. Infection with enterovirus 71 or expression of its 2A protease induces apoptotic cell death. *J. Gen. Virol.* **83**, 1367–1376 (2002).
21. Hsueh, C. *et al.* Acute encephalomyelitis during an outbreak of enterovirus type 71 infection in Taiwan: report of an autopsy case with pathologic, immunofluorescence and molecular studies. *Mod. Pathol.* **13**, 1200–1205 (2000).
22. Wong, K.T. *et al.* The distribution of inflammation and virus in human enterovirus 71 encephalomyelitis suggests possible viral spread by neural pathways. *J. Neuropathol. Exp. Neurol.* **67**, 162–169 (2008).
23. Nagata, N. *et al.* Pyramidal and extrapyramidal involvement in experimental infection of cynomolgus monkeys with enterovirus 71. *J. Med. Virol.* **67**, 207–216 (2002).
24. Nagata, N. *et al.* Differential localization of neurons susceptible to enterovirus 71 and poliovirus type 1 in the central nervous system of cynomolgus monkeys after intravenous inoculation. *J. Gen. Virol.* **85**, 2981–2989 (2004).
25. Ong, K.C. *et al.* Pathologic characterization of a murine model of human enterovirus 71 encephalomyelitis. *J. Neuropathol. Exp. Neurol.* **67**, 532–542 (2008).
26. Chang, S.C., Lin, J.Y., Lo, L.Y.C., Li, M.L. & Shih, S.R. Diverse apoptotic pathways in enterovirus 71–infected cells. *J. Neurovirol.* **10**, 338–349 (2004).
27. Chen, C.S. *et al.* Retrograde axonal transport: a major transmission route of enterovirus 71 in mice. *J. Virol.* **81**, 8996–9003 (2007).
28. World Health Organization. Outbreak news. Enterovirus, China. *Wkly. Epidemiol. Rec.* **83**, 169–170 (2008).

ONLINE METHODS

Cells. L929 cells were generously provided by H. Sakata P3U1 cells were generously provided by H. Akashi. 293T cells were generously provided by Y. Matsuura. GP2-293 cells were obtained from Clontech. Jurkat cells were obtained from Riken Cell Bank. U937 cells were obtained from the Japanese Collection of Research Biosources. MOLT-4 and MT-2 cells were generously provided by H. Shirato. RD cells were obtained from the US Centers for Disease Control. HEp-2 cells were obtained from the Victorian Infectious Diseases Reference Laboratory. SK-N-MC cells were obtained from DS Pharma Biomedical. Vero cells were obtained from Japan Poliomyelitis Research Institute. We cultured L929, 293T and GP2-293 cells in DMEM (Sigma) supplemented with 10% FCS. We maintained P3U1, Jurkat, U937, MOLT-4 and MT-2 cells in RPMI-1640 medium (Sigma) with 10% FCS. We maintained RD, HEp-2c, SK-N-MC and Vero cells in Eagle's minimal essential medium (Nissui) supplemented with 10% FCS.

Viruses. We propagated all EV71 strains (**Supplementary Table 1**) in RD or Vero cells. Because some of the strains produced diffuse plaques on RD cells, we determined the viral titers by a microtitration assay using 96-well plates and RD cells as previously described[23]. Briefly, we used ten wells for each viral dilution and expressed the viral titers as 50% cell culture infectious dose ($CCID_{50}$).

Monoclonal antibodies and recombinant proteins. We generated the EV71-specific mAbs MA105 (mouse IgG2b) and MA35 (mouse IgG2a) from mice immunized with EV71-1095 (Y. Tano *et al.*, unpublished data). We purchased the EV71-specific mAb 10F0 from Biogenesis. We purchased the mAbs to human CD34 (clone 563), human CD43 (L60), human PSGL-1 (KPL1), and mouse Psgl-1 (4RA10) from BD Biosciences, and we purchased human PSGL-1–specific mAb PL2 from Beckman-Coulter. For negative controls, we purchased human (MOPC-21) and rat IgG1 (R3-34) and human IgG2b (27-35) from BD Biosciences. We used NaN_3-free mAbs, MOPC-21 (BioLegend) and NCG01 (Lab Vision) in cell culture as negative controls. We purchased soluble recombinant forms of human proteins fused to the Fc region of human IgG1 (PSGL-1–Fc and cytotoxic T lymphocyte antigen-4–Fc) from R&D Systems.

Preparation of EV71-1095–coated dishes and selection of Jurkat T cell clones. We incubated a polystyrene Petri dish (Ina-optika) with 10 μg ml^{-1} MA35 in 10 ml of 50 mM Tris-HCl (pH 9.4) at 4 °C overnight. We rinsed the dishes twice with PBS containing 2% FCS (PBS-2FCS) and blocked them with PBS-2FCS at 25 °C for 30 min. We then applied EV71-1095 (10 ml, $1 \times 10^{8.6}$ $CCID_{50}$ ml^{-1}) to the MA35-coated dishes and incubated at 25 °C for 30 min. We replaced the supernatant with a fresh viral preparation, and we repeated replacing the supernatant three times. We fixed the dishes with a viral preparation containing 1% paraformaldehyde for 30 min at 25 °C. Finally, we washed the dishes with PBS-2FCS five times and used them for selection and expression cloning.

We selected Jurkat T cell clones that expressed high levels of EV71-binding molecules on the cell surface with the panning method with EV71-1095–coated dishes. We added Jurkat T cells to the EV71-coated dishes in RPMI-1640 medium and incubated them at 4 °C for 90 min. We removed nonadherent cells by washing with RPMI-1640 medium, and we cultured the adherent cells for a week. We used the Jurkat T cell colonies on the EV71-coated dishes for preparation of the cDNA library.

Retroviral cDNA library. Using the Jurkat T cell colonies on the EV71-coated dishes, we prepared a retroviral cDNA library as previously described[29] with minor modifications. We used the Pantropic Retroviral Expression System (Clontech) to prepare vesicular stomatitis virus G protein–pseudotyped retroviruses. We ligated the Jurkat cDNA with the EcoRI adaptor and cloned it into the EcoRI site of the pLIB retroviral vector (Clontech). We transfected the pLIB plasmid along with a vesicular stomatitis virus G protein expression plasmid into GP2-293 cells with Lipofectamine 2000 (Invitrogen). We harvested the culture supernatant 2 d after transfection and used it to infect P3U1 cells.

Expression cloning of the EV71-binding receptor. We preformed retrovirus-mediated expression cloning using EV71-coated dishes and P3U1 cells as described previously[5]. We isolated genomic DNA from each P3U1 cell colony bound to the EV71-coated dishes, subjected it to PCR amplification with pLIB-specific primers and sequenced the amplification product.

Expression plasmids. We cloned the cDNAs encoding human *SELPLG*, *CD34*, *SPN* and mouse *Selplg* into pEF6-Flag-3S to produce pEF-PSGL-1, pEF-CD34, pEF-CD43 and pEF-mPsgl-1, respectively. For hmPSGL-1 expression, we constructed chimeric *SELPLG* cDNA by PCR and cloned it into pEF6-Flag-3S. The primers used for cDNA cloning and chimeric protein construction and mutation are shown in **Supplementary Tables 3** and **4**. More details are provided in the **Supplementary Methods**.

Detection of cell surface molecules by flow cytometry. We washed cells once with flow cytometry buffer (PBS supplemented with 2 mM EDTA, 2% FCS and 0.1% NaN_3) and incubated them with mAb on ice for 30 min. After washing with flow cytometry buffer, we incubated the cells with secondary antibodies conjugated to Alexa Fluor 488 (Invitrogen). We washed and analyzed the cells by FACSCalibur (BD Biosciences).

EV71-binding assay by flow cytometry. We collected 293T cells 24 h after transfection with expression plasmids, washed them once with flow cytometry buffer and incubated them 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 flow cytometry buffer. In the binding inhibition assay with mAbs, we treated cells with mAb for 30 min on ice before exposing them to the virus. We washed the cells and stained them with Alexa Fluor 488–conjugated MA105, washed them and then analyzed them by FACSCalibur.

Establishment of the L-PSGL-1.1 cell line. To establish a mouse L929 cell line expressing human PSGL-1, we transfected L929 cells with pEF-PSGL-1 and selected stable transfectants with 5 μg ml^{-1} blasticidin S HCl (Invitrogen). Through a limiting dilution of the various cell colonies, we selected three of the 16 cell lines on the basis of high PSGL-1 expression. Finally, we established L-PSGL-1.1 as the single cell clone that supported the most efficient EV71-1095 replication. As a PSGL-1–negative control, we also established an L-bsd cell line by transfecting L929 cells with pEF6-Flag-3S followed by selection with 5 μg ml^{-1} blasticidin.

Immunofluorescence microscopy. We fixed cells with 4% paraformaldehyde in PBS for 30 min at 25 °C, washed them, permeabilized them with PBS containing 1% Triton X-100 for 10 min at 25 °C, blocked them with PBS containing 1% BSA for 10 min at 25 °C and incubated them with VP1-specific mAb 10F0 labeled with Alexa Fluor 488 for 30 min at 37 °C. After a final wash, we analyzed the cells with a fluorescence microscope (Keyence).

Immunoprecipitation and western blotting. We diluted concentrated viruses (0.5 μg VP1 protein) in 300 μl of immunoprecipitation buffer (20 mM Tris-Cl, 135 mM NaCl, 1% Triton X-100 and 10% glycerol; pH 7.4) and incubated them with 1 μg of mAb or chimeric proteins for 2 h at 4 °C. We added Dynabeads Protein G (Invitrogen) and incubated the mixture for an additional 2 h. We washed the beads and subjected the immunoprecipitates to 12.5% SDS-PAGE. For western blotting, we transferred the proteins onto nitrocellulose membranes and blotted with MA105.

Viral infection assays. We inoculated cells (4×10^4 cells per 200 μl in a 48-well plate) with viruses at 10 $CCID_{50}$ per cell for 1 h, washed them and incubated them in medium at 34 °C (for L-PSGL-1.1, Jurkat, U937, MOLT-4 or MT-2 cells) or 37 °C (for RD, HEp-2c, SK-N-MC or Vero cells). For mAb inhibition, we pretreated the cells with 10 μg ml^{-1} mAb for 1 h, washed them and maintained them in the medium with 10 μg ml^{-1} mAb. For inhibition with PSGL-1–Fc, we pretreated EV71-1095 (1×10^5 $CCID_{50}$) with 1 μg PSGL-1–Fc per 100 μl for 1 h and then inoculated them into L-PSGL-1.1 cells. We incubated the cells for 1 h in the presence of PSGL-1–Fc, washed them and maintained them in the absence of PSGL-1–Fc. We subjected the culture supernatants and infected cells to three cycles of freeze-thawing before titration.

Statistical analyses. We carried out all infection assays in triplicate and compared the mean viral titers with Student's *t* test (two-tailed). We considered *P* values of <0.01 statistically significant.

29. Kitamura, T. & Morikawa, Y. Isolation of T-cell antigens by retrovirus-mediated expression cloning. *Methods Mol. Biol.* **134**, 143–152 (2000).

nature medicine

Scavenger receptor B2 is a cellular receptor for enterovirus 71

Seiya Yamayoshi[1,2,5], Yasuko Yamashita[1], Jifen Li[1,5], Nobutaka Hanagata[3,4], Takashi Minowa[3], Taro Takemura[3] & Satoshi Koike[1,5]

Enterovirus 71 (EV71) belongs to human enterovirus species A of the genus *Enterovirus* within the family *Picornaviridae*[1]. EV71, together with coxsackievirus A16 (CVA16), are most frequently associated with hand, foot and mouth disease (HFMD)[1]. Although HFMD is considered a mild exanthematous infection, infections involving EV71, but not CVA16, can progress to severe neurological disease, including fatal encephalitis, aseptic meningitis and acute flaccid paralysis[2]. In recent years, epidemic and sporadic outbreaks of neurovirulent EV71 infections have been reported in Taiwan, Malaysia, Singapore, Japan and China[3–7]. Here, we show that human scavenger receptor class B, member 2 (SCARB2, also known as lysosomal integral membrane protein II or CD36b like-2) is a receptor for EV71. EV71 binds soluble SCARB2 or cells expressing SCARB2, and the binding is inhibited by an antibody to SCARB2. Expression of human SCARB2 enables normally unsusceptible cell lines to support EV71 propagation and develop cytopathic effects. EV71 infection is hampered by the antibody to SCARB2 and soluble SCARB2. SCARB2 also supports the infection of the milder pathogen CVA16. The identification of SCARB2 as an EV71 and CVA16 receptor contributes to a better understanding of the pathogenicity of these viruses.

Virus receptors have essential roles in the early steps of viral infection. They are, therefore, a primary determinant of host range and tissue tropism. EV71 is known as one of the most important pathogens of emerging infectious disease in humans. However, the receptor for EV71 has not been identified yet. Thus, we set out to identify a receptor on human cells for this key pathogen.

RD cells, established from human rhabdomyosarcoma and frequently used to isolate EV71 from clinical specimens, are highly susceptible to EV71. Mouse L929 cells, however, allow only highly inefficient EV71 infection, as we observed only a few viral protein–positive cells (approximately 1 cell in 1×10^5 cells) and no development of cytopathic effects in the cells even when infected at a high multiplicity of infection (MOI) (data not shown). However, we efficiently recovered infectious particles from L929 cells transfected with EV71 genomic RNA (data not shown). These results suggest that the restriction occurs at one of the early steps of infection (attachment, possible internalization or viral uncoating), but not at the replication or assembly steps. Accordingly, we pursued a strategy used in the identification of the poliovirus receptor[8,9]. Briefly, we established L929 transformant (Ltr) cells by transfection of L929 cells with genomic DNA from RD cells. We then detected EV71-susceptible cells via infection with EV71-GFP, which expressed GFP upon infection. Repeated screens led to the generation of two monoclonal cell lines, Ltr051 and Ltr246. We detected a number of GFP-positive cells in Ltr051 and Ltr246 cell cultures upon EV71-GFP infection (**Fig. 1a**). To examine EV71 viral growth kinetics, we infected Ltr051, Ltr246, RD and L929 cells with EV71 strain SK-EV006, isolated from the rectal swab of an individual with encephalitis, at an MOI of 0.01 (**Fig. 1b**). EV71 SK-EV006 grew in Ltr051 cells as efficiently as it did in RD cells (**Fig. 1b**). Ltr246 cells could also support EV71 infection, but the final virus titer was approximately 2.7% of that obtained in Ltr051 cells (**Fig. 1b**). We observed cytopathic effects in Ltr051, Ltr246 and RD cells at 48 h after infection (**Supplementary Fig. 1**). The appearance of cytopathic effects in Ltr246 cells was slower and weaker than in Ltr051 and RD cells (**Supplementary Fig. 1**). Collectively, these results suggest that Ltr051 and Ltr246 cells express a human gene(s) that is essential in the early steps of EV71 infection.

To identify the putative human gene(s) in Ltr051 and Ltr246 cells, we performed a transcriptome analysis comparing the two transformants and L929 cells via human microarray analysis. A total of 14 genes in Ltr051 cells and 33 genes in Ltr246 cells showed a more than twofold increase in expression in Ltr051 or Ltr246 cells compared with L929 cells (**Supplementary Tables 1** and **2**). We selected protein-coding genes with more than fourfold increased signals or putative transmembrane protein-coding genes with more than twofold increased signals for further analysis. To determine whether these 22 selected genes were integrated into the genomes of Ltr051 or Ltr246 cells, we performed PCR with genomic DNA from Ltr051 and Ltr246 cells as the template. We amplified the *SCARB2* gene from the genomic DNA of Ltr051 cells, whereas we amplified the *CCL2*

[1]Department of Microbiology and Immunology, Tokyo Metropolitan Institute for Neuroscience, Tokyo Metropolitan Organization for Medical Research, Tokyo, Japan. [2]Japan Health Sciences Foundation, Tokyo, Japan. [3]Nanotechnology Innovation Center, National Institute for Materials Science, Ibaraki, Japan. [4]Biomaterials Center, National Institute for Materials Science, Ibaraki, Japan. [5]Present addresses: Neurovirology Project, Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, Tokyo, Japan (S.Y. and S.K.) and Center for Translational Medicine, Department of Medicine, Jefferson Medical College, Philadelphia, Pennsylvania, USA (J.L.). Correspondence should be addressed to S.K. (koike-st@igakuken.or.jp).

Received 26 November 2008; accepted 26 May 2009; published online 21 June 2009; doi:10.1038/nm.1992

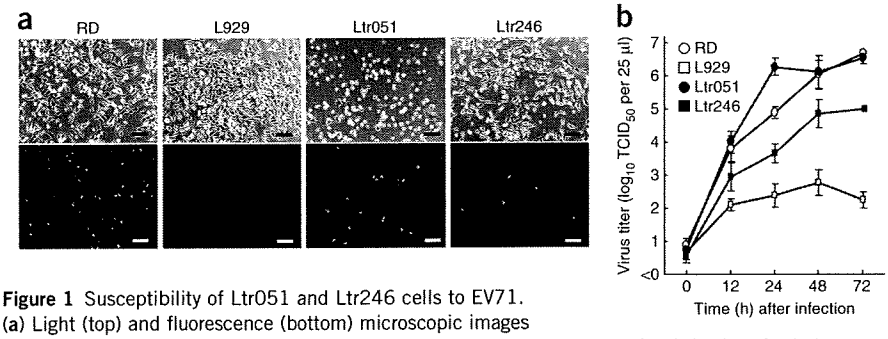

Figure 1 Susceptibility of Ltr051 and Ltr246 cells to EV71. (**a**) Light (top) and fluorescence (bottom) microscopic images of Ltr051, Ltr246, RD and L929 cells infected with EV71-GFP at 24 h after infection. Scale bar, 200 μm. (**b**) Virus titers in Ltr051 cells, Ltr246 cells, RD cells and L929 cells infected with EV71 SK-EV006 at an MOI of 0.01. Virus titers were determined at 0, 12, 24, 48 and 72 h after infection. The data are shown as mean virus titers ± s.d. ($n = 3$). $TCID_{50}$, 50% tissue culture infective dose.

(encoding CC chemokine ligand-2) and *MEPCE* (methylphosphate capping enzyme) genes from Ltr246 cells (**Supplementary Tables 1** and **2**). We did not amplify the other 19 genes from either genomic DNA.

To determine whether expression of SCARB2, CCL2 or BCDIN3 makes L929 cells susceptible to EV71, we transfected L929 cells with a plasmid encoding SCARB2, CCL2 or BCDIN3 (pCA-SCARB2, pCA-CCL2 or pCA-BCDIN3) and then infected the cells with EV71-GFP. We detected GFP-positive cells in L929 cell cultures transfected with pCA-SCARB2 alone (**Supplementary Fig. 2**). We obtained similar results when we used BHK-21 and CHO cells as recipient cells (**Supplementary Fig. 2**). In contrast, L929 cells did not become susceptible to EV71-GFP by individual transfection of pCA-CCL2 and pCA-BCDIN3 or by transfection of both plasmids together (data not shown). These results suggest that human SCARB2 is sufficient to permit EV71 infection of Ltr051 cells. We presume that the susceptibility of Ltr246 cells to EV71 may be due to an unlisted human protein-coding gene. Further studies are required to identify the gene.

SCARB2 belongs to the scavenger receptor class B subfamily, which also includes *SCARB1* (*Cla-1*) and *CD36*[10,11]. SCARB2 is a type III double-transmembrane protein with N- as well as C-terminal cytoplasmic tails and is located primarily in lysosomes and endosomes[11]. SCARB2 participates in membrane transportation and the reorganization of the endosomal-lysosomal compartment[12]. Deficiency of SCARB2 in mice causes ureteric pelvic junction obstruction, deafness and peripheral neuropathy[13].

We established L-SCARB2 and L-Empty cells, which stably retained pCA-SCARB2 and pCAGGS-PUR, respectively. Because SCARB2 is known to localize mainly to the lysosomal membrane, we evaluated whether SCARB2 is also expressed in the plasma membrane. L-SCARB2 and L-Empty cells were stained with an antibody to human SCARB2 without permeabilization (**Fig. 2a**). We also stained RD and L929 cells as a positive and a negative control, respectively. We clearly detected cell surface expression of human SCARB2 on L-SCARB2 and RD cells (**Fig. 2a**). Next, to evaluate the growth kinetics of EV71, we infected these cells with EV71 SK-EV006. The growth kinetics of EV71 SK-EV006 in L-SCARB2 cells was similar to those in RD cells (**Fig. 2b**). In accordance with viral growth, we found cytopathic effects at 48 h after infection in both L-SCARB2 and RD cells (**Fig. 2c**). These data indicate that EV71 enters and propagates in L-SCARB2 cells and spreads efficiently.

To determine whether L-SCARB2 cells are susceptible to other EV71 strains, we infected L-SCARB2 cells with EV71 strains belonging to genogroups A, B and C (**Supplementary Fig. 3**). At 48 h after infection, all tested strains induced obvious cytopathic effects in both L-SCARB2 and RD cells (**Supplementary Fig. 3**). To evaluate the viral growth kinetics of representative strains from each genogroup, we infected these cells with strain BrCr (genogroup A), strain Nagoya (genogroup B) and strain Isehara (genogroup C). BrCr, Nagoya and Isehara grew in L-SCARB2 cells with a similar efficiency to that observed in RD cells (**Fig. 2d**). These results suggest that SCARB2 is able to serve as an efficient cellular receptor for several, and possibly all, EV71 strains. Strains BrCr and Isehara hardly propagated in L929 cells, whereas strains SK-EV006 and Nagoya inefficiently

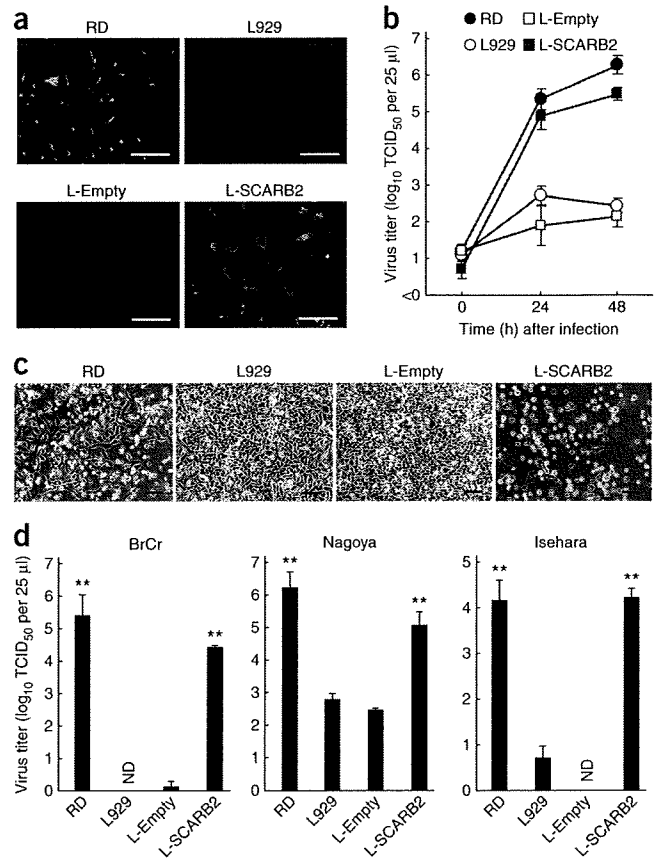

Figure 2 SCARB2 confers susceptibility to EV71 to mouse L929 cells. (**a**) Cell surface expression of SCARB2 on L-SCARB2, L-Empty, RD and L929 cells stained with antibody to SCARB2 without permeabilization. Scale bar, 200 μm. (**b**) Virus titers in L-SCARB2 cells, L-Empty cells, RD cells and L929 cells infected with SK-EV006 at an MOI of 0.01. Virus titration was performed at 0, 24 and 48 h after infection. The data are shown as mean virus titers ± s.d. ($n = 3$). (**c**) L-SCARB2, L-Empty, RD and L929 cells were infected with SK-EV006 at an MOI of 0.01. These cells were imaged via light microscopy at 48 h after infection. Scale bar, 200 μm. (**d**) Virus titers in L-SCARB2, L-Empty, RD and L929 cells infected with BrCr, Nagoya or Isehara at an MOI of 0.01. Virus titers were determined at 48 h after infection. The data are shown as mean virus titers ± s.d. ($n = 3$). $^{**}P < 0.01$ by Student's t test compared with the virus titer in L929 cells. ND, not detected.

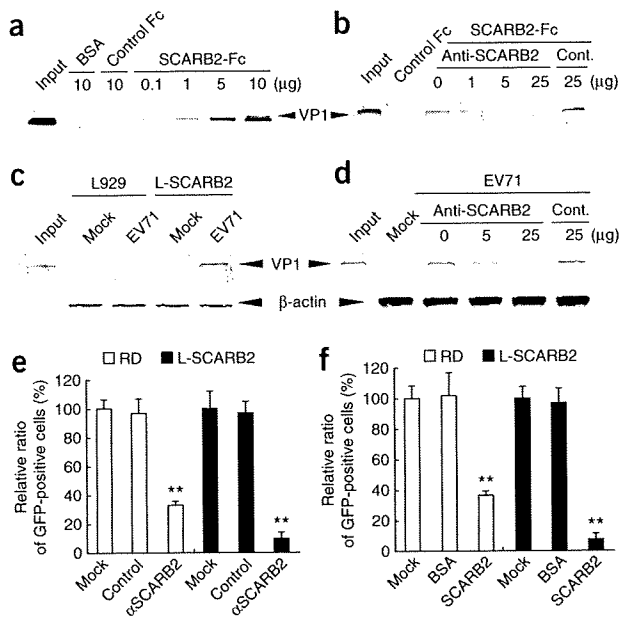

Figure 3 Interaction of SCARB2 with EV71. **(a)** Western blot analysis of EV71 incubated with BSA, control Fc or SCARB2-Fc bound to protein G–agarose. The precipitated proteins were analyzed with antibody to EV71 (recognizing mainly VP1). **(b)** Western blot analysis of SCARB2-Fc (2 μg) bound to protein G–agarose pretreated with indicated amount of antibody to SCARB2 (anti-SCARB2) or a normal goat IgG (Cont.) before incubation with EV1. The precipitated proteins were analyzed with antibody to EV71. **(c)** Western blot analysis of L-SCARB2 or L929 cells incubated with EV71 strain SK-EV006 (EV71) or 5% FCS-DMEM (Mock). After washing steps, these cells were lysed and then analyzed with antibody to EV71 or to β-actin. **(d)** Western blot analysis of L-SCARB2 cells treated with the indicated amount of antibody to SCARB2 or a normal goat IgG before incubation with EV71. Total cell lysates were analyzed with antibody to EV71 or to β-actin. **(e)** GFP-positive cell counts among L-SCARB2 cells and RD cells pretreated with 50 μg ml^{-1} of antibody to SCARB2 or a normal goat IgG before EV71-GFP infection. Mock treatment is set at 100%. The data are shown as mean percentages ± s.d. ($n = 5$). $^{**}P < 0.01$ by Student's t test when compared with the mock infection. **(f)** GFP-positive cell counts among L-SCARB2 cells and RD cells infected with EV71-GFP that had been mixed with 12 μg ml^{-1} of SCARB2-Fc or BSA. Mock treatment is set at 100%. The data are shown as mean percentages ± s.d. ($n = 5$). $^{**}P < 0.01$ by Student's t test when compared with the mock infection.

propagated in these cells (**Fig. 2b,d**). Some strains may be able to infect L929 cells via mouse Scarb2, as overexpression of mouse Scarb2, which has an 85.8% amino-acid sequence identity to human SCARB2, makes mouse cells susceptible to EV71 although not as efficiently as human SCARB2 (data not shown).

To determine whether EV71 directly binds SCARB2, we conducted pull-down assays with soluble SCARB2-Fc (extracellular region of SCARB2 fused to the Fc region of human IgG) (**Fig. 3a**). We incubated EV71 SK-EV006 with SCARB2-Fc and protein G–agarose beads, and we analyzed the precipitated proteins by western blotting with an antibody to EV71. The antibody to EV71 predominantly detected VP1 (**Fig. 3a**). We detected an EV71-VP1 band in a SCARB2-Fc concentration–dependent manner (**Fig. 3a**). The binding of EV71 to SCARB2-Fc was inhibited by the antibody to SCARB2 in a dose-dependent manner (**Fig. 3b**). Binding of SCARB2-Fc to poliovirus, whose receptor is CD155, was not observed under similar conditions (data not shown). These data indicate that SCARB2 directly and specifically binds EV71.

To assess whether EV71 binds SCARB2 in the plasma membrane, we conducted a virus attachment assay with L-SCARB2 or L929 cells. We incubated these cells with EV71 SK-EV006 and detected attached virus by western blotting (**Fig. 3c**). EV71 SK-EV006 was attached to L-SCARB2 cells (**Fig. 3c**), and the attachment was inhibited by the antibody to SCARB2 in a dose-dependent manner (**Fig. 3d**). These data indicate that SCARB2 mediates the attachment of EV71 to the cells.

To ascertain whether the binding of SCARB2 to EV71 mediates EV71 infection, we next performed an infection inhibition assay with either the antibody to SCARB2 or the SCARB2-Fc. We preincubated L-SCARB2 and RD cells with the antibody and then infected them with EV71-GFP. EV71 infection was blocked by pretreatment of L-SCARB2 and RD cells with the antibody in a dose-dependent manner (**Supplementary Fig. 4a**). The inhibition efficiency for L-SCARB2 cells was higher than that for RD cells, in which inhibition was not complete even at the highest antibody concentration (**Fig. 3e**). We next tested the infection inhibition assay with SCARB2-Fc. We infected L-SCARB2 and RD cells with EV71-GFP after we incubated the virus with SCARB2-Fc. We found that the inhibition effect was dose dependent (**Supplementary Fig. 4b**). Again, the inhibition efficiency for L-SCARB2 cells was higher than that for RD cells at the highest SCARB2-Fc concentration (**Fig. 3f**). These data indicate that EV71 enters cells through a SCARB2-dependent pathway. In addition, the difference in the extent of infection inhibition by the antibody and SCARB2-Fc between the L-SCARB2 and RD cells suggests that RD cells might have a separate, minor path for the early steps of EV71 infection.

To confirm the importance of SCARB2 in EV71 infection, we examined whether EV71 also uses SCARB2 in infecting other human cell lines. We detected SCARB2 on the surface of HeLa, HEp-2, 293T and HepG2 cells, and the infection efficiency of EV71-GFP was well correlated with the level of cell surface SCARB2 expression (**Supplementary Fig. 5a,b**). By infection inhibition assay, the infection of these cells by EV71-GFP was clearly inhibited by pretreatment with the antibody at a similar degree to RD cells (**Supplementary Fig. 5c**). These data indicate that SCARB2 has a dominant role in the early steps of EV71 infection of the tested human cell lines.

We next tested whether SCARB2 mediates infection of other human enterovirus species A viruses. For this purpose, we infected L-SCARB2 cells and L-Empty cells with CVA2, CVA3, CVA4, CVA5, CVA6, CVA7, CVA8, CVA10, CVA12, CVA14 and CVA16 (**Table 1** and **Supplementary Fig. 6**). At 48 h after infection, CVA16 induced cytopathic effects in both L-SCARB2 and RD cells (**Supplementary Fig. 6**). CVA2, CVA3, CVA4, CVA5, CVA6, CVA8 and CVA12 induced

Table 1 Induction of cytopathic effects by coxsackieviruses

Virus	RD cells	L929 cells	L-Empty cells	L-SCARB2 cells
CVA2	+	–	–	–
CVA3	+	–	–	–
CVA4	+	–	–	–
CVA5	+	–	–	–
CVA6	+	–	–	–
CVA7	+	+	+	+
CVA8	+	–	–	–
CVA10	+	+	+	+
CVA12	+	–	–	–
CVA14	+	+	+	+
CVA16	+	–	–	+

cytopathic effects only in RD cells (**Supplementary Fig. 6**). CVA7, CVA10 and CVA14 induced cytopathic effects in all four cell lines (**Supplementary Fig. 6**); thus, it was impossible to determine whether the cytopathic effects induced by these viruses were due to human SCARB2–mediated infection. We examined the viral growth kinetics of these viruses in L-SCARB2 cells. CVA16 grew efficiently in L-SCARB2 and RD cells, whereas CVA2, CVA3, CVA4, CVA5, CVA6, CVA8 and CVA12 grew at a high efficiency only in RD cells (**Supplementary Fig. 7**). These data indicate that CVA16 is also able to infect cells through a SCARB2-dependent pathway and that infection with most other human enterovirus species A is not dependent upon SCARB2.

In this study, we obtained two L929 cell transformants susceptible to EV71 infection and revealed that Ltr051 cells express human SCARB2. EV71 propagation in both L-SCARB2 and Ltr051 cells was as efficient as it is in RD cells. EV71 infection of RD cells was mostly blocked by antibody to SCARB2 and by SCARB2-Fc. Ltr246 cells were also susceptible to EV71, but their infection efficiency was lower than that of Ltr051 cells and RD cells. These results indicate that SCARB2 has a dominant role in EV71 infection of RD cells, although another minor receptor presents in Ltr246 cells might also contribute to EV71 infection.

During fatal cases of human EV71 infection, EV71 has been recovered from the central nervous system, lymph nodes, skin, intestinal mucosa and infectious lesions found in nearly all organs[14]. Because SCARB2 is expressed ubiquitously[11], it is possible that SCARB2 may be involved in systemic EV71 infections. SCARB2 serves as a receptor for EV71 strains isolated from individuals with HFMD as well as from individuals with encephalitis. SCARB2 also serves as a receptor for CVA16. It is noteworthy that although SCARB2 may be involved in the pathogenesis of HFMD caused by these viruses, EV71 occasionally induces neurologic symptoms, and CVA16 rarely does. Further study should clarify the involvement of SCARB2 in EV71 and CVA16 infection *in vivo* and may elucidate the mechanism behind the different levels of pathogenicity observed for these similar viruses.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Accession codes. Microarray data have been deposited in the Gene Expression Omnibus database with accession code GSE16358.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank H. Shimizu (Japan National Institute of Infectious Diseases) for providing us with all viruses used in this report and critical reading of this manuscript, Y. Kawaoka (University of Tokyo) for providing us with pCAGGS-PUR, Y. Yanagi for critical reading of this manuscript and Y. Matsumoto and K. Kohyama for supporting flow cytometry. This work was supported in part by a grant-in-aid for Scientific Research (C) (20590243) from the Japan Society for the Promotion of Science and in part by a grant-in-aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare, Japan.

AUTHOR CONTRIBUTIONS

S.Y. designed and performed the majority of the experiments, analyzed the data and wrote the manuscript; Y.Y. constructed pSVA-EV71-GFP and established Ltr051 and Ltr246 cells; J.L. constructed pSVA-EV71; N.H., T.M. and T.T. performed microarray analysis; S.K. designed the project, assisted with the experiments, analyzed the data and wrote the manuscript.

Published online at http://www.nature.com/naturemedicine/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Pallansch, M. & Roos, R. *Fields Virology* Ch. 25, 839–893 (Lippincott Williams & Wilkins, Philadelphia, 2006).
2. McMinn, P.C. An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS Microbiol. Rev.* **26**, 91–107 (2002).
3. Qiu, J. Enterovirus 71 infection: a new threat to global public health? *Lancet Neurol.* **7**, 868–869 (2008).
4. Fujimoto, T. *et al.* Outbreak of central nervous system disease associated with hand, foot, and mouth disease in Japan during the summer of 2000: detection and molecular epidemiology of enterovirus 71. *Microbiol. Immunol.* **46**, 621–627 (2002).
5. Chan, L.G. *et al.* Deaths of children during an outbreak of hand, foot, and mouth disease in Sarawak, Malaysia: clinical and pathological characteristics of the disease. For the Outbreak Study Group. *Clin. Infect. Dis.* **31**, 678–683 (2000).
6. Ho, M. *et al.* An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. *N. Engl. J. Med.* **341**, 929–935 (1999).
7. Ahmad, K. Hand, foot, and mouth disease outbreak reported in Singapore. *Lancet* **356**, 1338 (2000).
8. Koike, S. *et al.* The poliovirus receptor protein is produced both as membrane-bound and secreted forms. *EMBO J.* **9**, 3217–3224 (1990).
9. Mendelsohn, C.L., Wimmer, E. & Racaniello, V.R. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell* **56**, 855–865 (1989).
10. Calvo, D., Dopazo, J. & Vega, M.A. The CD36, CLA-1 (CD36L1), and LIMPII (CD36L2) gene family: cellular distribution, chromosomal location, and genetic evolution. *Genomics* **25**, 100–106 (1995).
11. Eskelinen, E.L., Tanaka, Y. & Saftig, P. At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol.* **13**, 137–145 (2003).
12. Kuronita, T. *et al.* A role for the lysosomal membrane protein LGP85 in the biogenesis and maintenance of endosomal and lysosomal morphology. *J. Cell Sci.* **115**, 4117–4131 (2002).
13. Gamp, A.C. *et al.* LIMP-2/LGP85 deficiency causes ureteric pelvic junction obstruction, deafness and peripheral neuropathy in mice. *Hum. Mol. Genet.* **12**, 631–646 (2003).
14. Yan, J.J., Wang, J.R., Liu, C.C., Yang, H.B. & Su, I.J. An outbreak of enterovirus 71 infection in Taiwan 1998: a comprehensive pathological, virological, and molecular study on a case of fulminant encephalitis. *J. Clin. Virol.* **17**, 13–22 (2000).

ONLINE METHODS

Viruses. We propagated EV71 (strains BrCr/USA/70 (BrCr; genogroup A), SK-EV006/Malaysia/97 (SK-EV006; genogroup B), Nagoya/Japan/73 (Nagoya; genogroup B), C7/Japan/97 (C7; genogroup B), Hungary/78 (Hungary; genogroup B), 258/Bulgaria/75 (258; genogroup B), 1095/Japan/97 (1095l; genogroup C) and Isehara/Japan/99 (Isehara; genogroup C)) in Vero cells for use in this study[15]. BrCr was isolated from an individual with meningitis; SK-EV006, C7, Hungary and 258 were from individuals with encephalitis; and Nagoya, 1095 and Isehara were from individuals with HFMD. All strains were provided by H. Shimizu.

Cloning of Ltr051 and Ltr246 cells. We transfected L929 cells with genomic DNA from RD cells and BamHI-digested pcDNA3.1.After 24 h, we divided the cells into 100–200 pools and selected with DMEM containing 5% FCS and 1 mg ml^{-1} G418. After 7 d, we split each pool, which contained approximately 100 G418-resistant clones, in two and cultured until the cells formed colonies. We infected one of the pools with EV71-GFP to test for EV71-susceptible cells and subjected the other, containing EV71-susceptible cells, to repeated cloning procedures and established it as Ltr051 cells. We established Ltr246 cells from an independent transfection.

Microarray analysis and genomic PCR. We extracted total RNA from Ltr051, Ltr246 and L929 cells with an RNeasy Mini Kit (Qiagen). We used each total RNA sample as a template to obtain amplified RNA (aRNA) using an Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion). We labeled each aRNA with Cy5 or Cy3 and competitively hybridized for 17 h at 65 °C with a Whole Human Genome Microarray kit 4x44K (Agilent) ($n = 3$). We analyzed the microarray images, obtained with a GenePix 4000B (Axon), by GenePix Pro (Axon). We calculated human gene expression levels in Ltr051 or Ltr246 cells as relative values to those in L929 cells. Increased signals would have been induced by the integration and expression of human genes. They would also have been elicited by the upregulation of mouse messenger RNAs, cross-hybridized with microarray probes target to human mRNA, owing to an incidental integration of human promoters, human enhancers or human signal transduction genes. We focused on the former cases. To examine whether increased signals were due to integrated human genes, we extracted genomic DNA from Ltr051 and Ltr246 cells and subjected it to PCR using AmpliTaq Gold (Applied Biosystems) and appropriate primers (**Supplementary Table 3**). We confirmed that all primer sets could amplify the target sequences from the genomic DNA of RD cells but not L929 cells. We analyzed the hydrophobicity of the candidate proteins with the SOSUI program[16].

Pull-down assay. We incubated EV71 strain SK-EV006 (8.55×10^6 $TICD_{50}$) or poliovirus (6×10^6 plaque-forming units) with BSA (10 μg), human IgG Fc (10 μg, R&D Systems) or human SCARB2-Fc (0.1 to 10 μg, R&D Systems) and protein G–agarose (25 μl, Sigma) in 1 ml of DMEM for 2 h at 4 °C. We then washed the beads twice with DMEM, suspended them in SDS sample buffer and incubated them for 5 min at 95 °C. After the beads were removed, we subjected the samples to 18% SDS-PAGE followed by western blotting with a rabbit antibody to EV71 (ref. 15) or a rabbit antibody to poliovirus[17]. In the binding inhibition test, we treated beads coated with SCARB2-Fc (2 μg) with antibody to SCARB2 (1–25 μg ml^{-1}, R&D Systems) for 1 h at 4 °C before the virus was mixed.

Virus attachment assay. We detached L-SCARB2 or L929 cells in PBS containing 0.05% EDTA and mixed them with EV71 strain SK-EV006 (8.55×10^6 $TCID_{50}$) for 1 h at 4 °C. We washed these cells twice with PBS, suspended them in SDS sample buffer and incubated them for 5 min at 95 °C. We then subjected them to 18% SDS-PAGE followed by western blotting with the antibody to EV71 or to β-actin (clone AC-74, Sigma). In the attachment inhibition test, we pretreated these cells with the antibody to SCARB2 (5 or 25 μg ml^{-1}) for 1 h at 4 °C before the virus was mixed.

Infection inhibition assays. To examine the effect of the antibody to SCARB2, we preincubated RD and L-SCARB2 cells with the antibody to SCARB2 (0.5, 1, 5, 10, 25 or 50 μg ml^{-1}) for 30 min at 37 °C. We subsequently added EV71-GFP to the cells. After 18 h, we counted GFP-positive cells under the microscope. To examine the effect of soluble SCARB2, we mixed EV71-GFP with the SCARB2-Fc (0.125, 0.25, 0.75, 1.5, 3, 6 or 12 μg ml^{-1}) and incubated for 60 min at 37 °C. We then added the mixture to L-SCARB2 and RD cells. After 18 h, we counted GFP-positive cells under the microscope. The experiments were repeated five times.

Flow cytometry. We performed flow cytometry as previously reported[18] with some modifications. Briefly, we detached cells in PBS containing 0.02% EDTA, stained them with the antibody to SCARB2 followed by the Alexa Fluor 488–conjugated donkey antibody to goat IgG (Invitrogen), and then analyzed the cells by using a FACSCalibur with Cell Quest Pro software (Becton Dickinson).

Statistical analyses. We analyzed data obtained from three to five independent experiments by Student's t test. We expressed results as means ± s.d. and considered them significant when $P < 0.05$.

Additional Methods. Detailed methodology is described in the **Supplementary Methods**.

15. Nagata, N. *et al.* Pyramidal and extrapyramidal involvement in experimental infection of cynomolgus monkeys with enterovirus 71. *J. Med. Virol.* **67**, 207–216 (2002).
16. Hirokawa, T., Boon-Chieng, S. & Mitaku, S. SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* **14**, 378–379 (1998).
17. Yanagiya, A., Jia, Q., Ohka, S., Horie, H. & Nomoto, A. Blockade of the poliovirus-induced cytopathic effect in neural cells by monoclonal antibody against poliovirus or the human poliovirus receptor. *J. Virol.* **79**, 1523–1532 (2005).
18. Yamayoshi, S. *et al.* Ebola virus matrix protein VP40 uses the COPII transport system for its intracellular transport. *Cell Host Microbe* **3**, 168–177 (2008).

Journal of Clinical Virology 44 (2009) 268–271

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Oral poliovirus vaccine type 3 from a patient with transverse myelitis is neurovirulent in a transgenic mouse model

Bruce Thorley[a,*], Heath Kelly[a], Yorihiro Nishimura[b], Yeon Kyung Yoon[a], Kerri Anne Brussen[a], Jason Roberts[a], Hiroyuki Shimizu[b]

[a] Polio Reference Laboratory and Epidemiology Unit, Victorian Infectious Diseases Reference Laboratory, 10 Wreckyn Street, North Melbourne, Australia
[b] Section of Enteroviruses, Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

ARTICLE INFO

Article history:
Received 30 October 2008
Received in revised form 27 January 2009
Accepted 28 January 2009

Keywords:
Neurovirulence
Oral polio vaccine
Poliovirus
Transgenic mice
Transverse myelitis

ABSTRACT

Background: It is accepted that oral poliovirus vaccine (OPV) can cause vaccine-associated paralytic poliomyelitis (VAPP) and that wild poliovirus infection can rarely present as transverse myelitis. It is therefore possible that OPV could cause transverse myelitis. We previously reported a case of transverse myelitis that developed in a 6-month-old boy, 7 days after receiving his second dose of OPV.
Objectives: Our aim was to test the virus from this patient with transverse myelitis for neurovirulence in a mouse model.
Study design: The TgPVR21 transgenic mouse line, which expresses the human poliovirus receptor CD155, was used to assess neurovirulence of the viruses tested. Neurovirulence was expressed as the PD_{50}, the dose of virus causing paralysis in 50% of the mice. Four type 3 polioviruses were tested: a prototype wild strain, a fully attenuated polio vaccine virus, a virus from a patient with VAPP and the virus from the patient with transverse myelitis.
Results: The PD_{50} for the wild poliovirus strain was 3.83 and for the fully attenuated vaccine strain, 7.63. The PD_{50} for the two clinical isolates were between these values, $\geq$4.96 for the poliovirus known to have caused VAPP and $\geq$4.81 for the virus from the patient with transverse myelitis.
Conclusions: The report of an OPV strain from a transverse myelitis case being neurovirulent in an *in vivo* mouse model provides further evidence for a causal association between OPV and transverse myelitis.

1. Introduction

The neurological presentation of poliovirus infection is characteristically of acute flaccid paralysis (AFP) in one or more limbs, or acute bulbar palsy. Poliovirus infection affects the motor neurons of the CNS. However, sensory deficits have been recorded and presentations of acute poliovirus infection with both sensory and motor deficits, manifesting as transverse myelitis, have been reported in a number of older case studies.[1–3] Standard textbooks record wild poliovirus as a cause of encephalomyelitis[4] and myelitis.[5]

We previously reported the case of a 6-month-old boy who, in 1996, developed transverse myelitis 7 days after the receipt of oral poliovirus vaccine (OPV).[6] The boy received his first scheduled OPV at the age of 4 months and developed immunity to poliovirus serotypes 1 and 2 but not to serotype 3 by the time he received his second dose of OPV. The onset of symptoms, confirmed later by a paediatric neurologist as transverse myelitis, occurred 7 days after receiving the second OPV dose. Two days later poliovirus serotype 3 was isolated from stool and throat specimens collected from the infant while CSF was negative for enterovirus culture. The virus tested as an OPV-like strain by nucleic acid probe hybridisation and enzyme-linked immunosorbent assay according to recommended procedures in a World Health Organization (WHO) poliovirus reference laboratory.[7,8] The boy subsequently developed immunity to poliovirus serotype 3.

We argued that there was strong evidence for a causal relationship between the OPV serotype 3 and transverse myelitis in this patient. From a review of the literature we estimated transverse myelitis to occur in 1:125–1:800 cases of symptomatic poliovirus infection.[6] It is also accepted that OPV can cause vaccine-associated paralytic polio (VAPP) with a frequency of approximately one case per 2.5 million doses of OPV distributed.[9] Based on the frequencies with which wild poliovirus infection causes transverse myelitis and vaccine virus causes VAPP, we estimated that a clinical entity of poliovirus vaccine-associated transverse myelitis could occur with a frequency of 1 in 300 million to 1 in two billion doses of OPV

Abbreviations: AFP, acute flaccid paralysis; CCID, cell culture infective dose; OPV, oral polio vaccine; PD, paralysis dose; VAPP, vaccine-associated paralytic poliomyelitis; WHO, World Health Organization.

* Corresponding author. Tel.: +61 3 9342 3910; fax: +61 3 9342 2665.
E-mail address: bruce.thorley@mh.org.au (B. Thorley).

1386-6532/$ – see front matter.
doi:10.1016/j.jcv.2009.01.014

distributed.[6] An event as rare as this would almost certainly be missed in any review of a population based program.

In a 1994 publication, the Institute of Medicine of the National Academies concluded in relation to adverse events associated with OPV, that while OPV causes poliomyelitis (that is, VAPP) the evidence was inadequate to accept or reject a causal relationship between OPV and transverse myelitis.[10] The authors indicated that they needed to place substantial reliance on case reports in order to establish likely causal relationships and illustrated this with the following scenario. "For example, if a patient receives a live viral vaccine, experiences a pathological condition known to be associated with the natural virus, and molecular biological techniques identify virus isolated from the patient as vaccine strain rather than the natural virus, causality between the vaccine and the pathological condition is established for this patient". In the case reported, the child received OPV, a live viral vaccine; developed transverse myelitis, a pathological condition known to be associated, albeit rarely, with wild virus infection; and the poliovirus type 3 isolated from stool and throat specimens obtained from the child was shown to be of vaccine origin. Although the causality criteria were satisfied, we did not claim the relationship was causal because of the possibility that another vaccine, or unidentified agent, may have been implicated[6] or that the cause of transverse myelitis was due to a vascular lesion, as has been suggested in the older literature.[11,12]

We now report that the poliovirus type 3 identified from the patient with transverse myelitis was of moderate neurovirulence in a transgenic mouse model, with similar activity as a type 3 poliovirus known to have caused VAPP in another patient.

2. Materials and methods

2.1. Intracerebral inoculation of TgPVR21 mice with poliovirus isolates

The TgPVR21 line of transgenic mice (Central Laboratory of Experimental Animals, Kanagawa, Japan) was used as a model for human poliovirus infection. Wild poliovirus naturally infects only humans and some other primates. Rodents are not permissive to natural infection, whereas the TgPVR21 transgenic mice express the human poliovirus receptor, CD155, and are susceptible to poliovirus infection. This line of mice has been widely used as a suitable replacement for monkeys to assess the neurovirulence of poliovirus strains.[13,14]

The experimental protocol for the TgPVR21 transgenic mice was as described previously.[15] Thirty microlitres of 10-fold serial dilutions of each poliovirus isolate were inoculated intracerebrally into 3-week-old TgPVR21 mice. Six mice, three males and three females, were used for each dilution point. The mice were observed daily for 14 days post-inoculation for limb weakness, paralysis or death. The dose of each virus causing paralysis in 50% of the mice (PD_{50}) was determined by the Karber formula[16] and reported as $CCID_{50}$/30 μl, where $CCID_{50}$ is the 50% cell culture infective dose.

2.2. Viruses

Four poliovirus type 3 isolates were selected for intracerebral inoculation: wild poliovirus prototype Leon strain (neurovirulent), Sabin 3 prototype (attenuated), the OPV poliovirus isolate from the transverse myelitis case and an OPV poliovirus from a case of VAPP.[17] The titre of the poliovirus isolates was determined in the HEp-2C cell line before and after intracerebral inoculation of the transgenic mice.

2.3. Nucleotide sequence of the poliovirus isolates from clinical specimens

RNA extraction of the poliovirus isolates was performed according to the manufacturer's instructions using either the QIAamp viral RNA kit (Qiagen) or the High Pure viral nucleic acid kit (Roche). Reverse transcription and PCR amplification from viral RNA was performed using the Superscript RT/Taq system (Invitrogen). The 5′ non-translated region of the VAPP case was amplified by PCR using oligonucleotide primers POL3 (5′-TTAAAACAGCTCTGGGGTTG-3′) and POL30 (5′-ACGTCCTTNAKKGGCTCGGTG-3′), while primers E1 (5′-CAAGCACTTCTGTTTCCCCGG-3′) and E4 (5′-CACYGGATGGCCAATCCAA-3′)[18] were used for the transverse myelitis case. Primers Y7 and Q8[19] were used for PCR amplification of the VP1 genomic region of both isolates. The nucleotide sequence of the respective amplicons was determined using the same primers as was used for PCR amplification but in separate reactions with BigDye Terminator (Applied Biosystems) followed by electrophoretic separation on an ABI Prism 3730S Genetic Analyzer (Applied Biosystems).

3. Results

3.1. Mouse neurovirulence test

The $CCID_{50}$ of the four poliovirus isolates was determined *in vitro* and an appropriate range of dilutions for intracerebral inoculation of the TgPVR21 mice was prepared (Table 1). The $CCID_{50}$ was repeated after the inoculation and all virus titres were within 0.25 log of the initial determination (data not shown). Indicating that the procedure of intracerebral inoculation was not in itself harmful, no paralysis or deaths occurred within 24 h of the inoculation procedure and no paralysis or deaths occurred in the six mice inoculated with the fully attenuated OPV reference strain. The number of mice with paralysis for each dilution point of each virus is presented in Table 1 as the PD_{50} (log_{10} $CCID_{50}$/30 μl/mouse) from observations over 14 days post-inoculation.

The PD_{50} for the wild poliovirus strain was 3.83 and for the Sabin fully attenuated vaccine strain was 7.63. The PD_{50} for the two OPV viruses were between these values, ≧4.96 for the virus known to have caused VAPP and ≧4.81 for the virus from the patient with transverse myelitis.

Table 1
Determination of PD_{50} of virus isolates by intracerebral inoculation into TgPVR21 mice.

Strain of poliovirus type 3	Initial virus titre in HEp-2C cells (log_{10} $CCID_{50}$/0.1 ml)	Number of mice with paralysis/number of mice inoculated for each virus dilution						PD_{50} (log_{10} $CCID_{50}$/30 μl/mouse)
		Virus dilution (10^{-x})						
		0	−1	−2	−3	−4	−5	
Sabin (OPV prototype)	7.65	0/6	–	–	–	–	–	≧7.63
Leon (wild prototype)	8.35	–	–	6/6	6/6	3/6	0/6	3.83
OPV from transverse myelitis case, 1996	7.50	–	–	3/6	1/6	0/6	0/6	≧4.81
OPV from VAPP case, 1986	7.65	–	–	2/6	1/6	1/6	0/6	≧4.96

3.2. Nucleotide sequence of the poliovirus isolates from clinical specimens

The 5′ non-translated and VP1 genomic regions of the poliovirus isolates from the transverse myelitis and VAPP cases were analysed at sites known to be critical for attenuation. The nucleotide sequence of both isolates had reverted to the wild poliovirus sequence at position 472 (472U→C) of the 5′ non-translated region—the major site of neurological attenuation in Sabin poliovirus type 3 strains.[20,21] A cytosine to uracil substitution at position 2493 of the VP1 genomic region encoded a threonine to isoleucine amino acid substitution in both isolates that has been associated with loss of attenuation.[22]

4. Discussion

We have previously demonstrated that the criteria for causality between a vaccine and a pathological condition, as specified by the Institute of Medicine of the National Academies of Science, were satisfied for a 6-month-old boy who developed transverse myelitis after his second dose of OPV in 1996. Because of the administration of other vaccines at the same time as OPV administration, we did not claim the association to be causal. However we have now shown that the virus from the patient with transverse myelitis has the same degree of neurovirulence in a transgenic mouse model as a virus of the same serotype known to have caused VAPP. Moreover, the neurovirulence of the two OPV viruses isolated from the clinical cases was between the activity of a wild virus and a fully attenuated virus.

Genomic sequencing confirmed that both viruses were of vaccine origin and that they had lost two critical genetic determinants of attenuation at nucleotide positions 472 and 2493. Rather than cytosine at nucleotide position 472, Sabin type 3 has a uracil residue that causes disruption of the local secondary structure of domain V of the internal ribosome entry site. This leads to decreased binding with polypyrimidine tract-binding protein and defective translation.[21] The relative abundance of 472-C to 472-U in preparations of Sabin 3 correlates with neurovirulence in monkey and TgPVR21 mouse models.[23,24] The detection of 472-C revertant polioviruses in the stools of healthy vaccinees and cases of VAPP[25,20,26] indicates that neurological disease associated with OPV depends upon host factors, exactly as it does for wild poliovirus infection.[27]

The strong circumstantial case for OPV causing transverse myelitis in a single patient has now been strengthened by the demonstration that the virus from this patient had the same neurovirulence in a transgenic mouse model as a virus of the same serotype known to have caused VAPP. The clinical, molecular biological and mouse model evidence all argue for a causal relationship between OPV and transverse myelitis.

AFP is the differential diagnosis for poliovirus infection and the WHO recommends surveillance for cases of AFP as the "gold standard" for detection of circulating or imported wild poliovirus. In countries conducting routine AFP surveillance as part of the global polio eradication initiative, cases of transverse myelitis should be investigated to exclude wild poliovirus as a cause of AFP. However not all countries conduct routine AFP surveillance and, of those that do, many do not attain the WHO AFP surveillance performance indicators.[28] Furthermore, clinicians may not consider the symptoms of transverse myelitis to be compatible with poliovirus infection. In a world where wild poliovirus is rare but OPV is not, this report of a neurovirulent strain of OPV isolated from a case of transverse myelitis highlights the importance of full clinical and laboratory investigation of cases of AFP as a differential diagnosis of poliovirus infection.

Conflict of interest

None declared.

Acknowledgements

The report was produced as part of Australia's program for the surveillance of cases with acute flaccid paralysis. The Commonwealth Department of Health and Ageing supports the surveillance program, which is currently coordinated by the Victorian Infectious Diseases Reference Laboratory and the Australian Paediatric Surveillance Unit. The important contributions of all clinicians involved in the care of this child and all clinicians notifying cases of acute flaccid paralysis are acknowledged. The views expressed are the authors' own and do not represent the views of the Polio Expert Committee in Australia or any Australian Government body. This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases and a Grant-in-Aid for the Promotion of Polio Eradication, from the Ministry of Health, Labour and Welfare, Japan. BT travelled to Japan under the Program for the Invitation of Foreign Scientists to Japanese Institutes by the Japan Health Sciences Foundation, whose support is gratefully acknowledged.

Funding: This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases and a Grant-in-Aid for the Promotion of Polio Eradication, from the Ministry of Health, Labour and Welfare, Japan.

Ethical approval: The case of transverse myelitis was notified and reviewed according to an ethics submission for acute flaccid paralysis surveillance approved by the Australian government. The child's parents provided written consent to publish the findings of this study.

References

1. Plum F. Sensory loss with poliomyelitis. *Neurology* 1956;**6**:166–72.
2. Lance JW, White SW, Dodgson MCH. Sensory loss in poliomyelitis. *Aust Ann Med* 1963;**12**:221–9.
3. Foley KM, Beresford HR. Acute poliomyelitis beginning as transverse myelitis. *Arch Neurol* 1974;**30**:182–3.
4. Griffin DE. Encephalitis, myelitis and neuritis. In: Mandell GL, Bennett JE, Dolin R, editors. *Principles and practice of infectious diseases*. 6th ed. Philadelphia: Elsevier Churchill Livingstone; 2005. p. 1143–50.
5. Cassady KA, Whitely RJ. Viral central nervous system infections. In: Richman DG, Whitley RJ, Hayden FG, editors. *Clinical virology*. 2nd ed. Washington: ASM Press; 2002. p. 27–44.
6. Kelly H. Evidence for a causal association between oral polio vaccine and transverse myelitis: a case history and review of the literature. *J Paediatr Child Health* 2006;**42**:157–61.
7. Lina D, Nottay B, Yang C-F, Holloway BP, Pallansch M, Kew O. Identification of vaccine-related polioviruses by hybridization with specific RNA probes. *J Clin Microbiol* 1995;**33**:562–71.
8. van Wezel AL, Hazendonk AG. Intratypic serodifferentiation of poliomyelitis virus strains by strain-specific antisera. *Intervirology* 1979;**11**:2–8.
9. The relationship between acute persisting paralysis and poliomyelitis vaccine (oral): results of a WHO enquiry. *Bull World Health Org* 1976;**53**:319–31.
10. Stratton KR, Howe CJ, Johnston RB. Adverse events associated with childhood vaccines other than pertussis and rubella: summary of a report from the Institute of Medicine. *JAMA* 1994;**271**:1602–5.
11. Paine RS, Byers RK. Transverse myelopathy in childhood. *Am J Dis Child* 1953;**85**:151–63.
12. Walley RV. Sensory loss in poliomyelitis. *Br Med J* 1961;**2**:33.
13. Koike S, Taya C, Kurata T, et al. Transgenic mice susceptible to poliovirus. *Proc Natl Acad Sci USA* 1991;**88**:951–5.
14. Abe S, Ota Y, Koike S, et al. Neurovirulence test for oral poliovaccine using poliovirus-sensitive transgenic mice. *Virology* 1995;**206**:1075–83.
15. Arita M, Shimizu H, Miyamura T. Characterization of in vitro and in vivo phenotypes of poliovirus type 1 mutants with reduced viral protein synthesis activity. *J Gen Virol* 2004;**85**:1933–44.
16. Hawkes RA. General principles underlying laboratory diagnosis of viral infections. In: Lennette EH, Schmidt NJ, editors. *Diagnostic procedures for viral, rickettsial and chlamydial infections*. 5th ed. Washington: American Public Health Association Inc.; 1979. p. 3–48.
17. Kennett ML, Brussen KA, Wood DJ, van der Avoort HG, Ras A, Kelly HA. Australia's last reported case of poliovirus infection. *Comm Dis Intell* 1999;**23**:77–9.

18. McIver CJ, Jacques CFH, Chow SSW, Munro SC, Scott GM, Roberts JA, et al. Development of multiplex PCRs for detection of common viral pathogens and agents of congenital infections. *J Clin Microbiol* 2005;**43**:5102–10.
19. Vinje J, Gregoricus N, Martin J, Gary HE, Caceres VM, Venczel L, et al. Isolation and characterization of circulating type 1 vaccine-derived poliovirus from sewage and stream waters in Hispaniola. *J Infect Dis* 2004;**189**:1168–75.
20. Westrop GD, Wareham KA, Evans DMA, Dunn G, Minor PD, Magrath DI, et al. Genetic basis of attenuation of the Sabin type 3 oral poliovirus vaccine. *J Virol* 1989;**63**:1338–44.
21. Guest S, Pilipenko E, Sharma K, Chumakov K, Roos RP. Molecular mechanisms of attenuation of the Sabin strain of poliovirus type 3. *J Virol* 2004;**78**:11097–107.
22. Tatem JM, Weeks-Levy C, Georgiu A, DiMichele SJ, Gorgacz EJ, Racaniello VR, et al. A mutation present in the amino terminus of Sabin 3 poliovirus VP1 protein is attenuating. *J Virol* 1992;**66**:3194–7.
23. Chumakov KM, Norwood LP, Parker ML, Dragunsky EM, Ran Y, Levenbook IS. RNA sequence variants in live poliovirus vaccine and their relation to neurovirulence. *J Virol* 1992;**66**:966–70.
24. Abe S, Ota Y, Doi Y, Nomoto A, Nomura T, Chumakov KM, et al. Studies on neurovirulence in poliovirus-sensitive transgenic mice and cynomolgus monkeys for the different temperature sensitive viruses derived from the Sabin type 3 virus. *Virology* 1995;**210**:160–6.
25. Laassri M, Lottenbach K, Belshe R, Rennels M, Plotkin S, Chumakov K. Analysis of reversions in the 5′-untranslated region of attenuated poliovirus after sequential administration of inactivated and oral poliovirus vaccines. *J Infect Dis* 2006;**193**:1344–9.
26. Macadam AJ, Arnold C, Howlett J, John A, Marsden S, Taffs F, et al. Reversion of the attenuated and temperature sensitive phenotypes of the Sabin type 3 strain of poliovirus in vaccinees. *J Virol* 1989;**172**:408–14.
27. Nathanson N, Martin JR. The epidemiology of poliomyelitis: enigmas surrounding its appearance, epidemicity, and disappearance. *Am J Epidemiol* 1979;**110**:672–92.
28. World Health Organization. Performance of acute flaccid paralysis (AFP) surveillance and incidence of poliomyelitis, 2008. *Wkly Epidemiol Rec* 2008;**83**:106–8.

Vaccine 27 (2009) 3153–3158

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Cross-antigenicity among EV71 strains from different genogroups isolated in Yamagata, Japan, between 1990 and 2007

K. Mizuta[a,*], Y. Aoki[a], A. Suto[a], K. Ootani[a], N. Katsushima[b], T. Itagaki[c], A. Ohmi[d], M. Okamoto[d], H. Nishimura[d], Y. Matsuzaki[e], S. Hongo[f], K. Sugawara[f], H. Shimizu[g], T. Ahiko[a]

[a] Department of Microbiology, Yamagata Prefectural Institute of Public Health, Tokamachi 1-6-6, Yamagata, 990-0031, Japan
[b] Katsushima Pediatric Clinic, Yamagata, 990-2461, Japan
[c] Yamanobe Pediatric Clinic, Yamagata, 990-0301, Japan
[d] Virus Research Center, Sendai Medial Center, Sendai, 983-8520, Japan
[e] Course of Clinical Nursing, Yamagata University Faculty of Medicine, Iida-Nishi, Yamagata, 990-9585, Japan
[f] Department of Infectious Diseases, Yamagata University Faculty of Medicine, Iida-Nishi, Yamagata, 990-9585, Japan
[g] Department of Virology II, National Institute of Infectious Diseases 4-7-1, Gakuen, Musashimurayama, Tokyo, 208-0011, Japan

ARTICLE INFO

Article history:
Received 22 December 2008
Received in revised form 16 March 2009
Accepted 23 March 2009
Available online 10 April 2009

Keywords:
Enterovirus type 71
Subgenogroup
Cross-antigenicity

ABSTRACT

We isolated and identified six subgenogroups (B2, B4, B5, C1, C2, and C4) of enterovirus 71 (EV71) between 1990 and 2007 in Yamagata, Japan. We measured neutralizing antibody (NT Ab) titers against those subgenogroup strains and the BrCr reference strain for antigenic analysis. Serological analysis of 83 residents in Yamagata in 2004 showed that differences in the NT Ab titer of each individual against the different subgenogroups were mostly within 4-fold. Furthermore, sera from guinea pigs, immunized with the B2 and C1 strains indicated cross-antigenicity among the seven different subgenogroups. In conclusion, our results showed that cross-antigenicity exists among EV71 strains from different subgenogroups circulating in the community through genomic evolution. Our results also suggest that eliciting neutralizing antibodies against one genotype is likely to confer cross-neutralization against other genotypes.

1. Introduction

Enterovirus 71 (EV71) is a small, non-enveloped virus belonging to the genus *Enterovirus* within the family *Picornaviridae*. Together with Coxsackievirus A16 (CoxA16) and CoxA10, EV71 is known to be a causative agent of hand-foot-and-mouth disease (HFMD). Although HFMD is typically a benign and common self-limiting rash illness in children, EV71 infection sometimes causes severe complications such as encephalitis and poliomyelitis-like paralysis, mainly in infants and young children [1]. The history of EV71-associated severe neurological diseases has been reviewed by Bible et al. [2]. Briefly, there were three separate waves of EV71 activity around the world between 1970 and 2000; the cases were reported from U.S.A., Australia, Japan and other countries in 1970s, from Hong Kong, Taiwan, U.S.A. and Brazil in 1980s, and that in the last decade of the 20th century was mainly observed in the Asia-Pacific region with outbreaks such as in Malaysia between 1997 and 2003, in Taiwan in 1998, in Singapore between 1998 and 2001, and in Australia in 1999 and 2000 [1–7]. Outbreaks of EV71 have also been reported in the 21st century and a case of adult encephalitis due to EV71 was also recently reported [2,5,8–10]. EV71 infection has become a greater concern due to the increased neuropathogenicity observed in recent outbreaks. According to an overview by McMinn, future research should focus on (a) understanding the molecular genetics of virulence, (b) identification of receptor(s), (c) development of antiviral agents to ameliorate the severity of neurological disease, and (d) vaccine development [11].

Since the reports of outbreaks in the 1990s, several epidemiological studies have clarified that EV71 is an active circulating agent capable of rapid evolution into several different genogroups and that EV71 consists of 11 subgenogroups (A, B1–5, C1–5) [2,10,12,13]. As both B and C genogroups have been associated with complicated and uncomplicated diseases, the relationship between severity and genogroup has not been confirmed and no direct correlation has been observed between the neurovirulence and EV71 genotype even in experimental infection studies on cynomolgus monkeys [2,14,15].

Neutralizing antibodies (NT Abs) against EV71 have been suggested as one of the most important factors in limiting the severity of EV71 infections, although viral inoculation dose, host immune response, such as cytokine reaction and cellular immunity, genetic resistance of the host, hygiene and nutritional status have been suggested as factors influencing the clinical course [15–18]. Yu et al. showed that passively transferred NT Abs provided protection

* Corresponding author. Tel.: +81 23 627 1109; fax: +81 23 641 7486.
E-mail address: mizutak@pref.yamagata.jp (K. Mizuta).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.03.060

against a lethal challenge with EV71 in neonatal mice, depending on the titer, and even human serum containing anti-EV71 NT Abs (1:1024) attenuated the disease in infant mice [19]. Using an infected mouse model, protection against EV71 challenge was demonstrated following administration of hyperimmune serum (1:128) 1 day after inoculation of the virus [20]. Thus, the protective role of anti-EV71 NT Abs is clear.

In reality, the development of a vaccine against EV71 has been pursued as a preventive measure. The main target region for vaccine development has been the VP1 region, which was defined as the neutralization determinant [21]. The VP1 recombinant protein, VP1 subunit vaccine and some peptides containing amino acid sequence in this region were reported as candidate targets for the development of a vaccine against EV71 [22–25]. Since EV71, possibly evolving and changing its antigenicity, circulates and causes HFMD and other severe illnesses, antigenic analysis of different subgenogroups is necessary in order to develop an effective vaccine. For this reason, the VP1 region is of primary importance for genetic and antigenic analysis. However, studies involving such antigenic analysis and measurement of NT Abs against different subgenogroups have been limited [26,27]. In particular, to our knowledge, no seroepidemiological survey of different subgenogroups has been reported.

We previously reported the molecular epidemiology of EV71 strains isolated from children in Yamagata, Japan, between 1998 and 2003, using sequence analysis of the VP1 region and genotyping [13]. In this study, we further analyzed the EV71 strains isolated between 1990 and 2007 in Yamagata, and carried out a seroepidemiological survey and antigenic analysis using guinea pig hyperimmune sera against representative EV71 strains from the different subgenogroups.

2. Materials and methods

2.1. Virus isolation and sequence analysis

Virus isolation and identification was carried out by means of a microplate method from nasopharyngeal samples from children with HFMD or respiratory illnesses, as described previously [28–30]. EV71 strains were isolated using HEF, Vero, Vero E6, RD-18S and GMK cell lines at the Virus Research Center, National Hospital Organization, Sendai Medical Center, Sendai, Japan, between 1990 and 1997 and thereafter at the Department of Microbiology, Yamagata Prefectural Institute of Public Health. Sequence analysis for the complete VP1 region (891 nucleotides) of the EV71 isolates was carried out as shown previously [13]. Sequence data for the isolates from Yamagata were registered with GenBank accession numbers AB433862–AB433892. Sequence data were analyzed with CLUSTAL W (version 1.83), and a phylogenetic tree was constructed by the neighbor-joining method using the same software [31].

2.2. Serum dilution-plaque reduction neutralization test

Serum samples were collected from residents in Yamagata, from whom informed consent was received (either from the individual or guardian) between June and September 2004 for the national epidemiological surveillance of vaccine-preventable diseases led by the Ministry of Health, Labour and Welfare, Japan. In 2004, there was no EV71-related HFMD outbreak. A total of 83 serum samples (1-year old: 11, 2 years: 5, 3 years: 6, 4 years: 4, 5 years: 3, 6–9 years: 22, 10–14 years: 3, 15–19 years: 4, 20–29 years: 5, 30–39 years: 5, 40–49 years: 5, 50–59 years: 5, above 60: 5) were enrolled. A plaque reduction neutralization test using human sera was performed in 6-well plates (Greiner, Bio-one, Frickenhausen, Germany). Serum samples were inactivated at 56 °C for 30 min and then serially diluted 2-fold from 8- to 2048-fold. We mixed and incubated (37 °C, 60 min) 200 μl of each diluted serum sample with 200 μl of virus fluid containing approximately 100 plaque forming unit (PFU) of EV71 representative strains for each subgenogroup (Y90-3205 for B2, 1067-Yamagata-00 for B4, 2542-Yamagata-03 for B5, Y90-2913 for C1, 1585-Yamagata-01 for C2 and 75-Yamagata-03 for C4) as well as a reference strain, BrCr, belonging to genogroup A. We prepared confluent monolayers of the GMK cell line in advance, washed the cells with phosphate-buffered saline without calcium or magnesium, and inoculated 200 μl of each incubated virus–serum mixture into each of two cultures. The inoculated plates were gently rocked every 15 min up to 2 h at 33 °C in a 5% CO_2 incubator. The cultures were overlaid with Eagle's minimum essential medium (MEM) containing 1% methyl cellulose 4,000 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The plates were then incubated in a 5% CO_2 incubator at 33 °C for 72 h. Cells were fixed with 10% formaldehyde (Wako Pure Chemical Industries) and stained with 0.04% methylene blue (Wako Pure Chemical Industries). The plaque counts of both cultures at each serum dilution were compared with the number of plaques in the control cultures and the serotiter was determined. The reciprocal value of the highest dilution of serum giving a 50% reduction in the number of plaques as compared to the control was taken to be the titer. Sero-positivity was defined as a serotiter $\geq$1:8.

2.3. Preparation of EV71 virus as immunogen, guinea pig immunization and antigenic analysis

The representative strains for genogroups B (Y90-3205) and C (Y90-2913), which were originally isolated in Vero cells and were passaged once in Vero and 3 times in GMK cells, were used as immunogens to immunize guinea pigs. The virus in the cultured fluid was purified by precipitation with 8% polyethylene glycol 6,000 (Wako Pure Chemical Industries) and then centrifuged on a 40% sucrose gradient at 40,000 rpm for 3 h. The virus titer was determined as PFUs on GMK cells. One specific pathogen-free, 5- and 14-week-old Hartley female guinea pig (Japan SLC, Inc., Shizuoka, Japan) were used for genogroup C and B, respectively. They were intraperitoneally and subcutaneously (1:1) injected with two doses of 2 ml virus fluid (approximately 10^7 PFU/ml) without being inactivated, and the same volume of adjuvant incomplete FREUND (DIFCO Laboratories, Detroit, Michigan) at an interval of 1 week. Two weeks after the second immunization, the guinea pigs were boosted by one intraperitoneal and one subcutaneous injection of the identical virus without the adjuvant. Serum samples were collected 1-week later and stored at −70 °C after heat inactivation (56 °C for 30 min). NT Ab titers for these sera were measured by the same method as described above for the seroepidemiological survey, except that serum dilutions were carried out from 1:100 or 1:400 to 1:25,600-fold.

3. Results

3.1. Virus isolation, genotyping, and phylogenetic and genetic analysis

A total of 154 EV71 strains were isolated between 1990 and 2007, in Yamagata. The numbers of EV71 strains isolated in Yamagata by month are shown in Fig. 1.

The phylogenetic tree for the VP1 region and the monthly distribution of subgenogroups are shown in Figs. 1 and 2, respectively. In 1990, three isolates were identified as belonging to subgenogroup C1 (C1) and one isolate was identified as B2. In 1993, one strain was identified as B2. Between 1997 and 1999, all analyzed strains were typed as C2. The details of the isolates obtained between 1998 and

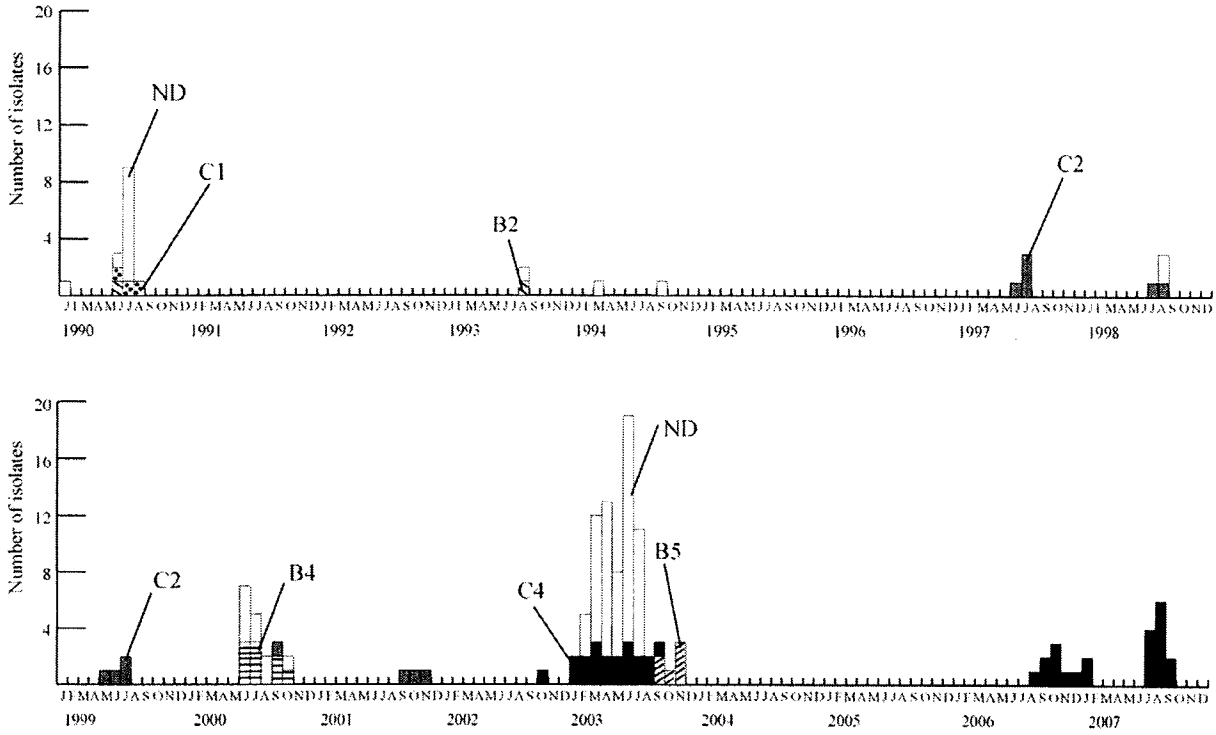

Fig. 1. Monthly distribution and subgenogroups of enterovirus 71 strains isolated in Yamagata, Japan between 1990 and 2007. The subgenogroups, C1, C2, B2, B4, C4 and B5, were grouped according to the phylogenetic analysis shown in Fig. 2. ND, not done.

2003 are described in our previous paper [13]. In 2006 and 2007, all strains were identified as C4. Genogroup C strains circulated for longer period with genomic variations, whereas genogroups B strains appeared only for one season (Figs. 1 and 2).

3.2. Seroepidemiological survey

The NT titers for 83 residents in Yamagata against the seven subgenogroups are shown in Table 1. Sero-positive rates were 24.1% (7/29), 44% (11/25), and 51.7% (15/29), among the residents under the age of 6, between the ages of 6 and 14, and above the age of 14, respectively. For most of the individuals, the titer range against the seven subgenogroups were within 2–4-folds. NT titers against genogroup B were slightly higher than those against A and C, especially among individuals having an NT Ab of less than 1:128.

3.3. Antigenic analysis

The NT titers of a guinea pig immunized with Y90-3205 (B2) ranged from 1:1600 to 1:12,800, whereas those for a guinea pig immunized with Y90-2913 (C1) ranged from 1:200 to 1:3200 (Table 2). The sera sets from both guinea pigs showed titers of <1:8 against the respective virus before immunization. For both sets of sera, NT Ab titers against genogroup B were higher than those against genogroups A and C. Ab titers against genogroups A and C were similar in both sets of sera.

4. Discussion

First, we showed the temporal distribution of EV71 subgenogroups over an 18-year period, in Yamagata, Japan. No such longitudinal analysis of the VP1 region of EV71 strains circulating in a local community has been reported except for one from Sydney, Australia between 1983 and 2001 [32]. Our study revealed several interesting epidemiological features. We found that six subgenogroups, B2, B4, B5, C1, C2, and C4, appeared one after another or concurrently during the study period, whereas B2, C1, and B4 were found in 1983, between 1986 and 1998, and between 2000 and 2001, respectively, in Sydney Australia [32]. C1 and B2 disappeared by the end of the 1990s in Yamagata, and this observation approximately coincides with the time-line observed in Sydney [32]. The sudden replacement of subgenogroup B5 for C4 found in 2003 in Yamagata provides important and interesting information regarding the mechanism of subgenogroup replacement in the community. There was a 2–16-fold antigenic difference in the human sera between these two subgenogroups in this study (Table 1). However, we are not sure whether the reason for this replacement was due to the antigenic difference or to the simple introduction of new strains from outside. The finding from Yamagata that sequence identity decreased over time even among strains belonging to the same subgenogroup, such as C2 or C4, suggests the active genomic evolution of EV71, particularly in genogroup C strains. As we reported previously, the fact that the detected subgenogroups in Yamagata (B2, B4, B5, C1, C2, C4) were also found in the Asia-Pacific region suggests the active circulation of EV71 in this area [3–5,10,32]. In this way, even surveillance studies in a local community such as Yamagata have the potential to function, on a wider level, such as a regional surveillance program.

The current efforts to develop a vaccine require the identification of any antigenic differences among different subgenogroups. Although it has been reported that sera from individuals infected with EV71 had high titers of ≥1:512 against both of B and C genogroups strains [27], our data showed that the residents who had previously been infected with EV71 had a fairly low NT Ab titer of less than 1:128 against the different subgenogroup strains (Table 1). Even at such low levels, the ranges of the NT Ab titers against different subgenogroup strains in the Yamagata residents

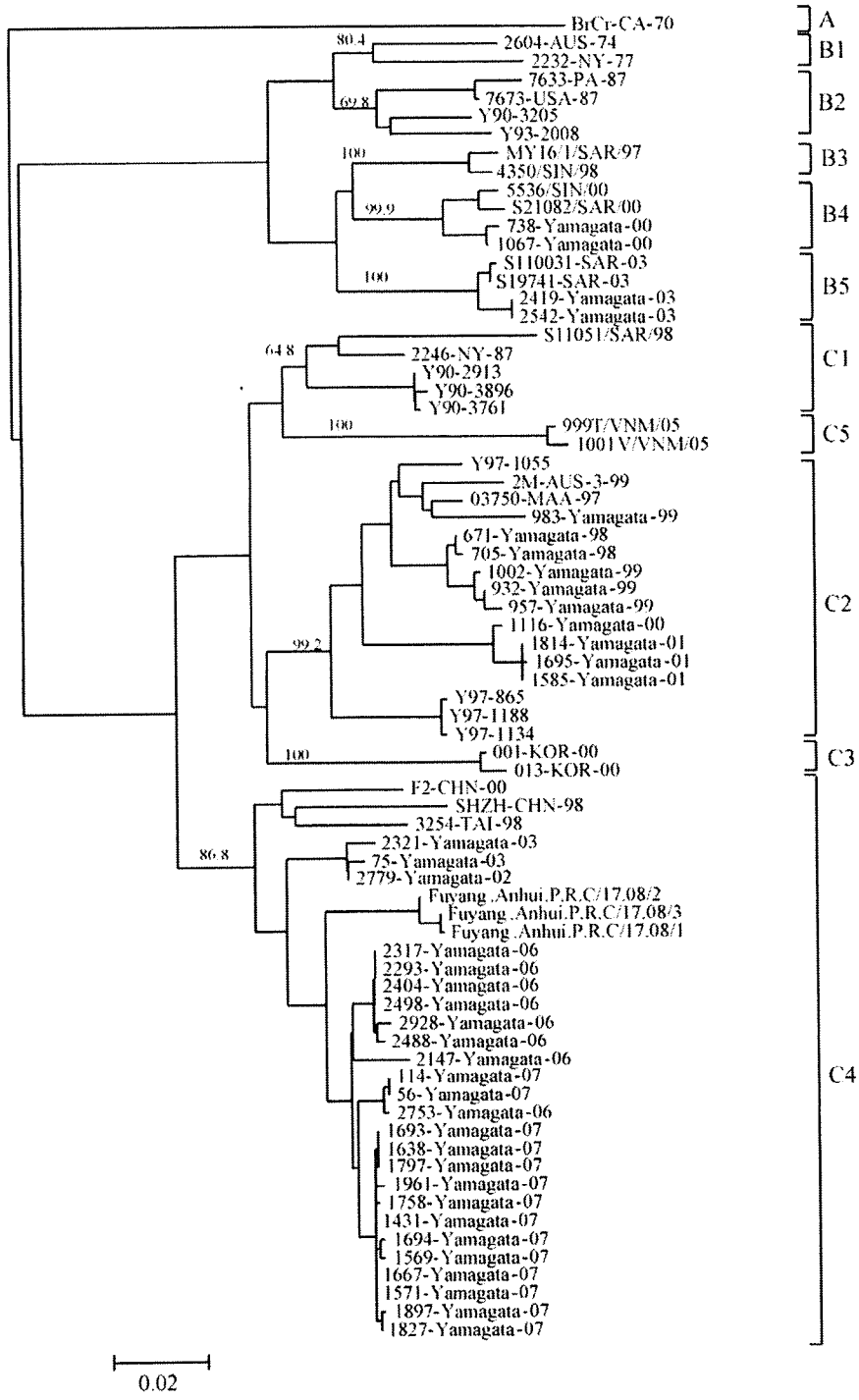

Fig. 2. Phylogenetic tree for the complete (891 nucleotide) VP1 region of enterovirus 71 (EV71) representative strains isolated in Yamagata, Japan between 1990 and 2007 and reference strains. Details of the EV71 strains belonging to subgenogroups A, B1–5, and C1–4 are provided in Table 1. Branch lengths are proportional to the number of nucleotide differences. Numbers above the branches are the bootstrap probabilities (%). The marker denotes a measurement of relative phylogenetic distance.

differed by up to almost 4-fold. The guinea pig sera immunized with strains from subgenogroups B2 and C1 succeeded in neutralizing strains from other subgenogroups (Table 2). Furthermore, for example, sera from 2-year-old children in 2004, who could only have been infected with B5 or C4 in 2003, succeeded in neutralizing other subgenogroup strains, that had circulated prior to their birth (Table 1). These results strongly indicate the cross-antigenicity among different subgenogroups. As shown in a previous study, NT Abs elicited by a synthetic peptide representing one of the VP1 epitopes of the B4 strain succeeded in protecting new born mice against a lethal challenge with EV71 strains belonging to the B2, B4, B5, C2 and C4 subgenogroups [23]. Kung et al. demonstrated that Abs raised in rabbits and sera collected from infected patients were able to neutralize EV71 strains, regardless of the subgenogroup used for the challenge [27]. Arita et al. reported that an attenuated strain of EV71 belonging to genogroup A showed a broad spectrum of neu-

Table 1
Neutralizing antibody titers against the seven distinct subgenogroup representative strains of EV71 among residents in Yamagata, Japan in 2004.

Enrolled ID[a]	Age (year)	Subgenogroup of EV71[b]						
		A	B2	B4	B5	C1	C2	C4
20041505	17	<1:8	<1:8	<1:8	<1:8	<1:8	<1:8	1:8
20040719	8	<1:8	1:8	1:16	1:16	<1:8	<1:8	1:8
20046003	63	<1:8	1:8	1:16	1:8	<1:8	1:8	1:8
20042004	20	1:8	1:8	1:8	1:16	<1:8	1:8	<1:8
20040715	7	<1:8	1:16	1:16	1:16	1:8	1:16	1:8
20040708	8	<1:8	1:16	1:32	1:64	<1:8	<1:8	<1:8
20042002	23	1:8	1:16	1:32	1:32	1:8	1:8	<1:8
20043002	32	1:8	1:16	1:16	1:16	1:16	1:16	1:8
20041506	15	1:8	1:16	1:32	1:32	1:16	1:16	1:8
20042005	24	1:8	1:16	1:32	1:32	1:16	1:16	1:16
20040705	7	1:8	1:16	1:32	1:32	1:32	1:32	1:16
20044001	40	1:16	1:16	1:16	1:16	1:8	1:16	1:8
20040725	9	1:16	1:16	1:32	1:32	1:16	1:32	1:32
20040609	6	1:8	1:32	1:32	1:64	1:8	1:16	1:16
20043004	31	1:8	1:32	1:32	1:32	1:16	1:8	1:8
20040718	9	1:8	1:32	1:16	1:16	1:16	1:16	1:32
20040509	5	1:8	1:32	1:32	1:32	1:32	1:16	1:32
20040702	9	1:8	1:32	1:32	1:32	1:32	1:32	1:32
20045004	57	1:16	1:32	1:32	1:32	1:16	1:32	1:16
20040505	5	1:16	1:32	1:64	1:64	1:32	1:8	1:32
20040713	7	1:16	1:32	1:64	1:64	1:32	1:32	1:64
20043005	31	1:32	1:32	1:64	1:32	1:16	1:32	1:32
20040207	3	1:64	1:32	1:32	1:32	1:32	1:64	1:64
20044004	40	1:8	1:64	1:32	1:32	1:16	1:32	1:16
20040009	1	1:16	1:64	1:64	1:32	1:16	1:16	1:16
20043003	34	1:16	1:64	1:32	1:64	1:32	1:64	1:32
20044002	46	1:32	1:64	1:32	1:32	1:16	1:32	1:32
20040608	6	1:32	1:64	1:128	1:256	1:64	1:128	1:256
20040716	7	1:64	1:128	1:256	1:128	1:128	1:256	1:256
20040210	2	1:128	1:128	1:256	1:128	1:128	1:256	1:256
20040213	2	1:128	1:128	1:128	1:128	1:128	1:256	1:256
20046005	72	1:128	1:128	1:128	1:512	1:256	1:512	1:256
20040201	2	1:2048<	1:1024	1:1024	1:2048<	1:1024	1:1024	1:2048

[a] Fifty residents from whom the serum was sero-negative (<1:8) against the seven subgenogroup antigens are not shown.
[b] BrCr, Y90-3205, 1067-Yamagata-00, 2542-Yamagata-03, Y90-2913, 1585-Yamagata-01, and 75-Yamagata-03 strains were used as representative of subgenogroups A, B2, B4, B5, C1, C2, and C4, respectively.

tralizing activity against different subgenogroups, A, B1, B4, C2 and C4, though the NT titers differed [33]. Thus, together with the above studies, our studies confirmed a close antigenic relationship among EV71 strains belonging to different subgenogroups.

Although we confirmed the cross-antigenicity among different subgenogroup strains, our data also suggested slight differences among the various genogroups. Antigenic analysis using guinea pig sera showed that (a) the antigenicity of the different subgenogroups within the same genogroup (B or C) is almost identical, (b) the antigenicity of genogroup A and that of genogroup C is very similar, and (c) there is a slight antigenic difference between genogroups A and C and genogroup B (Table 2). We also found that B5 isolates were readily and completely neutralized by EV71-specific antiserum, whereas C4 isolates were only partially neutralized in 2004 in Yamagata [13]. Thus, our data and observations indicated that genogroup C strains might be more difficult to neutralize than genogroup B strains or that genogroup B strains readily induce higher NT Ab titers rather than do genogroup C strains. Interestingly, genogroup C strains circulated for 5–6 years, whereas genogroup B strains appeared only for one season. (Fig. 1) These findings suggested that genogroup C strains, which are difficult to be neutralized, are more highly transmissible than subgenogroup B strains.

Finally, the results of our study supported those of previous studies in showing that there is a cross-antigenicity among different EV71 subgenogroups that circulate one after another or concurrently in the community through genomic evolution. For this reason, we can prevent severe illnesses due to EV71 infections through the development of a vaccine, most likely based on a subgenogroup B strain that effectively induces NT Abs against EV71, as has been shown with the measles vaccine strategy, in which the measles vaccine strain has not been changed even though a number of subgenogroups have been reported [34].

Table 2
Neutralizing antibody titers of guinea pig antisera against EV71 strains from distinct subgenogroups.

EV71 strain (subgenogroup)	Serum from guinea pigs immunized with:	
	Y90-3205	Y90-2913
BrCr (A)	1:3,200	1:400
Y90-3205 (B2)	1:6,400–1:12,800	1:1,600–1:3,200
1067-Yamagata-00 (B4)	1:6,400–1:12,800	1:1,600–1:3,200
2542-Yamagata-03 (B5)	1:12,800	1:3,200
Y90-2913 (C1)	1:1,600–1:3,200	1:200–1:400
1585-Yamagata-01 (C2)	1:1,600	1:200–1:400
75-Yamagata-03 (C4)	1:3,200–1:6,400	1:200–1:400

Acknowledgements

We thank the medical staff and people of Yamagata Prefecture for their collaboration in specimen collection for the surveillance of viral infectious diseases. We also thank Dr. T. Ito, Laboratory Animal Center, Yamagata University, Faculty of Medicine, and Dr. H. Yoshida, Department of Virology II, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan for their excellent technical assistance. This work was partially supported by Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare, Japan.

References

[1] Pallansch MA, Roos RP. Enteroviruses: polioviruses, coxsackieviruses, echoviruses and newer enteroviruses. In: Knipe DM, Howley PM, editors. Fields virology. 4th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2001. p. 723–75.

[2] Bible JM, Pantelidis P, Chan PKS, Tong CYW. Genetic evolution of enterovirus 71: epidemiological and pathological implications. Rev Med Virol 2007;17:371–9.

[3] Herrero LJ, Lee CSM, Hurrelbrink RJ, Chua BH, Chua KB, McMinn PC. Molecular epidemiology of enterovirus 71 in peninsular Malaysia, 1997–2000. Arch Virol 2003;148:1369–85.

[4] McMinn P, Lindsay K, Perera D, Chan HM, Chan KP, Cardosa MJ. Phylogenetic analysis of enterovirus 71 strains isolated during linked epidemics in Malaysia, Singapore, and Western Australia. J Virol 2001;75:7732–8.

[5] Podin Y, Gias EL, Ong F, Leong Y, Yee S, Yusof MA, et al. Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years. BMC Public Health 2006;6:180.

[6] Shimizu H, Utama A, Yoshii K, Yoshida H, Yoneyama T, Sinniah M, et al. Enterovirus 71 from fatal and nonfatal cases of hand, foot and mouth disease epidemics in Malaysia, Japan and Taiwan in 1997–1998. Jpn J Infect Dis 1999;52:12–5.

[7] Shimizu H, Utama A, Onnimala N, Li C, Li-Bi Z, Yu-Jie M, et al. Molecular epidemiology of enterovirus 71 infection in the western pacific region. Pediatr Int 2004;46:231–5.

[8] Hamaguchi T, Fujisawa H, Sakai K, Okino S, Kurosaki N, Nishimura Y, et al. Acute encephalitis caused by intrafamilial transmission of enterovirus 71 in adult. Emerg Infect Dis 2008;14:828–30.

[9] The Chinese Center for Disease Control and Prevention, the Office of the World Health Organization in China. Report on the hand, foot and mouth disease outbreak in Fuyang city, Anhui province and the prevention and control in China, 2008.

[10] Tu PV, Thao NTT, Perera D, Huu TK, Tien NTK, Thuong TC, et al. Epidemiologic and virologic investigation of hand, foot, and mouth disease, Southern Vietnam 2005. Emerg Infect Dis 2007;13:1733–41.

[11] McMinn PC. An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol Rev 2002;26:91–107.

[12] Brown BA, Oberste MS, Alexander Jr JP, Kennett ML, Pallansch MA. Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J Virol 1999;73:9969–75.

[13] Mizuta K, Abiko C, Murata T, Matsuzaki Y, Itagaki T, Sanjoh K, et al. Frequent importation of enterovirus 71 from surrounding countries into the local community of Yamagata, Japan, between 1998 and 2003. J Clin Microbiol 2005;43:6171–5.

[14] Nagata N, Shimizu H, Ami Y, Tano Y, Harashima A, Suzaki Y, et al. Pyramidal and extrapyramidal involvement in experimental infection of cynomolgus monkeys with enterovirus 71. J Med Virol 2002;67:207–16.

[15] Singh S, Poh CL, Chow VTK. Complete sequence analyses of enterovirus 71 strains from fatal and non-fatal cases of the hand, foot and mouth disease outbreak in Singapore (2000). Microbiol Immunol 2002;46:801–8.

[16] Chang L, Hsiung CA, Lu C, Lin T, Huang F, Lai Y, et al. Status of cellular rather than humoral immunity is correlated with clinical outcome of enterovirus 71. Pediatr Res 2006;60:466–71.

[17] Lin T, Hsia S, Huang Y, Wu C, Chang L. Proinflammatory cytokine reactions in enterovirus 71 infections of the central nervous system. Clin Infect Dis 2003;36:269–74.

[18] Witsø E, Palacios G, Rønningne KS, Cinek O, Janowitz D, Rewers M, et al. Asymptomatic circulation of HEV71 in Norway. Virus Res 2007;123:19–29.

[19] Yu C, Chen C-C, Chen C-L, Wang J, Liu C, Yan J, et al. Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J Biomed Sci 2000;7:523–8.

[20] Wang Y, Chou C, Lei H, Liu C, Wang S, Yan J, et al. A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection. J Virol 2004;78, 7916–1924.

[21] Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol 1999;73:1941–8.

[22] Chen H, Chang M, Chiang B, Jeng S. Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. Vaccine 2006;24:2944–51.

[23] Foo DGW, Alonso S, Phoon MC, Ramachandran NP, Chow VTK, Poh CL. Identification of neutralizing linear epitopes from the VP1 capsid protein of enterovirus 71 using synthetic peptides. Virus Res 2007;125:61–8.

[24] Foo DGW, Alonso S, Chow VTK, Poh CL. Passive protection against lethal enterovirus 71 infection in newborn mice by neutralizing antibodies elicited by a synthetic peptide. Microbes Infect 2007;9:1299–306.

[25] Wu C, Lin Y, Fann C, Liao N, Shih S, Ho M. Protection against lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. Vaccine 2002;20:895–904.

[26] Castro CMO, Cruz ACR, Da Silva EE, Gomes MLC. Molecular and seroepidemiologic studies of enterovirus 71 infection in the state of Pará, Brazil. Rev Inst Med Trop S Paulo 2005;47:65–71.

[27] Kung S, Wang S, Huang C, Hsu C, Liu H, Yang J. Genetic and antigenic analyses of enterovirus 71 isolates in Taiwan during 1998–2005. Clin Microbiol Infect 2007;13:782–7.

[28] Mizuta K, Abiko C, Aoki Y, Suto A, Hoshina H, Itagaki T, et al. Analysis of monthly isolation of respiratory viruses from children by cell culture using a microplate method: a two-year study from 2004 to 2005 in Yamagata, Japan. Jpn J Infect Dis 2008;61:196–201.

[29] Mizuta K, Abiko C, Goto H, Murata T, Murayama S. Enterovirus isolation from children with acute respiratory infections and presumptive identification by a modified microplate method. Int J Infect Dis 2003;7:138–42.

[30] Numazaki Y, Oshima T, Ohmi A, Tanaka A, Oizumi Y, Komatsu S, et al. A microplate method for isolation of viruses from infants and children with acute respiratory infections. Microbiol Immunol 1987;31:1085–95.

[31] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–25.

[32] Sanders SA, Herrero LJ, McPhie K, Chow SSW, Craig ME, Dwyer DE, et al. Molecular epidemiology of enterovirus 71 over two decades in an Australian urban community. Arch Virol 2006;151:1003–13.

[33] Arita M, Nagata N, Iwata N, Ami Y, Suzaki Y, Mizuta K, et al. An attenuated strain of enterovirus 71 belonging to genotype A showed a broad spectrum of antigenicity with attenuated neurovirulence in cynomolgus monkeys. J Virol 2007;81:9386–95.

[34] Zhou J, Fujino M, Inou Y, Kumada A, Aoki Y, Iwata S, et al. H1 genotype of measles virus was detected in outbreaks in Japan after 2000. J Med Virol 2003;70:642–8.