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

Acknowledgements

Thanks are expressed to B. Simizu, S. Hashizume (Japan Poliomyelitis Research Institute), and Y. Nagai (Riken Yokohama Institute Center of Research Network for Infectious Diseases) for helpful discussions.

References

[1] Takatsu T, Tagaya I, Hirayama M. Poliomyelitis in Japan during the period 1962–68 after the introduction of mass vaccination with Sabin vaccine. Bulletin WHO 1973;49:129–37.

[2] Shimojo H. Poliomyelitis control in Japan. Rev Infect Dis 1984;6(Suppl 2):S427–30.

[3] Infectious Agents Surveillance Center. Poliomyelitis, Japan, 1962–1995. Infect Agents Surveillance Rep 1997;18:1–2.

[4] Lago PM, Bravo JR, Andrus JK, Comellas MM, Galindo MA, Quadros CA, et al. Lessons from Cuba: mass campaign administration of trivalent oral poliovirus vaccine and seroprevalence of poliovirus neutralizing antibodies. Bulletin WHO 1994;72:221–5.

[5] Ramsay M, Begg N, Gandhi J, Brown D. Antibody response and viral excretion after live polio vaccine or a combined schedule of live and inactivated polio vaccines. Pediatr Infect Dis J 1994;13:1117–21.

[6] World Health Organization Collaborative Study Group on Oral and Inactivated Poliovirus Vaccines. Factors affecting the immunogenicity of oral poliovirus vaccine: a prospective evaluation in Brazil and the Gambia. J Infect Dis 1995;171:1097–106.

[7] World Health Organization Collaborative Study Group on Oral and Inactivated Poliovirus Vaccines. Combined immunization of infants with oral and inactivated poliovirus vaccines: results of a randomized trial in the Gambia, Oman, and Thailand. J Infect Dis 1997;175 (Suppl 1): S215–27.

[8] Vaccine Administration Subcommittee. Evaluation of Sabin live poliovirus vaccine in Japan. II. Clinical, virologic and immunologic effects of vaccine in children. Japan J Med Sci Biol 1966;19:277–91.

[9] Vaccine Administration Subcommittee. Evaluation of Sabin live poliovirus vaccine in Japan. III. Studies on the method of administration of Sabin vaccine for babies. Japan J Med Sci Biol 1967;20:151–66.

[10] World Health Organization. 2003. Global polio eradication initiative strategic plan 2004–2008.World Health Organization, Geneva.

[11] Kew OM, Wright P, Agol VI, Delpeyroux F, Shimizu H, Nathanson N, et al. Circulating vaccine-derived polioviruses: current state of knowledge. Bull WHO 2004;82: 16–23.

[12] Centers for Disease Control and Prevention. Update on vaccine-derived polioviruses. MMWR 2006;55:1093–7.

[13] World Health Organization. Weekly Epidemiological Record 2006;81:61–8.

[14] Matsuura K, Ishikura M, Yoshida H, Nakayama T, Hasegawa S, Ando S, et al. Assessment of poliovirus eradication in Japan: genomic analysis of the polioviruses isolated from the river water and the sewage in Toyama Prefecture. Appl Environ Microbiol 2000;66:5087–91.

[15] Chumakov KM, Powers LB, Noonan KE, Roninson IB, Levenbook IS. Correlation between amount of virus with acceptability of oral poliovirus vaccine. Proc Natl Acad Sci USA 1991;88:199–203.

[16] Chumakov KM, Dragunsky EM, Norwood LP, Douthitt MP, Ran Y, Taffs RE, et al. Consistent selection of mutations in the 5′-untranslated region of oral poliovirus vaccine upon passaging in vitro. J Med Virol 1994;42:79–85.

[17] Horie H, Yoshida H, Matsuura K, Miyazawa M, Ota Y, Nakayama T, et al. Neurovirulence of type 1 polioviruses isolated from sewage in Japan. Appl Environ Microbiol 2002;68:138–42.

[18] Horie H, Yoshida H, Matsuura K, Miyazawa M, Wakabayashi K, Nomoto A, et al. Isolation of vaccine-derived type 1 polioviruses displaying similar properties to virulent wild strain Mahoney from sewage in Japan. J Med Virol 2002;68: 445–51.

[19] Yoshida H, Horie H, Matsuura K, Kitamura T, Hashizume S, Miyamura T. Prevalence of vaccine-derived polioviruses in the environment. J Gen Virol 2002;83:1107–11.

[20] Yoshida H, Horie H, Matsuura K, Miyamura T. Characterization of polioviruses isolated from sewage and river water in Japan. Lancet 2000;356:1461–3.

[21] Iwai M, Nakayama T, Matsuura K, Hasegawa S, Ando S, Obara M, et al. Assessment of efficacy of a live oral poliovirus vaccine for virulent Sabin-like poliovirus 1 strains in Japan. Acta Virologica 2006;50:139–43.

[22] World Health Organization. Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization. 1995. WHO publication no. WHO/BLG/95.1. World Health Organization, Geneva.

[23] Shimizu B. Poliovaccine. Rinsho to Biseibutsu 2004; 31:349–53. (In Japanese.)

[24] Modlin FJ, Halsey NA, Thomas ML, Meschievitz CK, Patriarca PA, Baltimore Area Polio Vaccine Study Group. Humoral and mucosal immunity in infants induced by 3 sequential inactivated poliovirus vaccine-live attenuated oral poliovirus vaccine immunization schedules. J Infect Dis 1997;175 (Suppl 1):S228–34.

[25] Nishio O, Ishihara Y, Sakae K, Nonomura Y, Kuno A, Yasukawa S, et al. The trend of acquired immunity with live poliovirus vaccine and the effect of revaccination: follow-up of vaccinees for 10 y. J Biol Stand 1984;12:1–10.

JOURNAL OF VIROLOGY, Oct. 2008, p. 9765–9769
0022-538X/08/$08.00+0 doi:10.1128/JVI.01051-08

Vol. 82, No. 19

NOTES

Aichi Virus 2A Protein Is Involved in Viral RNA Replication[▽]

Jun Sasaki* and Koki Taniguchi

Department of Virology and Parasitology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan[1]

Received 19 May 2008/Accepted 11 July 2008

The Aichi virus 2A protein is not a protease, unlike many other picornavirus 2A proteins, and it is related to a cellular protein, H-rev107. Here, we examined the replication properties of two 2A mutants in Vero cells and a cell-free translation/replication system. In one mutant, amino acids 36 to 126 were replaced with an unrelated amino acid sequence. In the other mutant, the NC motif conserved in the H-rev107 family of proteins was changed to alanine residues. The two mutations abolished virus replication in cells. The mutations affected both negative- and positive-strand synthesis, the defect in positive-strand synthesis being more severe than that in negative-strand synthesis.

The picornavirus nonstructural 2A protein varies among viruses in amino acid sequence and function. In entero- and rhinoviruses, 2A is a protease responsible for cleavage of the polyprotein at its own N terminus (14, 15). It also cleaves cellular proteins, including eukaryotic translation initiation factor 4G (5). In addition, poliovirus 2A is involved in regulation of viral RNA stability, translation, and negative-strand synthesis (4). Aphthovirus 2A is ~18-amino-acids (aa) long, and cardiovirus 2A is about 140-aa long. The conserved amino acids, Asn-Pro-Gly (NPG), at the C termini of the aphtho- and cardiovirus 2A proteins, together with a proline at the N terminus of 2B, are required for the processing at the 2A/2B junction through a mechanism different from a proteolytic reaction (2). It has been reported for Theiler's murine encephalomyelitis virus, a cardiovirus, that a large deletion within the 2A-coding region does not affect RNA replication significantly (6). Parechovirus 2A, which has no proteolytic activity (12) nor the NPGP motif, shows specific binding activity to both single- and double-stranded forms of the 3′ untranslated region (UTR), suggesting its involvement in viral RNA replication (10).

Aichi virus (AiV), which is associated with acute gastroenteritis in humans (17), is a member of the genus *Kobuvirus* of the family *Picornaviridae* (18). AiV 2A, which is 136-aa long, does not have the protease motif characteristic of enterovirus 2A or the NPGP motif. AiV 2A, as well as parechovirus and avian encephalomyelitis virus 2A, has been reported to be related to a cellular protein, H-rev107, a candidate tumor suppressor protein (3, 13).

First, we investigated whether AiV 2A has a proteolytic activity required for the polyprotein processing. We had previously constructed a plasmid, pMAL-3CDmut, which contains the 3CD-coding region with mutations T6492G and G6493C to abolish the 3C protease activity (9). A Csp45I-PstI fragment (nucleotides [nt] 6480 to 6771) of pMAL-3CDmut was substituted for the corresponding fragment of an Aichi virus replicon, pAV-FL-Luc-5′rzm, in which the capsid-coding region was replaced with a firefly luciferase (Luc) gene and a hammerhead ribozyme sequence was inserted upstream of the viral sequence (7, 8), yielding pAV-FL-Luc-5′rzm-3Cmut (Fig. 1A). pAV-FL-Luc-5′rzm and pAV-FL-Luc-5′rzm-3Cmut were subjected to in vitro translation in the presence of L-[^{35}S]methionine and L-[^{35}S]cysteine (Amersham), using a TNT quick coupled transcription/translation system (Promega). After being incubated at 30°C for 90 min, translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and radioactive signals were detected with a BAS2000 bioimaging analyzer (Fujifilm). The predicted molecular mass of the polyprotein is approximately 240 kDa. As shown in Fig. 1B, the polyprotein processing was observed for pAV-FL-Luc-5′rzm but not for pAV-FL-Luc-5′rzm-3Cmut. This result indicates that in AiV, 3C is the only protease involved in the polyprotein processing and that 2A is not a protease.

To investigate the function of AiV 2A in virus replication, we constructed two kinds of 2A mutants, using an infectious cDNA clone, pAV-FL (11), and a replicon, pAV-FL-Luc-5′rzm (Fig. 2A). Of the two kinds of introduced mutations, one is a frameshift mutation within the 2A-coding region caused by a 1-nt deletion of nt 3895 and a 1-nt insertion between nt 4170 and 4171. By these mutations, aa 36 to 126 were replaced with an unrelated amino acid sequence encoded by another reading frame. The other mutation is a change of the NC (Asn-Cys) motif, one of the motifs of the H-rev107 family of proteins, to AA (Ala-Ala).

A SacI-XhoI fragment of pAV-FL-Luc-5′rzm was subcloned into pGEM-11Zf, and PCR-based mutagenesis was performed using the derived clone. For the frameshift mutation, a DNA fragment derived by inverse PCR with primers 3894 M (5′-GGCCACCTTGCGGATGGCCCAGTG-3′; nt 3894 to 3871) and 4171P (5′-GTGAAAGCGCTCCCAGGCATCAGG-3′; nt 4171 to 4191) and a DNA fragment amplified by PCR

* Corresponding author. Mailing address: Department of Virology and Parasitology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan. Phone: 81-562-93-2486. Fax: 81-562-93-4008. E-mail: jsasaki@fujita-hu.ac.jp.

▽ Published ahead of print on 23 July 2008.

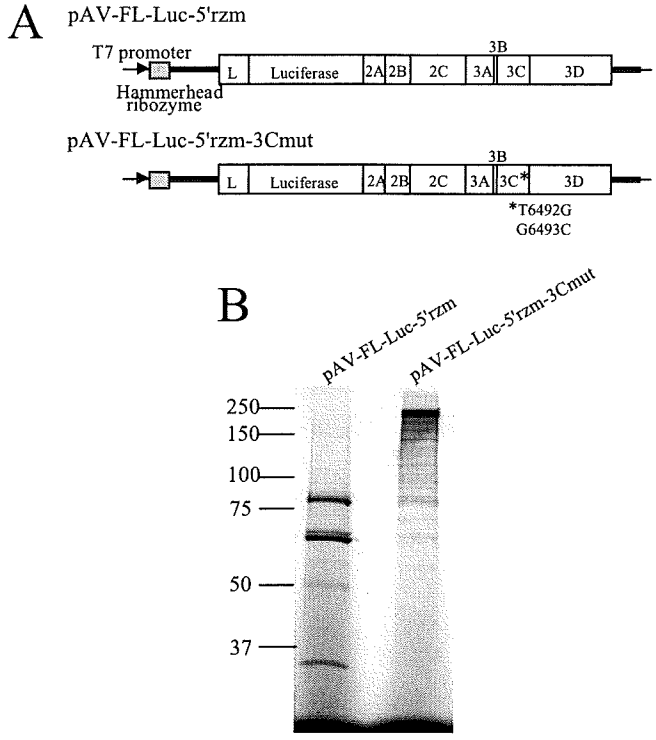

FIG. 1. (A) Schematic diagram of pAV-FL-Luc-5′rzm and pAV-FL-Luc-5′rzm-3Cmut. The virus sequences were cloned downstream of the T7 promoter. An asterisk indicates mutations (T6492G and G6493C) in the 3C-coding region. (B) In vitro transcription/translation of pAV-FL-Luc-5′rzm and pAV-FL-Luc-5′rzm-3Cmut in rabbit reticulocyte lysate. The translation products labeled with L-[^{35}S]methionine and L-[^{35}S]cysteine were analyzed by SDS-PAGE, and radioactive signals were detected. The positions of the molecular weight markers are indicated on the left.

with 3896P (5′-CCGACGGCAGTGCCAAACAGATCT-3′; nt 3896 to 3919) and G4170M (5′-CGGCAACAGCAGCCG AGGCTGCGAT-3′; nt 4170 to 4147; the inserted nucleotide is underlined) were ligated. For the NC-to-AA mutation, inverse PCR was performed using primers 4056 M (5′-GTTGG TGGCGCTGTACTCCCACTTG-3′; nt 4056 to 4032) and NC-AA4057P (5′-GCCGCTACCCACTTCGTCAGCTCCAT CACT-3′; nt 4057 to 4086; mutated nucleotides are underlined), and the PCR product was self-ligated. After the nucleotide sequences of the derived plasmids had been checked, the BclI fragment and the SacI-XhoI fragment with each mutation were replaced with the corresponding regions of pAV-FL and pAV-FL-Luc-5′rzm, respectively, yielding pAV-FL-2Afs, pAV-FL-NC-AA, pAV-FL-Luc-5′rzm-2Afs, and pAV-FL-Luc-5′rzm-NC-AA (Fig. 2A). In addition, the EcoRI fragments of pAV-FL-Luc-5′rzm, pAV-FL-Luc-5′rzm-2Afs, and pAV-FL-Luc-5′rzm-NC-AA, which contain the T7 promoter, the hammerhead ribozyme sequence, and the 5′-end 391 nt of the genome, were replaced with the corresponding fragment of pAV-FL-mut9 (11), in which the 6-nt stretches nt 3 to 8 and nt 39 to 44 were exchanged with each other. The generated plasmids were called pAV-FL-Luc-mut9, pAV-FL-Luc-mut9-2Afs, and pAV-FL-Luc-mut9-NC-AA, respectively (Fig. 2A). As negative controls for RNA replication, pAV-FL-3Dmut and pAV-FL-Luc-5′rzm-3Dmut, in which 3D RNA polymerase was inactivated (8), were used. The plasmids were linearized by digestion with HindIII, and in vitro transcripts were synthesized with T7 RNA polymerase.

The growth properties of these 2A mutants were examined. First, the abilities of the mutants to generate viable viruses were investigated. One microgram of an in vitro transcript derived from pAV-FL, pAV-FL-2Afs, or pAV-FL-NC-AA was transfected into Vero cells, using a lipofectin reagent (In-

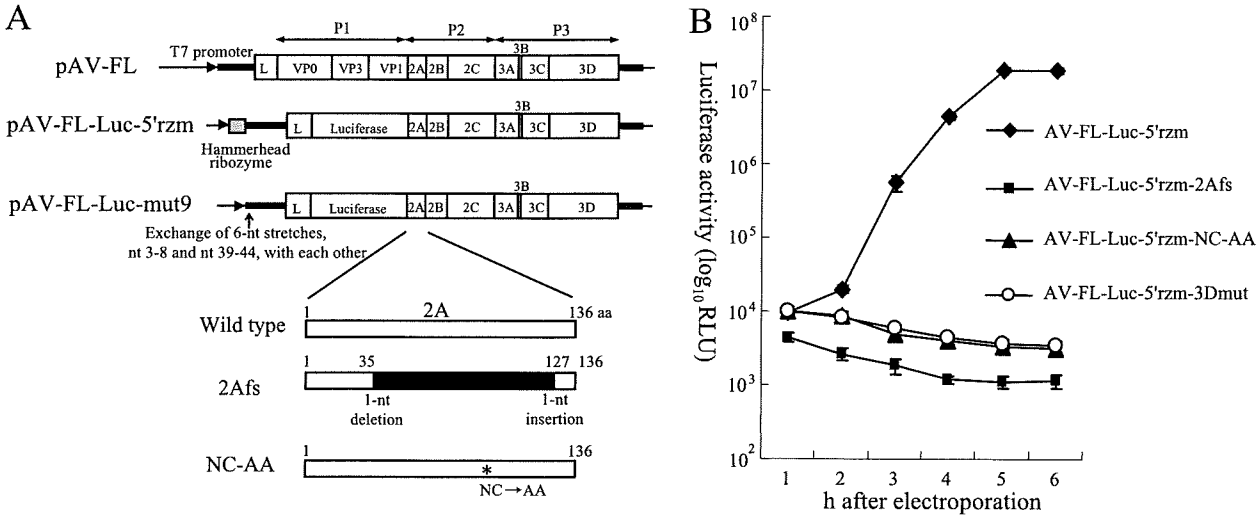

FIG. 2. (A) Organization of pAV-FL, pAV-FL-Luc-5′rzm, and pAV-FL-Luc-mut9. pAV-FL is an AiV infectious cDNA clone. pAV-FL-Luc-5′rzm is a replicon harboring the Luc gene and a hammerhead ribozyme sequence (shaded box). pAV-FL-Luc-mut9 is a replicon containing the indicated mutations at the 5′ end of the genome. The thick lines and open boxes show the UTRs and coding regions, respectively. The thin lines indicate the vector sequence. The virus sequences were cloned downstream of the T7 promoter. Two mutations were introduced into the 2A-coding region of pAV-FL, pAV-FL-Luc-5′rzm, and pAV-FL-Luc-mut9. In the 2Afs mutant, the sequence unrelated to the wild-type 2A sequence is indicated by a filled box. In the NC-AA mutant, an asterisk indicates the position of the mutated amino acids. (B) Replication of the replicon RNAs in Vero cells. Lysates of Vero cells transfected with each RNA were prepared at the indicated time points after transfection, and the Luc activity in each lysate was measured. Error bars represent the standard deviation for triplicate experiments. RLU, relative light units.

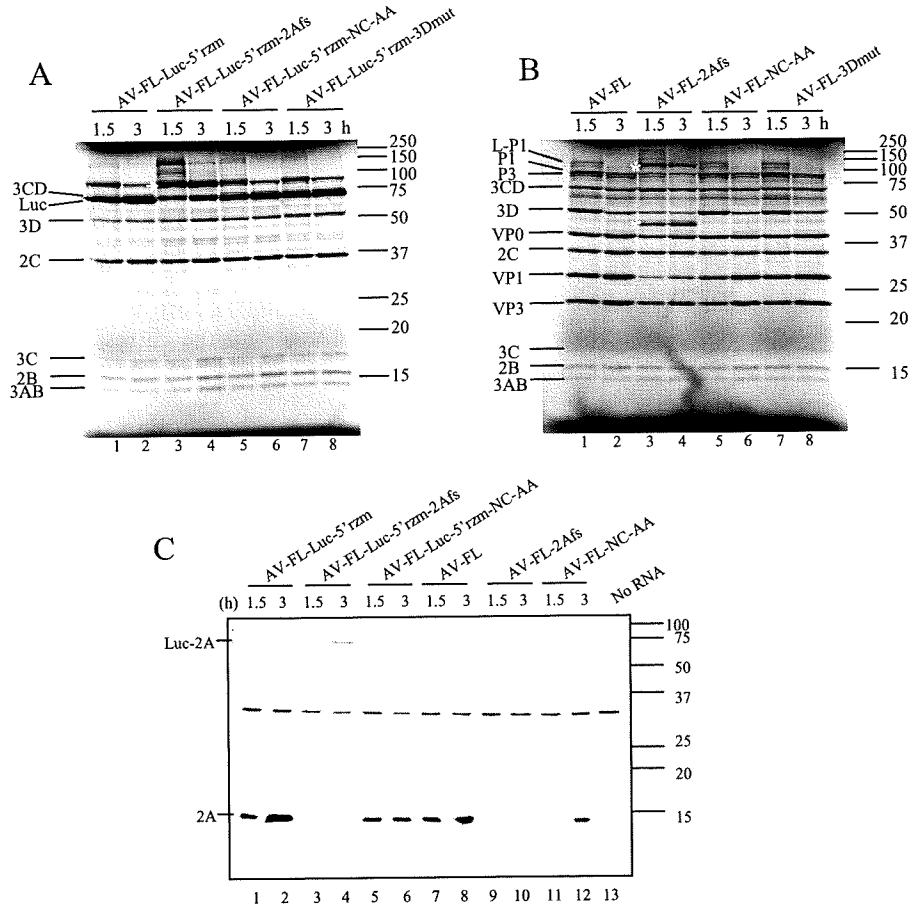

FIG. 3. Translation of AV-FL-Luc-5'rzm RNA and its mutant RNAs (A), and AV-FL RNA and its mutant RNAs (B), using Vero cell S10 extracts. For each translation reaction, aliquots collected at 1.5 and 3 h after incubation were analyzed by SDS-PAGE. (C) Immunoblot analysis of in vitro translation products, using anti-2A antiserum. The positions of the molecular weight markers and viral proteins are indicated on the right and left of each panel, respectively. An arrowhead in panel (A) indicates Luc-2A, and asterisks in panel (B) show the protein bands unique to AV-FL-2Afs.

vitrogen), and the virus titer at 72 h after transfection was determined by plaque assay as described previously (11). AV-FL RNA generated viable viruses at a titer of 10^5 PFU/ml, whereas the two mutant RNAs produced no plaques (data not shown).

Next, we examined RNA replication of the 2A mutants in Vero cells. Ten micrograms of AV-FL-Luc-5'rzm RNA, AV-FL-Luc-5'rzm-2Afs RNA, AV-FL-Luc-5'rzm-NC-AA RNA, or AV-FL-Luc-5'rzm-3Dmut RNA was electroporated into Vero cells as described previously (11), and the Luc activities of cell lysates prepared at various times were measured by using a luminometer (Lumat LB9507; Berthold) (Fig. 2B). At 1 h, no difference in the Luc activities between AV-FL-Luc-5'rzm and AV-FL-Luc-5'rzm-3Dmut was found, indicating that at this time point, RNA replication had not been initiated and that the Luc activity at this time represents the translation efficiency of the RNA. AV-FL-Luc-5'rzm-NC-AA RNA showed almost the same translation efficiency as AV-FL-Luc-5'rzm RNA. On the other hand, the Luc activity of AV-FL-Luc5'rzm-2Afs RNA at 1 h was approximately 50% lower than those of other RNAs. At 2 h, the Luc activity of AV-FL-Luc-5'rzm RNA was increased, whereas those of AV-FL-Luc-5'rzm-2Afs RNA and AV-FL-Luc-5'rzm-NC-AA RNA, as well as of AV-FL-Luc-5'rzm-3Dmut RNA, were gradually decreased, showing that the two mutants did not replicate in transfected cells.

Furthermore, translation and negative- and positive-strand syntheses of the mutants were examined by using a cell-free translation/replication system as described previously (8). The translation reaction mixture with a mixture of ~70% L-[^{35}S]methionine and ~30% L-[^{35}S]cysteine (Amersham) was incubated for 1.5 or 3 h, and then the labeled translation products were analyzed by SDS-PAGE and detected as described above (Fig. 3A and B). Additionally, proteins in the translation reaction mixtures without labeled amino acids were separated by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, immunodetected with rabbit antiserum raised against recombinant His-tagged 2A expressed in *Escherichia coli*, and then visualized by chemiluminescence (Fig. 3C). The predicted molecular mass of 2A is 14.4 kDa, and 2A must be found between 2B (17.5 kDa) and 3AB (13.7 kDa) on SDS-PAGE. However, the predicted amino acid sequences of wild-type 2A and 2A with the 2Afs mutation contain no methionine and only one cysteine, and for 2A with the NC-AA mutation, the cysteine

was changed to an alanine. Probably because of this, 2A and the 2A mutant proteins could not be detected when labeled with [^{35}S]methionine and [^{35}S]cysteine (Fig. 3A and B). Upon immunoblot analysis (Fig. 3C), wild-type 2A and 2A with the NC-AA mutation were detected (Fig. 3A and B, lanes 1, 2, 5 to 8, 11, and 12), but the processed 2A with the frameshift mutation was not (Fig. 3A and B, lanes 3, 4, 9, and 10).

There was no significant difference in the amount of the processing products such as 3D, 2C, 2B, or 3AB among the RNAs (Fig. 3A and B), indicating that the translation efficiencies of the two 2A mutant RNAs were comparable to that of the wild-type RNA. For AV-FL-Luc-5′rzm-2Afs RNA, polyprotein processing was slower than in the other RNAs (Fig. 3A). In addition, the amount of Luc was decreased, and an approximately 80-kDa protein was accumulated (Fig. 3A, lanes 3 and 4). Also for AV-FL-2Afs RNA, an abnormal processing pattern was observed: a reduced amount of VP1 and two unique protein bands were found (Fig. 3B, lanes 3 and 4). Of the two unique bands, the smaller one (approximately 45 kDa) exhibited an electrophoretic mobility (41.4 kDa) corresponding to that of VP1 (27 kDa) plus 2A (14.4 kDa). Upon immunoblotting, the 80-kDa protein was recognized by anti-2A antiserum (Fig. 3C, lane 4), and the 45-kDa protein was also detected on prolonged exposure (data not shown). These results suggest that the 2Afs mutation affects cleavage at the N terminus of 2A. The 80-kDa protein from AV-FL-Luc-5′rzm-2Afs RNA would be Luc-2A (63 kDa plus 14.4 kDa), and the 45-kDa protein from AV-FL-2Afs RNA would be VP1-2A. The failure to detect the processed 2A with the 2Afs mutation upon immunoblot analysis was probably due to inefficient processing as well as the reduced reactivity of the antiserum to the 2A protein containing the mutation. The decrease in the Luc activity observed in AV-FL-Luc-5′rzm-2Afs RNA-transfected cells (Fig. 2B) may have resulted from the reduced amount of properly processed Luc. The protein that is slightly smaller than L-P1 and that is accumulated only in AV-FL-2Afs (Fig. 3B, lanes 3 and 4) would be P1-2A. The accumulation of P1-2A suggests that the proper processing of P1 requires cleavage at the P1/2A junction beforehand. A conformational change in 2A would affect cleavage at the VP1/2A junction; in turn, the accessibility of the cleavage sites to 3C in the resulting P1-2A may be reduced compared with that in P1. It has been reported for mengovirus that mutations introduced into 2A affect the processing at the VP1/2A junction and the processing of P1-2A (19).

RNAs transcribed from plasmids harboring the ribozyme sequence were subjected to the cell-free translation/replication reaction to analyze RNA replication (Fig. 4A). RNA synthesized in the cell-free translation/replication reaction was labeled with [α-^{32}P]CTP at 3 to 5 h after the start of the reaction. Then total RNA was extracted and analyzed by nondenaturing agarose gel electrophoresis, and radioactive signals were detected. Negative- and positive-strand syntheses were evaluated as the production of the double-stranded replicative form and single-stranded RNA, respectively. In addition, AV-FL-Luc-mut9 RNA, AV-FL-Luc-mut9-2Afs RNA, and AV-FL-Luc-mut9-NC-AA RNA were analyzed to compare the efficiency of negative-strand synthesis among the RNAs in detail (Fig. 4B). The mutation introduced into mut9 has been shown to abolish positive-strand synthesis without affecting negative-strand synthesis (8). Negative-strand synthesis in the 2Afs mutant and the NC-AA mutant was decreased to 18% and 42% of that in the wild type, respectively (Fig. 4B). On the other hand, positive-strand synthesis in the 2Afs mutant was not detected and that in the NC-AA mutant was markedly reduced (Fig. 4A). Thus, the two mutations affected both negative- and positive-strand synthesis, the defect in positive-strand synthesis being more severe than that in negative-strand synthesis. Since the 2Afs mutation prevented cleavage at the N terminus of 2A (Fig. 3), in addition to the loss of function of 2A caused by the mutation, the decrease in the amount of the properly processed 2A may affect RNA synthesis. On the other hand, the mutation of the NC motif had only a moderate effect on negative-strand synthesis and mainly impaired the function of 2A required for positive-strand synthesis.

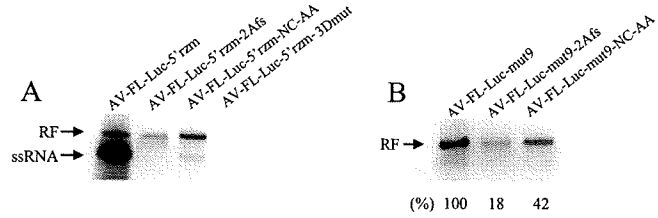

FIG. 4. Negative- and positive-strand synthesis of AV-FL-Luc-5′rzm RNA and its mutant RNAs (A) and AV-FL-Luc-mut9 RNA and its mutant RNAs (B) in the cell-free translation/replication system. The positions of replicative form (RF) and single-strand RNA (ssRNA) are indicated. In panel B, the signal intensities of products are quantitated and expressed as percentages of the product in the wild type.

Of the picornavirus 2A proteins related to H-rev107, parechovirus 2A has been studied as to its biochemical properties and its intracellular localization in infected cells (10), but direct evidence of the importance of 2A in virus replication has not been reported. This study showed that AiV 2A is essential for virus replication. AiV 2A was involved in both negative- and positive-strand synthesis, and the two mutations examined affected positive-strand synthesis more severely than negative-strand synthesis. Parechovirus 2A has been reported to interact with the 3′ UTR of the genome (10). There have been studies showing that the picornavirus 3′ UTR is involved in negative-strand synthesis (16) and positive-strand synthesis (1). It is possible that AiV 2A plays roles in negative- and positive-strand synthesis through interaction with the 3′ UTR. To understand the roles of AiV 2A in negative- and positive-strand synthesis, it will be necessary to study the interaction not only with viral RNA but also with viral and cellular proteins. The NC-AA mutant obtained in this study may be useful for such analyses because of its positive strand-specific synthesis defect.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. **Brown, D. M., S. E. Kauder, C. T. Cornell, G. M. Jang, V. R. Racaniello, and B. L. Semler.** 2004. Cell-dependent role for the poliovirus 3′ noncoding region in positive-strand RNA synthesis. J. Virol. **78:**1344–1351.
2. **Donnelly, M. L. L., G. Luke, A. Mehrotra, X. Li, L. E. Hughes, D. Gani, and M. D. Ryan.** 2001. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. J. Gen. Virol. **82:**1013–1025.

3. **Hughes, P. J., and G. Stanway.** 2000. The 2A proteins of three diverse picornaviruses are related to each other and to the H-rev107 family of proteins involved in the control of cell proliferation. J. Gen. Virol. **81**:201–207.
4. **Jurgens, C. K., D. J. Barton, N. Sharma, B. Joan Morasco, S. A. Ogram, and J. B. Flanegan.** 2006. $2A^{pro}$ is a multifunctional protein that regulates the stability, translation and replication of poliovirus RNA. Virology **345**:346–357.
5. **Kräusslich, H. G., M. J. H. Nicklin, H. Toyoda, D. Etchinson, and E. Wimmer.** 1987. Poliovirus proteinase 2A induces cleavage of eukaryotic initiation factor 4F polypeptide p220. J. Virol. **61**:2711–2718.
6. **Michiels, T., V. Dejon, R. Rodrigus, and C. Shaw-Jackson.** 1997. Protein 2A is not required for Theiler's virus replication. J. Virol. **71**:9549–9556.
7. **Nagashima, S., J. Sasaki, and K. Taniguchi.** 2003. Functional analysis of the stem-loop structures at the 5′ end of the Aichi virus genome. Virology **313**:56–65.
8. **Nagashima, S., J. Sasaki, and K. Taniguchi.** 2005. The 5′-terminal region of the Aichi virus genome encodes *cis*-acting replication elements required for positive- and negative-strand RNA synthesis. J. Virol. **79**:6918–6931.
9. **Nagashima, S., J. Sasaki, and K. Taniguchi.** 2008. Interaction between polypeptide 3ABC and the 5′-terminal structural elements of the genome of Aichi virus: implication for negative-strand RNA synthesis. J. Virol. **82**:6161–6171.
10. **Samuilova, O., C. Krogerus, T. Pöyry, and T. Hyypiä.** 2004. Specific interaction between human parechovirus nonstructural 2A protein and viral RNA. J. Biol. Chem. **279**:37822–37831.
11. **Sasaki, J., Y. Kusuhara, Y. Maeno, N. Kobayashi, T. Yamashita, K. Sakae, N. Takeda, and K. Taniguchi.** 2001. Construction of an infectious cDNA clone of Aichi virus (a member of the family *Picornaviridae*) and mutational analysis of a stem-loop structure at the 5′ end of the genome. J. Virol. **75**:8021–8030.
12. **Schultheiss, T., S. U. Emerson, R. H. Purcell, and V. Gauss-muller.** 1995. Polyprotein processing in echovirus 22 (EV22): a first assessment. Biochem. Biophys. Res. Commun. **217**:1120–1127.
13. **Sers, C., U. Emmenegger, K. Husmann, K. Bucher, A. C. Andres, and R. Schafer.** 1997. Growth-inhibitory activity and downregulation of the class II tumor-suppressor gene H-rev 107 in tumor cell lines and experimental tumors. J. Cell Biol. **136**:935–944.
14. **Sommergruber, W., M. Zorn, D. Blaas, F. Fessl, P. Volkmann, I. Maurer-Fogy, P. Pallai, V. Merluzzi, M. Matteo, T. Skern, and E. Kuechler.** 1989. Polypeptide 2A of human rhinovirus type 2: identification as a protease and characterization by mutational analysis. Virology **169**:68–77.
15. **Toyoda, H., M. J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer.** 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell **45**:761–770.
16. **van Ooij, M. J. M., C. Polacek, D. H. R. F. Glaudemans, J. Kuijpers, F. J. M. van Kuppeveld, R. Andino, V. I. Agol, and W. J. G. Melchers.** 2006. Polyadenylation of genomic RNA and initiation of antigenomic RNA in a positive-strand RNA virus are controlled by the same *cis*-element. Nucleic. Acids Res. **34**:2953–2965.
17. **Yamashita, T., S. Kobayashi, K. Sakae, S. Nakata, S. Chiba, Y. Ishihara, and S. Isomura.** 1991. Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. J. Infect. Dis. **164**:954–957.
18. **Yamashita, T., K. Sakae, H. Tsuzuki, Y. Suzuki, N. Ishikawa, N. Takeda, T. Miyamura, and S. Yamazaki.** 1998. Complete nucleotide sequence and genetic organization of Aichi virus, a distinct member of the *Picornaviridae* associated with acute gastroenteritis in humans. J. Virol. **72**:8408–8412.
19. **Zoll, J., F. J. M. van Kuppeveld, J. M. D. Galama, and W. J. G. Melchers.** 1998. Genetic analysis of mengovirus protein 2A: its function in polyprotein processing and virus reproduction. J. Gen. Virol. **79**:17–25.

Acute Encephalitis Caused by Intrafamilial Transmission of Enterovirus 71 in Adult

Tsuyoshi Hamaguchi,*† Hironori Fujisawa,‡ Kenji Sakai,† Soichi Okino,† Naoko Kurosaki,§ Yorihiro Nishimura,¶ Hiroyuki Shimizu,¶ and Masahito Yamada*

Enterovirus 71 (EV71) is a common cause of hand, foot, and mouth disease and sometimes causes severe neurologic complications, mainly in children. We report a case of adult-onset encephalitis caused by intrafamilial transmission of a subgenogroup C4 strain of EV71. This case elucidates the risk for EV71 encephalitis even in adults.

In children, enterovirus 71 (EV71) is a common cause of hand, foot, and mouth disease (HFMD), and most patients recover within 4–6 days. However, severe neurologic complications, such as acute encephalitis and poliolike paralysis, develop in some patients with EV71 infection. In the largest and most severe EV71-associated HFMD outbreak occurring in Taiwan in 1998, 405 children had severe neurologic complications, pulmonary edema, or both; 78 children died (*1*). In adults, transmission of EV71 within households is common, but EV71 infection is commonly limited to mild illness, and neurologic complications are uncommon in adults (*2–4*). We report a case of acute EV71 encephalitis in a mother and cases of HFMD in her 3 sons due to intrafamilial transmission of EV71.

The Case

In November 2006, a 37-year-old woman without serious past illness sought treatment at our hospital with hand tremor, unsteadiness, and a 2-day history of headache (day 1). Examination showed high fever (39.3°C), neck stiffness, intentional tremor of bilateral upper extremities, and truncal ataxia. Brain magnetic resonance images (MRI) and results of laboratory blood tests were normal. A cerebrospinal fluid (CSF) tap showed 305 leukocytes/mm^3 (82.5% polymorphonuclear leukocytes and 17.5% lymphocytes) and total protein concentration of 62 mg/dL with normal glucose levels. Empirical therapy with acyclovir and cefotaxime was initiated. On day 4, the patient reported diplopia and slurred speech. Ocular movements were not obviously restricted, and the extremities showed ataxia without weakness. She could not sit on the bed without support because of severe unsteadiness. Deep tendon reflexes were absent, and the patient had no pathologic reflexes. Brain MRI showed hyperintense lesions in the tegmentum of the medulla oblongata, pons, and midbrain in T2-weighted and fluid attenuated inversion recovery images (Figure 1). No abnormalities of the cervical spinal cord were detected on MRI. Results of nerve conduction studies were within normal ranges except for the absence of an F-wave in the median and ulnar nerves. Methylprednisolone (1 g/day) was administered for 3 days. From day 5 and forward, the patient gradually improved. A CSF tap on day 15 showed 14 leukocytes/mm^3 (100% lymphocytes) and a total protein concentration of 50 mg/dL. On day 22, MRI showed that the brain had normalized. Three months after the onset of disease, she had completely recovered.

During the illness, CSF was negative for bacteria and viruses. Enterovirus-specific RNA was detected from a stool sample on day 16 by a seminested reverse transcription–PCR (RT-PCR) with consensus-degenerative primers from Nix et al. (*5*); the virus was identified as EV71 by sequence analysis of the partial VP1 region (*5*). Serum neutralizing antibody titer against EV71 increased, from 8 on day 1 to 128 on day 15. There was no increase in serum antibodies against other viruses, including herpes simplex virus, cytomegalovirus, varicella-zoster virus, Epstein-Barr virus, rubella virus, and mumps virus by enzyme immunoassay, and against Japanese encephalitis virus by hemagglutination-inhibition test. Results for antinuclear antibody and antiganglioside antibodies were also negative.

Three days before the patient sought treatment, her 1-year-old son was affected with HFMD. This disease also developed in her other 2 sons, 5 and 7 years of age, on day 2. Her 3 children recovered within several days without any neurologic complications. Enterovirus-specific RNA was also detected in the stool samples from the 3 children by seminested RT-PCR (*5*), and all 3 viruses were identified as EV71 by sequence analysis. In addition, EV71 was isolated in Vero cells from stool samples from 2 of the 3 sons with HFMD. Stool and other clinical samples from the mother were all negative for virus isolation on Vero and RD cells.

The partial VP1 sequences (150 bp) of the PCR products directly amplified from stool samples of all 4 cases were 100% identical. The entire VP1 sequences (891 bp) of the EV71 isolates from 2 of her sons with HFMD (07-Ishikawa and 08-Ishikawa) were also 100% identical. Phy-

*Kanazawa University Graduate School of Medical Science, Kanazawa, Japan; †Ishikawa Prefecture Central Hospital, Kanazawa, Japan; ‡Fujii Neurosurgical Hospital, Kanazawa, Japan; §Ishikawa Prefectural Institute of Public Health and Environmental Science, Kanazawa, Japan; and ¶National Institute of Infectious Diseases, Tokyo, Japan

logenetically, by using VP1-based genetic classification, the isolates were classified as subgenogroup C4 (Figure 2) (*6*). The subgenogroup C4 of EV71 has recently been identified in Japan and might have emerged in the surrounding countries, mainland People's Republic of China and Taiwan (*6–9*). The 07-Ishikawa strain shows a close genetic relationship to recent subgenogroup C4 strains in mainland China (97.4% nt identity to the SHZH04–38 strain) and those in Japan (97.0% nt identity to the 2779-Yamagata strain, Figure 2) (*7,9*).

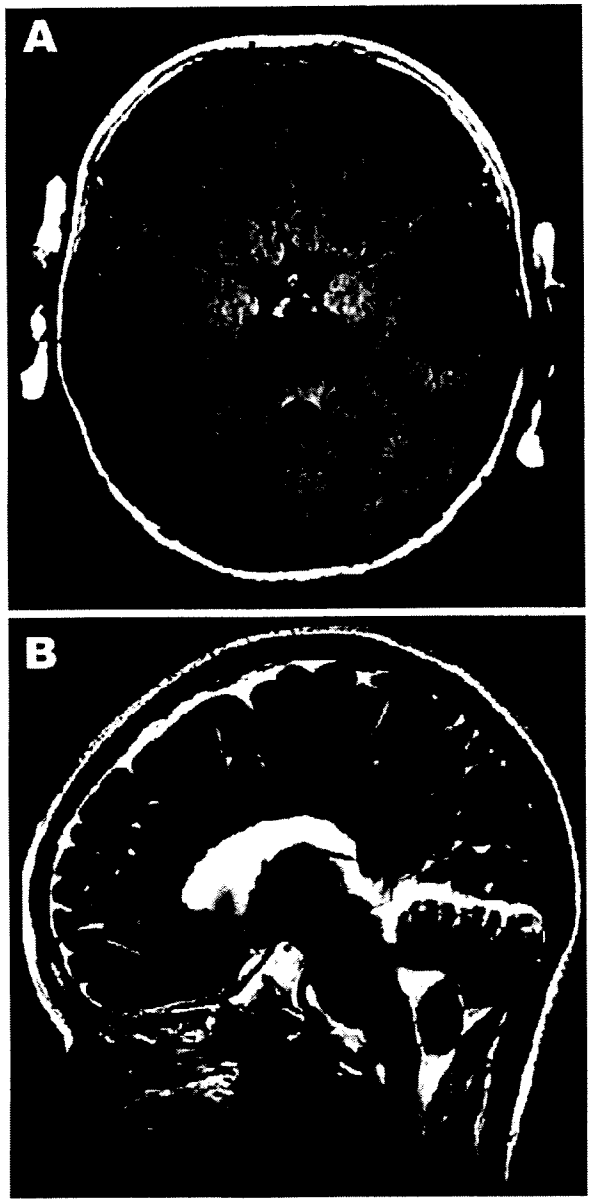

Figure 1. Magnetic resonance images of the brain. A) Hyperintense lesions in the tegmentum of the pons in the axial section of the fluid-attenuated inversion recovery image. B) In the sagittal section of the T2-weighted image, hyperintense lesions are present in the tegmentum of the midbrain, pons, and medulla oblongata.

Conclusions

Several EV71 outbreaks have been documented throughout the world, and clinical manifestations of EV71 infections can be diverse, including HFMD, herpangina, central nervous system (CNS) complications, and pulmonary edema. Recently, EV71-associated HFMD outbreaks with severe CNS complications have frequently been reported, especially in the Asian-Pacific region (*2,3*). In children, the CNS complications associated with EV71 manifest clinically in various ways, such as aseptic meningitis, acute flaccid paralysis, and rhombencephalitis; rhombencephalitis is one of the most common severe neurologic symptoms (*2*).

We diagnosed the mother's illness as EV71 encephalitis because the clinical features were similar to those of EV71 rhombencephalitis in children (*2,10*), although there has not previously been a detailed case report of adult-onset EV71 encephalitis. EV71 was not identified in the mother's CSF sample by virus isolation or direct molecular detection by RT-PCR, but EV71 was identified in her stool sample. We could not exclude the possibility of para- or post-infectious encephalitis during the initial stage of her illness. However, rhombencephalitis subsequently developed in this patient, which is common in children with EV71 encephalitis but is far less common from other infections and para- or post-infectious encephalitis. Clinical symptoms, MRI, and CSF findings of her illness were similar to those reported in children with EV71 encephalitis.

Several previous studies have demonstrated a rather low virus isolation rate in CNS specimens compared with that in other clinical samples, such as throat swab, rectal swab, and stool samples from EV71-associated cases with HFMD, encephalitis, or both (*2,3,11*). Along with the identification of EV71, the increase in serum neutralizing antibody titer against EV71 supports the diagnosis of acute EV71 infection. In addition, the lack of abnormalities of the spinal cord on MRI, the absence of an F-wave on nerve conduction study (a possible sign of radiculopathy), and the absence of deep tendon reflexes without weakness support the possibility of radiculitis as a complication. In a previous report, some patients with rhombencephalitis showed hyporeflexia or areflexia, but nerve conduction study findings were not reported (*2*).

Genetic analysis among 4 different EV71 isolates from the patients indicated probable intrafamilial transmission of EV71. In a recent study, EV71 transmission rate to household contacts was 52%, and the transmission rate from children to parents was 41% (*4*). Twenty-one percent of EV71-infected children experienced serious complications,

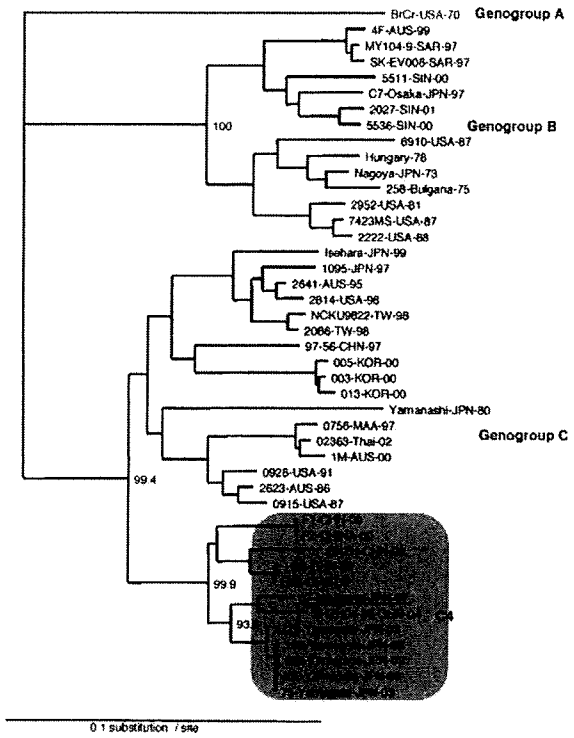

Figure 2. Phylogenetic analysis of EV71 based on the entire VP1 sequences. The tree was prepared by the neighbor-joining method by using the EV71 strains in the world as described previously (*6*) and newly identified subgenogroup C4 strains (*7,8*) were also included in the analysis.

including CNS or cardiopulmonary failure. By contrast, 53% of adults were asymptomatic, and all symptomatic adults recovered completely from uncomplicated illnesses (*4*). Considerable attention has been paid to EV71 infection in children because young age was considered the major risk factor associated with severe CNS complications, such as encephalitis resulting in severe neurologic sequelae and deaths (*2–4,12*). Thus, less attention has been paid to the adult-onset EV71 encephalitis. Our patient showed a good prognosis; however, a 19-year-old man died from EV71 encephalitis in Singapore (*3*). More careful disease surveillance, even for adults, will be needed during EV71-associated HFMD outbreaks.

Dr Hamaguchi is clinical research fellow of the Department of Neurology and Neurobiology of Aging, Kanazawa University Graduate School of Medical Science. He has a broad interest in neurologic infectious diseases.

References

1. Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, et al. An epidemic of enterovirus 71 infection in Taiwan. N Engl J Med. 1999;341:929–35.
2. Huang CC, Liu CC, Chang YC, Chen CY, Wang ST, Yeh TF. Neurologic complications in children with enterovirus 71 infection. N Engl J Med. 1999;341:936–42.
3. Chan KP, Goh KT, Chong CY, Teo ES, Lau G, Ling AE. Epidemic hand, foot, and mouth disease caused by human enterovirus 71, Singapore. Emerg Infect Dis. 2003;9:78–85.
4. Chang LY, Tsao KC, Hsia SH, Shih SR, Huang CG, Chan WK, et al. Transmission and clinical features of enterovirus 71 infections in household contacts in Taiwan. JAMA. 2004;291:222–7.
5. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol. 2006;44:2698–704.
6. 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.
7. 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.
8. Lin KH, Hwang KP, Ke GM, Wang CF, Ke LY, Hsu YT, et al. Evolution of EV71 genogroup in Taiwan from 1998 to 2005: an emerging of subgenogroup C4 of EV71. J Med Virol. 2006;78:254–62.
9. Li L, He Y, Yang H, Zhu J, Xu X, Dong J, et al. Genetic characteristics of human enterovirus 71 and coxsackievirus A16 circulating from 1999 to 2004 in Shenzhen, People's Republic of China. J Clin Microbiol. 2005;43:3835–9.
10. Shen WC, Chiu HH, Chow KC, Tsai CH. MR imaging findings of enteroviral encephalomyelitis: an outbreak in Taiwan. AJNR Am J Neuroradiol. 1999;20:1889–95.
11. Chang LY, Lin TY, Huang YC, Tsao KC, Shin SR, Kuo ML, et al. Comparison of enterovirus 71 and coxsackievirus A16 clinical illnesses during the Taiwan enterovirus epidemic, 1998. Pediatr Infect Dis J. 1999;18:1092–6.
12. Chang LY, Huang LM, Gau SS, Wu YY, Hsia SH, Fan TY, et al. Neurodevelopment and cognition in children after enterovirus 71 infection. N Engl J Med. 2007;356:1226–34.

Address for correspondence: Tsuyoshi Hamaguchi, Department of Neurology and Neurobiology of Aging, Kanazawa University Graduate School of Medical Science, 13-1, Takara-machi, Kanazawa 920-8640, Japan; email: gom56@med.kanazawa-u.ac.jp

Journal of Medical Virology 80:670–679 (2008)

Molecular Typing and Epidemiology of Non-Polio Enteroviruses Isolated From Yunnan Province, the People's Republic of China

Tian Bingjun,[1] Hiromu Yoshida,[2]* Wu Yan,[1] Lu Lin,[1] Takao Tsuji,[3] Hiroyuki Shimizu,[2] and Tatsuo Miyamura[2]

[1]*Polio Laboratory, Yunnan Center for Disease Control & Prevention, Kunming, Yunnan Province, The People's Republic of China*
[2]*Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan*
[3]*Department of Microbiology, School of Medicine, Fujita Health University, Toyoake, Aichi, Japan*

This report presents an overview of human enteroviruses in Yunnan Province, the People's Republic of China. A total of 210 non-polioviruses isolated under acute flaccid paralysis (AFP) surveillance during a total study period of 5 years—1997 to 2000 and 2004—were examined. Of the 210 non-poliovirus isolates, 12 adenoviruses were serologically identified, and the remaining 198 isolates were used for molecular typing. The viral genomes of 195 non-polio enteroviruses (NPEVs) on VP1 partial region of virus capsid were translated to the corresponding amino acid sequences; these were compared with those of prototype strains. Based on molecular typing, 5 isolates were classified into 5 serotypes of the *human enterovirus A* species, 158 isolates, into 35 serotypes of the *human enterovirus B* species; and 32 isolates, into 6 serotypes of the *human enterovirus C* species. Viruses belonging to the *human enterovirus D* species were not isolated. Thus, under AFP surveillance, the *human enterovirus B* species accounted for 75.2% of the 210 isolates, and it was considered the predominant species. This was followed by *human enterovirus C* (12.2%), adenovirus (5.7%), and *human enterovirus A* (2.4%). Further, molecular analysis suggested that several serotypes of *human enteroviruses B* and *C* that exhibited genetic polymorphism were indigenous. Molecular typing methods may aid in understanding the epidemiology of NPEVs in Yunnan Province. ***J. Med. Virol. 80:670–679, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: enterovirus; molecular typing; epidemiology

INTRODUCTION

Human enteroviruses (HEVs) are small non-enveloped RNA viruses that belong to the family *Picornaviridae*. Although polioviruses are known to be the causative agents of poliomyelitis, 64 other immunologically distinct non-polio enteroviruses (NPEVs) are also recognized to cause a wide variety of clinical diseases, particularly in children. Most of these clinical diseases are mild or asymptomatic; however, some NPEVs may cause serious illnesses such as aseptic meningitis, paralysis, encephalitis, and acute myocarditis [Wang et al., 1993; Xu et al., 1996; Peng et al., 2000; Pallansch and Roos, 2001]. Enterovirus infections are also associated with other illnesses with chronic courses, such as type I diabetes mellitus [Pallansch and Roos, 2001].

Recently, a new classification of HEVs has been adapted based on their molecular characterization. The genus *human enterovirus* was subgrouped into 5 species: *poliovirus* (3 serotypes), HEV-A (13 serotypes), HEV-B (41 serotypes), HEV-C (9 serotypes), and HEV-D (2 serotypes) [Stanway et al., 2005]. Moreover, several methods based on molecular approaches have been reported, and subsequently, new serotypes have been confirmed [Oberste et al., 2001, 2'004, 2005, 2006; Norder et al., 2003; Junttila et al., 2007].

Grant sponsor: Japan-China Medical Association; Grant sponsor: Ministry of Health and Welfare, Japan (Promotion of Polio Eradication and Research on Re-emerging Infectious Diseases).

*Correspondence to: Hiromu Yoshida, Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan.
E-mail: hyoshida@nih.go.jp

Accepted 30 November 2007
DOI 10.1002/jmv.21122
Published online in Wiley InterScience (www.interscience.wiley.com)

Yunnan Province is located in the southwestern part of China, and it shares its borders with Myanmar, Laos, and Vietnam; it has a 4,060-km border that accounts for one-sixth of the total border of China. The area of this province is 394,000 km^2, of which 94% is mountainous. In 1999, the total population of Yunnan Province was 42 million, of which 11 million (26.9%) were 15-year-old children. Further, 25 ethnic groups (minority nationalities) with a population of 15 million (35.5% of the total population) also reside in Yunnan Province.

Following the polio eradication program, the Western Pacific Region was declared free from the transmission of wild-type polioviruses in 2000. Since 1995, no indigenous wild-type polioviruses have been detected in China [Centers for Disease Control and Prevention, 1996; Zhang et al., 1997]. Historically, poliomyelitis has been one of the most common childhood diseases in Yunnan Province. With the adoption of "Expanded Program on Immunization" (EPI) in 1983, the vaccine coverage rate increased annually and has been maintained at more than 85%. The program has achieved tremendous success, particularly with the adoption of the polio eradication initiative in 1990. Since 1994, no indigenous polioviruses have been identified in Yunnan Province [Chiba et al., 2001].

On the other hand, many NPEVs have been isolated as "side products" during acute flaccid paralysis (AFP) surveillance [Bahri et al., 2005]; however, the serotypes and epidemiology of the NPEVs isolated from Yunnan Province remain unknown. In fact, NPEVs are not always the corresponding causative agents of AFP. Further information on NPEVs may play a role in controlling infectious enteroviral diseases.

This paper presents the results of the isolation of enteroviruses from Yunnan Province during AFP surveillance in the 5-year study period—from 1997 to 2000 and 2004—and the identification of these viruses by molecular typing. It also provides a genetic overview of the detected serotypes and their patterns of occurrence.

MATERIALS AND METHODS

Non-Polioviruses

During the study period of 5 years from 1997 to 2000 and 2004, a total of 1,260 AFP cases in children aged <15 years were reported from Yunnan Province; no specimens from 2001 to 2003 were available. Out of these 1,260 cases, at least 1 stool specimen was collected from 1,219 cases. These samples were processed according to the standard procedures of the World Health Organization (WHO) [World Health Organization, 2000] and inoculated into cell lines. For the 1997–2000 samples, RD, HEp-2, and L20B cells were used. From 2001 onward, only RD and L20B cells were used. After a complete cytopathic effect (CPE) was obtained, the infected cells were harvested and maintained under frozen conditions (−20°C). A total of 299 viruses were isolated and used for poliovirus identification according to the WHO standard procedures. A total of 89 isolates were identified as polioviruses in the poliovirus neutralization assay. All poliovirus isolates were forwarded to the Regional Reference Polio Laboratory and confirmed to be Sabin-like strains (data not shown). The remaining 210 non-polioviruses (NPVs) were used in our study.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Serotype Identification of NPEV by Partial Sequencing of the VP1 Capsid Region

Viral RNA was extracted from 140 μl of virus-infected culture supernatant by using the QIAamp viral RNA mini kit (Qiagen, Inc., Hilden, Germany) and eluted in 60 μL of sterile water according to the manufacturer's instructions. RT-PCR was carried out using the Access RT-PCR system (Promega, Madison, WI). Primers used for cDNA synthesis, RT-PCR, and sequencing procedures were the same as those previously described by Oberste et al. [2000]. RT-PCR reactions were carried out as follows: incubation at 48°C for 45 min, followed by incubation at 94°C for 2 min. Thermocycling was performed for 35 cycles of 94°C for 10 sec; 50°C for 10 sec, and 65°C for 1 min by using a model 9700 thermal cycler (Applied Biosystems, Foster City, CA). This was followed by incubation at 65°C for 5 min. The reaction products were analyzed by electrophoresis in a 2% agarose gel and identified by staining with 0.5 μg/ml ethidium bromide. These products were purified using the QIAquick PCR purification kit (Qiagen), and their sequences were determined by automated methods using dideoxy chain terminators (Applied Biosystems). Both strands were sequenced simultaneously and edited with the Sequencer software package (Gene Codes Corporation, Ann Arbor, MI).

For the typing of each isolate, VP1 RT-PCR with primer pairs 187–222, 188–222, and 189–222 that corresponded to the 5′ end of VP1 was initially attempted, as previously described by Oberste et al. [2000]. For isolates that could not be amplified using the primer pairs 187–222, 188–222, and 189–222, amplification was attempted by using the primer pairs 012–011 and 040–011 that corresponded to the 3′ end of VP1. As recommended by Oberste et al. [1999a,b], the serotype was determined by comparing the sequences obtained from the 5′ end of the VP1 region with the sequences of the same region of prototype strains existing in the GenBank database. The same procedure was followed for the sequences obtained from the 3′ end of the VP1 region.

In this study, a serotype was considered to be homologous when its VP1 amino acid (aa) sequence showed at least 88% [Oberste et al., 1999a] identity to the VP1 aa sequence of any of the HEV prototype strains. This means that the cutoff value for serotyping was a 12% divergence of the aa sequence of the partial VP1 region of the isolate from that of the prototype strain.

To determine the genetic polymorphism of the isolates from the prototype strains and among the isolates themselves, the values of both—aa identity with the

prototype strains and nucleotide identity among the isolates—were calculated.

Phylogenic Analysis of Sequence Data

As for representative isolates in this study, phylogenic trees were constructed for each HEV-A, HEV-B, and HEV-C species by using the neighbor-joining algorithms of the MEGA software version 3.1 [Kumar et al., 2004]. The nucleotide sequence data for prototype and available strains from neighboring countries in GenBank were used for comparison. Genetic distances were calculated using the Kimura-2 parameter model [Kimura, 1980]. The transition/transversion rate was set as 2.0. The robustness of the constructed phylogenies was estimated by bootstrap analysis with 1,000 pseudoreplicate data sets [Felsenstein, 1985].

Accession Numbers of the Nucleotide Sequences

The sequences reported in this study have been deposited in the nucleotide sequence database under accession numbers from AB268121 to AB268314 and AB268548.

Serological Confirmation of Common NPEVs Identified by Partial Sequencing

Representative isolates were selected from the isolates that formed a cluster with any of the prototype strains and used for a neutralization assay with a type-specific antiserum according to the procedure described by Arita et al. [2005]. For 2 hr at 37°C, 50 μl of 100 CCID50 enterovirus isolates were incubated with 50 μl of 20–40 units of antiserum. This was followed by the addition of 100 μl of HEp-2 or RD cell suspension (1.5×10^5 cells/ml) in 10% fetal calf serum-minimum essential medium and incubation at 36°C [World Health Organization, 2000; Arita et al., 2005]. The inoculated cells were observed daily for CPE, and observation was continued until 24 hr after the complete appearance of CPE.

Identification of Adenoviruses

Some NPVs that were isolated on HEp-2 cells with typical adenovirus CPE such as the "grape" form could neither be typed by the polio antiserum pool nor be amplified by the enterovirus PCR primers. Adenovirus test (SA Scientific, Inc., San Antonio, TX) was used to determine the type of the isolates, that is, if the isolates were adenoviruses.

RESULTS

NPVs Isolated From 1997 to 2000 and 2004

During the 5-year study period—1997 to 2000 and 2004—a total of 210 NPVs were isolated from stool samples collected from 1,219 AFP cases. The number of reported AFP cases, specimen collection rates, and NPV isolation rate for each year are shown in Table I. The NPV isolation rate varied from year to year and ranged from 11.3% to 26.4% per year with a mean rate of 17.2%.

Of the 210 NPVs, 12 adenoviruses were identified. This included five isolates from 1997, 4 from 2000, and 1 each from 1998, 1999, and 2004. The remaining 198 isolates were used for molecular typing. RT-PCR and sequencing analysis were performed for these isolates by using pan-enterovirus primers, and the viral genomes of 195 isolates that were translated into corresponding aa sequences were compared with the prototype strains. Since the sequencing analysis of three isolates failed to yield desired results, they were not used for typing (unknown NPVs).

Based on the cutoff value of 12% divergence of aa sequence of the isolates from those of the prototype strains, the 195 isolates were classified into 3 species: HEV-A, HEV-B, and HEV-C. Of these, 5 isolates belonged to HEV-A species and were classified into 5 serotypes (Table IIa) while 158 and 32 isolates belonged to HEV-B and HEV-C and were classified into 35 and 6 serotypes, respectively (Table IIb and c, respectively). The viruses that belonged to HEV-D were not isolated. Thus, under AFP surveillance, a total of 210 NPVs were isolated, of which 75.2% belonged to HEV-B species, followed by 12.2% to HEV-C, 5.7% to adenovirus, and 2.4% to HEV-A.

Genetic Divergence of the Isolates From the Prototypes and Among Each Other

HEV-A species. For 5 isolates, the nucleotide sequences of the amplicon that were amplified using the 188–222 primer pair corresponding to the 5′ end of their VP1 genomic region were translated into aa sequences and compared with those of the prototype strains (Table IIa). The number of isolates obtained each year is shown in Table IIa. The divergence of the aa

TABLE I. Reported AFP Cases, Specimen Collection Rate and Virus Isolation, Yunnan Province, P.R. China, 1997–2000 and 2004

Year	Reported AFP cases	Cases with ≥1 stool collected	No. of NPVs[a]	NPVs isolation rate (%)
1997	288	275	37	13.5
1998	228	220	31	14.1
1999	227	224	45	20.1
2000	276	269	71	26.4
2004	241	231	26	11.3
Total	1,260	1,219	210	17.2

[a]Numbers of nonpolioviruses

TABLE II. NPEVs Isolation in Each Species, and Their Distribution by Year, Yunnan Province

Serotype	Numbers of isolates					Total	Range of nt (aa) divergenceamong isolates (%)[a]	nt divergence (aa) between prototype strain and isolates (%)[a]
	1997	1998	1999	2000	2004			
(a) Human enterovirus A (HEV-A) species								
CA4	0	0	0	0	1	1	nc	13.8 (0.9)
CA6	0	0	0	0	1	1	nc	14.0 (0.9)
CA8	0	0	0	1	0	1	nc	15.0 (0.9)
EV71	0	0	0	0	1	1	nc	17.6 (3.6)
EV76	0	0	1	0	0	1	nc	12.7 (1.8)
Total	0	0	1	1	3	5	—	—
(b) Human enterovirus (HEV-B) species								
E13	4	1	2	3	3	13	0–24.0 (0–8.7)	20.0–24.4 (3.9–8.7)
E14	2	0	3	5	1	11	0–21.5 (0–3.8)	19.0–23.7 (1.9–4.7)
E12	0	3	0	5	3	11	0–12.4 (0–3.0)	16.1–17.7 (2.0–5.1)
CB3	1	0	3	4	1	9	0–7.0 (0–3.2)	23.3–24.7 (5.3–6.3)
E2	2	1	2	3	1	9	0–18.8 (0–4.5)	20.8–24.4 (7.2–9.0)
E6	3	4	2	0	0	9	0–21.5 (0–4.9)	21.9–25.7 (5.8–8.7)
E1	2	0	4	2	0	8	0.3–25.6 (0–6.7)	17.0–27.4 (0–7.6)
E7	1	3	0	3	0	7	1.7–10.3 (0.8–2.3)	20.3–21.7 (4.3–5.0)
E29	1	0	2	0	4	7	0–8.3 (0–2.8)	16.3–17.5 (2.8)
E11	0	1	1	0	4	6	0–15.4 (0–2.8)	20.5–22.5 (4.8–6.2)
E3	2	0	3	0	0	5	0–7.8 (0)	14.2–14.6 (1.03)
E19	2	3	0	0	0	5	0–6.5 (0–0.9)	19.6–20.8 (8.4–9.4)
E20	2	3	0	0	0	5	1.6–10.9 (0–1.9)	20.6–21.3 (4.7)
E27	0	0	0	5	0	5	0.3–1.7 (0–0.9)	22.4–23.5 (9.4–10.3)
CB5	2	0	0	2	0	4	4.8–20.4 (0–5.6)	17.8–18.5 (4.5–5.6)
CA9	1	1	0	2	0	4	5.0–12.1 (0–1.8)	18.3–19.8 (3.6–5.4)
E21	0	0	4	0	0	4	0.3–0.6 (0–1.7)	20.0–20.3 (2.6–3.5)
E24	3	0	0	1	0	4	0.7–7.6 (0–3.1)	20.8–21.5 (3.1–4.2)
E30	0	0	0	1	2	3	0–22.1 (0–7.7)	17.6–22.4 (7.7–10.3)
CB1	0	0	2	1	0	3	0.7–2.6 (0–1.0)	23.5–25.1 (9.9–10.9)
E25	0	1	0	2	0	3	15.8 (3.5)	19.5–19.8 (5.2–6.0)
EV75	0	0	0	3	0	3	0 (0)	19.5 (6.1)
CB4	0	1	0	1	0	2	nc	13.1 (0.9)
E9	0	0	0	2	0	2	5.1 (4.0)	17.5–20.1 (5.0–7.0)
E18	0	0	0	2	0	2	8.0 (1.7)	16.6–17.5 (3.5)
E33	0	0	0	0	2	2	0.3 (0)	24.7 (3.6)
EV83	0	0	2	0	0	2	22.3 (3.0)	16.5–23.3 (2.0)
EV93	0	0	1	1	0	2	1.7 (5.5)	NA
Not typed[b]	0	0	0	1	0	1	nc	nc
E15	0	0	0	1	0	1	nc	24.1 (8.1)
CB2	0	0	0	1	0	1	nc	16.7 (0.7)[d]
CB6	0	0	0	1	0	1	nc	22.1 (8.0)[d]
E4	0	0	0	1	0	1	nc	18.2 (6.6)[d]
EV80	0	0	1	0	0	1	nc	NA
E31	1	0	0	0	0	1	nc	17.7 (1.8)
EV81	0	1	0	0	0	1	nc	17.5 (3.0)
Total	29	23	32	53	21	158	—	—
(c) Human enterovirus (HEV-C) species								
CA24	1	2	5	3	0	11	8.3–18.1 (1.0–8.7)	17.1–21.0 (1.9–7.7)
EV96	0	1	3	4	1	9	10.7–16.1 (1.7–7.7)	18.4–23.5 (3.4–6.8)
CA20	2	0	0	4	0	6	5.7–19.4 (1.0–3.7)	20.9–22.4 (4.6–6.4)
Not typed[c]	0	3	0	1	0	4	0.9–7.9 (0–2.6)	nc
CA18	0	0	1	0	0	1	nc	22.1 (7.0)
CA17	0	0	1	0	0	1	nc	24.6 (9.2)
Total	4	6	9	12	1	32	—	—

CA, coxsackievirus A; EV, enterovirus; nt, nucleotide; aa, amino acid; nc, not calculated.
[a]Nucleotide (amino acid) divergence was calculated using 5′ end of VP1.
[b]Described in the Results Section of text.
[c]Not typed four isolates made a cluster, and considered as the same serotype.
[d]3′ end of VP1 sequence was used.

sequence of the HEV-A species from each prototype strain ranged from 0.9% to 3.6% (Table IIa).

HEV-B species. Table IIb shows 158 HEV-B isolates that were classified into 35 serotypes. The divergence of the aa sequence of the HEV-B species from that of each prototype strain ranged from 0% to 10.9% (Table IIb), but 1 isolate could not be classified into either serotype because its sequence diverged from that of the prototype by over 12%. The number of isolates obtained each year is shown in Table IIb. Coxsackievirus

B2 (CB2), CB6, and echovirus 4 (E4) isolates were subjected to molecular typing using amplicon sequences that were amplified using the 012–011 primer pair corresponding to the 3′ end of VP1, and the other serotypes were typed using the 187–222 primer pair. Isolate 75–99 was considered as enterovirus 80 (EV80), and both isolates 113-00 and 133-99 were considered as EV93 (Dr G. Stanway, personal communication). Since information on the sequences of the prototype strains was not available, the divergence of these serotypes from the prototype strains is not shown in Table IIb.

The representative isolates were confirmed by neutralization assays using type-specific antisera, except in the case of 7 serotypes (CB4, E31, EV75, EV80, EV81, EV83, and EV93).

The ranges of nucleotide divergence among the isolates in each serotype are shown in Table IIb. Of the 35 serotypes, 14 (E13, E14, E12, E2, E6, E1, E7, E11, E20, CB5, CA9, E30, E25, and EV83) demonstrated relatively high nucleotide divergence ranging from 0% to 10–25% Of these 14 serotypes, 8 (E1, E2, E6, E7, E11, E12, E13, and E14) were frequently isolated for 3 to 5 years during the 5-year study period (Table IIb)and provided high nucleotide divergence among the isolates; therefore, they were assumed to be common viruses in Yunnan Province.

In contrast, five serotypes (CB5, E20, E25, E30, and EV83) were isolated only for 1–2 years. Since they exhibited nucleotide divergence ranging from 1.6% to 22.3%, they might also be potentially indigenous viruses, similar to the eight above mentioned serotypes.

Of the 14 serotypes isolated for only 1 year (Table IIb), E27, E21, EV75, E9, E18, E33, and EV83 had multiple isolates, namely, 5, 4, 3, 2, 2, 2, and 2, respectively. However, EV83, E27, E21, EV75, E9, E18, and E33 exhibited less than 8% nucleotide divergence and were considered to belong to the same group.

We could not identify the 65-00 isolate by partially sequencing the VP1 region because it exhibited over 12% aa divergence from the prototype. The closest relative to isolate 65-00 was the E26 prototype strain, showing 28.0% nucleotide divergence and 16.7% aa sequence divergence with regard to a 325-bp amplicon that was amplified using the 187–222 primer pair. Moreover, since the 65-00 isolate was not neutralized by type-specific antisera, it was classified as "untyped HEV-B species."

HEV-C species. There were 32 isolates belonging to the HEV-C species. The nucleotide and aa sequences of the amplicon that were amplified using the 188–222 or 189–222 primer pair were compared between these isolates and prototype strains. Based on this comparison, 28 isolates were classified into 5 serotypes (Table IIc). The aa sequence divergence of HEV-C from that of each prototype strain ranged from 1.9% to 9.2% (Table IIc).

Although four isolates could not be classified into any HEV-C serotype, they were closely related to each other because they exhibited 0.9–7.9% nucleotide divergence (aa divergence, 0–2.6%) with regard to the 353-bp partial sequence of the VP1 region. The relative serotypes of these four isolates were CA13 and CA18. These isolates exhibited 30–30.3% nucleotide divergence (aa divergence, 17.1–18%) from the CA13 prototype and 29.5–30.6% nucleotide divergence (aa divergence, 16.2–17.1%) from the CA18 prototype. Since they could not be distinguished, they were tentatively classified as "untyped HEV-C."

The range of nucleotide divergence among the isolates of each serotype is shown in Table IIc. A relatively high nucleotide divergence was exhibited by three serotypes (CA24, EV96, and CA20); 11 CA24 isolates exhibited nucleotide divergence in the range of 8.3–18.1%; nine EV96 isolates, 10.7–16.1%; and six CA20 isolates, 5.7–19.4%.

Both the CA24 and EV96 serotypes were isolated for 4 years (Table IIc). Thus, these two serotypes were frequently isolated, and they overlapped with the three above mentioned serotypes, namely, CA24, EV96, and CA20, based on genetic polymorphism. Therefore, CA24 and EV96 were assumed to be common viruses in Yunnan Province with respect to frequency and polymorphism (Fig. 1c).

Relationship Between Yunnan Isolates and Other Asian Strains

Representative 5, 81, and 29 isolates of HEV-A, HEV-B, and HEV-C strains, respectively, were used for phylogenetic analysis (Fig. 1a–c), and then compared with 9, 26, and 8 strains in HEV-A, HEV-B, and HEV-C, respectively from neighboring countries. Because nucleotide sequences of the 5′ and 3′ ends of the VP1 region could not be obtained for isolates belonging to EV75, E15, CB4, E4, CB2, and CB6, these isolates were omitted from the analysis. In order to ensure the robustness among isolates, the nucleotide sequence data sets using the 5′ and 3′ ends of the VP1 partial region were used for analysis in HEV-A and HEV-B. As for HEV-C, data available regarding the 5′ end of the VP1 region was used. The results indicated that the Yunnan Province isolates demonstrated a tendency to have a similarity to other strains belonging to EV71, CA9, E11, and E13 in China (Fig. 1a,b).

DISCUSSION

This report presents an overview of NPEVs in Yunnan Province, the People's Republic of China, during AFP surveillance over a total study period of 5 years. Since 1992, many NPVs had been isolated as "side products" of poliovirus surveillance. At that time, although a number of enterovirus-positive samples were obtained, there might have been a bias in the proportion of these enteroviruses due to the cell lines that were used for isolation (RD and HEp-2 cell lines) and due to the sampling strategies used in the surveillance [Heim, 2005]. First, some circulating enteroviruses that cannot grow in these cell lines may have been overlooked, and the growth of other specific serotypes may have been favored [Pallansch and Roos, 2001]. For example, the

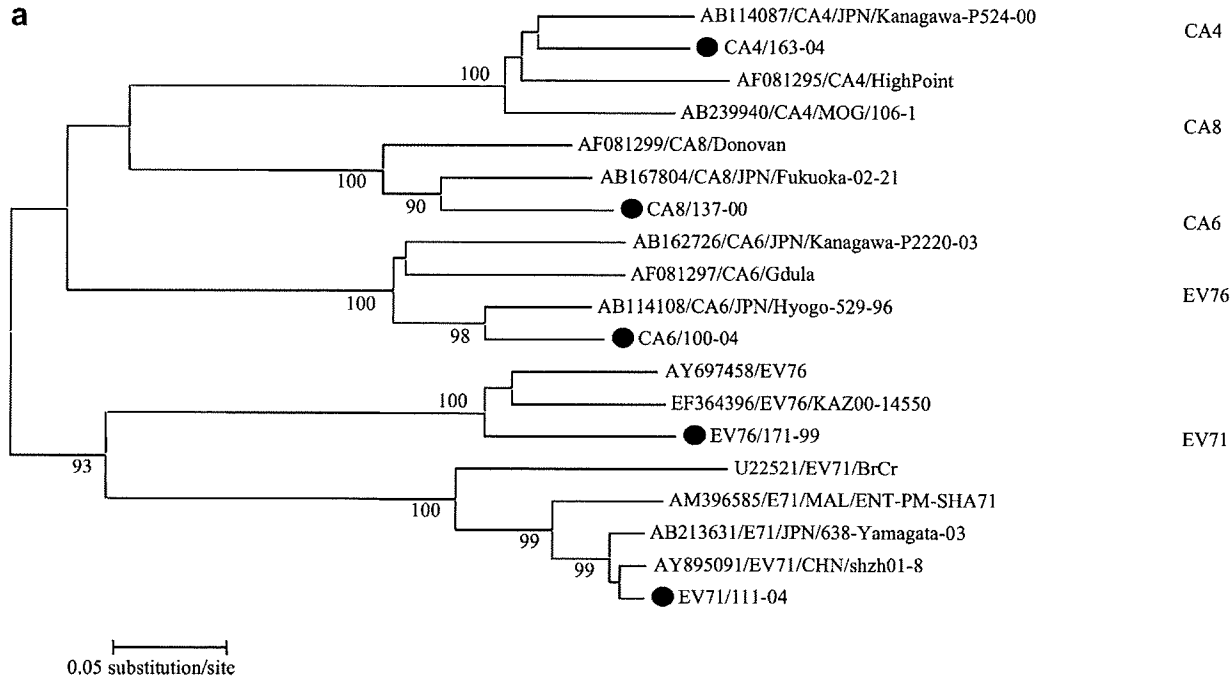

Fig. 1a. Phylogenic tree generated by neighbor-joining method using the nucleotide sequence data set; viruses isolated from Yunnan Province (serotype/strain code) are indicated by circles. Each serotype is shown on the right. Bootstrap values (in percentages) for 1,000 replicated trees are indicated. For analysis, closely related isolates were not included. Each strain is represented as accession No./serotype/country code /strain name, if available, using the web data from GenBank. The country codes used were as follows: AUS, Australia; BAN, Bangladesh; CAM, Cambodia; CHN, China; JPN, Japan; KOR, Korea; MAL, Malaysia; MOG, Mongolia; and SIN, Singapore. **a**: Phylogenic tree for HEV-A species. Analysis was performed by using 685 bp-sized nucleotide sequence data set on VP1 partial region, and five isolates that belonged to five serotypes from this study were compared with nine other strains in each serotype. **b**: HEV-B. Analysis was performed by using 654 bp on VP1 partial region, and 81 representative isolates that belonged to 30 serotypes were compared with 26 other strains. **c**: HEV-C. Analysis was performed by using 322 bp on the 5′ end of VP1 region, and 29 representative isolates that belonged to five serotypes were compared with eight other strains.

effective isolation of some HEV-A species requires that these species be inoculated in newborn mice rather than in cell lines [Pallansch and Roos, 2001].

Second, E13, E30, and EV71 are well known among the typical serotypes of enteroviruses that cause clinical diseases such as aseptic meningitis and hand-foot-mouth disease; further, they are capable of causing large outbreaks of infection. During the 5-year study period, the infection outbreaks caused by these serotypes were often reported not only from China [Li et al., 2005; Zhao et al., 2005] but also other East Asian countries such as Japan, Korea, Taiwan, and Malaysia [Centers for Disease Control and Prevention, 1998]. However, as shown in this study, the number of isolates of the causative agent of these diseases was small in Yunnan Province (Table II). The sampling strategy used in the AFP surveillance and the epidemiology of NPEVs may not be always related.

Interestingly, the E13 isolates were assumed to be indigenous viruses as a result of phylogenetic analysis. For example, although AB239084/E13/JPN/Toyama-2002-257NP belonged to the same clade of E13 that was responsible for a world outbreak during 2000–2002, as described in a previous study [Iwai et al., 2006], most isolates from Yunnan Province were genetically different from other strains based on available sequence data for these strains that could be causative agents for the outbreaks in the recent decade (Fig. 1b).

The isolation rate of NPVs during the study period was 17.2% as per the AFP surveillance in Yunnan Province. In other studies, the results of a surveillance of healthy children showed that NPEVs were silently circulating in the human community [Patti et al., 2000; Kuramitsu et al., 2005]. Thus, the isolation of NPEVs in our study was not always associated with symptoms of AFP. However, obtaining a rough estimate of the number of NPEVs in Yunnan Province was an additional benefit of the AFP surveillance. In China, a virus surveillance system for diseases caused by NPEVs has not been well established; therefore, the baseline data of NPEV distribution reported in our study could contribute to the construction of a laboratory-based surveillance system in the future.

Interestingly, we isolated the E12, E2, E1, and E29 serotypes from Yunnan Province for over 3 years during the study period (Table IIb). Based on epidemiological data on enteroviruses from England and Wales, Spain, Tunisia, USA, and Japan [Maguire et al., 1999; Trallero

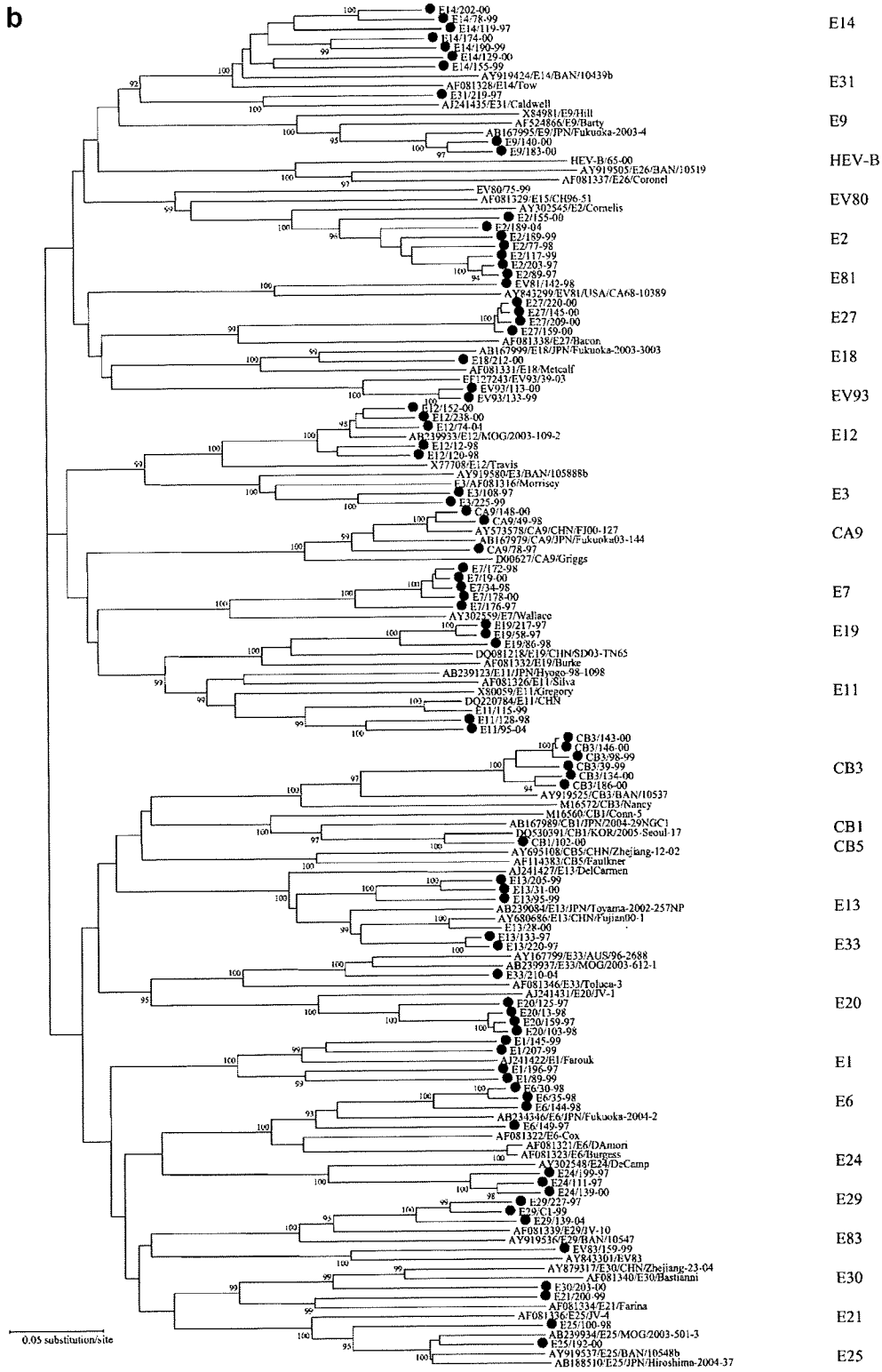

Fig. 1b. (*Continued*)

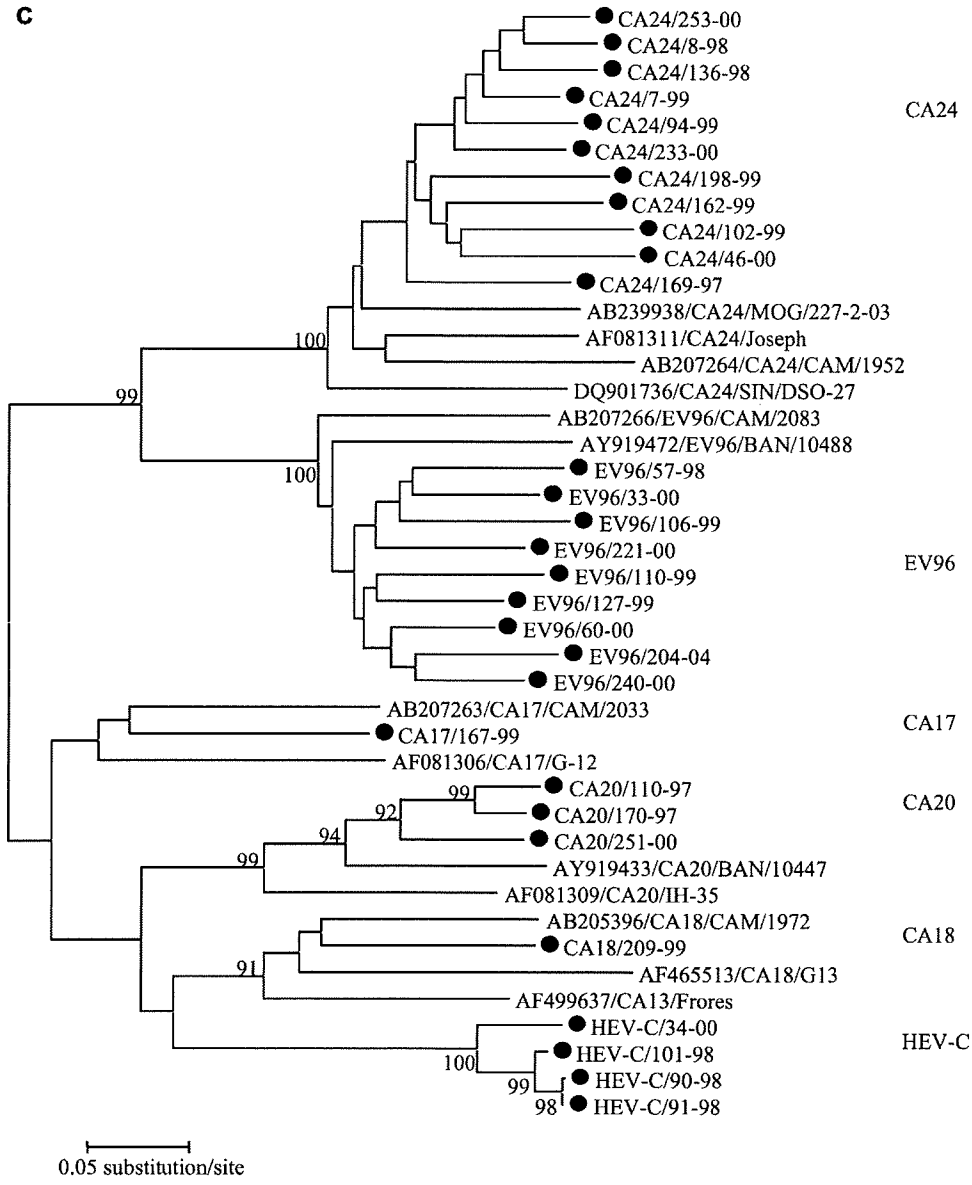

Fig. 1c. (*Continued*)

et al., 2000; Centers for Disease Control and Prevention, 2000, 2002, 2006; Bahri et al., 2005; Infectious Disease Surveillance Center, 2006a,b], it was observed that these four serotypes were rarely reported from these parts of the world. Moreover, CA24 and EV96 were frequently isolated, but limited information was available regarding the isolation of viruses belonging to the HEV-C species in other countries [Arita et al., 2005; Rakoto-Andrianarivelo et al., 2005].

The salient features of this isolation study are as follows: rare serotypes such as E12, E2, E1, and E29 were distributed in Yunnan Province, and several serotypes of the HEV-B and HEV-C species that exhibited genetic polymorphisms were assumed to be common (Fig. 1b,c).

A previous study reported that NPEVs such as EV71, CA9, CA7, and E9 might cause transient paralysis [Gear, 1984]. In some cases, the paralysis caused by NPEVs could not be easily clinically distinguished from poliomyelitis that is caused by polioviruses [Hida et al., 1999]. It was also reported that CA24 could be a causative agent of polio-like paralysis [Chaves et al., 2001]. Although detailed clinical information was not available in our study, further studies are required to elucidate the paralysis caused by NPEVs.

Although the neutralization assay for NPEV typing is generally reliable, it is labor intensive and time consuming; further, it may fail to identify the isolate because of aggregation of virus particles, antigenic drift, or the presence of multiple viruses in the specimen. Most importantly, the antiserum pool for NPEV identification is not always available to the provincial polio laboratories in China; thus, many NPEVs isolated during the laboratory surveillance of AFP cases remain untyped.

Several molecular methods based on the sequencing of the partial or complete genomic regions encoding the VP1 capsid protein are currently available; further, these sequences are compared with those of the prototype enterovirus strains. In this study, we used 1 of these molecular typing methods to identify the 195 NPEVs isolated during the 5-year study period. This method enabled the identification of not only most of the common enteroviruses but also some newly classified enteroviruses (such as EV75, EV76, EV80, EV81, EV83, EV93, and EV96). Molecular typing methods may help in understanding the epidemiology of NPEVs in Yunnan Province. With the progress of the polio eradication program in China, more attention should be paid to the etiology and epidemic pattern of NPEVs.

ACKNOWLEDGMENTS

The authors would like to thank the staff from all the Prefecture, County, and City Centers for Disease Control & Prevention in Yunnan Province. This work was supported by the Japan-China Medical Association and Grants in Aid for Promotion of Polio Eradication and Research on Re-emerging Infectious Diseases from the Ministry of Health and Welfare, Japan.

REFERENCES

Arita M, Zhu SL, Yoshida H, Yoneyama T, Miyamura T, Shimizu H. 2005. A Sabin 3-derived poliovirus recombinant contained a sequence homologous with indigenous human enterovirus species C in the viral polymerase coding region. J Virol 79:12650–12657.

Bahri O, Rezig D, Nejma-Oueslati BB, Yahia AB, Sassi JB, Hogga N, Sadraoui A, Triki H. 2005. Enteroviruses in Tunisia: Virological surveillance over 12 years (1992–2003). J Med Microbiol 54:63–69.

Centers for Disease Control and Prevention. 1996. Progress toward poliomyelitis eradication–People's Republic of China, 1990–1996. MMWR Morb Mortal Wkly Rep 45:1076–1079.

Centers for Disease Control and Prevention. 1998. Deaths among children during an outbreak of hand, foot, and mouth disease–Taiwan, Republic of China, April-July 1998. MMWR Morb Mortal Wkly Rep 47:629–632.

Centers for Disease Control and Prevention. 2000. Enterovirus surveillance–United States, 1997–1999. MMWR Morb Mortal Wkly Rep 49:913–916.

Centers for Disease Control and Prevention. 2002. Enterovirus surveillance—United States, 2000–2001. MMWR Morb Mortal Wkly Rep 51:1047–1049.

Centers for Disease Control and Prevention. 2006. Enterovirus surveillance—United States, 2002–2004. MMWR Morb Mortal Wkly Rep 55:153–156.

Chaves SS, Lobo S, Kennett M, Black J. 2001. Coxsackie virus A24 infection presenting as acute flaccid paralysis. Lancet 357:605.

Chiba Y, Hikita K, Matuba T, Chosa T, Kyogoku S, Yu J, Wang Z. 2001. Active surveillance for acute flaccid paralysis in poliomyelitis high-risk areas in southern China. Bull World Health Organ 79:103–110.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791.

Gear JH. 1984. Nonpolio causes of polio-like paralytic syndromes. Rev Infect Dis 6:S379–S384.

Heim A. 2005. From poliovirus surveillance to enterovirus surveillance: A complete picture? J Med Microbiol 54:1–2.

Hida C, Yamamoto T, Chiba Y, Jingjin Y. 1999. Differentiation of non-polio acute viral myelitis is mandatory for polio eradication in China. Pediatr Infect Dis J 18:388–389.

Infectious Disease Surveillance Center. 2006a. 25 May, 2006. Yearly reports of enterovirus isolation from human sources, 1982–1999.

Infectious Disease Surveillance Center. 2006b. Yearly reports of enterovirus isolation from human sources, 2000-2006. Infectious Disease Surveillance Center. Available at http://idsc.nih.go.jp/iasr/virus/graph/ev-2a.html.

Iwai M, Yoshida H, Matsuura K, Fujimoto T, Shimizu H, Takizawa T, Nagai Y. 2006. Molecular epidemiology of echoviruses 11 and 13, based on an environmental surveillance conducted in Toyama Prefecture, 2002–2003. Appl Environ Microbiol 72:6381–6387.

Junttila N, Leveque N, Kabue JP, Cartet G, Mushiya F, Muyembe-Tamfum JJ, Trompette A, Lina B, Magnius LO, Chomel JJ, Norder H. 2007. New enteroviruses, EV-93 and EV-94, associated with acute flaccid paralysis in the Democratic Republic of the Congo. J Med Virol 79:393–400.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120.

Kumar S, Tamura K, Nei M. 2004. MEG A3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5:150–163.

Kuramitsu M, Kuroiwa C, Yoshida H, Miyoshi M, Okumura J, Shimizu H, Narantuya L, Bat-Ochir D. 2005. Non-polio enterovirus isolation among families in Ulaanbaatar and Tov province, Mongolia: Prevalence, intrafamilial spread, and risk factors for infection. Epidemiol Infect 133:1131–1142.

Li L, He Y, Yang H, Zhu J, Xu X, Dong J, Zhu Y, Jin Q. 2005. Genetic characteristics of human enterovirus 71 and coxsackievirus A16 circulating from 1999 to 2004 in Shenzhen, People's Republic of China. J Clin Microbiol 43:3835–3839.

Maguire HC, Atkinson P, Sharland M, Bendig J. 1999. Enterovirus infections in England and Wales: Laboratory surveillance data: 1975 to 1994. Commun Dis Public Health 2:122–125.

Norder H, Bjerregaard L, Magnius L, Lina B, Aymard M, Chomel JJ. 2003. Sequencing of 'untypable' enteroviruses reveals two new types, EV-77 and EV-78, within human enterovirus type B and substitutions in the BC loop of the VP1 protein for known types. J Gen Virol 84:827–836.

Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. 1999a. Typing of human enteroviruses by partial sequencing of VP1. J Clin Microbiol 37:1288–1293.

Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. 1999b. Molecular evolution of the human enteroviruses: Correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol 73:1941–1948.

Oberste MS, Maher K, Flemister MR, Marchetti G, Kilpatrick DR, Pallansch MA. 2000. Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. J Clin Microbiol 38:1170–1174.

Oberste M, Schnurr D, Maher K, al-Busaidy S, Pallansch M. 2001. Molecular identification of new picornaviruses and characterization of a proposed enterovirus 73 serotype. J Gen Virol 82:409–416.

Oberste MS, Michele SM, Maher K, Schnurr D, Cisterna D, Junttila N, Uddin M, Chomel JJ, Lau CS, Ridha W, al-Busaidy S, Norder H, Magnius LO, Pallansch MA. 2004. Molecular identification and characterization of two proposed new enterovirus serotypes, EV74 and EV75. J Gen Virol 85:3205–3212.

Oberste MS, Maher K, Michele SM, Belliot G, Uddin M, Pallansch MA. 2005. Enteroviruses 76, 89,90 and 91 represent a novel group within the species Human enterovirus A. J Gen Virol 86:445–451.

Oberste MS, Maher K, Williams AJ, Dybdahl-Sissoko N, Brown BA, Gookin MS, Penaranda S, Mishrik N, Uddin M, Pallansch MA. 2006. Species-specific RT-PCR amplification of human enteroviruses: A tool for rapid species identification of uncharacterized enteroviruses. J Gen Virol 87:119–128.

Pallansch MA, Roos RP. 2001. Enteroviruses: Polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, editors. Fields virology. 4th edition. Philadelphia: Lippincott Williams & Wilkins. pp 723–775.

Patti AM, Santi AL, Fiore L, Vellucci L, De Stefano D, Bellelli E, Barbuti S, Fara GM. 2000. Enterovirus surveillance of Italian healthy children. Eur J Epidemiol 16:1035–1038.

Peng T, Li Y, Yang Y, Niu C, Morgan-Capner P, Archard LC, Zhang H. 2000. Characterization of enterovirus isolates from patients with heart muscle disease in a selenium-deficient area of China. J Clin Microbiol 38:3538–3543.

Rakoto-Andrianarivelo M, Rousset D, Razafindratsimandresy R, Chevaliez S, Guillot S, Balanant J, Delpeyroux F. 2005. High

frequency of human enterovirus species C circulation in Madagascar. J Clin Microbiol 43:242–249.

Stanway G, Brown F, Christian P, Hovi T, Hyypiä T, King AMQ, Knowles NJ, Lemon SM, Minor PD, Pallanch MA, Palmenberg AC, Skern T. 2005. Picornaviridae in virus taxonomy. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Eighth report of the international committee for the taxonomy of viruses. San Diego: Elsevier Academic Press. 757–763 p.

Trallero G, Casas I, Tenorio A, Echevarria JE, Castellanos A, Lozano A, Brena PP. 2000. Enteroviruses in Spain: Virological and epidemiological studies over 10 years (1988–97). Epidemiol Infect 124:497–506.

Wang DM, Zhao GC, Zhuang SM, Zhang YC. 1993. An epidemic of encephalitis and meningoencephalitis in children caused by echovirus type 30 in Shanghai. Chin Med J (Engl) 106:767–769.

World Health Organization. 2000. Manual for the virological investigation of poliomyelitis. Geneva: World Health Organization.

Xu Y, Zhaori G, Vene S, Shen K, Zhou Y, Magnius LO, Wahren B, Linde A. 1996. Viral etiology of acute childhood encephalitis in Beijing diagnosed by analysis of single samples. Pediatr Infect Dis J 15: 1018–1024.

Zhang J, Zhang LB, Otten MW Jr, Jiang T, Zhang XL, Zhang RZ, Wang KA. 1997. Surveillance for polio eradication in the People's Republic of China. J Infect Dis 175:S122–S134.

Zhao YN, Jiang QW, Jiang RJ, Chen L, Perlin DS. 2005. Echovirus 30, Jiangsu Province, China. Emerg Infect Dis 11:562–567.

JOURNAL OF VIROLOGY, Feb. 2008, p. 1787–1797 Vol. 82, No. 4
0022-538X/08/$08.00+0 doi:10.1128/JVI.01798-07

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[▽]

Minetaro Arita,[1]* Yasushi Ami,[2] Takaji Wakita,[1] and Hiroyuki Shimizu[1]

Department of Virology II[1] and Division of Experimental Animals Research,[2] National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan

Received 16 August 2007/Accepted 24 November 2007

Enterovirus 71 (EV71) is a causative agent of hand, foot, and mouth disease and is also associated with serious neurological disorders. An attenuated EV71 strain [EV71(S1-3′)] has been established in the cynomolgus monkey infection model; this strain contains the attenuation determinants derived from the type 1 poliovirus vaccine strain, Sabin 1 [PV1(Sabin)], in the 5′ nontranslated region (NTR), 3D polymerase, and 3′ NTR. In this study, we analyzed the effect of the attenuation determinants of PV1(Sabin) on EV71 infection in a NOD/SCID mouse infection model. We isolated a mouse-adapted EV71 strain [EV71(NOD/SCID)] that causes paralysis of the hind limbs in 3- to 4-week-old NOD/SCID mice by adaptation of the virulent EV71(Nagoya) strain in the brains of NOD/SCID mice. A single mutation at nucleotide 2876 that caused an amino acid change in capsid protein VP1 (change of the glycine at position 145 to glutamic acid) was essential for the mouse-adapted phenotype in NOD/SCID mice. Next, we introduced attenuation determinants derived from PV1(Sabin) along with the mouse adaptation mutation into the EV71(Nagoya) genome. In 4-week-old mice, the determinants in the 3D polymerase and 3′ NTR, which are the major temperature-sensitive determinants, had a strong effect on attenuation. In contrast, the effect of individual determinants was weak in 3-week-old NOD/SCID mice, and all the determinants were required for substantial attenuation. These results suggest that a cooperative effect of the attenuation determinants of PV1(Sabin) is essential for attenuated neurovirulence of EV71.

Enterovirus 71 (EV71) is a small nonenveloped virus with a genome of single-strand positive RNA of about 7,500 nucleotides (nt); it belongs to the genus *Enterovirus* of the family *Picornaviridae* (10, 60). EV71 is classified as *Human enterovirus species A*, along with some coxsackie A viruses, such as CA10 and CA16, which cause hand, foot, and mouth disease and herpangina (10, 54). However, EV71 infection is sometimes associated with severe neurological diseases, such as brain stem encephalitis and poliomyelitis-like paralysis (16, 44, 69). Fatal effects of an EV71 outbreak in Taiwan were mostly due to pulmonary edema and/or pulmonary hemorrhage, which may have been caused by direct destruction of the vasomotor and respiratory centers in the brain stem by EV71 infection (11, 23, 25, 36, 40, 70). The case severity rate of EV71 in the outbreak was <0.3% (23). This suggests a high neuropathogenicity of EV71 similar to that of poliovirus (PV), which causes poliomyelitis in 0.1 to 1.0% of infected individuals (reviewed in reference 46). Currently, various vaccines and treatments against EV71 infection are being developed (13, 15, 38, 39, 61, 64, 66, 72, 73).

Experimental infection models of EV71 have been established in the monkey and mouse (14, 21, 22, 49, 50, 71). The advantage of the monkey model is that the monkeys inoculated with clinical isolates of EV71 show neurological disorders similar to those observed in human cases of EV71 infection, including tremor, ataxia, and poliomyelitis-like paralysis; these disorders are critical for the evaluation of the neurovirulence of EV71 strains (3, 5). However, the limited availability of these animals has hampered characterization of the isolates in the monkey infection model. Mouse infection models of EV71 have been established with young mice (1 to 7 days old) (14, 71). For these mouse models, a sufficient number of animals are available, and some clinical symptoms, including rash and paralysis of the hind limbs, were observed in inoculated animals. However, an adaptive mutation(s) of EV71 is required for the infection of mice (14, 71); thus, the model is not useful for the characterization and evaluation of virulence of clinical isolates, but it can be used to evaluate some specific features of EV71 infection (12, 72). The neurological symptoms observed in young mice are difficult to evaluate, in contrast to those observed in adult mice infected with PV (4). Moreover, host range mutations that contribute to attenuation in mice but not in monkeys have been reported for PV (62), suggesting that intrinsic differences in the character of the animals underlie the pathogenesis of enterovirus.

To date, two kinds of mouse models have been established for the analysis of PV infection: one is with transgenic mice (35, 55) which express human PV receptor (34, 45), and another is a surrogate receptor model with nontransgenic mice and PV mutants with a BC loop of type 2 PV Lansing strain, which infects mice via an uncharacterized murine receptor (42, 47). The former mouse model is currently used for the evaluation of the neurovirulence of PV as well as the monkey infection model (48). The latter mouse model was used to de-

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan. Phone: 81-42-561-0771. Fax: 81-42-561-4729. E-mail: minetaro@nih.go.jp.

▽ Published ahead of print on 5 December 2007.