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

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

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

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

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

Recently, we generated an attenuated strain of EV71 [EV71(S1-3′)] derived from the prototype BrCr strain by defined genetic manipulation (5). The manipulation was based on the temperature-sensitive determinants of the type 1 PV vaccine strain (Sabin 1) (45), some of which are located in the...
conserved regions of the enterovirus genome, i.e., the 5' non-translated region (NTR), the 3'NTR, and the 3' NTR (24, 43, 53). Monkeys inoculated with EV71(S1-3') exhibited a mild, nonlethal neurological disorder with limited spread in the CNS on day 10 postinoculation (p.i.).

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

MATERIALS AND METHODS

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

General methods of molecular cloning. Escherichia coli strain XL1gold (Stratagene) was used for the preparation of plasmids. Ligation of DNA fragments was performed using a Quick Ligation kit (New England BioLabs). Site-directed mutagenesis (SDM) was performed using KOD plus DNA polymerase (Toyobo).

RNA extraction, RT-PCR, and sequencing. Viral genomic RNA was extracted from the culture fluid of infected cells using a High-pure viral RNA purification kit (Toyobo) (47).

Virus titration. The virus titer was determined by measuring the CCID_{50} of an EV71 strain, followed by incubation at 37°C for 3 h. Then, 100 μl of RD cell suspension (containing 2.0 × 10^{5} cells) was added to each well, followed by culturing at 37°C for 7 days for observation of CPE. The reciprocal of the highest dilution of serum that protected cells from infection in at least one of the two inoculated wells (per dilution) was recorded as the neutralization titer. For the IF method, we used the following infectious doses of EV71 in 50 μl of 10% FCS–DMEM, which each yielded about 100 infected cells, as detected by indirect immunofluorescence: EV71(BrCr-TR), 3.8 × 10^{5} CCID_{50}; EV71(Nagoya), 1.9 × 10^{5} CCID_{50}; EV71(C7-Osaka), 3.3 × 10^{5} CCID_{50}; EV71(1095), 1.1 × 10^{5} CCID_{50}; EV71(75-Yamagata-2003), 2.1 × 10^{5} CCID_{50}; EV71(1530-Yamagata-2003), 2.3 × 10^{5} CCID_{50}. These doses were incubated with 10-fold dilutions of monkey sera in 50 μl of 10% FCS–DMEM, or were incubated with 50 μl of 10% FCS–DMEM without monkey sera (control), in 96-well plates (Falcon; two wells per dilution), 50 μl of each dilution was mixed with 50 μl of 10% FCS–DMEM containing 20 CCID_{50} of an EV71 strain, followed by incubation at 37°C for 3 h. Then, 100 μl of RD cell suspension (containing 2.0 × 10^{5} cells) was added to each well, followed by culturing at 37°C for 7 days for observation of CPE. The reciprocal of the highest dilution of serum that protected cells from infection in at least one of the two inoculated wells (per dilution) was recorded as the neutralization titer. For the IF method, we used the following infectious doses of EV71 in 50 μl of 10% FCS–DMEM, which each yielded about 100 infected cells, as detected by indirect immunofluorescence: EV71(BrCr-TR), 3.8 × 10^{5} CCID_{50}; EV71(Nagoya), 1.9 × 10^{5} CCID_{50}; EV71(C7-Osaka), 3.3 × 10^{5} CCID_{50}; EV71(1095), 1.1 × 10^{5} CCID_{50}; EV71(75-Yamagata-2003), 2.1 × 10^{5} CCID_{50}; EV71(1530-Yamagata-2003), 2.3 × 10^{5} CCID_{50}. These doses were incubated with 10-fold dilutions of monkey sera in 50 μl of 10% FCS–DMEM, or were incubated with 50 μl of 10% FCS–DMEM without monkey sera (control), in 96-well plates (Falcon) at 37°C for 3 h. After incubation, 100 μl of RD cell suspension (2.0 × 10^{5} cells) was added, followed by culturing at 37°C for 16 h, except for cells incubated with 75-Yamagata-2003, which were cultured at 37°C for 8 h because 75-Yamagata-2003 fails to grow faster than other strains in RD cells. Cells were fixed in paraformaldehyde in phosphate-buffered saline [PBS(-); 10 mM phosphate buffered, pH 7.0, 137 mM NaCl, and 2.6 mM KCl] at room temperature for 10 min and were then incubated with rabbit anti-EV71(C7-Osaka) hyperimmune serum [1:100 dilution in 0.1% Triton X-100–PBS(-)] at 37°C for 1 h. After washing three times with PBS(-), goat anti-rabbit immunoglobulin G (IgG; H+L) conjugated with fluorescein isothiocyanate [1:300 dilution in 0.1% Triton X-100–PBS(-); Zymed] was added to the cells, followed by incubation at 37°C for 20 min. The cells were observed with a Biozero fluorescence microscopy system (KEYENCE). The number of infected cells obtained in the control wells (without serum) was designated as 100%, and the percentage of neutralization was calculated for each dilution. The 50% neutralization units (NU_{50}) for 50 μl of 10% monkey serum was calculated according to the Behrens-Kärber method (23).

Construction of infectious cDNA clones of EV71(Nagoya) and EV71(Nagoya-HIS). A DNA fragment, containing 4 kb of the 5' region of the viral genome of EV71(Nagoya), was amplified by RT-PCR using the primers SnaBl-T7-Nagoya+ and Nagoya3800- (Table 1). The resultant cDNA fragment was cloned into plasmid pEV71(BrCr-TR), which contains the infectious cDNA of strain BrCr-TR (5), after digestion by SnaBl and AgeI. Next, the 3' end of the above construct was then cloned into the pSport1 vector digested with SpeI and MluI. The resultant construct was sequenced and then differences in nucleotides between this construct and the parental Nagoya strain were corrected by SDM using appropriate primers. This infectious clone of EV71(Nagoya) was designated as pEV71(Nagoya).

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

<table>
<thead>
<tr>
<th>Primer</th>
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<tr>
<td>EcoRI-3END-</td>
<td>ACTGGAATCTTTTTTTTTTTTTTTTTTTT</td>
</tr>
<tr>
<td>SnaBl-T7-Nagoya+</td>
<td>TTAATACGAGTATCAAACACTCACAATAGGTTAACAGCTTGTTGCTT</td>
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<tr>
<td>Nagoya2200+</td>
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<tr>
<td>Nagoya3800-</td>
<td>CTAGCTTTCACATAATAGGGCTTGG</td>
</tr>
<tr>
<td>A2MluF-</td>
<td>AAAAAAGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTT</td>
</tr>
<tr>
<td>NagoyaVP1-HIS+</td>
<td>CACCATCATACATCACACTAACACATTTGATTCGTAACCTCCCAAAGAGGGAGGCTTCTATCTCC</td>
</tr>
<tr>
<td>NagoyaVP1-HIS-</td>
<td>GTGTTGATGGTGTATGTTGAGTACCCTTAAGAGGGAGGCTTCTATCTCC</td>
</tr>
</tbody>
</table>

*The variable sequence position in the primer is expressed according to the IUPAC system. Sequences read from the 5' position on the left.*
We constructed an infectious clone of EV71(Nagoya-HIS), which is a mutant of EV71(Nagoya) that contains a histidine tag in the BC loop of VP1 protein between amino acid residues 100 and 101 on the VP1 protein. The clone was constructed from pEV71(Nagoya) by SDM using the primers NagoyaVP1-HIS- and NagoyaVP1-HIS-. This infectious clone of EV71(Nagoya-HIS) was designated as pEV71(Nagoya-HIS).

Detection of anti-EV71 IgM and IgG in monkey serum. A virus solution of EV71(Nagoya-HIS) (1.0 × 10⁶ CCID₅₀/ml) was diluted with PBS(-) (1:10 dilution) and then added to HisGrab nickel-coated plates (Pierce) (100 μl per well). The plates were incubated at 4°C overnight for the adsorption of EV71(Nagoya-HIS) virions to the plates. After adsorption, 1% skim milk–PBS(-) was added to the plates (100 μl per well), which were then incubated at room temperature for 3 h. The plates were washed three times with 0.05% Tween 20–PBS(-) for detection of IgM or IgG, respectively and incubation at room temperature for 1 h. The plates were washed three times with T-PBS(-), followed by addition of 100 μl of diluted monkey serum [1:10,000 or 1:20,000 dilution with 0.5% skim milk–0.5% Tween 20–PBS(-)], for detection of IgM or IgG, respectively and incubation at room temperature for 1 h. The plates were washed three times with T-PBS(-), followed by addition of 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-monkey IgM or IgG antibody per well [1:10,000 dilution with 0.5% skim milk–0.5% Tween 20–PBS(-)].

Nordic Immunology for the detection of IgM or IgG, respectively and incubation at room temperature for 1 h. The plates were washed three times with T-PBS(-), for detection of IgM or IgG, respectively and incubation at room temperature for 1 h. The plates were washed three times with T-PBS(-), followed by addition of 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-monkey IgM or IgG antibody per well [1:10,000 dilution with 0.5% skim milk–0.5% Tween 20–PBS(-)].

Monoclonal antibodies was evaluated using EV71(Nagoya) (genotype B1), PV1(Mahoney), PV1(Sabin), and EV71(BrCr-TR) were inoculated with EV71(S1-3/H11032), we inoculated five cynomolgus monkeys with 10⁷ CCID₅₀ of EV71(S1-3/H11032) via an intravenous route (Fig. 1). Three monkeys were used to examine antigenicity (Fig. 2A, Exp. 1), and two monkeys were used to examine tissue specificity in the early phase of infection as indicated by clinical symptoms (day 4 p.i.) (Fig. 2A, Exp. 2).

Clinical symptoms of cynomolgus monkeys inoculated with EV71(S1-3’). To characterize the antigenicity and tissue specificity of EV71(S1-3’), we inoculated five cynomolgus monkeys with 10⁷ CCID₅₀ of EV71(S1-3’/H11032) via an intravenous route. Induction of anti-EV71 IgM antibodies in sera from the five monkeys inoculated with EV71(S1-3’/H11032) was not observed in the first- or second-round cultures, the result of the virus isolation was recorded as negative.

RESULTS

Clinical symptoms of cynomolgus monkeys inoculated with EV71(S1-3’). To characterize the antigenicity and tissue specificity of EV71(S1-3’), we inoculated five cynomolgus monkeys with 10⁷ CCID₅₀ of EV71(S1-3’/H11032) via an intravenous route. Induction of anti-EV71 IgM antibodies in sera from the five monkeys inoculated with EV71(S1-3’/H11032) was not observed in the first- or second-round cultures, the result of the virus isolation was recorded as negative.

Humoral immune response of cynomolgus monkeys inoculated with EV71(S1-3’). To examine the humoral immune response of monkeys inoculated with EV71(S1-3’), we measured the neutralizing and binding activities of anti-EV71 IgG and IgM antibodies in sera from the five monkeys inoculated with EV71(S1-3’/H11032) via the intravenous route. Induction of anti-EV71 antibodies was evaluated using EV71(Nagoya) (genotype B1), which is a prime strain of EV71 (16). We also used a genetically engineered EV71(Nagoya) mutant carrying a histidine tag in the BC loop of the VP1 protein [EV71(Nagoya-HIS)] (Fig. 3A and C). The neutralizing activity of the monkey sera was comparable to that of a monkey (number 4507) inoculated

FIG. 1. Schematic diagram of the EV71(S1-3’) genome. The sequences derived from the parental EV71(BrCr-TR) genome are represented by the black regions, and the mutations derived from PV1(Sabin) and PV1(BrCr-TR) are shown. The numbers in parentheses are the nucleotide positions in the genome or the amino acid positions in the 3Dpol protein. The figure was adapted from a previously published figure with the permission of the Society for General Microbiology (5).
with virulent parental EV71(BrCr-TR) in a previous study (Fig. 3A, 0 to 6 days p.i.) (5) and peaked (neutralization titer, 1,024 to 2,048) at 14 to 21 days p.i., followed by a gradual decline in activity (primary immune response). After the lethal challenge with EV71(BrCr-TR) on day 45 p.i., the neutralizing activity of the monkey sera markedly increased as a consequence of the secondary immune response. In the secondary immune response, the neutralizing activity peaked (neutralization titer, 16,384 to 32,768) at 7 to 14 days after the lethal challenge (52 to 59 days p.i.) (Fig. 3A).

The binding activity and/or the amounts of anti-EV71 IgG and anti-EV71 IgM antibodies were measured by ELISA. Pre-
viously, we developed an IgM capture ELISA for the detection of anti-EV71 IgM in human serum (52). However, that system is not suitable for monkey serum because of the strong cross-reactivity between the antibodies, which results in high background levels (data not shown). To minimize cross-reactivity between the antibodies, we developed a new ELISA system using histidine-tagged EV71(Nagoya-His) virus as the antigen (Fig. 3B and C). In this ELISA system, histidine-tagged EV71 virions are adsorbed on a nickel-coated plate and then monkey serum is added to the plate. Monkey anti-EV71 antibodies bound to the histidine-tagged virions were detected by HRP-conjugated goat anti-monkey IgM or anti-monkey IgG antibodies. OD450, optical density at 450 nm.

FIG. 3. Anti-EV71 antibody titers of monkeys inoculated with EV71(S1-3’). The time point of lethal challenge with EV71(BrCr-TR) is indicated by an arrow. (A) Anti-EV71 neutralization activity of monkey serum. The neutralization titer of 50 μl of the serum, determined by the CPE method, is shown over the course of the infection. The serum of a monkey (number 4507) inoculated with virulent EV71(BrCr-TR) was collected in a previous study (5). (B and C) Measurement of anti-EV71 IgM (B) or anti-EV71 IgG (C) in monkey serum. Histidine-tagged EV71 virions were adsorbed on a nickel-coated plate and then monkey serum was added to the plate. Monkey anti-EV71 antibodies bound to the histidine-tagged virions were detected by HRP-conjugated goat anti-monkey IgM or anti-monkey IgG antibodies. OD450, optical density at 450 nm.

Next, we examined the genotype specificity of the neutralizing activity of the monkey sera. We examined neutralizing activity against EV71 strains belonging to genotypes A, B1, B4, C2, and C4 (Tables 2 and 3). First, we determined the neutralization titer by observing the CPE caused by virus that escaped neutralization (CPE method; see Materials and Methods). The highest neutralization titer was against homotypic strain EV71(BrCr-TR) [the parental strain of EV71(S1-3’)]. The order of decreasing neutralization activity in the CPE assay was as follows: BrCr-TR (genotype A) > Nagoya (genotype B1) > 75-Yamagata-2003 (genotype C4) and 2399-Yamagata-2003 (genotype C4) > C7-Osaka (genotype B4) and 1095 (genotype C2). However, we were unable to determine the neutralization titer against strain 1530-Yamagata-2003 (genotype C4) using this method because of the late appearance of CPE, attributable to the nonneutralizable aggregated form of virus (54). Therefore, we determined the neutralization titer against strain 1530-Yamagata-2003 (genotype C4) using this method because of the late appearance of CPE, attributable to the nonneutralizable aggregated form of virus (54). Therefore, we determined the neutralization titer against strain 1530-Yamagata-2003 (genotype C4) using this method because of the late appearance of CPE, attributable to the nonneutralizable aggregated form of virus (54).
incomplete neutralization by the antibody. The monkey sera had substantial neutralizing activity against strain 1530-Yamagata-2003 (Table 3). The order of decreasing neutralization activity in the IF assay was as follows: BrCr-TR (genotype A) > Nagoya (genotype B1) and 75-Yamagata-2003 (genotype C4) > 1530-Yamagata-2003 (genotype C4) > C7-Osaka (genotype B4) > 1095 (genotype C2) and 2399-Yamagata-2003 (genotype C4).

Thus, the monkey sera exhibited a broad spectrum of neutralization activity against EV71 strains belonging to different genotypes, with the highest activity against the homotypic genotype A and the lowest activity against genotype C2 (1.6% of neutralization titer against homotypic strain).

**Protection of cynomolgus monkeys from lethal challenge by virulent EV71 (BrCr-TR).** Three monkeys inoculated with EV71(S1-3') were challenged with a lethal dose (10⁹ CCID₅₀) of EV71(BrCr-TR) via an intravenous route (Fig. 2) (5, 39). After the challenge, no clinical symptoms were observed in any of the three monkeys. Virus was not isolated from swab samples obtained after the challenge (Fig. 2B). These results indicate that EV71(S1-3') acted as an effective antigen that protected the monkeys from lethal infection with the homotypic virulent strain.

**Tissue specificity of EV71(S1-3') in cynomolgus monkeys.** We examined the tissue specificity of EV71(S1-3') in the cynomolgus monkeys on day 4 p.i., in the early phase of the infection as indicated by clinical symptoms (Tables 4 and 5). In our previous study (5), in the late phase of the infection (day 10 p.i.), virus was isolated only from the CNS of the monkeys inoculated with EV71(S1-3'), although inflammation was observed in a broad area of the CNS.

In the CNS of the inoculated monkeys, inflammation (perivascular cuffing with mononuclear cells) was observed in a limited area, mainly in gray matter, on day 4 p.i. (Table 4). In one monkey (number 4652), inflammation was observed in the pons, the medulla, and the cervical and lumbar spinal cord. In another monkey (number 4699), inflammation was observed only in the lumbar spinal cord. Virus was isolated from the lumbar spinal cords of both monkeys. In one monkey (number 4513) on day 10 p.i., no virus was isolated and no inflammation was observed. In another monkey (number 4514), virus was isolated only from the lumbar spinal cord, but inflammation was observed in all CNS tissues. These results suggest that EV71(S1-3') infection can cause inflammation in a broad range of CNS tissues with inefficient replication. Efficient replication of EV71(S1-3') was limited to the spinal cord, predominantly the lumbar spinal cord.

Next, we isolated virus from extraneural tissues of the monkeys inoculated with EV71(S1-3') or with the virulent parental EV71(BrCr-TR) (Table 5). Virus was isolated from the spleen, kidney, deep cervical lymph node, and dorsal root ganglion. There was no clear difference in extraneural tissue specificity between EV71(S1-3') and EV71(BrCr-TR). In the kidney and dorsal root ganglion, inflammation was observed, suggesting

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**TABLE 2. Neutralization titers of monkey sera against EV71 strains as determined by CPE assay**

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Day p.i.</th>
<th>BrCr-TR (A)</th>
<th>Nagoya (B1)</th>
<th>C7-Osaka (B4)</th>
<th>1095 (C2)</th>
<th>75-Yamagata-2003 (C4)</th>
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<td>4659</td>
<td>14</td>
<td>3.6 (100)</td>
<td>3.3 (50)</td>
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<td>59 (14)</td>
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<td>3.9 (25)</td>
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<td>4700</td>
<td>14</td>
<td>3.3 (100)</td>
<td>3.0 (50)</td>
<td>2.4 (13)</td>
<td>2.4 (13)</td>
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<td>2.4 (25)</td>
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<td>4.2 (25)</td>
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*Numbers in parentheses represent the day postinfection at which a lethal challenge with EV71(BrCr-TR) was administered to the monkeys.

**TABLE 3. Neutralization titers of monkey sera against EV71 strains as determined by IF assay**

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Day p.i.</th>
<th>BrCr-TR (A)</th>
<th>Nagoya (B1)</th>
<th>C7-Osaka (B4)</th>
<th>1095 (C2)</th>
<th>75-Yamagata-2003 (C4)</th>
<th>2399-Yamagata-2003 (C4)</th>
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<td>4.2 (43)</td>
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<td>3.9 (25)</td>
<td>4.4 (60)</td>
<td>3.7 (13)</td>
<td>4.6 (92)</td>
</tr>
<tr>
<td></td>
<td>59 (14)</td>
<td>5.5 (100)</td>
<td>5.0 (37)</td>
<td>4.6 (16)</td>
<td>4.4 (8.1)</td>
<td>5.1 (43)</td>
<td>5.1 (38)</td>
<td>5.0 (33)</td>
</tr>
<tr>
<td>4701</td>
<td>21</td>
<td>3.9 (100)</td>
<td>4.3 (230)</td>
<td>3.5 (36)</td>
<td>3.6 (41)</td>
<td>3.7 (50)</td>
<td>3.5 (36)</td>
<td>3.9 (88)</td>
</tr>
<tr>
<td></td>
<td>59 (14)</td>
<td>5.8 (100)</td>
<td>5.6 (63)</td>
<td>5.1 (18)</td>
<td>5.2 (21)</td>
<td>5.1 (20)</td>
<td>4.7 (7.6)</td>
<td>4.9 (12)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses represent the percentage of the neutralization titer relative to the titer against the BrCr-TR strain, which was taken as 100%.
that viral replication occurred in these tissues. However, on
day 10 p.i. after inoculation of EV71(S1-3\textsuperscript{3}), virus was not
isolated from extraneural tissues (data not shown). Thus,
EV71(S1-3\textsuperscript{3}) infected extraneural tissues of cynomolgus
monkeys in the early phase of the infection, but the infection of
extraneural tissues was transient.

**DISCUSSION**

To evaluate the antigenicity of EV71(S1-3\textsuperscript{3}) in cynomolgus
monkeys, we used an intravenous inoculation route instead of
the oral route. This is because in a previous study EV71 infec-
tion via the oral route did not efficiently cause neurological
disorders in the inoculated monkeys (1 out of 10 inoculated
monkeys) (18).

In the present study, the monkeys inoculated with EV71(S1-
3\textsuperscript{3}) via the intravenous route exhibited tremor followed by a
slight weakness of the legs (Fig. 2C and data not shown). One
monkey (number 4699) clearly exhibited tremor on day 3 and
day 4 p.i. However, in the CNS of that monkey, the only lesions
that were observed were in the lumbar spinal cord, which had
a low lesion score (0.3, perivascular cuffing with mononuclear
cells) (Table 4). Also in that monkey, no extrapyramidal le-
sions were detected in the CNS (40). Extrapyramidal lesions
directly cause tremor, suggesting that extrapyramidal lesions
present at undetectable levels can cause tremor in EV71-in-
fected monkeys and that detectable pyramidal lesions would
exacerbate such tremors. This suggests that the tremors ob-
served in the present monkeys inoculated with EV71(S1-3\textsuperscript{3})
have a different origin than those observed in monkeys inocu-
lated with the virulent parental strain EV71(BrCr-TR) in pre-
vious studies, in which extrapyramidal lesions were detected
(39).

The spinal cord is a niche of enterovirus infection (4, 13). Actually,
a PV replicon caused severe poliomyelitis-like paralysis in inoculated mice with limited lesions of the lumbar spinal
cord (<1.4% of motor neurons) (3). However, EV71 replica-
ction in the spinal cord of cynomolgus monkeys was not suf-
icient to cause tremor. In the young monkeys (ages, 7 and 12
years), EV71 replication was detected in the lumbar spinal
cord, but the animals did not exhibit tremor, in contrast to the
older monkeys (ages, 20 to 23 years). Interestingly, the lumbar
spinal cord of monkey 4699 had a lower lesion score (0.3) than

### Table 4. Tissue specificity of EV71(S1-3\textsuperscript{3}) in the CNS of cynomolgus monkeys

| Tissue         | Virus isolation (inflammation) for monkey no. [treatment]\textsuperscript{a}:
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4652 [EV71(S1-3\textsuperscript{3}), day 4 p.i.]</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>- (−)</td>
</tr>
<tr>
<td>Midbrain</td>
<td>- (−)</td>
</tr>
<tr>
<td>Pons</td>
<td>- (−)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>- (−)</td>
</tr>
<tr>
<td>Medulla</td>
<td>- (−)</td>
</tr>
<tr>
<td>Cervical cord</td>
<td>+ (+) (10\textsuperscript{3.75}) [0.9]</td>
</tr>
<tr>
<td>Lumbar cord</td>
<td>+ (+) (10\textsuperscript{3.5}) [0.9] + (+) (10\textsuperscript{3.6}) [0.3]</td>
</tr>
<tr>
<td>[lesion score\textsuperscript{b}]</td>
<td>+ (+) [1.3] + (+) [2.9] + (+) [3.2]</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The virus isolation results are shown as positive or negative. The presence of inflammation in the tissues is shown in parentheses as positive or negative. The inoculated viruses and the day sacrificed are also indicated. The virus titer (CCID\textsubscript{50}) in 100 \mu l of the homogenate is shown, in parentheses following the isolation and inflammation results, for the samples collected from two monkeys, numbers 4652 and 4699.

\textsuperscript{b} Samples of monkeys numbers 4507 and 4508 were collected in a previous study (5). ND, not determined.

### Table 5. Tissue specificity of EV71(S1-3\textsuperscript{3}) and EV71(BrCr-TR) in extraneural tissues of cynomolgus monkeys

| Tissue     | Virus isolation (inflammation) for monkey no. [treatment]\textsuperscript{a}:
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4652 [EV71(S1-3\textsuperscript{3}), day 4 p.i.]</td>
</tr>
<tr>
<td>Tonsil</td>
<td>- (−)</td>
</tr>
<tr>
<td>Heart</td>
<td>- (−)</td>
</tr>
<tr>
<td>Lung</td>
<td>- (−)</td>
</tr>
<tr>
<td>Liver</td>
<td>- (−)</td>
</tr>
<tr>
<td>Spleen</td>
<td>- (−)</td>
</tr>
<tr>
<td>Kidney</td>
<td>- (−) + (+) (10\textsuperscript{3.75})</td>
</tr>
<tr>
<td>Muscle</td>
<td>- (−) ND</td>
</tr>
<tr>
<td>Deep cervical lymph node</td>
<td>+ (+) (&lt;10\textsuperscript{6.5})</td>
</tr>
<tr>
<td>Dorsal root ganglion</td>
<td>+ (+) (10\textsuperscript{2.25})</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The virus isolation results are shown as positive or negative. The presence of inflammation in the tissues is shown in parentheses as positive or negative. The inoculated viruses and the day sacrificed are also indicated. The virus titer (CCID\textsubscript{50}) in 100 \mu l of the homogenate is shown, in parentheses following the isolation and inflammation results, for the samples collected from two monkeys, numbers 4652 and 4699.

\textsuperscript{b} Samples of monkeys numbers 4507 and 4508 were collected in our previous study (5). ND, not determined.
that of monkey 4652 (lesion score, 0.9), which exhibited no clinical symptoms. In poliomyelitis, adults are much more likely than children to be severely affected by infection in the spinal cord (42). Thus, detectable lesions and viral replication are not the only determinants of the occurrence of tremor in monkeys inoculated with EV71(S1-3’); other factors are critical.

Consistent with the mild neurological symptoms in the present cynomolgus monkeys inoculated with EV71(S1-3’), the distribution of EV71(S1-3’) in the CNS of the monkeys was limited to a small region on day 4 p.i. in the early phase of the infection (Table 4). Inflammation (perivascular cuffing with mononuclear cells) was observed in a broad area of the CNS without a detectable level of viral antigen. The virus was isolated from only the lumbar spinal cord on day 10 p.i. (5). Therefore, the inflammation observed in a broad area on day 10 p.i. was caused by inefficient infection of EV71(S1-3’) and was not caused by the vigorous infection in the early phase. Previously, we isolated an EV71 mutant [EV71(3’)] which contains temperature-sensitive mutations in the 3Dpol and 3’ NTR, from both the lumbar spinal cord and brain stem of inoculated monkeys (5). Thus, it appears that suppressed infection of EV71(S1-3’) in the brain stem is promoted by mutation in the 5’ NTR or by a combination of mutations in the 5’ NTR, 3Dpol, and 3’ NTR. Interestingly, studies indicate that PV1 and EV71 viruses with a mutation in the 5’ NTR (PV1(Sabin) and EV71(S1-3’)) are fairly stable in the lumbar spinal cord of inoculated monkeys (5, 24). The lesions of the brain stem are critical causes of fatality from EV71 infection (10, 21, 25, 31, 57), suggesting that the introduced attenuation determinant of PV1(Sabin) contributes to EV71-specific pathogenesis.

In the present study, we observed transient infection of EV71(S1-3’) in extraneural tissues, including the kidney, deep cervical lymph node, and dorsal root ganglion (Table 5). The infection in the dorsal root ganglion may simply reflect EV71 infection in sensory neurons (40). The deep cervical lymph node is thought to play a central role in establishment of the viremic phase of PV infection, according to a model proposed by Bodian (6; reviewed in reference 35). The kidney may also play an important role in the establishment of viremia as a transiently susceptible extraneural tissue in EV71 infection, as Sabin has proposed for PV infection, although the identity of the extraneural tissue involved in PV infection remains unknown (46).

The monkeys inoculated with EV71(S1-3’) were protected from a lethal challenge with EV71(BrCr-TR) (Fig. 2). We immunized monkeys with EV71(S1-3’) via an intravenous route with a high dose (10⁷ CCID₅₀) intended to cause experimentally transient high-level viremia (2.9 × 10⁴ to 5.1 × 10⁴ CCID₅₀ per ml). Even the viremogenic PV1(Mahoney) has been shown to cause viremia on the order of 10⁵ CCID₅₀ per ml in cynomolgus monkeys (6). Therefore, if EV71(S1-3’) were inoculated via the oral route (i.e., the natural route of EV71 infection), a much weaker humoral immune reaction would be expected in terms of levels of anti-EV71 IgG and IgM in the serum. The effectiveness of EV71(S1-3’) inoculation via the oral route, which depends on the induction of anti-EV71 secretory IgA for protection, remains to be further evaluated.

The neutralizing titers of the sera of monkeys immunized with EV71(S1-3’) were highest against the homotypic strain EV71 (genotype A) and were lowest against strains belonging to genotype C2, with a maximum difference of 60-fold between neutralization titers for different strains (Tables 2 and 3). It should be noted that genotype specificity was not eliminated after a single booster with the homotypic strain. Antigenic heterogeneity within a single serotype is a typical feature of enterovirus; for example, for echovirus 30, differences in the neutralization titer of >100-fold of the sera of rhesus monkeys have been observed (58). There have also been reports of antigenic heterogeneity among EV71 isolates, including a maximum difference in neutralization titers of 32-fold between different rabbit sera after EV71 inoculation (19). Frequent changes in the genotype of circulating EV71 may in part reflect antigenic variation in cross-neutralization between genotypes that may be critical for the perpetuation of EV71 (27, 37, 41, 55).

EV71(S1-3’) is a promising vaccine candidate, although it is still neurovirulent, at least when inoculated via the intravenous route. Several strategies have been proposed for attenuation and stabilization of the phenotype of PV (4, 9, 12, 15, 22, 32, 38). However, in general, attenuated PV strains exhibit impaired virus growth, even in cells cultured in vitro. Therefore, evaluation of the fitness of EV71(S1-3’) required for effective antigenicity is necessary to ensure that further attenuation produces effective vaccine strains.

In summary, we found that the attenuated strain EV71(S1-3’), which belongs to genotype A, acts as an effective antigen against several genotypes of EV71 and has attenuated neurovirulence in cynomolgus monkeys.

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