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Molecular biological characterization of enterovirus variant isolated from patients with aseptic meningitis

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Abbreviations: 5'-NCR, 5'-noncoding region; RGD, arginine-glycine- aspartic acid; RT-PCR, reverse transcription-polymerase chain reaction; RFLP, restriction fragment length polymorphism; OPT, oligopyrimidine tract; CSF, cerebrospinal fluid

Abstract

In Korea, there was a big outbreak of aseptic meningitis in 1993. Six clinical isolates of enterovirus were obtained from patients with aseptic meningitis and were identified as echovirus type 9 by serotyping with a pool of neutralizing antisera. For molecular characterization of the isolates, the nucleotide sequences of 5'-noncoding region (NCR), VP4, VP2, VP1, 2A and 2C regions of the isolates were compared with the corresponding regions of echovirus type 9 Hill and Barty strains. Unlike Hill strain, Barty strain contained a C-terminal extension to the capsid protein VP1 with an RGD (argnine-glycine-aspartic acid) motif. To determine whether similar structural features were present in our isolates, their nucleotide sequences including the VP1 region were analyzed. All isolates exhibited the VP1 extension with the RGD motif. We concluded the Korean isolates in the year of 1993 as the echovirus type 9 Barty strain although the isolates showed 15-20% nucleotide sequence differences in the several genomic regions.

Keywords: Aseptic Meningitis, enterovirus, RT-PCR, RFLP assay

Introduction

Enterovirus belongs to the picornavirus family which includes poliovirus, coxsackievirus A and B, echovirus and human enterovirus (Melnick, 1990). Many enteroviruses cause diseases ranging from a common cold to more severe pathogenesis such as paralysis, myocarditis, encephalitis and aseptic meningitis (Grist *et al*., 1978). The aseptic meningitis is generally considered mild in that neurological sequale and mental impairments are accompanied rarely in children under the age of one. In a temperate zone, aseptic meningitis caused by enteroviral infection is prevalent in summer and autumn, and are observed approximately five times more severe patho-genesis in children under the age of ten (Feigin and Cherry, 1987; Rotbart *et al*., 1991). Nonpolio enterovirus accounts for 90% of all cases of aseptic meningitis (Berlin *et al*., 1993).

From April to August of 1993, there was a big outbreak of aseptic meningitis in Korea (Kim *et al*., 1997). More than 4,000 patients under the age of ten were hospitalized during the spread of this country-wide epidemic. Echovirus type 3 was isolated from the patients of aseptic meningitis in 1994, and echovirus type 7 and coxsackie virus type B3 were identified as the culprit for aseptic meningitis in 1995 (Kim *et al*., 1996). Although enterovirus infection can be diagnosed by isolating virus from cerebrospinal fluids (CSF), blood, and other affected organs, the cul-turing of enterovirus are frequently unsuccessful due to poor growth (Lipson *et al*., 1988). Recently, PCR has been used to detect the enterovirus RNA for a direct and rapid clinical diagnosis of enteroviral infections (Hyypia *et al*., 1989; Rotbart *et al*., 1990; Zoll *et al*., 1992). In the earlier study, we identified the isolates obtained from CSF of patients with aseptic meningitis in 1993 to be echovirus type 9 based on serotyping with a panel of neutralizing antisera (Kim *et al*., 1997). We also showed that the isolates exhibited pathogenicity for newborn mice. The two echovirus type 9 strains, Hill and Barty, have been previously shown to be different each other in pathogenicity for new born mice as well as in a number of *in vitro* characteristics which depend on viral capsid structure (Rosenwirth *et al*., 1986). The complete nucleotide sequences of the both strains have been recently reported (Zimmermann *et al*., 1996).

Since serotyping with a panel of neutralizing antisera lacks the sensitivity to distinguish the different strains with the same serotype, we have presently examined the genetic diversity and molecular characteristics of the isolates by sequence analysis of different regions of the viral genome: the nucleotide sequences of cDNA clones representing the 5'-noncoding region (NCR), capsid regions, 2A protease regions and 2C helicase regions of six isolates were determined and compared with both Hill and Barty strains of echovirus type 9.

Materials and Methods

Viruses and cells

Clinical isolates were obtained in 1993 in our laboratory (Kim *et al*., 1997). Vero E6 cell (ATCC C1008 CRL 1586), coxsackie virus B3 (Nancy strain, ATCC VR-30), coxsackie virus A9 (Griggs strain, ATCC VR-1311), and echovirus type 9 (Hill strain, ATCC VR-39) were purchased from American Type Culture Collection (Rockville, MD, USA). Maintenance medium consisted of Eagle's minimum essential medium with 10% fetal calf serum, 4% L-glutamine (200 mM, 100×: Gibco, Grand Island, NY, USA) and 1% non-essential amino acid (10 mM of each amino acids: MA. Bioproducts, Walkersville, MD, USA) (Kim and Mckee, 1985). Coxsackie virus B3, coxsackie virus A9, echovirus type 9 and the six isolates named as AMC 2, 3, 9, 11, 14, 16 were propagated in the monolayers of Vero E6 cells, and were used as reference viruses.

RNA extraction

When nearly all of the cells displayed viral specific immunofluorescence by immunofluorescence antibody staining, the cells were harvested and then the RNAs from virus infected Vero E6 cells were extracted by using a Ultraspec-II RNA kit (Biotecx Laboratories, Inc., Huston, Texas, USA). RNA was prepared according to the procedures provided by the suppliers.

Primers

Primer sequences were selected from the conserved regions of the enterovirus genome, and their sequences and locations are shown in Table 1 (Samuelson *et al*., 1995; Olive *et al*., 1990; Kopecka *et al*., 1995). 5'-NCR was found to be highly conserved in the majority of the enterovirus serotypes which have been sequenced to date. To identify the isolates by restriction fragment length polymorphism (RFLP) assay, 5'-NCR of the six isolates were amplified. For the whole sequencing of 5'-NCR and VP4-VP2 region, the 643 bp products were obtained by using primer combination of 5NC and EU2 (Pair A, Table 1), and the 652 bp by EU3 and EU4 (Pair C, Table 1).

RT-PCR (reverse transcription-polymerase chain reaction)

The cDNA from RNA was synthesized at 42°C for 30 min in a 20 μl solution containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 3 mM DTT, 20 units of RAV2 reverse transcriptase (Boehringer Mannheim GmBH, Germany), 5 μg of RNA and 4 μg of random hexamers. Thirty cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C and 1 min extension at 72°C were carried out in a thermal cycler (Thermolyne Barnstead, Pubuque, IA, USA) (Zoll *et al*., 1992). The reaction mixture for PCR contained 25 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT and 1.3 units of Ampli-*Taq* DNA poly-merase (Perkin-Elmer, Norwalk, CT, USA)

Restriction endonuclease digestion

Table 1. List of primers for cDNA synthesis and RT-PCR

Primers	Position[1]	Sequences
Pair A		
5NC	1-17	5'- TCA GCG GCC GCT TAA AAC AGC CTG TGG G -3'
EU2	626-643	5'- CAC CGG ATG GCC AAT CCA -3'
Pair B		
UE1	160-180	5'- CAA GCA CTT CTG TTT CCC CGG -3'
UE3	580-599	5'- ATT GTC ACC ATA AGC AGC CA -3'
Pair C		
EU3	542-558	5'- CTA CTT TGG GTG TCC G -3'
EU4	1176-1195	5'- GGT AAC TTC CAC CAC CAG CC -3'
Pair D		
Evp1	2187-2206	5'- GGI ACI CAT GTI ATI TGG GA -3'
Evp3	2587-2606	5'- TGT CTI GTT TGC ATI GTG TC -3'
Pair E		
2A1	3179-3198	5'- ACC AAA GCT TGG GTG CCG CG -3'
2A2	3635-3661	5'- CCT TAG GAT CCC ACC ACA GTC CCC TGG -3'
Pair F		
2C1	4396-4419	5'- CAT ACA GTT CAA GTC CAA ATG CCG -3'
2C2	5009-5033	5'- TGT CTA GCG AGT ATC TGA CCT GTG -3'

[1] Positions refer to the echovirus type 9 (Hill strain) sequence.

For restriction enzyme analysis, 10 μl of the DNA solution obtained by PCR was digested by subgroup specific restriction enzymes in a final volume of 20 μl with restriction buffer. The reactions were carried out by the method recommended by the supplier (Boehringer Mannheim GmbH, Germany) and the digests were analyzed on a 2% agarose gel (Ultra Pure, Bethesda Research Laboratories, Bethesda, MD, USA) in 0.5 × TBE buffer containing 0.5 μg/ml of ethidium bromide (Jung and Kim, 1995).

Cloning of PCR product

To determine the nucleotide sequence of the 5'-NCR, capsid protein, 2A protease and 2C helicase, PCR amplicons generated by using each primers were cloned into pCRII vector (Invitrogen, Carlsbad, CA, USA).

Nucleotide sequence analysis

The plasmid DNA was sequenced by the dideoxy-chain-termination method using Sequenace version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH, USA) (Sanger *et al*., 1977). Annealing mixture containing 7μl of DNA (3-5 μg), 2 μl of reaction buffer and 1 μl of primer was heated for 2 min at 65˚C, prior to being cooled to 35˚C for 15-30 min. One μl of 0.1 M DTT, 2 μl of labelling mix (diluted 1:5), 0.5 μl of [^{35}S] dATP, and 2 μl of polymerase were added to the annealed mixture for labelling. The labelled mixture (3.5 μl) was transferred to each termination tube and then incubated at 37˚C for 5-10 min. The Reaction was stopped by adding 4 μl of stop solution. Samples were electrophoresed on 6% denaturing gel at 1600V.

Results

Virus identification and detection of viral genome

Cytopathic effect of Vero E6 cells was observed on the day 2 after the inoculation. More than 90% of the cells showed cytopathic effect on the day 5. All of the isolates were identified as echovirus type 9 by using pools of neutralizing antisera; monovalent neutralizing antisera were used against echovirus type 9 (by Dr. W. L. Lim at the Virus Unit, Queen Mary Hospital, Clinical Pathology, Hong Kong). Bacteriological cultures from each CSF were sterile, and serological studies by immunofluorescence antibody staining for leptospira or rickettsia infection were negative. For the molecular identification of the isolates, we used the RT-PCR method. RNAs of three reference viruses and six isolates were extracted in two to three days after the inoculation. The PCR products of 440 bp were amplified from nine viruses with the 5'-NCR primer pair B (Table 1, Figure 1A). RNA extracted from the uninfected normal Vero E6 and RNA virus such as Hantaan and Seoul viruses were not amplified with the same primer set B (data not shown). These results indicated that six AMC isolates (AMC 2, 3, 9, 11, 14, 16) from CSF specimens were enteroviruses, however, the enterovirus species were not differentiated. To differentiate the species of enteroviruses, the RFLP pattern of the 5'-NCR amplified products were analyzed. When the 5'-NCR products of the six isolates were digested with *Taq*I, the two generated fragments were 281 bp and 91 bp sizes as echovirus type 9 (Figure 2A). The RFLP patterns (340 bp and 100 bp fragments) of the six isolates upon digestion with *Dde*I was also similar to that of echovirus type 9 (Figure 2B). The pattern generated with *Sty*I , however, was not identical with that of echovirus type 9 (Figure 1B). To confirm and analyze the base substitutions of the isolates, we sequenced the 5'-NCR of AMC 2 and compared the sequence with that of other enteroviruses (Figure 3 and Figure 4, discussed below). The results indicated that they belonged to echovirus type 9, even though there were many U to C and A to G substitutions and other

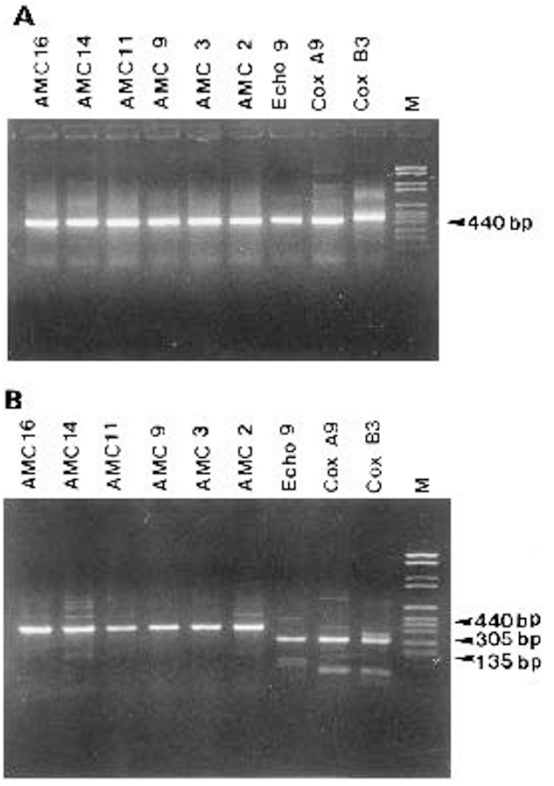

Figure 1. A. Agarose gel electrophoresis of PCR product resulting from amplification of 5'-NCR using primer pair B was carried out on 2% agarose gel. B. Restriction enzyme digestion pattern of PCR products from three different prototype strains and six clinical isolates with *Sty*I. Cox B3, coxsackievirus B3; Cox A9, coxsackievirus A9; Echo 9, echovirus type 9;AMC2, 3, 9, 11, 14, 16, the isolates; M, DNA size marker VI (Boehringer Mannheim GmbH, Germany).

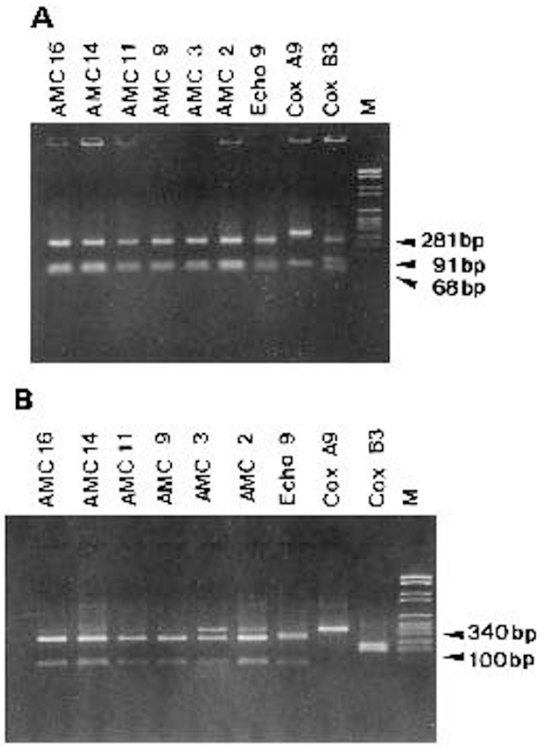

Figure 2. Restriction enzyme digestion pattern of PCR products from thereference strains and six clinical isolates with *Taq*I (A) and *Dde*I (B). Cox B3, coxsackievirus B3; Cox A9, coxsackievirus A9; Echo 9, echovirus type 9; AMC 2, 3, 9, 11, 14, 16, the isolates; M, DNA size marker VI (Boehringer Mannheim GmBH, Germany).

Figure 3. Sequence alignment of the echovirus down stream of 5'-NCR.Upstream half of the oligopyrimidine tract (OPT) is shown in bold and shade. 5'-TTTCC-3' domain may base pair with complementary sequence within the host 18S rRNA.

mutations in the isolates resulting losses of restriction sites.

5'-NCR

5'-NCR of enterovirus contains the internal ribosome entry site (IRES) element and oligopyrimidine tract (OPT) sequence of 5'-TTTCCTTTTA-3' which is crucial for efficient initiation of translation (Nicholson *et al*., 1991; Meerovitch *et al*., 1991). Sequences of the downstream 5'-NCR of seven echoviruses with different neurovirulence phenotype were reported (Romero *et al*., 1995). The neurovirulent serotype echovirus 2 and echovirus 12 showed little variations at this position. The 5'-NCR of six isolates were amplified, sequenced, and found that they possessed the upstream OPT sequence of 5'-TTTC CTTTTA-3', the sequences being identical to those of the five echoviruses having the neurovirulent phenotype (Figure 3).

Analysis of 5'-NCR and VP4-VP2 sequence

The 1,366 bp of AMC 2 isolate were sequenced from the 5'-NCR to VP4-VP2 region (GenBank accession #U77070). It showed 85% nucleotide sequence homology to that of echovirus 9 (Barty strain). The homology in the 5'-NCR was higher with about 87% with echovirus 9 (Barty strain). The sequence was less homologous with other enterovirus types. AMC 2 isolate had 82% nucleotide sequence homology and 92% amino acid sequence homology with VP4 region of the Barty strain. Prototype (Hill strain) and AMC 2 isolate shared 76% nucleotide sequence homology and 87% amino acid sequence homologies in VP4 region. The VP2 region of AMC 2 isolate shared an 82% nucleotide sequence homology with Hill and Barty strain, and 98% and 96% amino acid sequence homologies with Hill and Barty strain, respectively (Table 2).

Partial sequence comparison of VP1/2A region

It has been shown previously that, unlike other enteroviruses, the coxsackievirus A9 prototype strain (Griggs) and echovirus type 9 (Barty strain) contain a C-terminal extension to the capsid protein VP1 and that there is an RGD (arginine-glycine-aspartic acid) motif within the extension (Zimmermann *et al*., 1996). To determine whether the VP1 C-terminal extension found in the Barty strain is also present in our isolates, PCR product (419 bp) derived from the region of VP1/2A junction was obtained from cDNA of the six isolates using primer Evp1 and Evp2 (Jung

Table 2. Comparison of nucleotide homology of AMC 2 isolate with otherenterovirus members (%)

	5'-NCR	VP4	VP2	VP3/VP1	VP1/2A	2C
Echo 6	84.6	74.4	73.2	66.8	73.7	80.3
Echo 9 (Hill)	83.3	76.3	82.6	79.8	76.7	80.3
Echo 9 (Barty)	87.6	82.0	82.6	83.4	77.7	83.3
Echo 11	82.5	75.8	76.8	65.3	66.5	80.7
Echo 12	81.4	80.2	77.9	65.0	73.1	82.6
Coxsackie B1	82.9	75.8	77.9	60.3	70.7	81.3
Coxsackie B3	83.8	77.8	75.8	62.5	67.4	80.0
Coxsackie B4	80.9	79.6	74.2	64.2	72.1	84.9
Coxsackie A9	83.3	78.3	74.2	64.6	71.6	79.9
Coxsackie A16	81.9	58.8	63.2	52.1	64.5	65.7
Polio 3	72.4	63.6	74.3	50.9	54.6	67.9

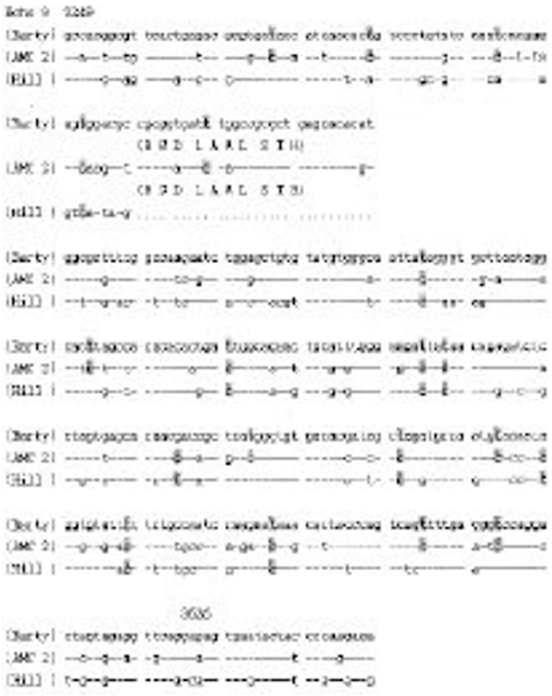

Figure 4. Nucleotide and amino acid sequence of VP1/2A region of AMC 2 isolate compared with the prototype strain Hill and Barty. Nucleotide sequences of AMC 2 isolate and echovirus 9 Hill strain are indicated by "(-)" for corresponding sequence; deletion depicted (.). The shade boxes denote for the substitutions of U to C or A to G.

et al., 1998). The amplified products were sequenced and compared with the published sequence of echovirus type 9 (Figure 4, GenBank accession #U77069). The result showed that both the extension and the RGD motif were retained in all isolates.

VP1 region

We amplified and analyzed the major recognition site for human IgG antibodies (Samuelson *et al*., 1995), which has been previously characterized by RT-PCR as the VP1 region of the isolates. The amino acid sequence crucial for antibody binding was completely conserved in all of the isolates (GenBank accession #U77071), providing an explanation for the observed serological cross reactivity in the patient's sera.

2C region

2C region may code for a helicase involved in viral RNA strand separation during replication (Dmitrieiva *et al*., 1991; Teterrina *et al*., 1992). Thus, a 580 bp fragment of the entire 2C helicase coding region was amplified with primer pair F (Table 1) and sequenced (GenBank accession #U77072). The 2C region of the isolates shared 80% and 83 % nucleotide sequence identity with Hill and Barty strains, respectively (Table 2). The amino acid sequences were identical in both strains.

Discussion

Echoviruses were discovered when cell culture techniques were first introduced into the virus laboratory. Although it is now widely known that echoviruses are common human pathogens, our knowledge about their molecular characteristics is relatively limited. Humans are the only known reservoir of echoviruses, and a close contact appears to be the mode of transmission via the fecal-oral route. The majority of infections probably remain limited to the initially infected cells in the alimentary or respiratory tract however, the viruses occasionally disseminate beyond the primary foci to the distant organs. Enterovirus infection is diagnosed by the isolation of the virus in cell culture, followed by neutralizing typing. RT-PCR has been recently used for

rapid and sensitive detection and identification of enteroviruses. There are numerous reports on applications of the PCR method such as multiplex PCR to distinguish between polio and nonpolio enterovirus, nested PCR for the rapid identification of human picornavirus, and the usefulness of the serotyping of PCR product by RFLP assay (Abraham *et al*., 1993; Egger *et al*., 1995; Kammerer *et al*., 1994; Schweiger *et al*., 1994).

In 1953, the prototype strain Hill of echovirus 9 isolated from rectal swab of a healthy child was found to be non-pathogenic to a newborn mice. In contrast, echovirus 9 strain Barty which was isolated during an epidemic in 1957 from the CSF of a child suffering from aseptic meningitis (Rosenwirth *et al*., 1986) was pathogenic. In a previous study, we showed that the isolates from CSF collected during 1993 were echovirus type 9 which exhibited pathogenicity for newborn mice. In order to identify the genetic difference of the 1993 Korea echovirus type 9 isolates comparing with Hill and Barty strain, we carried out partial sequencing of 5'-NCR, VP4-VP2, VP1 capsid protein coding region, and 2C helicase region. Furthermore we combined RT-PCR and RFLP assays for molecular characterization of the isolates. Based on the size and the pattern of restriction enzyme digestion of 440 bp PCR products of the 5'-NCR region (Figures 1 and 2), we concluded that the isolates were echovirus type 9 which was consistent with our earlier identification by a neutralizing test.

However, the restriction enzyme fragment patterns were not identical to those expected of the echovirus 9 prototype strains (Figure 1B and Figure 2). To analyze the genetic variability of the isolates, we sequenced the 5'-NCR of AMC 2 and compared the sequence with that of other enteroviruses. AMC 2 isolate share a high level (87.6%) of nucleotide sequence identity with echovirus 9 Barty strain, suggesting that the restriction enzyme site may have been lost by mutation.

It has been demonstrated with poliovirus that 5'-terminal 90 nucleotides forms a cloverleaf-like secondary structure which is essential for replication (Skinner *et al*., 1989; Toyoda *et al*., 1993). However, such unique structure has not been observed in the isolates (data not shown). Further study is in need to clarify the significance of this variable secondary structural change. Recently, neuro-virulent echovirus serotypes are shown to possess an upstream OPT sequence of 5'-TTTCCTTTT-3' (Romero *et al*., 1995), and the less virulent serotype, echovirus type 2 and 12 have changes in this sequence. We found that the six isolates shared an identical nucleotide sequence with neurovirulent echovirus serotype (Figure 3), providing an evidence that these isolates exhibited neurovirulent phenotype.

Although we had identified the six isolates as echovirus type 9 by neutralizing test, we did not clarify exactly which strain causes aseptic meningitis. In the case of strain Barty, an additional 10 amino acid fragment including an RGD motif is inserted at the C-terminus of the capsid protein VP1 (Zimmermann *et al*., 1996). When we com-pared the nucleotide sequence of VP1 region of AMC 2 isolate with those of echovirus type 9 Hill and Barty strain, we found that AMC 2 isolate belonged to the Barty strain. AMC 2 isolate had a RGD motif (Figure 4), which was known to be involved in receptor interaction in a number of cellular systems, including attachment of food-and-mouth-disease virus (FMDV) to its cellular receptor. Contrary to other sequenced enteroviruses, the coxsackie virus A9 sequence has an insertion in the VP1-encoding region, which encodes an apparent 17 amino acid exten-sion containing RGD sequence (Chang *et al*., 1992). Thus, the RGD sequence provides a possibility that coxsackie virus A9 and the isolates recognize their respective receptors by the same mechanism. This is consistent with the finding that echovirus type 9 produced paralysis in a new born mice with muscle lesions similar to those which are associated with coxsackie A virus.

We have observed a significant number of U to C and A to G transitions in the genomic sequence of enteroviruses (Figure 4). These transitions have been frequently observed in many RNA viruses. During the replication of antigenomes or of newly generated genomes containing U to C substitutions, A to G substitutions could be introduced in the genome template. After a round of replication, this would give rise to certain genomes having U to C changes and to others having both U to C and A to G changes. Biased hypermutations may be a common feature during replication of RNA viruses. The U to C and A to G transitions in echoviruses may have no significant effects on the viral RNA replication. Thus, clinical isolates with these mutations might be found frequently.

We also found that the antigenic region known to elicit cross-reactivity with enterovirus IgG antibodies in VP1 of the isolates was highly conserved as poliovirus and coxsackievirus A and B. Genomic sequences in 2C region of the six isolates were compared with the published sequences of enteroviruses, finding the isolates being genetically closely related to echoviruses, coxsackie B virus and coxsackie virus A9. The AMC 2 isolate shared about 80% nucleotide sequence homology of the 2C region with Hill and Barty stains, whereas their amino acid sequences were identical. This result suggests that nonstructural proteins are not under the selection pressure caused by host antibodies.

In summary, we showed in the present study that echovirus type 9 was responsible for the outbreak of aseptic meningitis in 1993 in Korea. Based on the analysis of the VP1 region sequence, the isolates were identified as echovirus type 9 Barty strain.

Acknowledgments

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