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Differences in Replication Capacity Between Enterovirus 71 Isolates Obtained From Patients With Encephalitis and Those Obtained From Patients With Herpangina in Taiwan

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The cellular-tropism and biological characteristics of enterovirus 71 (EV71) isolates in Taiwan (TW) were studied. Growth curve experiments were conducted using cell lines that were possibly exhibited pathogenesis, and RT-PCR and sequencing tests were undertaken to amplify the 5′ non-coding region (5′-NCR). The encephalitis isolate EV71 TW98NTU2078 was PBMC-tropic, temperature-resistant (Tr) at 40°C, and easier to replicate in HTB-14 (astrocytoma) than the herpangina isolate EV71 TW98NTU1186 (The viral yields were 100-fold higher than those of the herpangina isolate EV71 TW98NTU1186 at 96 hr post infection.). The herpangina isolate EV71 TW98NTU1186 was non-PBMC-tropic, and temperature-sensitive (Ts) at 40°C. The replication of EV71 TW98NTU1186 in HTB-14 was lower. No EV71 isolate infected HTB-37 (human colon adenocarcinoma cells). The encephalitis EV71 isolate exhibited better replication and transmission in PBMCs and astrocytes than did the EV71 isolate without CNS involvement. ***J. Med. Virol. 79:60–68, 2007.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: enterovirus 71; virus variation; cellular tropism

INTRODUCTION

Enterovirus 71 (EV71), a member of the genus *Enterovirus* and the family *Picornaviridae*, is a main cause of hand-foot-mouth disease (HFMD), causing various serious neurological diseases, such as aseptic meningitis, encephalitis, poliomyelitis-like paralysis, and fatal meningoencephalitis in young children [Muir and Van Loon, 1997]. Since it was described in California in 1969, EV71 has been reported in several epidemics in Bulgaria, Australia, Europe, Malaysia, Japan, and Taiwan (TW) [Shindafov et al., 1979; Freymuth et al., 1981; Abubakar et al., 1999; Ho et al., 1999]. In particular, the two largest epidemics of EV71 in TW were more severe than earlier epidemics, with 78 fatalities in 1998 and 26 pediatric deaths in 2000 [Ho et al., 1999]. Severe clinical features included meningitis, encephalitis, poliomyelitis-like paralysis, and meningoencephalitis, and the most frequent clinical complications were pulmonary edema and hemorrhage [Chang et al., 1999a]. However, the mechanism of viral pathogenesis remains unclear.

Like poliovirus, EV71 transmitted by the fecal-oral route has an affinity for cells in the central nervous system (CNS), and manifests as poliomyelitis-like paralysis [Huang et al., 1999; Nagata et al., 2004]. During the most serious epidemic of EV71 in TW in 1998, EV71 invaded the midbrain, the brain stem, the pons, the medulla oblongata, and the dentate nucleus of the cerebellum. In the most serious cases, the virus

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invaded the spinal cord [Shen et al., 2000]. However, how the cellular and molecular mechanisms of EV71 cause neurological damage, and whether some strains of EV71 in CNS cases are more neurovirulent than others, are worthy of study. Two principal EV71 isolates obtained from the 1998 outbreak in TW were used. These were the TW98NTU2078 isolate derived from a patient with CNS involvement and the TW98NTU1186 isolate from a patient with herpangina (with mild symptoms). Their cellular tropisms have been demonstrated to be associated with viral replication [Bienkowska-Szewczyk and Ehrenfeld, 1988; Nugent et al., 1999], and were studied during this investigation. The disease progressed rapidly in fatal cases, and therefore several cell lines, including enteric, lung, neurological cells, and blood-adherent cells that are associated with viral transmission and clinical outcomes were used to test the growth yield of viruses. The results showed that the EV71 TW98NTU2078 isolate was temperature-resistant (Tr), able to grow in human peripheral blood mononuclear cells (PBMCs), and replicated more efficiently in human astrocytoma (HTB-14) cells than was EV71 TW98NTU1186 isolate (>2 $\log_{10}$ PFU/ml). Additionally, no isolate of EV71 could infect human colon adenocarcinoma cells (HTB-37).

MATERIALS AND METHODS

Cells

African green monkey kidney (Vero) cells were propagated in Eagle's minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) (Gibco BRL, Life Technologies, Grand Island, NY). Human pharyngeal epidermoid carcinoma cells (HEp-2) and human lung carcinoma cells (A549) were replicated in Eagle's MEM supplemented with 10% FBS (MEM-10). PBMCs were propagated in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) with 10% FBS. Human neuroblastoma cells (HTB-11, ATCC, Rockville, MD) and human astrocytoma cells (HTB-14, ATCC) were grown in Eagle's MEM supplemented with 1% L-glutamic acid, 1% non-essential amino acid, and 20% FBS. Human colon adenocarcinoma cells (HTB-37, ATCC) were grown in DMEM with 1% pyruvic acid, 1% L-glutamic acid, 1% non-essential amino acid, and 20% FBS. Vero cells were used for plaque assay and plaque purification.

Virus

Two EV71 isolates, TW98NTU2078 from a patient with CNS and TW98NTU1186 from a patient with herpangina, were isolated at National Taiwan University Hospital during a single EV71 outbreak from April to June of 1998. Another nine EV71 isolates, including fivefrom patients with CNS involvement(TW80NTU3100, TW98NTU1107, TW98NTU1311, TW98CH35, TW98 PT142) and four from patients with herpangina (TW98NTU1334, TW99NTU1183, TW2KNTU0652, TW2KNTU1148), and other enteroviruses (poliovirus type 1, poliovirus type 2, coxsackie A16, coxsackie B2, and coxsackie B3) were also utilized to confirm the results. Table I shows the details of all EV71 isolates used in the study. Viruses were isolated originally in RD cells, and identified by indirect fluorescent antibody staining (IFA) with EV71 specific monoclonal antibody (3324, Chemicon International, Temecula, CA). The viruses were collected from infected cells in three freeze-thawing cycles, centrifuged at 3,000 rpm for 10 min to remove cell debris, and then treated with chloroform for 10 min. The harvested virus stocks were stored at −80°C. In all growth curve experiments, viruses were plaque-purified on Vero cells triplely and their serotypes were confirmed by a micro-neutralization assay.

Growth Curves of EV71 Strains in Cells

HEp-2, A549, HTB-11, HTB-14, and HTB-37 infected with EV71 isolates at a multiplicity of infection (MOI) of 0.01. The reason for using a low MOI of the added enterovirus is to elucidate the entire process of infection of the cycle of enterovirus propagation in cell culture, and particularly the steps involved in the release of the virus and the infection of new cells. Following adsorption for 2 hr at 37°C, non-adsorbed viruses were removed, and the maintenance medium that contained 2% fetal calf serum (MEM-2) was added. The cells were incubated at 37°C in CO_2 for 5 days. During incubation, the viruses were harvested at the stated times (0, 6, 12, 24, 48, 72, and 96 hr post infection)

TABLE I. The Year of Isolation, Clinical Manifestations, Genotypes, Type of Clinical Specimens and Geographical Location Isolated of EV71 Isolates Used

EV71 isolates	Year of isolation	Clinical manifestations	Genotype	Type of specimen	Geographical location isolated
TW80NTU3100	1980	Polio-like syndrome	B	Throat swabs	Taipei
TW98NTU2078	1998	Encephalitis	C	Throat swabs	Taipei
TW98NTU1107	1998	Meningitides	C	Throat swabs	Taipei
TW98NTU1311	1998	Meningitides	C	Throat swabs	Taipei
TW98C035	1998	Encephalitis	C	Throat swabs	Taichung
TW98PT142	1998	Meningitides	C	Throat swabs	Pin-tong
TW98NTU1186	1998	Herpangina	C	Throat swabs	Taipei
TW98NTU1334	1998	Herpangina	C	Throat swabs	Taipei
TW99NTU1183	1999	Herpangina	C	Throat swabs	Taipei
TW2kNTU0652	2000	Herpangina	B	Throat swabs	Taipei
TW2kNTU1148	2000	Herpangina	B	Throat swabs	Taipei

in three freeze-thawing cycles, and then quantified by plaque assay on Vero cells. The viral incubation temperature was also increased to 40°C, to study the viral sensitivity or resistance to high temperatures.

Infectious Center Assay of EV71 on Human PBMC-Adherent Cells

The infectious center (IC) assay [Freistadt and Eberle, 1996] in vitro was conducted to determine whether human PBMCs can carry the EV71 isolates and thus play a very important role in viral pathogenesis. PBMCs obtained from the venous blood of healthy persons were prepared by Ficoll-Paque™ plus (Amersham Pharmacia Biotech, AB, Sweden) gradient centrifugation. The mononuclear cell fraction collected was washed three times in RPMI-1640 with 1% FBS and adhered to the plastic Petri plates (Costar. 3513. Corning, NY) at 37°C for 30 min. The adherent cells were then washed three times to remove non-adhering cells. These adhering cells were collected using cell scrapers (Costar. 3010. Corning, NY). The viability of these scraped cells was determined using trypan blue to be over 95%. The harvested PBMC-adherent cells were counted and resuspended in RPMI-1640 that contained 10% FBS at a concentration of 2×10^6 cells/ml. PBMC-adherent cells were infected with EV71 isolates (at an MOI of 10) at 37°C for 30 min. The non-adsorbed virus was removed by washing three times in a phosphate buffer saline (PBS, pH 7). The infected PBMC-adherent cells were diluted serially from 10^{-1} to 10^{-6} in 0.5% gelatin (in PBS). Each diluent (100 μl) was added to confluent Vero cells in duplicate. The non-infected PBMC-adherent cells were diluted serially and added to confluent Vero cells as described above for the "mock-infection" control group. Following adsorption at 37°C for 2 hr, RPMI-1640 (with 2% methylcellulose) medium was placed on the monolayer, and incubated in a 5% CO_2 incubator at 37°C for 5 days. The same batch of PBMC-adherent cells was infected with various isolates of EV71 to improve the consistence of the comparison. The cells were fixed in 10% formalin for 60 min and stained using 5% crystal violet for 10 min; plaques were counted. The average number of plaques of duplicate wells represented the number of ICs/ml.

Replication of EV71 in Human PBMC-Adherent Cells

Human PBMCs were attached to 6-well plastic Petridishes (Costar. 3513 Corning, NY) at 37°C for 30 min. The non-adherent cells were washed three times and the adherent cells were infected with EV71 isolates at an MOI of 0.1. Following adsorption at 37°C for 2 hr, the non-adsorbed viruses were removed by washing, and RPMI-1640 that contained 2% FBS medium was added to the cells and incubated at 37°C in a 5% CO_2 incubator for 4 days. The viruses were harvested at 72 and 96 hr post infection in three cycles of freeze-thawing and plaque forming unit were quantified by plaque assay on Vero cell.

RNA Extraction

Viral RNA was extracted using the TRI-ZOL method. Briefly, TRI-ZOL® Reagent (Gibco BRL: 15596) was added to a solution of the virus, mixed vigorously, and held at room temperature for 3–5 min. Chloroform was added to the mixture, which was mixed weakly and centrifuged at 14,000*g* and 4°C for 15 min. Thereafter, the aqueous phase that contained the viral RNA was transferred to another fresh Eppendorff tube. Ice-cold isopropanol was added to the supernatant, mixed for 15 sec, and left to stand at room temperature for 10 min. Following centrifugation at 14,000*g* and 4°C for 15 min, the resulting pellet was washed in cold 75% ethanol. The RNA pellets were dried by centrifugation, dissolved in 20 μl of distilled water with 0.01% diethyl pyrocarbonate, and stored at −80°C.

RT-PCR and DNA Sequencing

RT-PCR was carried out using a Ready-to-go RT-PCR single-tube kit (Amersham Pharmaceutical Biotech, Amersham, UK). The total volume of the reaction mixture was 100 μl. Universal primers EV-1 (5′-TCCTCCGGCCCCTGAATGCG-3′) (position 449–468 upstream) and EV-2 (5′-ATTGTCACCATAAGCAGCCA-3′) (position 569–603 downstream) were employed to amplify the 154 bps-long part of the 5′NTR NTR region [Fujioka et al., 1995]. EVP-4 (5′-CTACTTTGGGTGTCCGTGTT-3′) (position 547–566 upstream) and OL68-71R (5′-GGGAACTTCCAGTACCAYCC-3′) (position 1182–1201 downstream) were utilized to flank a 654 bps-long part of the VP4–VP2 region [Shimizu et al., 1999]. Briefly, the first reverse transcription from RNA to cDNA was undertaken at 42°C by incubation for 1 hr, denatured at 94°C for 4 min, and amplified in 32 cycles (model 480; Perkin-Elmer Cetus, Foster City, CA) under the following conditions; 94°C for 40 sec, 55°C for 30 sec, 72°C for 45 sec, and a final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis in a 2% agarose gel and visualized by staining the gels with ethedium bromide. They were purified further and sequenced using an ABI Model 373A automated fluorescence sequencer with a Prism Ready Reaction Dideoxy Terminator Cycle sequencing kit (Perkin-Elmer, Norwalk, CT). Nucleotide sequence analysis and alignment were carried out using the GeneWork (2.01 edition, Perkin-Elmer) and the DNAMAN software systems (4.15 edition, Lynnon BioSoft, Quebec, Canada).

RESULTS

Cellular Tropism and Growth Curve of EV71

EV71 isolates, TW98NTU2078 (encephalitis) and TW98NTU1186 (herpangina) were harvested on several pathogenically related cell lines at the stated times post infection and the viral yields were quantified to determine whether the cellular tropisms of EV71 in severe CNS and mild cases were similar. Human pharyngeal epidermoid carcinoma cells (HEp-2) were

chosen because they were isolated from throat swabs. Human colon adenocarcinoma cells (HTB-37) were used to study possible local infection in the colon by viruses such as polioviruses [Masson et al., 2001]. The role of human PBMCs in the transmission of a virus after viremia has been studied [Persidsky et al., 1999]. Human lung carcinoma cell (A549) was chosen as a candidate since it was associated with a high fatality rate in cases of severe lung edema in TW [Ho et al., 1999]. The importance of human neuroblastoma cells (HTB-11) and human astrocytoma cells (HTB-14) in other viral diseases of the CNS has been documented [Brack-Werner and Bell, 1999].

Figure 1 shows the growth curves obtained from both isolates. The two principal strains of EV71 grew in all of the cells used in the experiments, except in the HTB-37 cells (human colon adenocarcinoma cells), which did not support the replication of both EV71 isolates. However, the TW98NTU2078 isolate from CNS patients grew much faster and generated almost 100-fold PFU/ml greater viral yields than TW98NTU1186 isolate in the mild case of herpangina in HTB-14 (astrocytoma cell line) at 12 hr post infection ($2 \log_{10}$ PFU/ml vs. $0.1 \times \log_{10}$ PFU/ml). The numbers of plaques of both isolates in HEp-2 cells (human epidermoid carcinoma from upper respiratory pharyngeal cells) and A549 cells (human lung carcinoma Cells) 72 hr post infection were 3.5 and 4.3 $\log_{10}$ PFU/ml, showing that the EV71 strains might multiply in both the upper and the lower respiratory tracts.

Another nine isolates of EV71 (including five isolates from patients with CNS and four isolates from patients with herpangina) and other enteroviruses (poliovirus type 1, poliovirus type 2, coxsackie A16, coxsackie B2, and coxsackie B3) were used to infect HTB-37 cells to confirm the above findings on undetectable viral growth in HTB-37 cells. The results demonstrate that no EV71 isolates and coxsackie A16 viruses grew in HTB-37 cells, whereas polioviruses types 1 and 2 and coxsackie viruses B2 and B3 grew very well in these cells.

Growth Phenotype of EV71 Isolates in Human PBMCs and Their Spreading Capacity

The growth of EV71 TW98NTU2078 isolated from a patient with severe encephalitis in human PBMC-adherent cells had high viral yields (5.8 ± 0.4 $\log_{10}$ PFU/ml at 96 hr post infection) whereas the EV71 TW98NTU1186 did not replicate in these cells (Fig. 2). Additionally, whether the virus can be carried by human PMBC adherent cells was determined using the IC assay. The results also showed that EV71 TW98NTU2078 was indeed carried by human adherent cells more efficiently than was the EV71 TW98NTU1186 isolate with a difference of over 100-fold plaque numbers per milliliter (4.2 ± 0.2 vs. 2.2 ± 0.1) (Fig. 3).

The CNS-Related Isolate of EV71 Was Temperature-Resistant at 40°C

The persistence of a high fever in EV71 patients has been documented [Ng et al., 2001]. The important biological characteristics of the sensitivity of EV71 to temperature in monkeys have also been reported [Hashimoto and Hagiwara, 1983]. TW98NTU2078 and TW98NTU1186 isolates of EV71 grown in Vero cells for 5 days at 37 and 40°C, respectively, and the formation of plaques were studied. The results indicated that the TW98NTU2078 isolate was a Tr strain that grew at both temperatures, whereas the TW98NTU1186 isolate was a temperature-sensitive (Ts) strain that grew only at 37°C. For further confirmation, another five EV71 isolates, three of herpangina and two from patients with CNS, were tested. The results showed that both the TW80NTU3100 isolate obtained from the patient with polio-like paralysis and the TW98NTUPT142 isolate obtained from a patient with CNS-involvement were Tr strains. In contrast, all three herpangina EV71 isolates were Ts strains.

Comparison of Nucleotide Sequences in 5′-NCR of EV71 Isolates

Comparing the characteristics of neurovirulence showed that the nucleotides in the 5′ non-coding region (5′-NCR) had the following four characteristics similar to poliovirus serotype 3; (1) neurovirulent cytidine nucleotide (at position 475), (2) polypyrimidine tracts (at positions 565–584), (3) upstream AUG codons (at positions 593–595), and (4) a distance between the AAUAAA motif (at positions 571–576) and the AUG codon in the G-loop of 22 nucleotides [Aurelia et al., 1992[. The other nine EV71 isolates from TW, including five from patients with CNS manifestations (TW80NTU3100, TW98NTU1107, TW98NTU1311, TW98C035, TW98PT142) and four herpangina patients (TW98NTU1334, TW99NTU1183, TW2KNTU0652, TW2KNTU1148), had all four phenomena described above. Restated, a total of 11 EV71 TW isolates, from severe encephalitis and mild herpangina patients, exhibited features similar to the four features shown by the poliovirus. When compared to the EV71 BrCr strain (ETU22521), an old isolate obtained from California, US in 1969, all 11 Taiwanese isolates had two additional inserted nucleotides of uridine and cytidine at positions 741 and 742. Interestingly, the 5′-NCR of all 8 of the 11 tested EV71 Taiwanese isolates exhibited a polyadenylation signal motif (AAUAAA) in the G loop, whereas the EV71 TW98NTU1186, TW2KNTU0652, and TW2KNTU1148 isolates, in the mild case of herpangina, had one point mutation (U → C) at positions of 575 or 576 at the polyadenylation site in the G loop (Fig. 4).

DISCUSSION

Cellular tropism of a virus not only offers a site for the replication of an infectious agent but also ensures that the extent of replication may cause further successful spread of the virus and viral pathogenesis [Dow et al., 1999]. EV71 causes sudden death and the disease progresses rapidly, therefore several isolates obtained from patients with and without CNS involvement in TW were studied. The following three unique characteristics

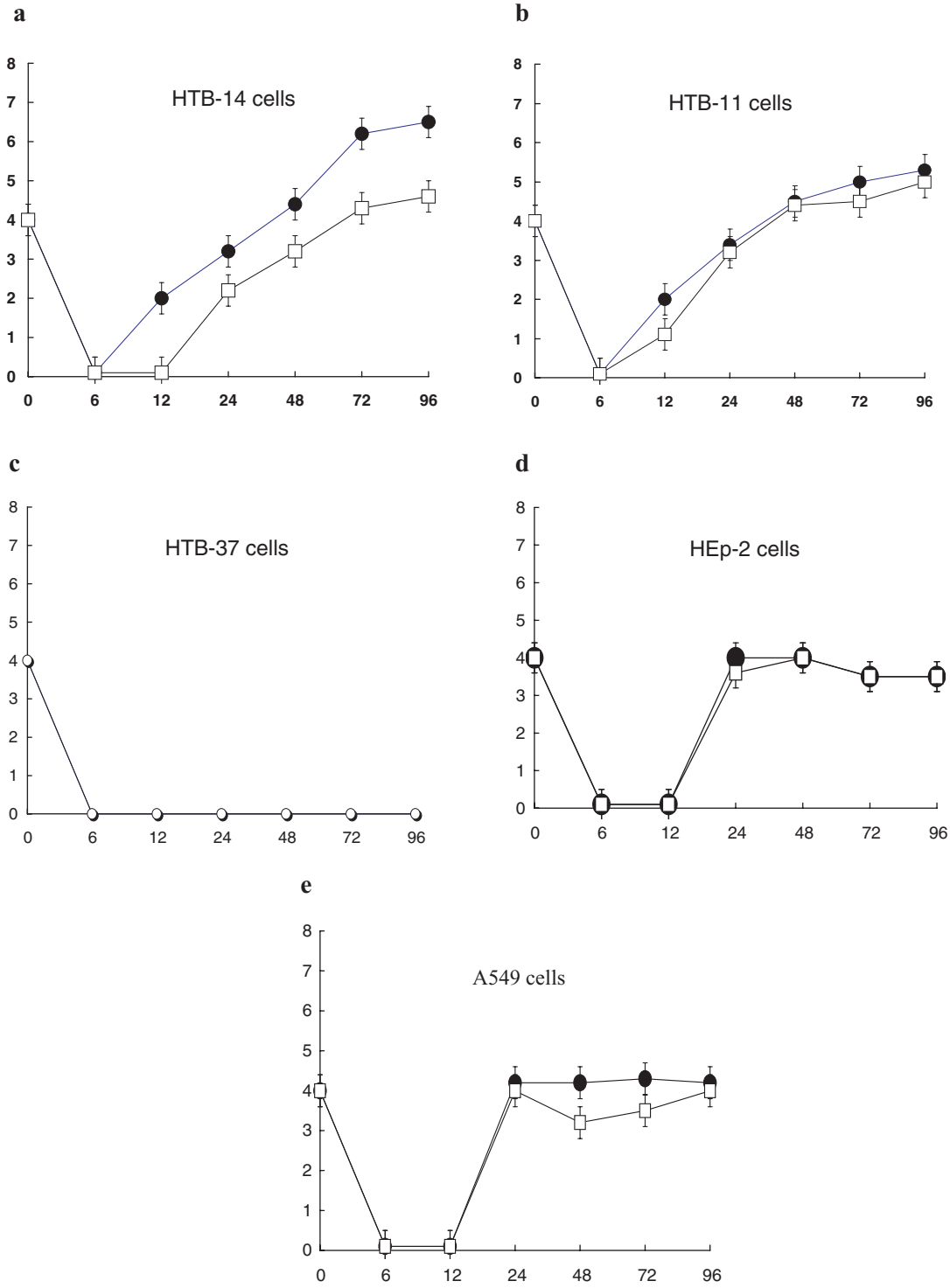

Fig. 1. Cellular tropism of EV71 isolates. Cells were infected with the TW98NTU1186 (—□—) isolate from herpangina and TW98NTU2078 (—●—) isolate from encephalitis at an MOI of 0.01, and the amounts of virus released at the indicated time points (0, 6, 12, 24, 48, 72, and 96 hr) were quantified by plaque assay. The X-axis represents number of hours post infection, and the Y-axis represents $\log_{10}$ PFU/ml. The growth curves of viruses in HTB-14, HTB-11, HTB-37, HEp-2, and A549 were shown in (**a**–**e**), respectively.

were noted, which may explain important aspects of the epidemiology, laboratory diagnosis, and the mechanism of pathogenesis. First, no EV71 isolate could replicate the HTB-37 human colon carcinoma cell-line. Second, the EV71 isolate TW98NTU2078 obtained from a patient with encephalitis yielded a high virus in both HTB-14 and human PBMC-adherent cells. Third, three EV71 isolates obtained from patients with CNS involvement

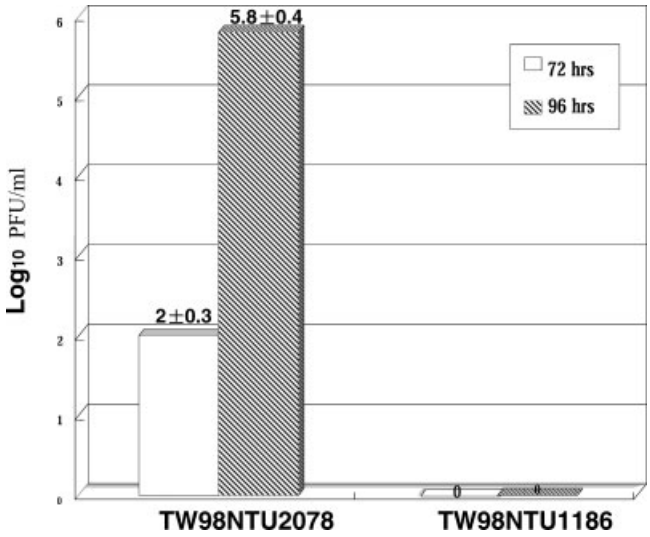

Fig. 2. Replication of two different EV71 isolates (TW98NTU2078 and TW98NTU1186) in human adherent cells infected with viruses with an MOI of 0.1. Incubated at 37°C in CO_2 for 5 days, the viruses were harvested at the indicated times (72 and 96 hr post infection) following three cycles of freeze-thawing, and the supernatants were then quantified with plaque assay on Vero cell monolayer.

exhibited greater resistance at 40°C than did the EV71 isolates obtained from the patient with herpangina.

The inability of all EV71 isolates to replicate in the HTB-37 cells demonstrated that EV71 may not invade or replicate in human colon cells. This result differs substantially from that obtained for poliovirus and

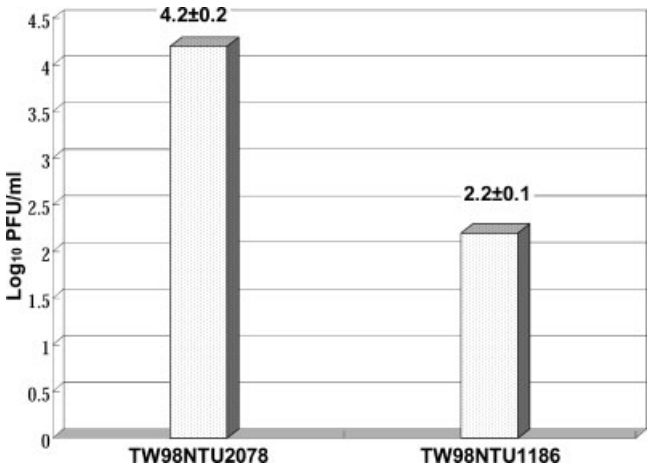

Fig. 3. Infectious center (IC) assay of Taiwan EV71 isolates (TW98NTU2078 and TW98NTU1186) in human blood mononuclear cells (PBMC). To investigate whether human PBMCs can carry EV71, the adhered PBMCs were infected with the EV71 virus (TW98NTU2078 and TW98NTU1186) at an MOI of 10 at 37°C for 30 min, and washed immediately in PBS three times. The adsorbed cells were serially diluted, by a factor of 10 each time, from 10^{-1} to 10^{-6} using 0.5% gelatin (in PBS), and then 100 μl of each diluent was added onto the confluent Vero cells, duplicated. The infected cells were incubated at 37°C with 5% CO_2 for 5 days. The cells were then fixed in 10% formalin solution, stained with 5% crystal violet, and the number of plaques were quantified and represented as numbers of infection centers (ICs)/ml. The means and standard deviations of the numbers of infectious centers in duplicated wells per milliliter were presented.

demonstrates that the binding of receptors of EV71 to the colon cells may differ from that of the poliovirus. Poliovirus can bind to such cells via the CD155 molecule on the cell membrane [Lange et al., 2001]. Further investigations must be conducted to identify the binding sites, the receptors and the possible co-receptors on the cells. Moreover, the high viral yields of all EV71 isolates in HEp-2 cells (a pharyngeal cell line) may explain why the rates of EV71 isolation from throat swabs exceeded markedly those from rectal swabs (91.7 vs. 64.8%) [Chuan et al., 1999], and differ from the high rates of isolation of polio viruses from fecal specimens [Wang et al., 2000].

EV71 is known to be an infectious agent causing CNS disease and leading to poliomyelitis-like paralysis [Lum et al., 1998; Abubakar et al., 1999; Huang et al., 1999; Shen et al., 2000]. The cellular and molecular mechanisms of neurological invasion by EV71 are unknown. However, the EV71 isolate TW98NTU2078 obtained from a patient with encephalitis was found in this study to exhibit three phenomena that were not shown by the TW98NTU1186 isolate obtained from the patient with herpangina. First, TW98NTU2078 yielded around 2 $\log_{10}$ PFU/ml more plaques than the TW98NTU1186 isolate on HTB-14 cells obtained from human astrocytoma. The other three EV71 isolates (TW98NTU3100, TW98CH35, and TW98PT142) from patients with encephalitis and two EV71 isolates (TW98NTU1183 and TW99NTU1334) from patients with herpangina showed a similar biological difference (Data no shown). In fact, astrocytes are one of the most important constituent cells in the blood–brain barrier, a very important vasoneurotic part of the CNS [Abbruscato and Davis, 1999]. Moreover, the data also indicate that EV71 can infect human neuroblasotma cells (HTB-11) (Fig. 1). Hence, the higher viral yield of EV71 on blood–brain barrier may allow the virus to break through the blood–brain barrier and then infect the neurons. Second, the TW98NTU2078 isolate in the severe CNS case was a PBMC-tropic strain, which could not only be carried by PBMCs but also able to replicate in adherent cells of PBMCs, unlike the EV71 TW98NTU1186 isolate in the mild case, which was transported only by human PBMCs without further replication. After EV71 viruses enter the blood stream, they may be carried by PBMCs and their subsequent productive infection may have the potential of infecting astrocytes in the blood–brain barrier and other neighboring neurons. Third, TW98NTU2078 isolate was a Tr strain, which has been reported as being a neurovirulent strain of poliovirus [Freistadt and Eberle, 1996]. EV71 patients have fever [Chang et al., 1999b], which is consistent with the fact that TW98NTU2078 replicated at 40°C, and so this isolate overcomes high body-temperature during fever in the host and continues to grow in the infected cells. Therefore, such marked temperature-resistance allows the severe EV71 strain to infect cells in the CNS. The other two isolates of EV71 obtained from patients with CNS involvement (TW80NTU3100 and TW98PT142) also exhibited this biological characteristic. EV71 can

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                449         459         469  *      479         489         499         509         519
EV71BrCr        TCCTCCGGCC CCTGAATGCG GCTAATCCTA ACTGCGGAGC ACATACCCTT AATCCAAAGG GCAGTGTGTC GTAACGGGCA
TW80NTU3100     ---------- ---------- ---------- ---------- ---cg--t-c --c---g-a- -t-------- ----------
TW98NTU1107     ---------- ---------- ---------- ---------- ----g-t-ac --a---gt-- -tg------- ----------
TW98NTU1311     ---------- ---------- ---------- ---------- ----g-t-ac --a---gt-- -tg------- ----------
TW98CH35        ---------- ---------- ---------- ---------- ----g-t-ac --a---gt-- -tg------- ----------
TW98PT142       ---------- ---------- ---------- ---------- ----g-t-ac --a---gt-- -tg------- ----------
TW98NTU2078     ---------- ---------- ---------- ---------- ----g-t-ac --a---gt-- -tg------- ----------
TW98NTU1186     ---------- ---------- ---------- ---------- ----g-t-ac --a---gt-- -tg------- ----------
TW98NTU1334     ---------- ---------- ---------- ---------- ----g-t-ac --a---gt-- -tg------- ----------
TW99NTU1183     ---------- ---------- ---------- ---------- ----g-t-ac --a---gt-- -tg------- ----------
TW2kNTU0652     ---------- ---------- ---------- ----t----- ----g--t-c ------g--- -t-------- ----t-----
TW2kNTU1148     ---------- ---------- ---------- ----t----- ----g--t-c ------g--- -t-------- ----t-----

                529         539         549         559         569         579         589         599
EV71BrCr        ACTCTGCAGC GGAACCGACT ACTTTGGGTG TCCGTGTTTC TTTTTATTCT TGTATTGGCT GCTTATGGTG ACAATTAAAG
TW80NTU3100     ---------- ---------- ---------- ---------- ---------- -a-------- ---------- -------c--
TW98NTU1107     ---------- ---------- ---------- ---------- c--------c -a-------- ---------- -----c---a
TW98NTU1311     ---------- ---------- ---------- ---------- c--------c -a-------- --------- ------c----
TW98CH35        ---------- ---------- ---------- ---------- c--------c -a-------- --------- ------c----
TW98PT142       ---------- ---------- ---------- ---------- c--------c -a-------- --------- ------c----
TW98NTU2078     ---------- ---------- ---------- ---------- c--------c -a-------- ---------- -----c----
TW98NTU1186     ---------- ---------- ---------- ---------- c-----c--c -a-------- ---------- -----c----
TW98NTU1334     ---------- ---------- ---------- ---------- c--------c -a-------- --------- ------c----
TW99NTU1183     ---------- ---------- ---------- ---------- c--------c -a-------- --------- ------c----
TW2kNTU0652     ---------- ---------- ---------- ---------- c------c-- -ac------- --------- -------t---
TW2kNTU1148     ---------- ---------- ---------- ---------- c------c-- -ac------- --------- -------t---

                609         619         629         639         649         659         669         679
EV71BrCr        AATTGTTACC ATATAGCTAT TGGATTGGCC ATCCAGTGTC AAACAGAGCT ATTGTATATC TCTTTGTTGG ATTCACACCT
TW80NTU3100     ---------- ---------- ---------- ----g----g c--t------ g--a----c- -g-------- t--tgtg--a
TW98NTU1107     -g-------- ---------- ---------- ----g----g c-----g--a -----t--c- -a---a---- t--tgt---a
TW98NTU1311     -g-------- ---------- ---------- ----g----g c-----g--a -----t--c- -a---a---- t--tgt---a
TW98CH35        -g-------- ---------- ---------- ----g----g c-----g--a -----t--c- -a---a---- t--tgt---a
TW98PT142       -g-------- ---------- ---------- ----g----g c-----g--a -----t--c- -a---a---- t--tgt---a
TW98NTU2078     -g-------- ---------- ---------- ----g----g c-----g--a -----t--c- -a---a---- t--tgt---a
TW98NTU1186     -g-------- ---------- ---------- ----g----g c-----g--a -----t--c- -a---a---- t--tgt---a
TW98NTU1334     -g-------- ---------- ---------- ----g----g c-----g--a -----t--c- -a---a---- t--tgt---a
TW99NTU1183     -g-------- ---------- ---------- ----g----g c-----g--a -----t--c- -a---a---- t--tgt---a
TW2kNTU0652     ---------- ---------- ---------- ----g----g c--t----t- --------c- -g-----g-- c--tgac--a
TW2kNTU1148     ---------- ---------- ---------- ----g----g c--t----t- --------c- -g-----g-- c--tgac--a

                689         699         709         719         729         739**       749         759
EV71BrCr        CTCACTCTTG AAACGTTACA CACCCTCAAT TACATTATAC TGCTGAACAC GA..AGCGAT GGGCTCCCAG GTCTCCACAC
TW80NTU3100     --a---t-aa --t-ta--gt t-----t--- -tt--at-gg ct--c----- a-tt-aac-- ---t--a--a --g-----t-
TW98NTU1107     t-at-a--ga -gt-tg-gat ---t-----a -t----t-ga cc--c----- a-tc-aac-- ------a--- --g-------
TW98NTU1311     t-at-a--ga -gt-tg-gat ---t-----a -t----t-ga cc--c----- a-tc-aac-- ------a--- --g-------
TW98CH35        t-at-a--ga -gt-tg-gat ---t-----a -t----t-ga cc--c----- a-tc-aac-- ------a--- --g-------
TW98PT142       t-at-a--ga -gt-tg-gat ---t-----a -t----t-ga cc--c----- a-tc-aac-- ------a--- --g-------
TW98NTU2078     t-at-a--ga -gt-tg-gat ---t-----a -t----t-ga cc--c----t a-tc-aac-- ------a--- --g-------
TW98NTU1186     t-at-a--ga -gt-tg-gat ---t-----a -t----t-ga cc--c----- a-tc-aac-- ------a--- --g-------
TW98NTU1334     t-at-a--ga -gt-tg-gat ---t-----a -t----t-ga cc--c----- a-tc-aac-- ------a--- --g-------
TW99NTU1183     t-at-a--ga -gt-tg-gat ---t-----a -t----t-ga cc--c----- a-tc-aac-- ------a--- --g-------
TW2kNTU0652     t-a--ct-aa --t-ta--ac -------g-- -tt--at-ta cc--c--t-- a-tc-aac-- ------a--- --g--t--t-
TW2kNTU1148     t-a--ct-aa --t-ta--ac -------g-- -tt--at-ta cc--c--t-- a-tc-aac-- ------a--- --g--t--t-
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Fig. 4. Alignment of one fragment of nucleotide sequences 5′-NCR from 11 Taiwan isolates with one reference California strain BrCr, obtained from the NCBI gene bank (ETU22521). Dashed positions correspond to the nucleotides conserved in all eleven Taiwan EV71 isolates. The asterisk (*) denotes the cystidine nucleotide associated with neurovirulent at position 475, the asterisks (**) denote the insertion position of two nucleotides 741 and 742, and the bold type indicates the AAAUAA polyadenylation signal motif and upstream AUG codons. The F-loop region is at nucleotide position from 449 to 564, and the G-loop area is at nucleotide position from 565 to 632. Six isolates (TW80NTU3100, TW98NTU1107, TW98NTU1311, TW98CH35, TW98PT142, and TW98NTU2078) were obtained from patients with encephalitis, and five isolates (TW98NTU1186, TW98-NTU1334, TW99NTU1183, TW2KNTU0652, and TW2KNTU1148) were from the herpangina patients.

also grow well in HEp-2 (human pharyngeal epidermoid carcinoma) and A549 (human lung carcinoma) cells, indicating that EV71 may cause viremia immediately following local replication in the pharyngeal cells, and would then cause a more severe lung edema and CNS involvement [Lum et al., 1998; Chuan et al., 1999].

The internal ribosome entry site (IRES) of the 5′-NCR in enteroviruses is important as an internal ribosomal landing pad for the replication of viruses [Andino et al., 1990; Lee and Young, 1998]. EV71 5′-NCR includes F loop (at positions 449–564) and G loop (at positions 565–632), and a polyadenylation signal motif (AAUAAA) is present in the G loop, like that in the 5′-NCR of the poliovirus [Jackson et al., 1990]. Although the eight EV71 isolates that are similar to the TW98NTU2078 isolate had similar polyadenylation signal motifs in their G loops, which have been documented to be the motif that participates in accelerating viral replication [Hwang et al., 1998; Terhune et al., 1999], the TW98NTU1186, TW2KNTU0652, and TW2KNTU1148 isolates did not have this similar motif because of the point mutation (U → C) of the polyadenylation signal motif in each G loop (Fig. 4). However, the mutation of polyadenylation sequences may affect the transduction of cellular sequences, reducing the number of viral transcripts [Swain and Coffin, 1989]. Accordingly, the point mutation in this polyadenylation signal motif may reduce the capacity of the TW98NTU1186 isolate to replicate in HTB-14 cells (Fig. 1a) and cause poor replication in the RD cells of TW2KNTU0652 and TW2KNTU1148 isolates (data not shown).

All 11 EV71 isolates, regardless of CNS outcome or whether they were involved in mild herpangina, had a neurovirulent nucleotide (cytidine) at position 475 in the F loop of the 5′-NCR. The region has been reported to be a neurovirulent region of wild-type poliovirus, as determined by comparison with the Sabin vaccine strain [Dildine and Semler, 1992]. However, EV71 like other enteroviruses, including coxsackie viruses and ECHO viruses, has such a cytidine nucleotide position, which is important in the formation of the stem of an F-loop, which may bind with other host factors; therefore, a single nucleotide at this position cannot determine viral virulence. This finding is unlike that of the neurovirulence of poliovirus type 3 that involve the mutation of C to U at position 472 in 5′-NCR (the same stem position of the F-loop as EV71) and the viral load in neurological cells [Evans et al., 1985].

Based on the results obtained concerning phenotypic differences between encephalitis and herpangina EV71 isolates in human PBMCs and astrocytes in vitro, a possible pathogenic pathway of EV71 from the oral route to CNS is hypothesized, although the cell culture experiments may not reflect completely the situation in vivo. The virus would enter from the oral route by person-to-person transmission by the fecal-oral route, and it would be very likely to replicate initially in pharyngeal cells, increasing replication in PBMCs and viremia with a higher viral load, even during the fever stage at a high body temperature; therefore, the EV71 pathogenic strain can infect the astrocytes (HTB-14) of the blood–brain barrier via PBMCs, finally invading the neurons (HTB-11) in the brain. Further animal model investigations based on various strains of EV71 integrated infectious clones may offer clues on the exact molecule that is responsible for viral pathogenesis.

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