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Neutralizing Antibody Provided Protection against Enterovirus Type 71 Lethal Challenge in Neonatal Mice

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Key Words
Enterovirus type 71 · Experimental infection · Mice · Neutralizing antibody · Vaccine

Abstract
Experimental infection with enterovirus type 71 (EV71) induced death in neonatal mice in an age- and dose-dependent manner. The mortality rate was 100% following intraperitoneal inoculation 1-day-old ICR mice and this gradually decreased as the age at the time of inoculation increased (60% in 3-day-old mice and no deaths occurred in mice older than 6 days of age). A lethal dose greater than 10^8 PFU was necessary. Lethargy, failure to gain weight, rear limb tremors and paralysis were observed in the infected mice before death. EV71 was isolated from various tissues of the dead mice. Using a reverse transcription polymerase chain reaction technique with a specific primer pair, a 332-bp product was detected in the tissues that produced a culture positive for EV71. Protection against EV71 challenge in neonatal mice was demonstrated following passive transfer of serum from actively immunized adult mice 1 day after inoculation with the virus. Pups from hyperimmune dams were resistant to EV71 challenge. Additionally, maternal immunization with a formalin-inactivated whole-virus vaccine prolonged the survival of pups after EV71 lethal challenge.

Introduction

Enterovirus type 71 (EV71) is a human enterovirus in the Picornaviridae family [3]. EV71 infection was first described in 1974 in the United States; it has subsequently been reported worldwide [1, 5, 6, 12, 16]. It has been associated with an array of clinical diseases, including hand-foot-and-mouth disease (HFMD), herpangina, aseptic meningitis, poliomyelitis-like paralysis and possibly fatal encephalitis [12, 13]. Two EV71 outbreaks manifesting in rapid clinical deterioration and death among young children were reported in Bulgaria in 1975 and in Malaysia in 1997 [4]. In 1998, there were more than 129,000 cases of HFMD or herpangina reported among young children in Taiwan. Approximately 400 children were hospitalized with complications of neurogenic shock and pulmonary edema that mostly affected infants under 5 years of age (a total of 78 deaths). EV71 was thought to be the causative agent [2, 7, 14, 17]. The pathogenesis of EV71, its host cell receptor and tissue tropism remain unclear. Furthermore, little is known of the factors that

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determine the different clinical manifestations. Results of the inoculation of suckling mice with EV71 culture isolates have been reported, however, further characterization has been absent [4, 20]. This study was undertaken to examine the relative susceptibilities of 1-day-old mice and adult mice to infection and disease induction following intraperitoneal inoculation with EV71. We also wanted to determine the role of EV71-specific antibody in the disease process.

Materials and Methods

Virus

EV71 was obtained from the National Health Research Institute Virology Laboratory for Diagnosis and Research at the National Cheng Kung University Hospital, Tainan, Taiwan; this isolate was made from tissues of a patient with EV71 encephalitis. The stock virus was grown in Vero cells or rhabdomyosarcoma (RD) cells with a resulting titer of 4.6×10^9 PFU/ml. These cell cultures were grown at 37°C in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 IU of penicillin, 100 μg of streptomycin and 0.25 μg of amphotericin B per milliliter. For preparation of the EV71 vaccine, the bulk culture of EV71 in Vero cells was disrupted by two freeze-thaw cycles. Cellular debris was removed using low-speed centrifugation (200 *g*, 10 min). Virus supernatant was further clarified using centrifugation (10,000 *g*, 30 min) and ultrafiltration (YM-10 membrane, Amico Inc., Beverly, Mass., USA). Virus particles were then pelleted using ultracentrifugation in an SW27 rotor at 100,000 *g* for 4 h through a 30% sucrose gradient. After determination of protein concentration, the virus was incubated with 0.1% (v/v) formaldehyde (Merck, Darmstadt, Germany) at 37°C for 30 min and absorbed with an equal volume of alum (Sigma, St. Louis, Mo., USA).

Mice

Outbred ICR mice were obtained from the Laboratory Animal Center of the National Cheng Kung University Medical College. All institutional guidelines for animal care and use were strictly followed. At 1,3, 6 and 9 days and 3 weeks of age, the mice were inoculated intraperitoneally with 100 μl of EV71 stock culture. In other experiments, the mice were given serial dilutions of EV71 1 day after birth. All mice were monitored daily for body weight and death until weaning or 21 days after inoculation. Dams and weanlings were euthanized and sera were collected and stored at −70°C until use. The mice in the control group were given 100 μl of Vero cell lysate and kept either in the same cage or in cages separate from the infected mice. All of the mice in the control group were healthy throughout the experiments. Preliminary studies showed that the adult mice (>8 weeks) were resistant to multiple EV71 administrations, and pooled sera obtained from these animals were used in protection studies. Briefly, heat-treated (56°C, 30 min) serially diluted sera (100 μl) were injected intraperitoneally into neonatal mice 1 day after inoculation with a lethal dose of EV71 (4.6×10^9 PFU/ml). Body weight and death were monitored as described above. The control animals were given sera from naïve mice. One-day-old pups from infected dams were also used in challenge studies to examine the role of maternal antibodies. For maternal immunization, adult female mice were injected subcutaneously with the EV71 vaccine (12.8 or 25.6 μg/mouse) twice at a 1-week interval. Seven days after the second injection, the animals were allowed to mate. Lethal challenges were performed on pups 1 day after birth.

Virus Isolation and Identification

Organs were aseptically removed from dead animals and aliquots were either homogenized or frozen at −70°C before RNA extraction, as described below. Tissue suspensions (10% w/v) were clarified using centrifugation and the supernatants were inoculated onto monolayers of Vero cells or RD cells. These cells were inspected daily for a minimum of 14 days for cytopathic effect (CPE) [8]. All cell cultures were scraped, then the resuspended cells were pelleted using centrifugation, fixed on slides and stained using indirect immunofluorescence with EV71 monoclonal antibody (Chemicon International Inc., Temecula, Calif., USA). Cultures with CPE and/or indirect immunofluorescence-positive cells were considered to be infected with EV71.

Reverse Transcription Polymerase Chain Reaction

Viral RNA was extracted from homogenized tissues using a GStract™ RNA isolation kit II (Maxim Biotech Inc., San Francisco, Calif., USA) according to the manufacturer's instructions. Total RNA was converted to cDNA with Strata Script™ H-reverse transcriptase (Stratagene, La Jolla, Calif., USA) and polymerase chain reaction (PCR) was performed using oligonucleotide primers flanking nucleotides 1623–1933 of the VP1 of EV71. The primer sequences were 5′-GTGGCAGATGTGATTGAGAG-3′ and 5′-GTTATGTCTATGTCCCAGTT-3′, which were based on genomic sequences of our EV71 strain, Tainan/5079/98 (AF177911) [18]. Forty cycles of PCR amplification were carried out on an automated thermal cycler (Hybrid Omnigene, UK). Temperatures were 94°C during denaturation (1 min), 55°C during annealing (1 min) and 72°C during polymerization (2 min). The final extension time was 10 min at 72°C. PCR products were subjected to a second series of DNA amplification using the same primers. In every PCR experiment, a negative control (without DNA) was run simultaneously as an additional control for reagent contamination.

Plaque Assay and Neutralization Test

Confluent monolayers of Vero cells or RD cells were prepared in 96-well plates at a density of 9×10^5 cells/well. Cells were infected with serial dilutions of EV71, overlaid with 0.75% methylcellulose in MEM and incubated at 37°C for 3 days before the plaques were visualized using staining with crystal violet. The 50% tissue culture infective doses ($TCID_{50}$) were determined with the method described by Hsiung [9] using the Reed and Muench formula. Neutralizing antibodies were determined using a microassay with RD cells [9]. Briefly, 50 μl of serial serum dilutions were mixed with 50 μl of 100 $TCID_{50}$ EV71 in a 96-well plate and RD cell suspensions (final concentration 8×10^3 cells) were added 2 h later. After incubation for 6 days at 37°C, neutralizing antibody titers were determined as the highest dilutions of serum that inhibited virus growth.

Results

Neonatal mice at different ages were inoculated with EV71 at 4.6×10^8 PFU per mouse. Death began to occur by day 5 after infection of 1-day-old mice, and 100% of

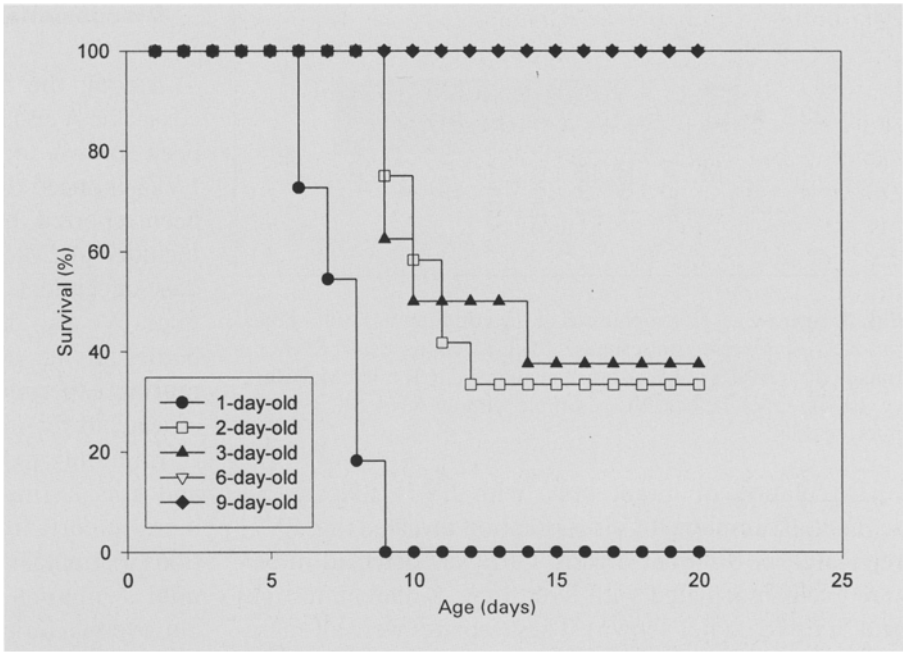

Fig. 1. Age dependence of EV71-induced death. ICR mice were inoculated intraperitoneally with 10^8 PFU of EV71 per animal at 1, 2, 3, 6 or 9 days of age. Death was then monitored daily after infection. Control animals were given Vero cell lysate instead of virus. Each group contained 6–8 mice.

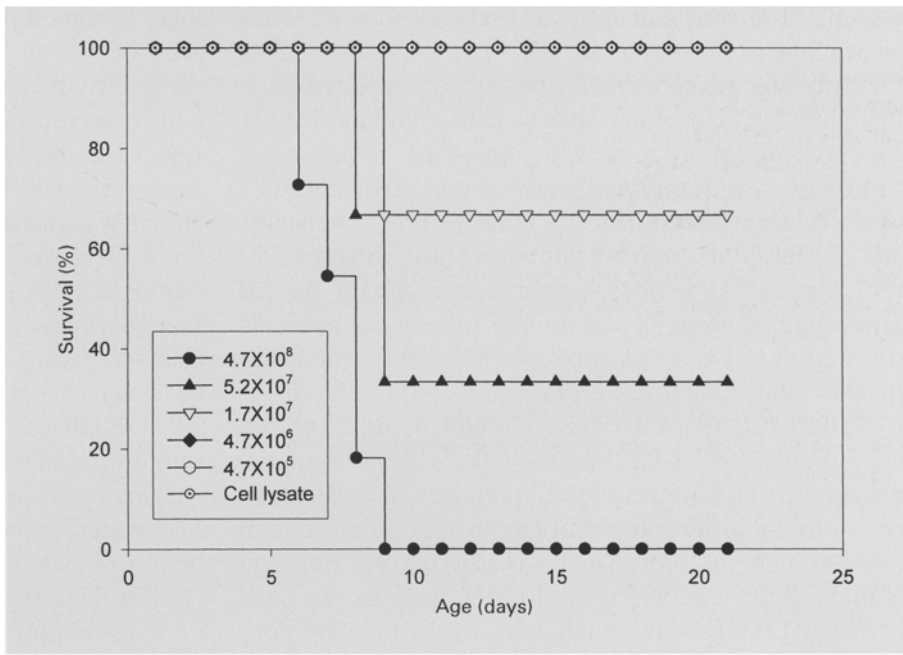

Fig. 2. Dose dependence of EV71-induced disease. One-day-old ICR mice were inoculated intraperitoneally with increasing doses of EV71 (from 4.7×10^5 to 4.7×10^8 PFU/mouse). Death was then monitored daily after infection. Control animals were given Vero cell lysate instead of virus. Each group contained 6–8 mice.

the mice had died by day 10. With infection at 2 or 3 days of age, death was delayed and the incidence of death fell to 60%. Mice older than 6 days at the time of inoculation, and the control mice and dams, showed no signs of disease and all survived (fig. 1). Furthermore, the sera of the control mice and dams did not contain neutralizing antibodies (data not shown). Mice inoculated before 3 days of age showed signs of disease followed by death. Weight loss, weakness, rear limb tremors and paralysis were usually observed 2 or 3 days before death. One-day-old pups died of EV71 in a dose-dependent manner. The death rate was 100% with a dose of $>10^8$ PFU per mouse. With 10^7 PFU per mouse, the mortality rate reduced by one third to two third. A dose of less than 10^7 PFU failed to induce death (fig. 2).

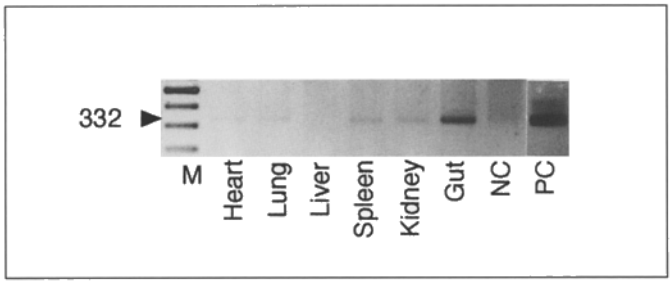

Fig. 3. Agarose gel electrophoresis of the ethidium bromide-stained products of reverse transcriptase PCR reactions using RNA extracted from tissues of EV71-infected neonatal ICR mice. M = Marker, 100-bp DNA ladder; NC = sample without RNA; PC = EV71-infected cells.

Inoculation of infant mice with EV71 followed by sequential sampling for virus isolation revealed that EV71 replicated in different organs. CPE was detected in cultured cells inoculated with liver, lung, kidney, intestines and brain (data not shown). These isolates were all subsequently identified as EV71 using immunofluorescence with EV71 monoclonal antibody (data not shown). Using a specific pair of primers for the VP1 of EV71, a 332-bp PCR product was generated from tissues of infected mice, including the heart, lung, spleen, kidney and gut (fig. 3). Tissues from control mice were consistently negative.

Preliminary experiments showed that adult mice (>8 weeks of age) were resistant to multiple EV71 challenges and produced neutralizing antibodies after infection. To test whether antibodies were protective, serially diluted sera obtained from hyperimmune mice were passively transferred to 1-day-old mice 24 h after EV71 challenge. Figure 4 shows that antiserum significantly reduced the EV71-induced mortality rate. Undiluted serum with a neutralizing titer of 1:128 exerted 100% protection. Approximately 60% of the infected pups survived when given twofold-diluted serum but serum diluted more than fourfold had no protective effect at all. In addition, human serum containing anti-EV71 neutralizing antibodies (1:1,024) also attenuated the disease in infant mice (data not shown). Pups delivered and fed by EV71-immunized dams (with neutralizing antibody titer 1:128) were also resistant to EV71 challenge 1 day after birth (fig. 4). Maternal immunization with a formalin-inactivated whole virus vaccine resulted in a rise in neutralizing antibody titer (1:64). Table 1 shows that after a lethal challenge, pups from vaccinated dams had a delayed mortality rate and increased survival rate compared to those from nonvaccinated dams.

Discussion

Among the human enteroviruses, polioviruses and coxsackie A and B viruses, but not echoviruses, have long been known to be pathogenic in newborn mice [10]. EV71-induced disease in newborn mice has occasionally been reported, but no further characterization has been included [4, 20]. In this report, we described the age- and dose-dependent manner of EV71-induced disease in mice. We also demonstrated the protective role of antibodies in the disease process. More importantly, this murine experimental infection model was found to be efficacious for vaccine assessment.

Immunity to enterovirus infections was type specific and long lasting. Antibodies appeared to be relatively more important in protection and recovery from infections. Perinatal infections tended to produce no disease or mild symptoms in neonates with maternal antibodies, but overwhelming disseminated disease occasionally occurred in those without antibodies [3, 11]. Seroepidemiologic studies showed that the disease prevalence of the recent EV71 outbreak in Taiwan was associated with low seropositivity in the extremely young [7]. Our results here confirmed the protective role of antibodies in EV71 infection. First, passive immunization fully protected newborn mice from EV71-induced death. Second, pups derived from EV71-infected dams with elevated neutralizing antibody titers were also resistant to EV71 challenge. The nature of EV71 infection, as well as our results, indicated that vaccination was the best way to prevent EV71 infection. We found that a formalin-inactivated whole virus vaccine partially protected pups of vaccinated dams from EV71 challenge. We chose maternal instead of neonatal immunization because it was difficult if not impossible to vaccinate suckling mice or perhaps infants as well. The inactivated vaccine induced a weaker neutralizing antibody response (1:64) than that of natural infection (1:128). This might be due to the dose of the vaccine, the number of administrations, the type of adjuvant or the loss of B cell epitope during the preparation. Currently, we are testing several other EV71 vaccine preparations, including VP1 subunit vaccine and DNA vaccine using this murine infection model.

The transmission of EV71 was unclear, and per os inoculation has been thought to be one of the naturally infectious routes. On the basis of seroconversion, contact transmission from infected pups to their cagemates or dam did not occur, and lethal doses of EV71 given orally to newborn mice failed to induce illness [unpubl. observation]. More studies are required to determine whether

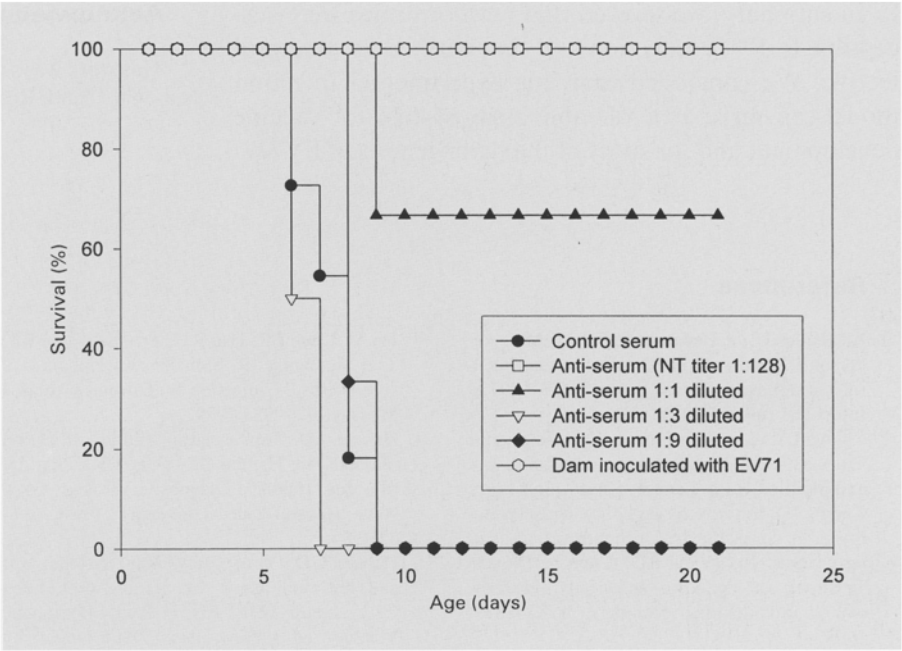

Fig. 4. Passive immunization protected pups from EV71 challenge. One-day-old ICR mice derived from EV71-infected or noninfected dams were intraperitoneally infected with 10^8 PFU of EV71. One day after infection, pups from the noninfected dams were given 100 µl of serially diluted mouse anti-EV71 serum or serum from healthy mice. Death was then monitored daily thereafter. Each group contained 6–8 mice.

virus genomes were present in these animals. Although histopathologic examination was not carried out in this study, the symptoms of the mice that died suggested involvement of the CNS. Chumakov et al. [4] demonstrated that infection of newborn mice with the Bulgarian strain of EV71 caused extensive lesions in the CNS. The demonstration of characteristic lesions in the CNS as well as other tissues will make this murine model an excellent tool for studying the pathogenesis of EV71.

Enterovirus infections are usually mild and subclinical. On the contrary, the clinical syndromes of the EV71 epidemic in 1998 in Taiwan involved the pulmonary, cardiac and nervous systems, suggesting a possible disturbance of the host-parasite interaction [7]. It has been postulated that differences in pathogenicity of EV71 strains contributed to the notable differences in the characteristics of associated clinical diseases. Serological differences have been shown to exist between the HFMD strain and aseptic meningitis strain of EV71 [6]. In addition, Zheng et al. [19] demonstrated that the 5′-noncoding regions (NCR) were different between EV71 isolates from HFMD patients and patients with aseptic meningitis. The 5′-NCR is an important locus that is associated with the neurovirulence of poliovirus, the process of viral RNA replication and cap-independent translation [15]. In a related study, we compared the full-length nucleotide sequences of various EV71 isolates and showed that there were 2 or 3 nucleotide differences within the 5′-NCR and 2 or 4 amino acid differences within the regions encoding viral polyproteins among viral strains with a different clinical pattern (HFMD vs. encephalomyelitis). However, none of these differences correlated with either clinical manifestation [18]. Thus, other potential factors, in addition to causative agents, might contribute to the disease progress [14].

Table 1. Maternal immunization with an inactivated whole-virus EV71 vaccine partially protected newborn mice against challenge

Vaccination	Pups	Challenge (LD_{100})	Deaths at days of age					Survival %
			6	7	8	9	10	
Vehicle	10	+	10	0	0	0	0	0
	6	–	0	0	0	0	0	100
Low dose	8	+	1	2	2	2	1	0
	8	–	0	0	0	0	0	100
High dose	12	+	4	2	2	2	1	8.3
	8	–	0	0	0	0	0	100

Adult female ICR mice were subcutaneously injected with an inactivated whole EV71 virus vaccine (low dose: 12.8 µg/mouse, high dose: 25.6 µg/mouse) twice at a 1-week interval, and allowed to mate at day 7 after the last injection. After delivery, pups were challenged on postnatal day 1.

In summary, we showed that newborn mice were susceptible to EV71 infection and that antibodies were protective. We concluded that this experimental infection model can serve as a valuable assay system for vaccine development and for study of the pathogenesis of EV71.

Acknowledgment

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