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In vitro and *in vivo* characterization of a new enterovirus type 71-specific human intravenous immunoglobulin manufactured from selected plasma donors

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ABSTRACT

Background: Enterovirus type 71 (EV71) causes large outbreaks with significant mortality among young children, and no specific antiviral treatment is currently available. Antibody-based therapy represents a promising alternative strategy for lethal EV71 infection. Our previous data has shown that anti-EV71 neutralization antibodies were present in a significant proportion of blood donors in China.
Objectives: To produce a new human intravenous immunoglobulin (IVIG) product containing high titer anti-EV71 neutralizing antibodies and investigate its therapeutic efficacy against lethal EV71 infection in a murine model.
Study design: Plasma units that contained high titer neutralizing antibodies from selected Chinese donors were pooled and processed into pharmaceutical grade IVIG preparations according to the standard procedure. The efficacy of these EV71-specific IVIG product was characterized *in vitro* by neutralization assay and *in vivo* by suckling mouse protection testing. The therapeutic effects against lethal EV71 challenge were further assayed in a suckling mouse model.
Results: About 12% of the normal plasma units were selected and pooled to manufacture the EV71-IVIG preparations, and *in vitro* and *in vivo* efficacy data showed that these EV71-specific IVIG preparations were enriched with neutralizing antibodies against EV71. Furthermore, treatment with EV71-specific IVIG was evidenced to confer protection against lethal EV71 challenge in a dose- and time-dependent manner in the suckling mouse model.
Conclusions: This preclinical study indicates that these "tailor-made" EV71-IVIG preparations manufactured from selected plasma donors in EV71-endemic areas may represent a promising therapeutic option for the lethal EV71 infections, and further clinical trials should be warranted in the future.

1. Background

Human enterovirus type 71 (EV71), a member of the genus *Enterovirus* of the family *Picornaviridae*, is one of the major pathogen of hand, foot and mouth diseases (HFMD) in young children. Since its first isolation in California, USA, in 1969,[1] EV71-associated outbreaks with high mortality have been reported throughout the world. Especially in Asian-Pacific counties, it has caused several wide spread epidemics since 1997 and is expected to continue to do so for a long time.[2] At present, there is no vaccine or specific antiviral therapy available for EV71 disease.[3]

For many infectious diseases that a specific therapy is not yet available, antibody-based therapy represents a promising alternative strategy. Intravenous immunoglobulin (IVIG) is a pharmaceutical preparation of human IgG that is pooled from thousands of healthy blood donors, thus containing antibodies against a large variety of pathogens. The IVIG mechanisms of action are complex, but the anti-infection effect has been attributed to the presence of pathogen-specific neutralizing antibodies.[4] Pathogen-specific IVIG preparations enriched with specific antibodies have been manufactured using plasma from vaccinated or selected donors for the treatment of rabies, smallpox, hepatitis A, West Nile virus infection and so on.[5–8]

Therefore, the use of IVIG with high titer anti-EV71 antibodies has been considered as a possible strategy for the treatment of severe EV71 infection. Previously, we have shown that commercial IVIG products manufactured from Chinese donors contained anti-EV71 antibodies,[9] while the relatively low levels and high batch-to-batch variance of specific antibodies might restrict its application.[10] The fact that a significant proportion of healthy Chinese blood donors contain anti-EV71 neutralization antibodies[9] makes it possible to develop the EV71-specific IVIG (termed EV71-IVIG) preparations enriched with high titer anti-EV71 antibodies.

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2. Objectives

The purpose of this study is to produce a new human IVIG product containing high titer anti-EV71 neutralizing antibodies and investigate its therapeutic efficacy against lethal EV71 infection in a murine model.

3. Study design

3.1. Manufacturing process of the EV71-IVIG preparations

Plasma units from healthy Chinese blood donors was screened for anti-EV71 neutralizing antibodies by a high-throughput microneutralization assay, and the positive plasma units (>1:50) were then selected and pooled for the production of pharmaceutical grade IVIG preparations according to the standard manufacturing procedure. The IVIG product was manufactured by the Tonrol Bio-Pharmaceutical, Hefei, China, and normal IVIG preparations manufactured in the same factory were used for comparison in this study.

3.2. In vitro and in vivo efficacy

The efficacy of EV71-IVIG was characterized *in vitro* by neutralization assay and *in vivo* by suckling mouse protection testing. The titer of neutralizing antibodies was determined in human rhabdomyosarcoma (RD) cells according to the standard protocol.[11] Briefly, 50 μL of sample dilutions and 50 μL of virus stock containing 100 $TCID_{50}$ EV71 was mixed and incubated with RD cells at 36 °C. EV71-IVIG samples were tested in serially twofold dilution from 1:8 to 1:2048, and cell and virus controls were run simultaneously. Cytopathic effects (CPE) were observed under an inverted microscope after 3–6 days. The neutralizing antibody titer was defined as the highest dilution of serum that would prevent the occurrence of CPE. EV71 prototype strain BrCr (genotype A) and local isolates AH/08/06, HN/08/08 and SD/09/18 (genotype C4) isolated from different provinces in China[12–14] were used for neutralization assay, respectively.

The *in vivo* efficacy of EV71-IVIG was assayed in the 1-day-old suckling mice model as previously described.[15] Kunming mice intraperitoneally infected with EV71 strain AH08/06 developed typical neurological manifestation, including movement disorientation, hind-limb paralysis and opisthotonus, and died within 7–10 days. The 50% lethal dose (LD_{50}) of EV71 stock was determined by the Reed–Muench method. 10 LD_{50} of EV71 was mixed with an equal volume of serial fourfold dilutions of EV71-IVIG and incubated for 60 min at 37 °C. Then the IVIG-virus mixture was injected intracerebrally into 1-day-old suckling mice. The control group received PBS-virus mixture. The animals were monitored daily for 2 weeks. The *in vivo* Effective Dose 50% (ED_{50}) value of EV71-IVIG was calculated according to the Reed–Muench method[16].

3.3. Treatment with EV71-IVIG

The therapeutic efficacy of EV71-IVIG was finally assayed in the 7-day-old suckling mice model. Briefly, mice were infected with 10 LD_{50} of EV71 by intraperitoneal inoculation, and then infected mice were treated by intraperitoneally injections of different doses of EV71-IVIG according to the specific experimental protocols. The mice were then followed for signs of disease and mortality for 21 days. All animal experimental procedures were approved and carried out in accordance with the guidelines of the Animal Experiment Committee of the State Key Laboratory of Pathogen and Biosecurity.

Table 1
Mouse protection testing of the EV71-IVIG preparation.

EV71-IVIG dilutions	No. of deaths/ total mice	Mean survival time, day	Mortality
1:4	2/10	13.0 ± 0.6	20%
1:16	3/10	12.6 ± 0.7	30%
1:64	3/10	12.7 ± 0.6	30%
1:256	6/10	11.3 ± 0.7	60%
PBS	10/10	9.4 ± 0.6	100%

3.4. Statistical analysis

Kaplan–Meier survival curves were used to display the mortality data, and log-rank analyses were performed to determine the statistical significance of the differences between groups.

4. Results

In the present study, about 12% of the plasma units from normal Chinese blood donors were selected and pooled to manufacture the EV71-IVIG preparations according to the same procedure as regular IVIG preparations. The physical and biochemical characterization of the new product, including appearance, purity, protein content, pH, thermostability, integrity and IgG subclass distribution, was performed in accordance with the guidelines of China Pharmacopeia, demonstrating the same quality with the regular commercial IVIG products.

The efficacy of the EV71-IVIG product was first characterized *in vitro* by neutralization test. The titer of neutralizing antibodies of EV71-IVIG preparations was calculated to 1:1024 by standard neutralizing test using AH08/06 strain, showing an eightfold increase in comparison with regular IVIG preparation (1:128) manufactured from normal plasma pools. Independent tests using another 3 EV71 strains, BrCr, HN08/08 and SD09/18, were performed and the results showed the same titer of neutralizing antibodies. This phenomenon confirmed that EV71 neutralizing antibodies were enriched by the screening process of plasma units.

Further, the suckling mouse protection testing was used to characterize the *in vivo* efficacy of the EV71-IVIG product. The results, summarized in Table 1, showed that all the mice injected with PBS-virus mixture died within 7–9 days as expected, while the presence of high concentrations of EV71-IVIG significantly prolonged the surviving time and decreased the mortality caused by EV71 in comparison with the control group. The ED_{50} of EV71-IVIG was calculated to 1:89, indicating a 1:89 dilution of EV71-IVIG can protect 50% of the infected mice in this model. Together, these *in vitro* and *in vivo* data demonstrated that these "tailor-made" EV71-IVIG preparations were endowed with strong neutralizing activity against EV71.

Then, the therapeutic efficacy of EV71-IVIG preparations was assessed in the 7-day-old suckling mouse model of EV71 infection. Firstly, a single injection of different concentrations of EV71-IVIG preparation was given to the mice 4 h after infection with 10 LD_{50} of EV71, respectively. As shown in Fig. 1, a dose-dependent therapeutic effect of EV71-IVIG was observed. Treatment with 7.5 mg of EV71-IVIG provided 50% protection against lethal EV71 infection, and 30% and 10% of mice treated with 5 and 2.5 mg of EV71-IVIG survived. No protection was observed when mice were mock treated with PBS or treated with the same dose of regular IVIG. Furthermore, neutralization test and ELISA for human IgG results demonstrated that human-derived anti-EV71 neutralizing antibodies were successfully transferred to the blood of mice (data not shown). These data indicate a single treatment of EV71-IVIG can provide a partial protection from lethal EV71 challenge in mice.

Next, to achieve a full protection, serially 3 injections of EV71-IVIG were administered to the mice challenged with the lethal dose