Immunity to avirulent enterovirus 71 and coxsackie A16 virus protects against enterovirus 71 infection in mice

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Enterovirus 71 (EV71) has caused significant morbidity and mortality in Taiwan since the 1998 outbreak. In fact, EV71 has been regarded as the most important neurotropic enterovirus after the eradication of the poliovirus (8).

Both live attenuated and inactivated whole virus vaccines have been used to successful control poliovirus (1). However, there is still no specific antiviral therapy or vaccine for EV71, although several experimental vaccines have been tested, including inactivated whole virus (14) and VP1 subunit DNA (13), as well as transgenic tomato fruit expressing VP1 protein (3).

In this study, we for the first time showed that live vaccine is feasible for EV71. EV71/4643, an avirulent strain for mice, when orally inoculated to mice at day 1 of age was tolerated and was able to induce both specific serum IgG (days 7 and 14 post-inoculation [p.i.]) and intestinal IgA antibody (day 14 p.i.) responses (Fig. 1A) with neutralizing activity (NT) of 1:16. Splenocytes isolated from the immunized mice proliferated upon EV71 antigen stimulation in a dose-dependent manner (Fig. 1B). A protection study showed that active live EV71 immunization reduced the mortality of the animals by 20 to 30% after lethal challenge (Fig. 2). Furthermore, those surviving mice gained weight normally and exhibited no sign of illness. These results indicated that EV71, like poliovirus infection, might be preventable by live vaccines.

Remarkably, active immunization with coxsackie A16 virus (CA16) not only induced CA16 specific but also EV71 cross-reactive antibody (Fig. 1A) with a weak NT of 1:2. Furthermore, CA16 immunization reduced the mortality of mice by 10 to 20% upon EV71 lethal challenge (Fig. 2). Type I interferons (IFNs), however, were probably not responsible for the protective effect of CA16 immunization. This view was supported by the fact that oral administration of CA16 did not induce type I IFN production. The protective effect of active immunization was restricted since it did not extend to CB3 immunization. CB3 neither shared in vitro cross-reactivity with EV71 (Fig. 1A and B) nor provided in vivo protection following active immunization (Fig. 2).
Fig 1. Active immunization with both live avirulent EV71 strain and CA16 induced EV71 specific immunity in newborn mice. One-day-old ICR mice were orally inoculated with either live EV71/4643, CA16, or CB3 (104 PFU/mouse). (A) EV71 specific serum IgG and intestinal IgA levels were determined by ELISA at 7 and 14 days pi. (b) Proliferation of splenocytes in response to EV71, CA16, or CB3 antigen stimulation was determined at 7 day pi. Results are the mean ± SEM. *P< 0.05 as compared with control mice. n=10 to 12 mice.

Enteroviruses, a large group of closely related RNA viruses that consisting more than 70 types, exhibit considerable intratypic and intertypic cross-reactivity and recombination (2, 7, 9, 11). Furthermore, exposure to and infection with multiple enteroviruses is thought to be very common, and thus immunity should prevail in the general population (6).

Based on their biological and molecular characteristics, both EV71 and CA16 have been grouped with human enterovirus group A (10). An early study demonstrated that EV71 and CA16 shared some common epitopes (4). Although both EV71 and CA16 can cause hand-foot-and-mouth disease and herpangina in the young peoples, infection with only EV71 occasionally leads to severe diseases such as aseptic meningitis, poliomyelitis-like paralysis, and possible fatal encephalitis in neonates (5).
In the present study, we clearly demonstrated that both humoral and cellular immunities induced by CA16 could cross-react with EV71. Cross-reactivity of the immune sera was observed between EV71 and CA16, but not CB3, in an ELISA-based assay (Fig. 3).

![Fig 3. Cross reactivity between EV71 and CA16.](image)

EV71 or CA16 viral antigen (10 μg/ml) were coated onto 96-well plates and incubated with serially diluted mouse anti-EV71, CA16 or CB3 immune serum. Immunoreactivity was revealed by incubation with secondary antibody and TMB substrate. Representative of two similar experiments is shown.

At 1:4 dilution, the CA16 immune serum neutralized EV71 but not CB3 or poliovirus (Fig. 4A). On the other hand, the CB3 immune serum at the same dilution neutralized neither EV71 nor CA16. Plaque formation assay with a serial dilution of CA16 immune serum confirmed its neutralization activity on EV71 (Fig. 4B). Furthermore, splenocytes isolated from the CA16-immunized mice at day 7 p.i. were proliferated in response to both CA16 and EV71 but not CB3 antigen stimulation (Fig. 1B).

Western blotting revealed that the common epitope of EV71 and CA16 has a MW of approximately 60 kDa (Fig. 5), and is probably located on the outer capsid proteins.
Fig 4. Anti-CA16 immune serum cross-neutralized EV71. (A) Left panel: Anti-CA16 immune serum reduced cytopathic effect (CPE) of EV71 on RD cells. RD cells (8×10^4 cells/well) were incubated with 2-fold dilution of anti-CA16 or anti-EV71 immune serum in duplicate before the addition of 100 TCID50 of EV71. The cells were then observed daily for CPE. Middle and right panels: Anti-CA16 immune serum neutralized EV71 but not CB3 or poliovirus. Anti-CA16 or anti-CB3 immune serum was added to RD cells in duplicate before the addition of equal volume of 100 TCID50 of CA16, EV71, CB3 or poliovirus. CPE was observed daily as described above. Only the results of 1:4 dilution are shown. Magnification, ×100. (B) Anti-CA16 immune serum (1:2 to 1:16 dilutions) for 2h. Viral titer in the cultures was detected with plaque assay. Results are the mean ± SEM of two experiments performed in triplicate. ND = not detectable.

Next we tested whether passive immunization of mice with CA16 immune serum which had an in vitro NT of 1:4 only could work in vivo. Mice were passively immunized with an intraperitoneal injection of normal sera, CA16, CB3, or EV71 immune sera 1 day before a lethal challenge with EV71/MP4 strain via oral route at day 7 of age. The mice that received normal or CB3 immune serum failed to gain weight and 70 to 80% of them died at day 5 p.i. (Fig. 6A) with viremia and heavy viral loads in intestines, leg muscles, and brain tissue (Fig. 6B). On the other hand, 100% of the EV71 immune serum-treated mice survived, and almost all of the tissues tested were free of the virus. Notably, the CA16 immune serum-treated mice had a higher survival rate (40%, 10 to 20% increases) and a lower clinical score than the normal serum-treated mice. Concomitantly, the viral titer in blood, intestine, muscle, and brain tissue of the CA16 immune serum-treated mice were also significantly decreased by more than 2 to 3 log-fold at days 3, 5, and 7 p.i. as compared to control mice (Fig. 6B).

Fig 5. Anti-EV71 and anti-CA16 immune serum recognized both EV71 and CA16 viral proteins. EV71, CA16, and CB3 viral proteins (3 mg) were separated by 12.5% SDS-polyacrylamide gel and transferred to a PDVF membrane. The transferred membrane was stained with anti-CA16 or anti-EV71 immune serum (1:500 dilution) followed by HRP-conjugated goat anti-mouse IgG (1:5,000 dilution). The reaction was developed with DAB expression system.
The circulation pattern of EV71 is not known (12). We hypothesize that the preexisting immunity, either specific or cross-reactive, to a certain extent may provide a way to determine the prevalence of EV71 in the general population. The findings that CA16 infection elicited cross-reactivity for EV71 and that memory to CA16 infection could be recalled by either CA16 or EV71 stimulation favor our argumentation. Limited epidemiology data from the Center for Disease Control in Taiwan also support this hypothesis since it has been shown that there was an inverse relationship between the infection rate of CA16 and EV71. It is interesting to know that this intertypic cross-reactivity is clinically significant in terms of protection. If this hypothesis is validated in a large cohort of patients, then it might provide a strategy for designing a vaccine regimen.


lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. Vaccine 20:895-904.