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Immunity to Avirulent Enterovirus 71 and Coxsackie A16 Virus Protects against Enterovirus 71 Infection in Mice▽

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In this study, we sought to determine whether intratypic and intertypic cross-reactivity protected against enterovirus 71 (EV71) infection in a murine infection model. We demonstrate that active immunization of 1-day-old mice with avirulent EV71 strain or coxsackie A16 virus (CA16) by the oral route developed anti-EV71 antibodies with neutralizing activity (1:16 and 1:2, respectively). Splenocytes from both EV71- and CA16-immunized mice proliferated upon EV71 or CA16, but not coxsackie B3 virus (CB3), antigen stimulation. Immunized mice became more resistant to virulent EV71 strain challenge than nonimmunized mice. There was an increase in the percentage of activated splenic T cells and B cells in the immunized mice 2 days after EV71 challenge. The CA16 immune serum reacted with EV71 antigens in an enzyme-linked immunosorbent assay and neutralized EV71 but not CB3 or poliovirus at a titer of 1:4. Passive immunization with the CA16 immune serum reduced the clinical score, diminished the organ viral load, and increased the survival rate of mice upon EV71 challenge. CB3 neither shared in vitro cross-reactivity with EV71 nor provided in vivo protection after both active and passive immunization. These results illustrated that live vaccine is feasible for EV71 and that intertypic cross-reactivity of enteroviruses may provide a way to determine the prevalence of EV71.

Based on their biological and molecular characteristics, both enterovirus 71 (EV71) and coxsackie A16 virus (CA16) have been grouped with human enterovirus group A, which also includes CA2 to CA8, CA10, CA12, and CA14 (15). Although both EV71 and CA16 can cause hand-foot-and-mouth disease and herpangina in young people, infection with only EV71 occasionally leads to severe diseases such as aseptic meningitis, poliomyelitis-like paralysis, and possible fatal encephalitis in neonates (7). In fact, EV71 has been regarded as the most important neurotropic enterovirus after the eradication of the poliovirus (13). There is no specific antiviral therapy or vaccine for EV71. We have demonstrated the effectiveness of Milrinone, a cyclic nucleotide phosphodiesterase inhibitor 3 (19) and intravenous immunoglobulin for the treatment of EV71 patients with autonomic nervous system dysregulation and pulmonary edema (20). Inactivated whole virus (24) and VP1 subunit DNA (22), as well as transgenic tomato fruit expressing VP1 protein (4), have been suggested as potential vaccine candidates.

Exposure to and infection with multiple enteroviruses is thought to be very common, and thus immunity should prevail in the general population (9). We reason that the preexisting immunity, either specific or cross-reactive, to a certain extent may provide a way to determine the prevalence of the viruses. The present study was designed to test whether immunity to avirulent EV71 strains, as a form of attenuated live vaccine, and non-EV71 enteroviruses could prevent the infection of EV71 virulent strain. We demonstrate that specific immunity and protection could be induced by mucosal exposure to EV71 and that CA16 immunity could cross-react with EV71. Both passive and active immunization with CA16 reduced the clinical score, diminished the organ viral load, and increased the survival rate of mice after EV71 challenge.

MATERIALS AND METHODS

Cells and viruses. Rhabdomyosarcoma (RD) cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) plus 2 mM L-glutamine, 100 IU of penicillin, and 100 μg of streptomycin per milliliter. Stock viruses of EV71/Tainan/4643/98 (GenBank accession number AF304458), mouse-adapted EV71 strain MP4 (21), CA16, CB3, and poliovirus type 2 (Department of Virology, National Cheng Kung University Hospital, Tainan, Taiwan) were grown in RD cells. Working stocks contain 10^7 PFU/ml. Both EV71/4643 and MP4 strains were tested by using anti-EV71 monoclonal antibody (MAb979; Chemicon, Temecula, CA) in indirect immunofluorescence staining of infected RD cell cultures. CA16, CB3, and poliovirus were confirmed by standardized serum pool typing (American Type Culture Collection) or PCR (23). To prepare viral antigens, the bulk culture of virus in RD cells was disrupted by two freeze-thaw cycles. Cellular debris was removed by using low-speed centrifugation (200 × *g*, 10 min). The virus supernatant was further clarified by centrifugation (10,000 × *g*, 30 min) and ultrafiltration (YM-10 membrane; Amico, Beverly, MA). Virus particles were then pelleted by using ultracentrifugation in an SW28 rotor at 24,000 × g for 6 h through a sucrose cushion.

Preparation of immune serum and experimental infection. Specific-pathogen-free, 8- to 10-week-old ICR female mice (Laboratory Animal Center, National Cheng Kung University College of Medicine, Tainan, Taiwan) were subcutaneously injected with two doses of EV71, CA16, or CB3 (10^5 PFU/mouse) or uninfected culture medium separated by a 3-week interval. One week after the second injection, mice were boosted by one intravenous injection of the virus or medium. The serum samples were collected 3 days later and stored at −70°C after heat inactivation (56°C for 30 min). Nonimmune sera were obtained from naive mice. For active immunization, 1-day-old ICR mice were orally inoculated with 10 μl of viral suspensions (10^4 PFU/mouse) by using a 24-gauge feeding tube

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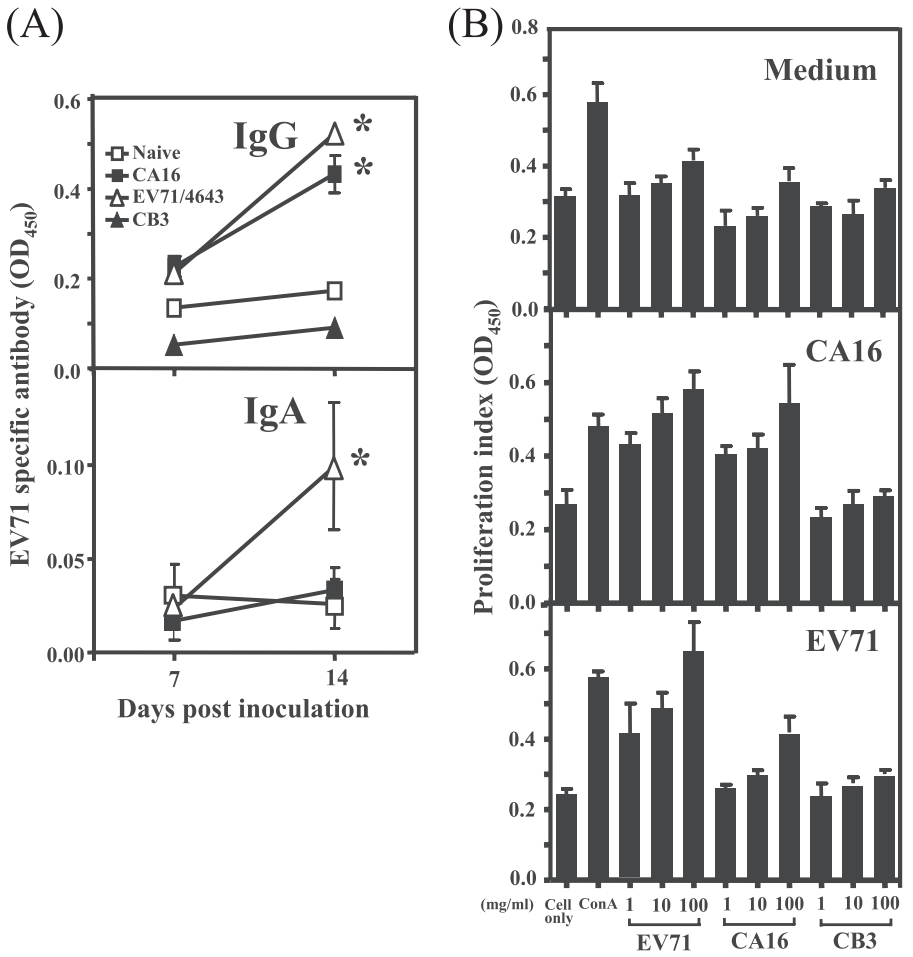

FIG. 1. Active immunization with both live avirulent EV71 strain and CA16 induced EV71 specific immunity in newborn mice. One-day-old ICR mice were orally inoculated with either live EV71/4643, CA16, or CB3 (10^4 PFU/mouse). (A) EV71 specific serum IgG and intestinal IgA levels were determined by ELISA at 7 and 14 days p.i. (B) Proliferation of splenocytes in response to EV71, CA16, or CB3 antigen stimulation was determined at 7 day p.i. The results are means ± the SEM (n = 10 to 12 mice). *, $P < 0.05$ compared to control mice.

after fasting for 8 h. Control mice were given with culture medium. For passive immunization, heat-treated immune or normal sera (100 μl) were intraperitoneally injected into mice 1 day before challenge. For challenge, 7-day-old mice were orally inoculated with EV71/MP4 (10^5 PFU/mouse) after fasting for 8 h (21). Animals were observed daily for clinical signs, weight gain or loss, and mortality. Clinical disease was scored as follows: 0, healthy; 1, ruffled fur and hunchbacked appearance; 2, wasting; 3, limb weakness; 4, limb paralysis; and 5, moribund and death. In an experiment to determine the type I interferon (IFN) response, 1-day-old mice were intraperitoneally injected with poly(I-C) (polyriboinosic-polyribocytidylic acid; 50 μg/mouse; Sigma-Aldrich, St. Louis, MO) (11) or orally inoculated with CA16 as described above. Serum samples were then collected at regular time intervals and stored at −70°C until assay. All animal protocols were approved by the institutional animal care and use committee.

Tissue virus titer. After anesthetization with pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL), blood samples were aseptically collected by heart puncture. After perfusion with isotonic saline containing EDTA, tissue samples were aseptically removed, weighed, homogenized in 1 ml of DMEM containing 2% FBS, and disrupted by three freeze-thaw cycles. In other experiments, the whole sections of the small intestine were homogenized in 0.05 M ETDA in phosphate-buffered saline for immunoglobulin A (IgA) antibody determination. The clarified supernatants of the tissue homogenates, blood, and serum samples were stored at −70°C until assay. For viral titration, the tissue and blood samples were inoculated onto monolayers of RD cells. These cells were inspected daily for a minimum of 14 days for cytopathic effect (CPE) (8). Virus titers were expressed as log PFU per mg of tissue or ml of blood. The lower limit of virus detection was 20 PFU.

ELISA. The total anti-EV71-specific serum IgG and intestinal IgA levels of immunized mice and cross-reactivity among enteroviruses were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, serum samples were added into microtiter plates coated with 100 μl of native virus (10 μg/ml) in 0.1 M phosphate carbonate-bicarbonate buffer and incubated at 4°C overnight, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000 dilution; Chemicon) or IgA (1:1,000 dilution; Serotec, Oxford, United Kingdom) at room temperature for 1 h. The reaction was developed by TMB substrate (KPL, Gaithersburg, MD), and the optical densities at 450 nm (OD_{450}) were determined. Concentrations of serum IFN-α of CA16-immunized mice were measured by a sandwich ELISA technique according to the manufacturer's instruction (Mouse-IFN-Alpha ELISA kit; R&D System, Minneapolis, MN). The detection limit was 7.5 pg per ml.

Plaque assay and neutralization test. Confluent monolayers of RD cells were prepared in 24-well plates (2×10^5 to 5×10^5 cells/well). The cells were infected with serial dilutions of viral suspensions, overlaid with 1.5% methylcellulose in DMEM plus 2% FBS, and incubated at 37°C for 3 days before the plaques were visualized using staining with crystal violet. The 50% tissue culture infective dose(s) ($TCID_{50}$) were determined according to the method of Hsiung (8) using the Reed and Muench formula. The neutralizing titer (NT) was determined by using a microassay. 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, the *NT* was determined as the highest dilutions of serum that inhibited virus growth.

Splenocyte proliferation and flow cytometric analysis. One-day-old mice were actively immunized with EV71/4643 or CA16 as described above. Splenocytes were collected at day 7 postinoculation (p.i.), and single cell suspensions (erythrocytes lysed) were seeded on a 96-well plate (2×10^5 cells/well). The cells were stimulated with concanavalin A (1 mg/ml; Sigma-Aldrich) or UV-inactivated EV71, CA16, or CB3 viral antigens for 72 h at 37°C. Cell proliferation was detected by measuring the absorbance at OD_{450} using the CCK-8 reagent according to the manufacturer's instruction (Dojindo, Gaithersburg, MD). In other experiments, EV71- or CA16-immunized mice were challenged with EV71/MP4 at 7 days of age. Two days later, the splenocytes (2×10^5 in 1 ml of Hanks balanced salt solution containing 2% fetal calf serum and 0.1% sodium azide) were incubated with anti-CD16/CD32 MAb (Fc blocker, 2.4G2; BD Pharmingen, San Diego, CA), followed by fluorescein isothiocyanate-conjugated anti-CD69 (H1.2F3) and phycoerythrin-conjugated anti-CD19 (1D3, both from BD Pharmingen) or CD3 (145-2C11; eBioscience, San Diego, CA) MAbs for 30 min on ice. After being washed, stained cells were quantified by flow cytometry (BD Immunocytometry Systems, San Jose, CA). Isotype-matched MAb-stained cells were used as a background control in all experiments.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. The EV71, CA16, and CB3 viral proteins (3 mg) were separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Temecula, CA). The transferred membrane was stained with CA16 or EV71 immune serum (1:500 dilution), followed by the addition of HRP-conjugated goat anti-mouse IgG (1:5,000 dilution). The reaction was developed with the DAB expression system (Vector, Burlingame, CA).

Statistics. The values of NT, tissue virus titer, cell proliferation, and flow cytometry were analyzed by using the Mann-Whitney U test or Kruskal-Wallis test, and survival rates were analyzed by log-rank analysis. The results are expressed as means ± the standard error of the mean (SEM). A P value of <0.05 was considered significant.

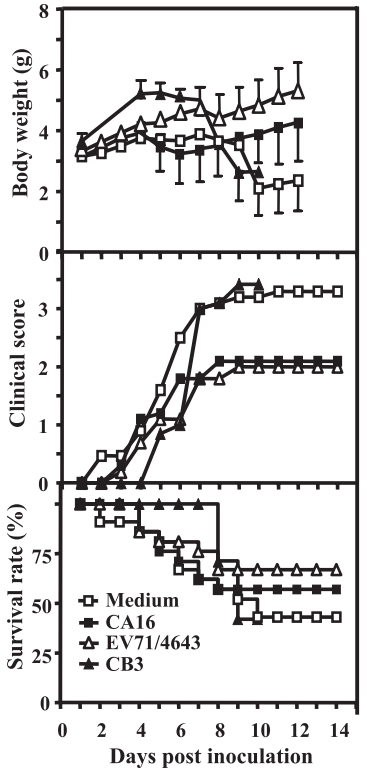

FIG. 2. Active immunization with both live avirulent EV71 strain and CA16 partly protected newborn mice against lethal EV71 challenge. One-day-old ICR mice were orally inoculated with either live EV71/4643, CA16, or CB3 (10^4 PFU/mouse) as described in Fig. 1. Mice were subsequently challenged with EV71/MP4 (10^5 PFU/mouse) by the same route at day 7 of age. The body weight, clinical score, and survival rate were then monitored daily after challenge. The results are the means ± the SEM (n = 10 to 12 mice).

RESULTS

Active immunization of avirulent EV71 strain elicited both humoral and cellular immunity and partly protected mice against EV71 lethal challenge. Active immunization of 1-day-old mice with mouse avirulent EV71 strain 4643 (10^4 PFU) via the oral route resulted in neither clinical disease nor mortality (data not shown). On the other hand, increased levels of serum-specific IgG antibody were observed at days 7 and 14 p.i. Increased levels of intestinal IgA antibody were also noted at day 14 p.i. (Fig. 1A). The sera of EV71-immunized mice had an NT of 1:16. At day 7 p.i., 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 compared to mice immunized with medium only (Fig. 2). Furthermore, these surviving mice gained weight normally and exhibited no sign of illness. Flow cytometric analysis showed that 2 days after EV71 challenge the percentage of $CD69^+$ $CD3^+$ T cells in the immunized mice was significantly increased compared to medium-immunized, EV71-challenged mice (medium versus EV71, 2.4% ± 0.5% versus 7.8% ± 0.7%, $n = 3$ mice, $P < 0.05$). These results indicated that EV71 infection, like poliovirus infection, might be preventable by live vaccines.

Active immunization of CA16 induced a weak protective cross-reactivity for EV71. Active immunization with CA16, as a control for the experiment, not only induced CA16 specific but also EV71 cross-reactive antibody (Fig. 1A) with a weak NT of 1:2. Furthermore, splenocytes isolated from the CA16-immunzied mice at day 7 p.i. proliferated in response to both CA16 and EV71 but not CB3 antigen stimulation. An increase in $CD69^+$ $CD3^+$ T cells was also observed in the CA16-immunized mice after EV71 challenge (medium versus CA16, 2.4% ± 0.5% versus 6.5% ± 1.1%, $P < 0.05$). Interestingly, the percentage of $CD69^+$ $CD19^+$ B cells in the CA16-immunized mice increased even higher than that of EV71-immunized mice after challenge (medium versus CA16 versus EV71, 1.7% ± 0.2% versus 5.0% ± 0.7% versus 2.0% ± 0.3%; medium versus CA16, $P < 0.05$). Furthermore, CA16 immunization reduced the mortality of mice by 10 to 20% upon EV71 lethal challenge (Fig. 2). The protective effect of CA16 immunization was unrelated to the induction of IFN-α since there was no increase in the serum IFN-α level in CA16-immunized mice. On the other hand, an intraperitoneal inoculation of poly(I-C) at 1 day of age augmented a rapid IFN-α production at 6 h p.i., which decreased thereafter, and the level returned to baseline by day 3 p.i. (data not shown). CB3 immunization neither elicited cross-reactivity for EV71 (Fig. 1) nor protected mice against EV71 challenge (Fig. 2).

Cross-reactivity between EV71 and CA16. The protective effect of CA16 immunization prompted us to clarify the extent of this intertypic cross-reactivity and its significance on EV71 infection. Immune sera generated in adult mice against avirulent EV71/4643 and CA16 contained high titers of specific antibodies to their corresponding antigen, and cross-reactivity of the immune sera was observed between EV71 and CA16,

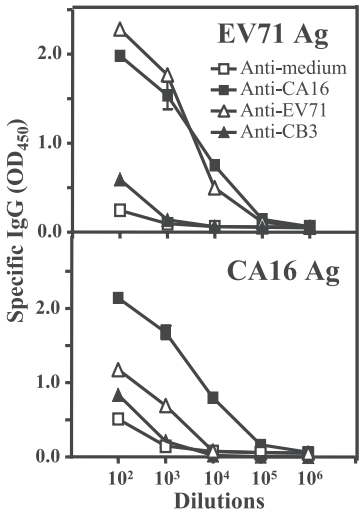

FIG. 3. Cross-reactivity between EV71 and CA16. EV71 or CA16 viral antigen (10 μg/ml) was coated onto 96-well plates, followed by incubation with serially diluted mouse anti-EV71, CA16, or CB3 immune serum. Immunoreactivity was revealed by incubation with secondary antibody and TMB substrate. Representative of two similar experiments is shown.

but not CB3, in an ELISA-based assay (Fig. 3). Both the EV71 and the CA16 immune sera had an NT of 1,024 to their corresponding virus. 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). Western blotting revealed that both EV71 and CA16 immune sera recognized a common band of the viruses with a molecular mass of approximately 60 kDa, which may represent the VP0-VP3 protein (Fig. 5).

Passive immunization of CA16 immune serum protected mice against EV71 lethal challenge. Next we tested whether the CA16 immune serum could work in vivo. Mice were passively immunized with an interperitoneal injection of normal sera, CA16, CB3, or EV71 immune sera 1 day before a lethal challenge with EV71/MP4 strain (10^5 PFU) 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 the 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 virus titers 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. compared to control mice (Fig. 6B). Subsequent experiments showed that the sera collected from mice passively immunized with the CA16 immune serum contained a high ELISA titer for EV71 (OD_{450} = 1.5) with an NT of 1:4, which was significantly higher than those induced by CA16 active immunization in 1-day-old mice (OD_{450} = 0.2 and NT = 1:2).

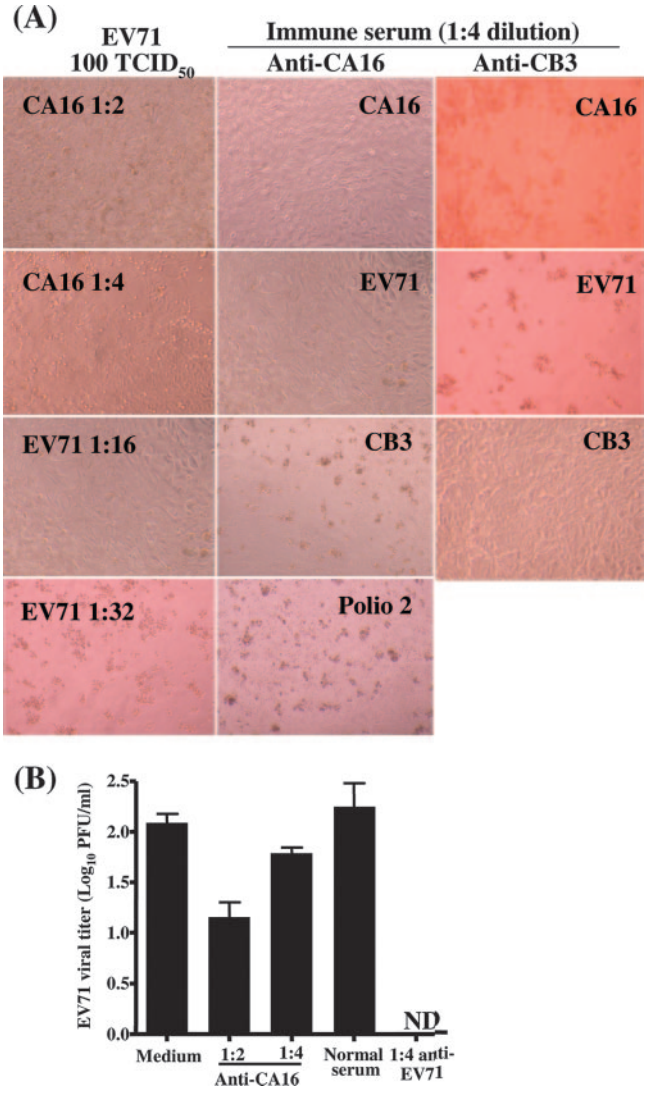

FIG. 4. Anti-CA16 immune serum cross-neutralized EV71. (A) The left panel shows that anti-CA16 immune serum reduced the CPE of EV71 on RD cells. RD cells (8×10^4 cells/well) were incubated with a twofold dilution of anti-CA16 or anti-EV71 immune serum in duplicate before the addition of 100 $TCID_{50}$ of EV71. The cells were then observed daily for CPE. The middle and right panels show that 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 $TCID_{50}$ of CA16, EV71, CB3, or poliovirus. The CPE was observed daily as described above. Only the results of 1:4 dilution are shown. Magnification, ×100. (B) Anti-CA16 immune serum neutralized EV71 in a dose-dependent manner. EV71 (100 $TCID_{50}$) was incubated with normal serum or anti-EV71 or anti-CA16 immune serum (1:2 to 1:16 dilutions) for 2 h. Virus titer in the cultures was detected by plaque assay. The results are means ± the SEM of two experiments performed in triplicate. ND, not detectable.

DISCUSSION

Both live attenuated and inactivated whole virus vaccines have been used to successfully control poliovirus (1). In our previous study we demonstrated the productive role of antibody and the efficacy of a formalin-inactivated whole virus vaccine for EV71 in susceptible newborn mice (24). In the

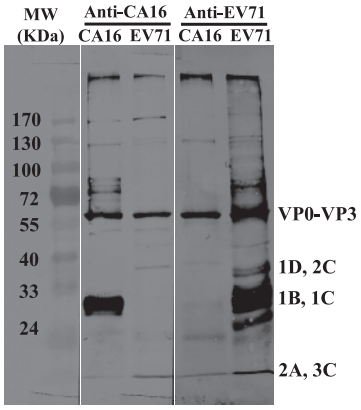

FIG. 5. Anti-EV71 and anti-CA16 immune serum recognized both EV71 and CA16 viral proteins. EV71, CA16, and CB3 viral proteins (3 mg) were separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The transferred membrane was 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 the DAB expression system.

present 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 systemic and mucosal antibody responses with neutralizing activity. Even though the initial antibody response remained weak, EV71 preexposure resulted in efficient immunological priming, since the acquired immunity could be recalled by both in vitro viral antigen stimulation and in vivo live virus challenge. The proliferation responses to subsequent stimulation clearly indicated the induction of memory cells rather than of neonatal tolerance upon this neonatal immunization. It is known that live replicating agents could enhance antigen-presenting cell activation and thus antigen-presenting cell–T-cell interactions (16). Indeed, live replicating EV71 was proven to be present in the guts of neonatal mice for a couple of days after oral inoculation (21). More importantly, live EV71 active immunization reduced the clinical disease and mortality of mice upon lethal challenge with the virulent mouse-adapted EV71 strain. Antibody-mediated neutralization and perhaps cell-mediated effects were probably essential to the viral clearance and hence clinical disease prevention. Since the susceptibility of the mouse-adapted EV71 strain is age dependent, only mice younger than 7 days of age could be infected orally (21). Thus, optimizing early vaccination condition with a suitable viral dose and the use of adjuvant or, if an adult model were available, increasing the number and duration of the immunization would improve the efficacy of the active immunization.

As a group of closely related RNA viruses, enteroviruses

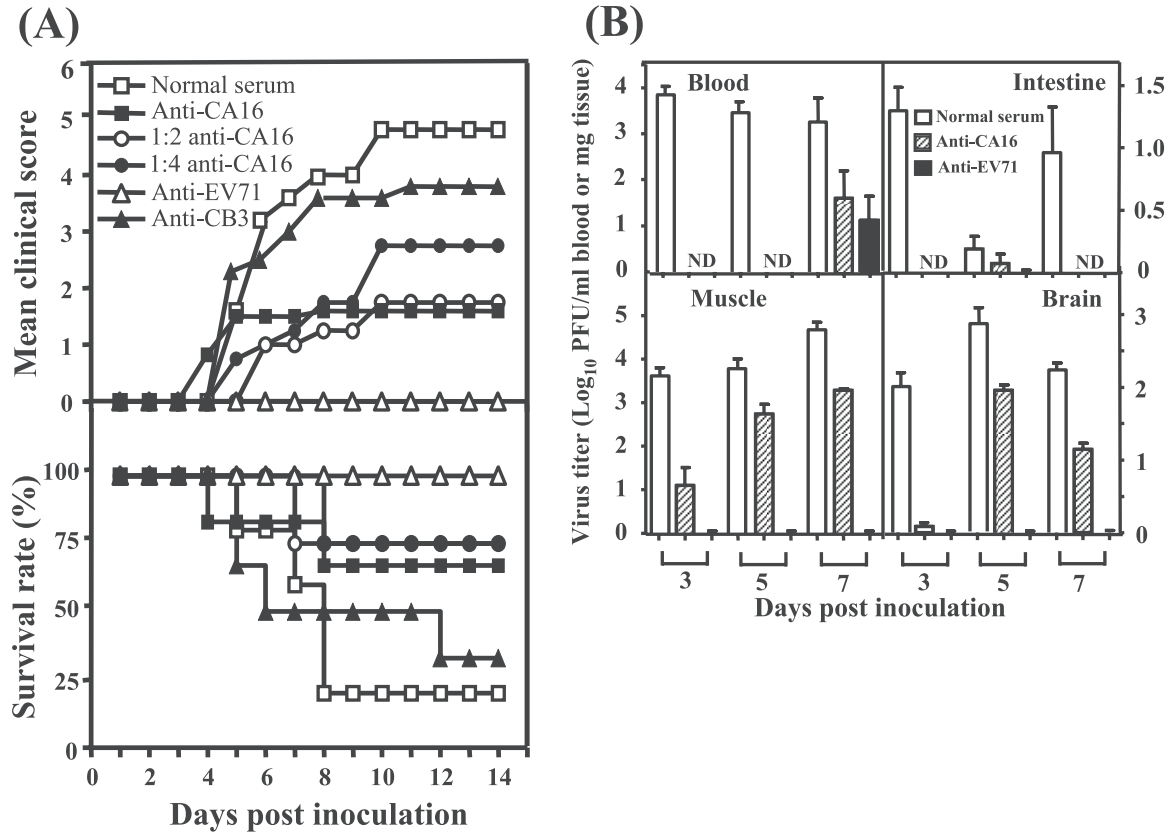

FIG. 6. Passive immunization with anti-CA16 immune serum partly protected mice against EV71 challenge and reduced the tissue viral load of challenged mice. Seven-day-old ICR mice were orally inoculated with EV71/MP4 (10^5 PFU/mouse) 1 day after an intraperitoneal injection of normal serum, anti-CB3, anti-CA16, or anti-EV71 immune serum. The clinical score and survival rate (A) and tissue virus titers (B) were determined. The results are means ± the SEM of 5 to 10 mice. ND, not detectable. Results representative of two similar experiments are shown.

exhibit considerable intratypic and intertypic cross-reactivity and recombination (12, 14, 17). An early study demonstrated that EV71 and CA16 shared some common epitopes, which could be demonstrated by immunofluorescence, complement fixation, and immunodiffusion assays (6). Indeed, a commercial anti-EV71 MAb, MAb979, shows cross-reactivity with CA16. In the present study, we clearly demonstrated that both humoral and cellular immunities induced by CA16 could cross-react with EV71 and that the common epitopes are probably located on the outer capsid proteins. It is not surprising that, since intertypic recombination has been reported between CA16 and EV71, the two viruses share 80% similarity (2).

Remarkably, animal studies revealed that active CA16 immunization also provided weak protection against EV71. It could be argued that innate immunity and especially type I IFN might be responsible for the phenotype since IFN levels during primary infection might be persisting through day 7, the time of EV71 challenge. We reported previously that type I IFNs represent an essential innate defense mechanism for controlling EV71 infection in mice. An early administration of recombinant mouse IFN-αA protected the mice against EV71 infection. In contrast, treatment with a neutralizing antibody for type I IFNs resulted in frequent deaths and higher tissue viral loads in infected mice (11). Type I 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 nor provided in vivo protection after active immunization.

Passive immunization of mice with CA16 immune serum, which had an in vitro NT of 1:4 only, could reduce the mortality by 10 to 20% of EV71-challenged mice. This result suggested that, in addition to viral neutralization, the Fc effector function might also play a role in the clearance of the virus. Antibody to coxsackie B4 virus (CB4) would bind to the viral particles and form immune complex, which could induce IFN-γ production of peripheral blood mononuclear cells (3). This effect was speculated to be able to reduce viral infection. On the other hand, antibody-dependent enhancement has been shown to be responsible for the augmentation of myocarditis in mice infected with CB3 (10). Furthermore, preexisting CB3 antibody could enhance the infectivity of other coxsackieviruses through cross-reactivity (5).

The circulation pattern of EV71 is not known (18). The finding that CA16 infection elicited cross-reactivity for EV71 and that memory to CA16 infection could be recalled by either CA16 or EV71 stimulation indicated that a preexisting immunity to CA16 or perhaps other non-EV71 enteroviruses may be considered as a way to determine the prevalence and circulation of EV71 in the general population. Limited epidemiology data from the Center for Disease Control in Taiwan 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 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.

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REFERENCES

1. **CDC.** 2005. Epidemiology bulletin, vol. 21. CDC, Taiwan, Republic of China.
2. **Chan, Y. F., and S. AbuBakar.** 2004. Recombinant human enterovirus 71 in hand, foot and mouth disease patients. Emerg. Infect. Dis. **10:**1468–1470.
3. **Chehadeh, W., A. Bouzidi, G. Alm, P. Wattre, and D. Hober.** 2001. Human antibodies isolated from plasma by affinity chromatography increase the coxsackievirus B4-induced synthesis of interferon-alpha by human peripheral blood mononuclear cells in vitro. J. Gen. Virol. **82:**1899–1907.
4. **Chen, H. F., M. H. Chang, B. L. Chiang, and S. T. Jeng.** 2006. Oral immunization of mice using transgenic tomato fruit expressing VP1 protein from enterovirus 71. Vaccine **24:**2944–2951.
5. **Girn, J., M. Kavoosi, and J. Chantler.** 2002. Enhancement of coxsackievirus B3 infection by antibody to a different coxsackievirus strain. J. Gen. Virol. **83:**351–358.
6. **Hagiwara, A., Tagaya, I., and T. Yoneyama.** 1978. Common antigen between coxsackievirus A16 and enterovirus 71. Microbiol. Immunol. **22:**81–88.
7. **Ho, M.** 2000. Enterovirus 71: the virus, its infections, and outbreaks. J. Microbiol. Immunol. Infect. **33:**205–216.
8. **Hsiung, G. D.** 1994. Hsiung's diagnostic virology. Yale University Press, New Haven, CT.
9. **Juhela, S., H. Hyoty, M. Lonnrot, M. Roivainen, O. Simell, and J. Ilonen.** 1998. Enterovirus infections and enterovirus specific T-cell responses in infancy. J. Med. Virol. **54:**226–232.
10. **Kishimoto, C., M. Kurokawa, and H. Ochiai.** 2002. Antibody-mediated immune enhancement in coxsackievirus B3 myocarditis. J. Mol. Cell. Cardiol. **34:**1227–1238.
11. **Liu, M. L., Y. P. Lee, Y. F. Wang, H. Y. Lei, C. C. Liu, S. M. Wang, I. J. Su, J. R. Wang, T. M. Yeh, S. H. Chen, and C. K. Yu.** 2005. Type I interferons protect mice against enterovirus 71 infection. J. Gen. Virol. **86:**3263–3269.
12. **Lukashev, A. N.** 2005. Role of recombination in evolution of enteroviruses. Rev. Med. Virol. **15:**157–167.
13. **McMinn, P. C.** 2002. An overview of the evolution of enterovirus 71 and its clinical and public health significance. FEMS Microbiol. Rev. **26:**91–107.
14. **Oberste, M. S., K. Maher, and M. A. Pallansch.** 2004. Evidence for frequent recombination within species human enterovirus B based on complete genomic sequences of all thirty-seven serotypes. J. Virol. **78:**855–867.
15. **Oberste, M. S., S. Penaranda, K. Maher, and M. A. Pallansch.** 2004. Complete genome sequences of all members of the species human enterovirus A. J. Gen. Virol. **85:**1597–1607.
16. **Siegrist, C. A.** 2001. Neonatal and early life vaccinology. Vaccine **19:**3331–3346.
17. **Uhlig, H., and R. Dernick.** 1988. Intertypic cross-neutralization of polioviruses by human monoclonal antibodies. Virology **163:**214–217.
18. **Wang, J. R., Y. C. Tuan, H. P. Tsai, J. J. Yan, C. C. Liu, and I. J. Su.** 2002. Change of major genotype of enterovirus 71 in outbreaks of hand-foot-and-mouth disease in Taiwan between 1998 and 2000. J. Clin. Microbiol. **40:**10–15.
19. **Wang, S. M., H. Y. Lei, M. C. Huang, J. M. Wu, C. T. Chen, J. N. Wang, J. R. Wang, and C. C. Liu.** 2005. Therapeutic efficacy of milrinone in the management of enterovirus 71-induced pulmonary edema. Pediatr. Pulmonol. **39:**219–223.
20. **Wang, S. M., H. Y. Lei, M. C. Huang, L. Y. Su, H. C. Lin, C. K. Yu, J. L. Wang, and C. C. Liu.** 2006. Modulation of cytokine production by intravenous immunoglobulin in patients with enterovirus 71-associated brainstem encephalitis. J. Clin. Virol. **37:**47–52.
21. **Wang, Y. F., C. T. Chou, H. Y. Lei, C. C. Liu, S. M. Wang, J. J. Yan, I. J. Su, J. R. Wang, T. M. Yeh, S. H. Chen, and C. K. Yu.** 2004. A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection. J. Virol. **78:**7916–7924.
22. **Wu, C. N., Y. C. Lin, C. Fann, N. S. Liao, S. R. Shih, and M. S. Ho.** 2001. Protection against lethal enterovirus 71 infection in newborn mice by passive immunization with subunit VP1 vaccines and inactivated virus. Vaccine **20:**895–904.
23. **Yan, J. J., I. J. Su, P. F. Chen, C. C. Liu, C. K. Yu, and J. R. Wang.** 2001. Complete genome analysis of enterovirus 71 isolated from an outbreak in Taiwan and rapid identification of enterovirus 71 and coxsackievirus A16 by RT-PCR. J. Med. Virol. **65:**331–339.
24. **Yu, C. K., C. C. Chen, C. L. Chen, J. R. Wang, C. C. Liu, J. J. Yan, and I. J. Su.** 2000. Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J. Biomed. Sci. **7:**523–528.