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Lymphocyte and Antibody Responses Reduce Enterovirus 71 Lethality in Mice by Decreasing Tissue Viral Loads▽

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Enterovirus 71 (EV71) infects the central nervous system and causes death and long-term neurological sequelae in hundreds of thousands of young children, but its pathogenesis remains elusive. Immunopathological mechanisms have been suspected to contribute to the pathogenesis of neurological symptoms, so anti-inflammatory agents have been used to treat patients with neurological symptoms. The present study was therefore designed to investigate the functions of lymphocyte and antibody responses in EV71 infection using a mouse model. Immunohistochemical staining analysis revealed virus and three types of lymphocytes, B cells, CD4 T cells, and CD8 T cells, in the spinal cord of an EV71-infected patient who died. A study of mice showed that the levels of virus and lymphocytes in brains and antibody titers in sera were elevated during the time when the mice succumbed to death in a phenomenon analogous to that observed in patients. Further studies demonstrated that after infection, the disease severity, mortality, and tissue viral loads of mice deficient in B, CD4 T, or CD8 T cells were significantly higher than those of wild-type mice. In addition, treatment with a virus-specific antibody, but not a control antibody, before or after infection significantly reduced the disease severity, mortality, and tissue viral loads of mice deficient in B cells. Our results show that both lymphocyte and antibody responses protect mice from EV71 infection. Our study suggests the use of vaccines and virus-specific antibodies to control fatal outbreaks and raises caution over the use of corticosteroids to treat EV71-infected patients with neurological symptoms.

Enterovirus 71 (EV71), a member of the family *Picornaviridae*, infects humans by the fecal-oral route and induces mild symptoms, such as herpangina and hand, foot and mouth disease. It can also infect the central nervous system (CNS) and induce fatal neurological manifestations, such as aseptic meningitis, brain stem encephalitis, encephalomyelitis, and acute flaccid paralysis, with cardiopulmonary complications, especially in young children. Most fatalities occur in cases with brain stem encephalitis and fulminant pulmonary edema complications (6, 7, 9, 12, 15, 19). Survivors of severe cases are often left with long-term neurologic sequelae (6, 14, 15).

EV71 outbreaks have been reported periodically throughout the world (7, 9, 16). In the past decade, the Asia-Pacific region has experienced more frequent and widespread fatal outbreaks (16). The largest and most severe outbreak occurred in Taiwan in 1998 when 129,106 cases of herpangina and hand, foot and mouth disease, 405 cases of neurological and cardiopulmonary complications, and 78 deaths were reported (7). Since then, EV71 infection has become endemic in Taiwan and caused >40, >40, and 14 deaths in 2000, 2001, and 2008, respectively (3, 11). In addition, 42 deaths have been reported in China by June in 2008 (16). Although it has been estimated that EV71 infects millions of children and causes thousands of cases of neurologic sequelae and >200 deaths in the past decade (3, 7, 11, 16), there are no effective vaccines and specific antiviral therapies available to control fatal outbreaks due in part to the lack of understanding of viral pathogenesis.

Infants and young children are very susceptible to EV71 infection. Immature immunity is therefore suspected to associate with increased morbidity and mortality (6, 7, 9). This is supported by the findings of lymphopenia, depletion of CD4 and CD8 T lymphocytes, and decreased cellular immunity in the peripheral blood of patients with brain stem encephalitis and pulmonary edema (4, 17). However, some clinical studies showed that elevated cellular immunity was linked with unfavorable outcomes (5, 8). High levels of white blood cell counts in blood and cerebrospinal fluid with a predominance of lymphocytes were detected in patients with fatal or severe sequelae (5, 8, 19, 22). In addition, autopsy reports revealed not only virus but also severe mononuclear cell infiltrates in the CNSs of patients who died (12, 22). Moreover, a clinical study reported that a patient developed opsomyoclonus syndrome, which is an autoimmune disease resulting from lesions in the dentate nucleus of the cerebellum (14). In this patient, the high titer of virus-specific antibodies detected at the onset of neurological disease and the responsiveness of the condition to anti-inflammatory agents (corticosteroids) provide further evidence of an autoimmune etiology. Besides corticosteroids, intravenous immunoglobulin (IVIG), which has several anti-inflammatory properties and often contains neutralizing antibodies to enteroviruses, has been a mandatory treatment for patients with neurological symptoms in Taiwan, because it has been shown to improve the conditions of patients infected

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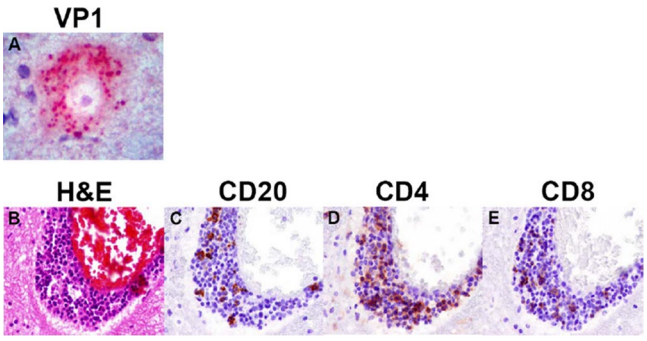

FIG. 1. Presence of virus and lymphocytes in the spinal cord of an EV71-infected patient who died. Sections of the spinal cord specimen from a patient who died were stained with antibody against the EV71 capsid protein, VP1 (A) or human leukocyte antigen, CD20 (C), CD4 (D), or CD8 (E), or hematoxylin and eosin (H&E) (B). The reddish brown color in panel A and panels C to E denotes a positive reaction (original magnification, ×1,000 for panel A and ×400 for panels B to E).

with other enteroviruses, coxackievirus B, and echovirus (1, 5, 13, 18).

Although corticosteroids have been used to treat EV71-infected patients with neurological symptoms (14, 15), the significance of lymphocyte and antibody responses in the pathogenesis of EV71 remains to be determined. The present study was therefore designed to address this issue using a mouse model.

MATERIALS AND METHODS

Immunohistochemical staining. A spinal cord specimen from an EV71-infected child who died and was autopsied was obtained, and informed consent was obtained from the child's parent. Formalin-fixed tissue sections were deparaffinized and stained with hematoxylin and eosin. In addition, deparaffinized sections were autoclaved at 121°C and 20 lb/in^2 for 7.5 min before incubating with biotinylated antibodies against the EV71 capsid protein, VP1 (clone 422-8D-4C-4D; Chemicon), or human leukocyte common antigens, CD4 (clone 1F6; Ventana Medical Systems, Inc.), CD8 (clone C8/144B; Dako), or CD20 (clone L26; Dako). Subsequently, sections were treated with peroxidase-conjugated streptavidin (LSAB+ kit; Dako) and 3-amino-9-ethylcarbazole and counterstained with hematoxylin.

Cells, virus, and mice. A human muscle (rhabdomyosarcoma [RD]) cell line was maintained in medium according to the instructions of the American Type Culture Collection. EV71 strain M2 was propagated and titrated in RD cells and used to infect mice as previously described (10). ICR and C57BL/6 mice and C57BL/6-derived mice deficient in B cells (B6.129S2-*Igh-6*^{tm1Cgn}/J), CD4 T cells (B6.129S2-*Cd4*^{tm1Mak}/J), or CD8 T cells (B6.129S2-*Cd8a*^{tm1Mak}/J) purchased from The Jackson Laboratory were bred and maintained under specific-pathogen-free conditions in the Laboratory Animal Center of our university. All mouse experiment protocols were approved by the Laboratory Animal Committee of our university.

Infection of mice. Seven-day-old ICR and 9-day-old C57BL/6 mice were infected with 5×10^6 and 3×10^7 PFU of EV71, respectively, by oral inoculation as previously described (20). Infected mice were monitored daily for signs of disease and survival. The disease score was graded as follows: 0, healthy; 1, ruffled hair; 2, weakness in hind limbs; 3, paralysis in a single hind limb; 4, paralysis in both hind limbs; 5, death. In separate experiments, mouse tissues were collected after infection to determine viral titers as previously described (10). Additionally, the brains of mice were harvested after infection to quantify the numbers of B cells, CD4 T cells, and CD8 T cells by flow cytometry.

Flow cytometry. Mice were perfused by tail vein injection of saline. Mouse brains were harvested and homogenized, and then the homogenates were passed through a strainer filter (BD Biosciences). Brain leukocytes were purified by Percoll (GE Healthcare) gradient centrifugation. Freshly isolated mouse brain leukocytes were stained with fluorescein isothiocyanate-, phycoerythrin-, or Cy5-conjugated control antibodies or antibodies (eBioscience) against mouse leukocyte antigens, CD4 (clone GK 1.5), CD8a (clone 53-6.7), or CD19 (clone 6D5) for 45 min on ice. The stained cells were analyzed by a FACSCalibur (BD Biosciences) using WinMDI software.

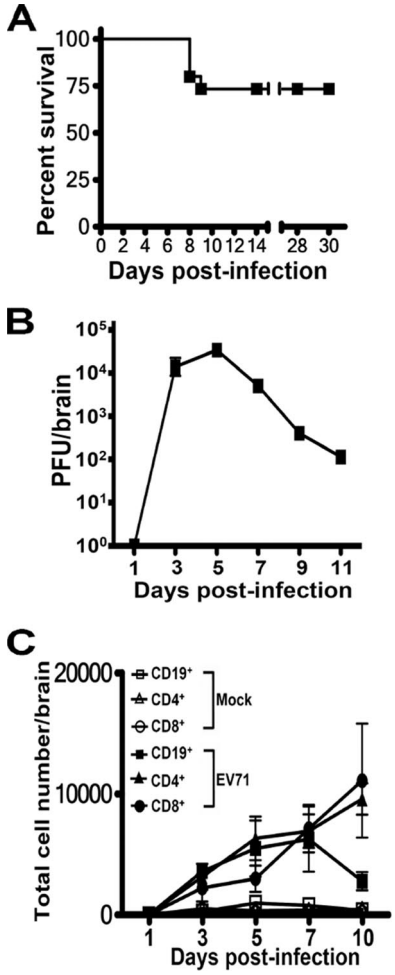

FIG. 2. Kinetics of viral replication and lymphocyte infiltration in the brains of EV71-infected mice. (A) The survival of EV71-infected ICR mice ($n = 20$) is shown. (B) The viral titers in the brains of EV71-infected mice at the indicated times after infection are shown. The data shown are the mean values ± SE values (error bars) for six samples per time point. (C) The numbers of CD4$^+$ T, CD8$^+$ T, and CD19$^+$ B cells in the brains of mock-infected or EV71-infected mice at the indicated times after infection are shown. The data shown are the mean values ± SE values (error bars) from three experiments.

Neutralization assay. The sera of C57BL/6 mice were collected 7 days after infection to determine neutralizing titers. Serial twofold dilutions of heat-inactivated serum were mixed with 100 50% tissue culture infective dose of EV71 as previously described (23). The serum and virus mixtures were incubated at 37°C for 1 h before transfer to RD cell monolayers seeded the day before. The cells were incubated for three more days and stained with crystal violet to observe the cytopathic effect. The highest dilution of serum that protected RD cell monolayers from infection was taken as the neutralizing titer.

Antibody treatment. Eight-week-old wild-type mice were immunized with 1×10^6 PFU of EV71 in complete Freund's adjuvant and then boosted with the same dose of EV71 in incomplete Freund's adjuvant 3 and 4 weeks later. Three days after the third immunization, mice were bled, and sera were collected. In addition, 9-day-old mice were immunized by oral inoculation with 3×10^7 PFU of EV71, and mouse sera were collected 2 weeks after infection. The antibodies in

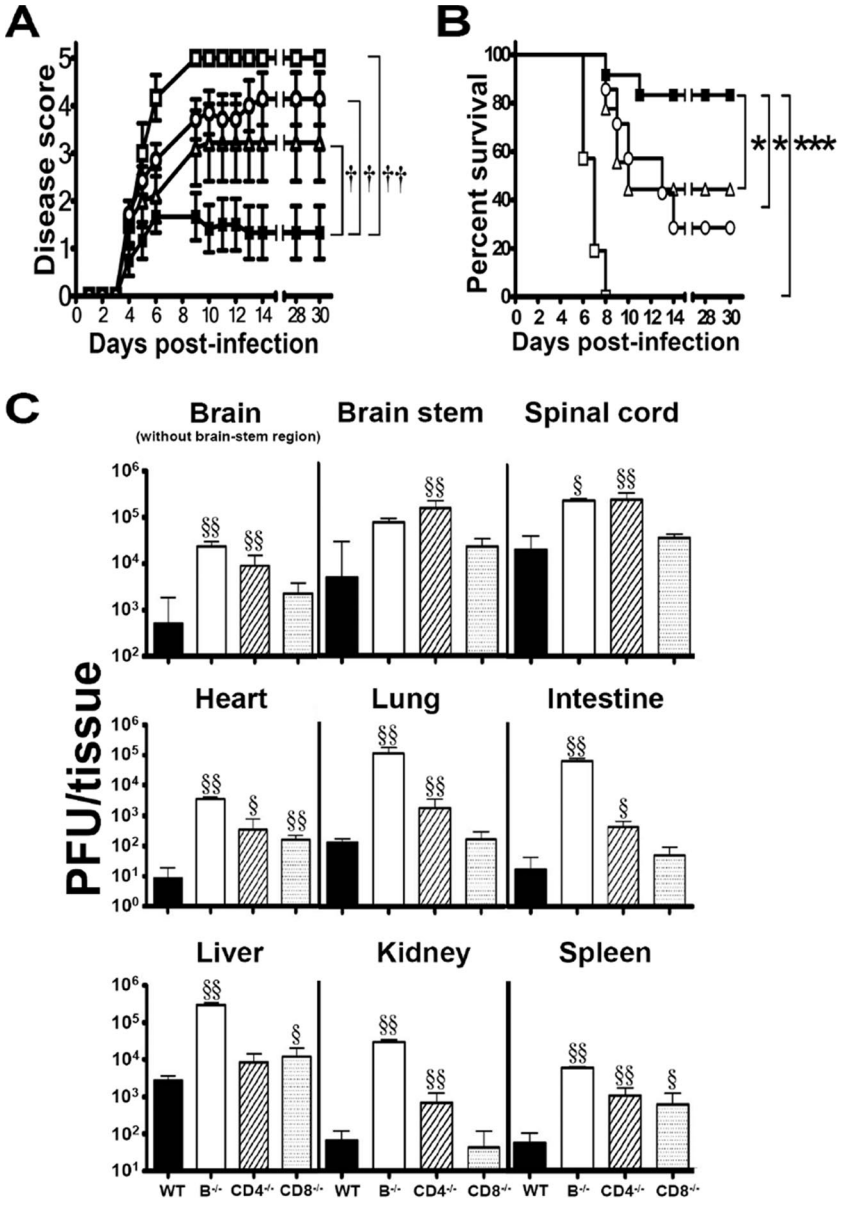

FIG. 3. Lymphocytes reduce the lethality of EV71-infected mice by decreasing viral loads in tissues. The disease scores (A) and survival rates (B) of EV71-infected wild-type mice (black squares; $n = 12$) or mice deficient in CD4 T cells (white triangles; $n = 9$), CD8 T cells (white circles; $n = 7$), or B cells (white squares; $n = 7$) are shown. Disease scores that are significantly different by the Wilcoxon test are indicated as follows: †, $P < 0.05$; ††, $P < 0.01$. Survival rates that are significantly different by the log-rank test are indicated as follows: *, $P < 0.05$; ***, $P < 0.001$. (C) The viral titers in the indicated tissues of wild-type mice (WT; $n = 6$) or mice deficient in B cells ($B^{-/-}$; $n = 6$), CD4 T cells ($CD4^{-/-}$; $n = 6$), or CD8 T cells ($CD8^{-/-}$; $n = 10$) 5 days after infection are shown. The data shown are the mean values plus SE values (error bars). Viral titers that are significantly different from those of wild-type mice by the Mann-Whitney U test are indicated as follows: §, $P < 0.05$; §§, $P < 0.01$.

mouse sera were purified by using a Melon gel immunoglobulin G purification kit (Pierce) according to the manufacturer's protocols and quantified by a spectrophotometer (Beckman). Mice deficient in B cells were given 25 μg of control antibody (Serotec) or anti-EV71 antibody by intraperitoneal injection 1 day before and 1 day after, 1 day after, or 2 and 4 days after infection with 1×10^7 PFU of EV71.

Statistical analyses. Data are expressed as mean ± standard error (SE) values. For statistical comparison, disease score curves were analyzed by the Wilcoxon test, Kaplan-Meier survival curves were analyzed by the log-rank test, and viral titers were analyzed by the Mann-Whitney U test. All *P* values are for two-tailed significance tests. A *P* value of <0.05 was considered statistically significant.

RESULTS

Presence of virus and three kinds of lymphocytes, B cells, CD4 T, and CD8 T cells, in the spinal cord of an EV71-infected patient who died. Autopsy studies of EV71-infected patients who died revealed virus and dense mononuclear cell infiltrates in the lesions of the CNSs (12, 22). To further determine lymphocyte constituents, we performed immunohistochemical staining on the cervical spinal cord specimen from an EV71-infected patient who died. After the spinal cord sections were

stained with a monoclonal antibody against the viral capsid protein, VP1, positive signals were detected in the cytoplasmic granules of ganglion neurons (Fig. 1A). Mononuclear cell infiltration was also observed, mainly in perivascular cuffs (Fig. 1B). After the sections were stained with antibodies against human leukocyte common antigens, $CD20^+$ B cells (Fig. 1C), $CD4^+$ T cells (Fig. 1D), and $CD8^+$ T cells (Fig. 1E) were present in the cuff in percentages of approximately 10, 30, and 10, respectively.

Presence of B cells, CD4 T cells, and CD8 T cells in the CNSs of infected mice. We established a neonatal mouse model of EV71 infection that can reproduce neurological symptoms and death analogous to human EV71 infection (20). We used this model to investigate the kinetics of viral replication and lymphocyte infiltration in the brain. Seven-day-old ICR mice were infected with 5×10^6 PFU of EV71 by oral inoculation (Fig. 2A). With this inoculum, 25% of infected mice died by day 8 or 9 postinfection (p.i.). Mouse brains were harvested after infection to determine viral titers by plaque assay. Virus was not detected in mouse brains until day 3 p.i. (Fig. 2B). Viral titers detected in mouse brains reached a peak at day 5 p.i. and then declined afterwards. In addition, leukocytes were isolated from mouse brains and analyzed by flow cytometry. In the brains of mock-infected mice, $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells were rarely detected (Fig. 2C). In the brains of infected mice, the infiltration of $CD4^+$ T, $CD8^+$ T, and $CD19^+$ B cells was not detected until day 3 p.i. The infiltration of $CD4^+$ and $CD8^+$ T cells increased progressively from days 5 through 10 p.i., but the infiltration of B cells declined from days 7 through 10 p.i. We also detected virus and three types of lymphocytes in the spinal cords of infected mice. In the CNSs of infected C57BL/6 mice, virus and three types of lymphocytes were also detected at day 5 p.i., suggesting that the phenomenon of lymphocyte infiltration is not specific to a particular mouse strain. Collectively, these results show that both virus and all three types of lymphocytes are detected in the CNSs of infected mice during the time when mice succumb to death in a phenomenon analogous to that observed in patients.

B cells, CD4 T cells, and CD8 T cells function to reduce the lethality of infected mice by decreasing viral loads in tissues. To assess the function of lymphocytes in EV71 infection, we compared the disease severity and survival rates of wild-type C57BL/6 mice and their congenic mice deficient in B, CD4 T, or CD8 T cells after inoculation with EV71. Infected lymphocyte-deficient mice developed hind limb weakness or paralysis and showed signs of encephalitis manifested by hunch posture, lethargy, and ataxia with disease scores significantly higher than that of wild-type mice (Fig. 3A; $P < 0.05$, Wilcoxon test). Later, the survival rates of infected mice deficient in B, CD4 T, or CD8 T cells (0, 44, and 29%, respectively) were significantly lower than that of wild-type mice (83%; Fig. 3B; $P < 0.05$, log-rank test). We next harvested nine tissues, including the CNSs (brains without the brain stem regions, brain stems, and spinal cords) and peripheral tissues (hearts, lungs, intestines, livers, kidneys, and spleens) from infected mice at day 5 p.i. to determine viral titers (Fig. 3C). With the exception of the brain stems, the mean viral titers in the rest of eight tissues of mice deficient in B cells were significantly higher than those of wild-type mice ($P < 0.05$, Mann-Whitney U test). The mean viral titer in the brain stems of mice deficient in B cells was about 10-fold higher than that of wild-type mice. With the exception of the livers, the mean viral titers in the rest of eight organs of mice deficient in CD4 T cells were significantly higher than those of wild-type mice ($P < 0.05$, Mann-Whitney U test). The mean viral titer in the livers of mice deficient in CD4 T cells was about threefold higher than that of wild-type mice. The mean viral titers in the hearts, livers, and spleens of mice deficient in CD8 T cells were significantly higher than those of wild-type mice ($P < 0.05$, Mann-Whitney U test). The mean viral titers in the brains without the brain stem regions, brain stems, spinal cords, lungs, and intestines of mice deficient in CD8 T cells were also higher than those of wild-type mice. These results collectively show that all three types of lymphocytes function to protect mice from infection by reducing viral loads in tissues.

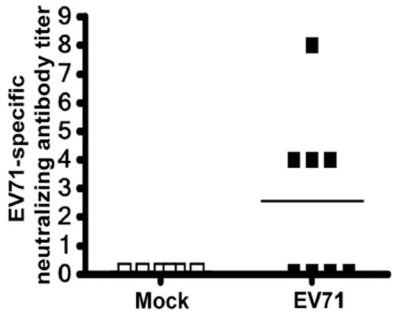

FIG. 4. Production of EV71-specific antibodies with neutralizing activity. The neutralizing antibody titers in the sera of mock-infected or EV71-infected C57BL/6 mice collected 7 days after infection were determined by neutralization assay. The highest dilution of serum that protected RD cell monolayers from infection was taken as the neutralizing titer. Each symbol on the scatter gram represents an individual sample, and the black horizontal line represents the mean value for the EV71-infected mice.

EV71-specific antibody functions to reduce the lethality of infected mice by decreasing viral loads in tissues. Our results showed that the survival rate of mice deficient in B cells was much lower than those of mice deficient in CD4 or CD8 T cells after infection, suggesting that B cells play a significant role in EV71 infection. B cells fight viral infections mainly by producing antibodies. We therefore investigated the production of virus-specific antibodies by examining neutralizing antibody titers in the sera collected from wild-type C57BL/6 mice at day 7 p.i. right before infected mice succumbed to death. All six serum samples collected from mock-infected mice failed to neutralize EV71 (Fig. 4). Of eight serum samples collected from infected mice, four were able to neutralize EV71 with titers of 4 or 8, but four failed to show neutralizing activity.

The antibody response has been suspected to contribute to the pathogenesis of neurological diseases in EV71-infected patients (14). We therefore tested the function of virus-specific antibody in EV71 infection using the most susceptible mouse strain, B-cell-deficient mice. Mice were given virus-specific antibody purified from the sera of wild-type mice immunized when they were 8 weeks old or control antibody by intraperitoneal injection 1 day before and 1 day after infection, and their survival was monitored. All six infected mice given control antibody developed severe hind limb paralysis, but all six

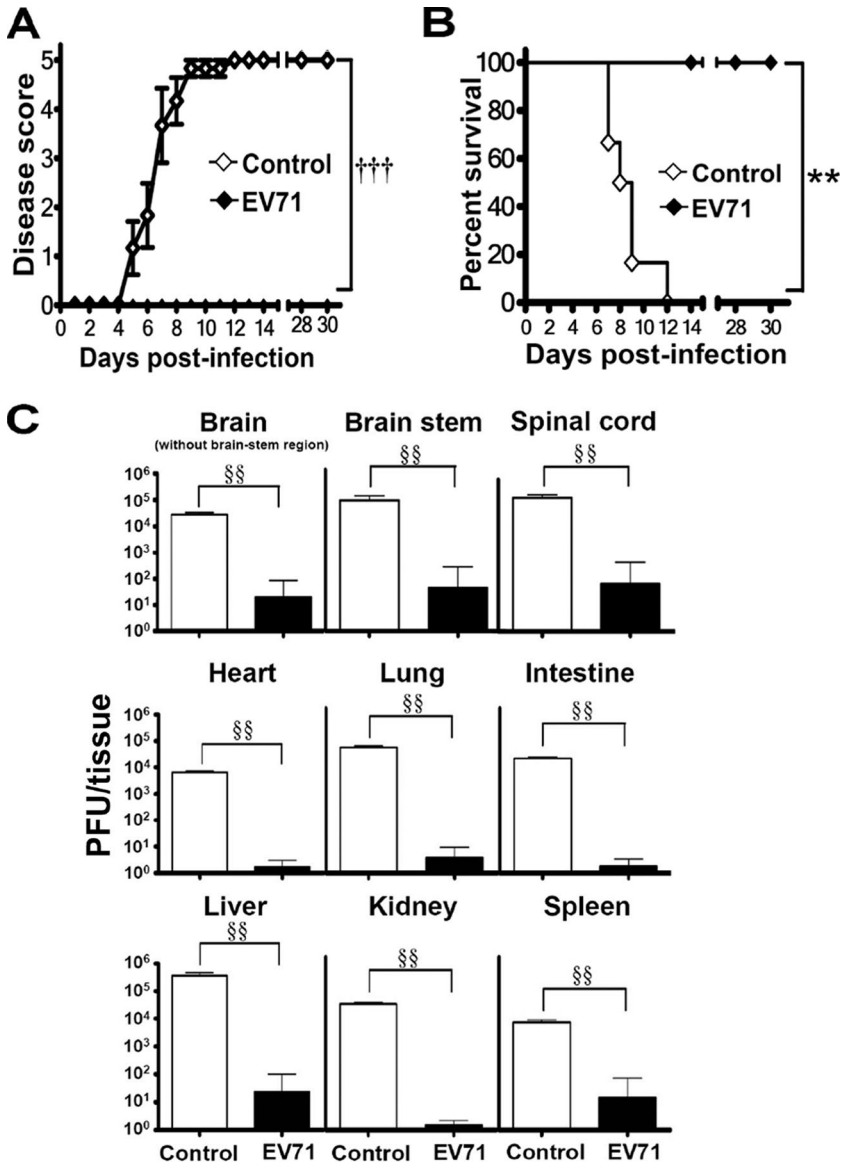

FIG. 5. EV71-specific antibody reduces the lethality of infected mice by decreasing viral loads in tissues. The disease scores (A) and survival rates (B) of EV71-infected, B-cell-deficient mice given control antibody ($n = 6$) or EV71-specific antibody ($n = 6$) are shown. Disease scores that are significantly different ($P < 0.001$) by the Wilcoxon test are indicated (†††). Survival rates that are significantly different ($P < 0.01$) by the log-rank test are indicated (**). (C) The viral titers in the indicated tissues of infected, B-cell-deficient mice given control antibody ($n = 6$) or EV71-specific antibody ($n = 6$) 5 days after infection are shown. The data shown are the mean values plus SE values (error bars). Viral titers that are significantly different ($P < 0.01$) by the Mann-Whitney U test are indicated (§§).

infected mice given virus-specific antibody appeared healthy (Fig. 5A) with a disease score significantly lower than that of mice given control antibody ($P < 0.001$, Wilcoxon test). Later, all six infected mice given control antibody died, but all six infected mice given virus-specific antibody survived (Fig. 5B) with a survival rate significantly higher than that of mice given control antibody ($P < 0.01$, log-rank test). Then, we harvested nine tissues, brains (without the brain stem region), brain stems, spinal cords, hearts, lungs, intestines, livers, kidneys, and spleens from infected mice at day 5 p.i. to determine viral titers (Fig. 5C). The mean viral titers in the nine tissues of infected mice given control antibody were all significantly higher than those of infected mice given virus-specific antibody ($P < 0.01$, Mann-Whitney U test). These results collectively show that infected mice produce antibodies with neutralizing activity to protect mice from infection by decreasing viral loads in tissues. We also tested mice given virus-specific antibody after infection. All five infected mice given virus-specific antibody at day 1 p.i. survived, but all four infected mice given virus-specific antibody at days 2 and 4 p.i. died. Virus-specific antibody given at day 1 p.i. also increased the survival of infected mice deficient in CD4 or CD8 T cells (unpublished data).

The above study showed that 8-week-old wild-type mice produced antibodies to protect B-cell-deficient mice from EV71 infection after immunization. We also tested whether

9-day-old wild-type mice could produce protective antibodies after infection. All six B-cell-deficient mice given antibodies purified from EV71-infected mice 1 day before and 1 day after infection survived, but all five B-cell-deficient mice given antibodies purified from mock-infected mice died. These data suggest that the T and B cells in the infected neonatal wild-type mice mount virus-specific antibody responses.

DISCUSSION

This is the first report to show that all three types of lymphocytes, B cells, CD4 T cells, and CD8 T cells are present in the CNSs of EV71-infected patient and mice. These three types of lymphocytes function to reduce the mortality and tissue viral loads of infected mice. Moreover, B cells produce antibodies with neutralizing activity to reduce the mortality of infected mice by decreasing tissue viral loads.

In EV71-infected patients with neurological symptoms, the appearance of clinical and tissue inflammation has raised the prospect that there is an immunopathological aspect to this infection (14, 15). Therefore, corticosteroids have been used to treat patients with neurological symptoms (14, 15). Our mouse study shows that although both lymphocyte and antibody responses are elevated during the time when mice succumb to death, these two responses actually function to protect mice from infection. Our results argue against immunopathological mechanisms in the infection and raise caution over the use of corticosteroids to treat patients with neurological symptoms. We are currently testing whether corticosteroids affect progression of the disease in infected mice by modulating lymphocyte and antibody responses, because corticosteroids are known to suppress T cells (2).

Currently, it remains unknown why some patients are highly susceptible to EV71 infection and develop fatal symptoms. In patients, lymphopenia and depletion of CD4 and CD8 T cells in peripheral blood are found in patients with fatal symptoms (17). Here we show that mice deficient in lymphocytes, especially B cells, are very susceptible to EV71 infection. This is very similar to a clinical report showing that patients with agammaglobulinemia are highly susceptible to chronic echovirus infection in the CNS (13). In the future, it will be worth determining whether EV71-infected patients with fatal symptoms have any defect in lymphocyte and antibody responses.

Although EV71 infection has become a new threat to global public health over the past decade (16), the limited knowledge of EV71 pathogenesis hinders the development of effective strategies to control fatal outbreaks. Besides corticosteroids and IVIG, there are no effective vaccines and specific antiviral therapies currently available to control fatal outbreaks. Our mouse results show that virus-specific antibody given before infection significantly reduces EV71 lethality and tissue viral loads in mice. This is consistent with previous reports showing that maternal antibodies protect mice from infection (21, 23). These results suggest the potential of using vaccines to control fatal EV71 outbreaks. Giving virus-specific antibody after infection also significantly reduces EV71 lethality in mice. In addition, administration of IVIG containing high neutralizing antibody titers to the patients' own coxackievirus B and echovirus isolates has been shown to reduce viremia in patients (1). Moreover, treatment with virus-specific antibodies is shown to improve the neurological symptoms of echovirus-infected patients with agammaglobulinemia (13). All these mouse and clinical results suggest the potential of using virus-specific antibody to treat EV71-infected patients with severe symptoms.

Taken together, the present findings enhance our understanding of EV71 pathogenesis and also provide strategies for a better control of fatal outbreaks. Our study is particularly important because based on the incidence of fatal outbreaks in the past decade, EV71 is very likely to continue to cause serious problems in hundreds of thousands of young children in the future.

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