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Complete protection of mice against a lethal dose challenge of western equine encephalitis virus after immunization with an adenovirus-vectored vaccine

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Abstract

Western equine encephalitis virus (WEEV) is an important pathogen for both humans and equines. The virus is also listed as a bioterrorism agent due to its ability for aerosol transmission with high mortality. No commercial vaccines or antiviral drugs are available for the prevention and treatment of WEEV infection in humans. In this paper, we constructed a recombinant WEEV vaccine, designated as Ad5-WEEV, using a replication defective, human adenovirus serotype 5 (HAd5) as a delivery vector. Ad5-WEEV contains the E3-E2-6K-E1 structural protein gene of the 71V-1658 strain of WEEV and the E1 and E2 proteins were synthesized in cells inoculated with Ad5-WEEV. After intramuscular immunization of mice with two doses of Ad5-WEEV, neutralizing antibodies against WEEV were generated and the mice were completely protected from a lethal dose challenge of 71V-1658. In addition, we showed that passive transfer of serum from the Ad5-WEEV-immunized mice could partially control WEEV infection. These results demonstrate that HAd5 vectors are promising for WEEV vaccine development.

Keywords: Western equine encephalitis virus; Adenovirus vector; Vaccine development

1. Introduction

Western equine encephalitis virus (WEEV) belongs to the *Alphavirus* genus in the *Togaviridae* family. The virus has been isolated mainly in Western North America and in Central and South Americas [1]. In North America, WEEV is maintained in a cycle involving wild birds as the natural reservoir and *Culex tarsalis* mosquitoes as vectors. The virus is transmitted to equines and humans by mosquitoes. Symptoms of WEEV infection in humans range from fever and headache to encephalitis with delirium, disorientation or coma. The overall case fatality rate is 3%, but increases to 8% in older adults [2]. In addition to natural transmission through mosquitoes, WEEV can be easily transmitted as aerosols with high mortality. This was demonstrated in several animal models [3–5] and in laboratory-acquired WEEV infections in which two out of five laboratory workers who were exposed to aerosolized WEEV died [6]. The ease of aerosol transmission with high mortality rates makes WEEV a potential bioterrorism agent [7].

Currently there are no licensed vaccines or antiviral drugs available for WEEV. An Investigational New Drug (IND) vaccine based on formalin-inactivated WEEV is available only for laboratory workers conducting researches on WEEV [8]. Long-term studies in humans found that only a 50% responder rate is achieved after immunization with three doses of the vaccine [9]. In addition, only 20% of recipients maintained the immunity one year after vaccination and an annual booster is required to offer protection [9]. Live-

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attenuated vaccine candidates for WEEV provide protection after a single-dose immunization [10,11], but there is concern of the reversion of the attenuated vaccine to virulent wild-type WEEV. Previously, we developed a DNA vaccine candidate that completely protected mice from a lethal dose challenge of WEEV [12]. The DNA vaccine candidate is safe and easy to produce; however, to be effective, it needs three immunizations and the ballistic method for the delivery [12]. To improve the current vaccines for WEEV, we investigated if viral vectors derived from human adenovirus serotype 5 (HAd5) could be used for the development of WEEV vaccine. We report here the construction of a HAd5-vectored WEEV vaccine expressing both the E1 and E2 structural proteins of WEEV and its immunogenicity and efficacy in mice.

2. Materials and methods

2.1. Cells and virus

Vero and 293 cells were purchased from American Type Culture Collection (Manassas, VA). Both cells were cultured in Dulbecco's modified Eagle media (DMEM; Invitrogen, Burlington, Ont., Canada) containing 10% fetal bovine serum (VWR International, Mississauga, Ont., Canada) and antibiotics. The 71V-1658 strain of WEEV contained in a 10% suckling mouse brain suspension was kindly provided by Nick Karabatsos (CDC, Fort Collins, CO). 71V-1658 was originally isolated in 1971 in Oregon from the brain tissue of a horse infected with WEEV [13] and the complete nucleotide sequence of the virus has been determined [14]. A seed stock of 71V-1658 was made by the infection of Vero cells with the suckling mouse brain suspension. The supernatants collected from the infected cells were aliquoted and stored at −70 °C. The titer of the 71V-1658 stock was determined by plaque assay in Vero cells. All in vitro and in vivo experiments with 71V-1658 were conducted using enhanced BSL-3 containment procedures according to the guidelines of Health Canada and Canadian Food Inspection Agency.

2.2. Generation of recombinant HAd5 vectors

Ad5-WEEV, a replication-deficient, recombinant HAd5 vector encoding the E3-E2-6K-E1 of the WEEV strain 71V-1658 was generated using AdEasy™ platform technology (Qbiogene, Carlsbad, CA). The cDNA encoding E3-E2-6K-E1 was cloned from plasmid pVHX-6 [12] through the following steps. First, the E3-E2 region was amplified from pVHX-6 by PCR using primers JQW3 (5′-CAC CAT GTC ACT AGT TAC AGC GCT ATG CGT GC-3′) and JQW5 (5′-TCA CTA AGC GTT GGT TGG CCG AAT GC-3′). Following a 2-min pre-incubation at 95 °C, PCR was carried out with *Pfu*Turbo DNA polymerase (Strategene, La Jolla, CA) by 25 cycles of a 30-s 94 °C denaturing step, a 30-s 55 °C annealing step, and a 1-min 70 °C extension step. The PCR product was cloned into pcDNA3.1D/V5-His-TOPO©(Invitrogen) to generate pcD3-WEE-E3-E2. The E3-E2 region was sequenced, which is agreed with the published sequence (GenBank accession no. NC_003908). Next, the cDNA encoding the N-terminal half of 6K-E1 was isolated from pVHX-6 as an *Eco*RI–*Eco*RV fragment and ligated to *Eco*RI–*Eco*RV-digested pcD3-WEE-E3-E2 to generate pcD3-EE157. Finally, the cDNA encoding the C-terminal half of 6K-E1 was excised from pVHX-6 and cloned into the *Xho*I and *Xba*I sites of pcD3-EE157 to produce pcD3-WEE-E3-E2-6K-E1 containing the E3-E2-6K-E1 gene of 71V-1658.

To generate Ad5-WEEV, the E3-E2-6K-E1 gene was excised from pcD3-WEE-E3-E2-6K-E1 with *Hin*dIII and *Xba*I and cloned into the *Hin*dIII–*Xba*I sites of pShuttle-CMV (Qbiogene). The resultant plasmid, pSCMV-WEE-E3-E2-6K-E1, was linearized with *Pme*I and co-transformed with pAdEasy-1 (Qbiogene) into *Escherichia coli* BJ5183 cells to generate plasmid pAd5-WEEV. Subsequently, pAd5-WEEV was digested with *Pac*I and transfected into 293 cells by Lipofectamine 2000 (Invitrogen) to produce Ad5-WEEV. Additionally, Ad5-EGFP expressing the enhanced green fluorescent protein (EGFP) gene was constructed as a control vector used throughout the study. Briefly, the EGFP gene was isolated from pEGFP-N3 (BD Biosciences, Mississauga, Ont., Canada) as a *Kpn*I–*Not*I fragment and cloned into the *Kpn*I and *Not*I sites of pShuttle-CMV to produce pSCMV-EGFP. pAdEasy-1 was mixed with *Pme*I-digested pSCMV-EGFP and the mixture was transformed into BJ5183 cells to generate pAd5-EGFP. Ad5-EGFP was obtained by transfection of 293 cells with *Pac*I-digested pAd5-EGFP. The expression of EGFP was confirmed by the observation of green autofluorescent in Ad5-EGFP-infected 293 cells under fluorescence microscopy. The recombinant HAd5 vectors were amplified in 293 cells, purified by double cesium chloride (CsCl) gradient centrifugation [15], and titrated by a 50% tissue culture infectious dose assay (TCID50) [16].

2.3. PCR detection of the E3-E2-6K-E1 gene in Ad5-WEEV

The presence of the E3-E2-6K-E1 gene in Ad5-WEEV was verified by PCR. The viral DNA of Ad5-WEEV was extracted from the purified virus using DNeasy Tissue Kits (QIAGEN, Mississauga, Ont., Canada). The E3-E2-6K-E1 gene was amplified from the viral DNA by PCR using primers S1 (5′-ACC ACG ACC ATG ACA TCA AG-3′) and JQW4 (5′-CCG CGC TCA GTC ATC TAC GTG TG-3′). PCR was performed with HotStarTaq DNA polymerase (QIAGEN) by a 15-min 95 °C initial activating step followed by 30 cycles of a 30-s 94 °C denaturing step, a 1-min 60 °C annealing step, and a 2-min 72 °C extension step. As a control, PCR was also carried out under the same condition for the viral DNA extracted from the Ad5-EGFP control vector. A 5-μl aliquot of the PCR mixture was analyzed by electrophoresis in a 0.8% agarose gel. The DNA was stained with ethidium bromide and visualized on a UV transilluminator.

2.4. Western immunoblot

The expression of E1 and E2 by Ad5-WEEV was determined by Western immunoblotting using WesternBreeze® Chromogenic Immunodetection Kit (Invitrogen). Briefly, 293 cells were infected with Ad5-WEEV at a multiplicity of infection of 1. Mock- and Ad5-EGFP-infected 293 cells were included as controls. At 24 h after infection, total cell proteins were extracted from the infected cells and electrophoresed on a 10% Bis–Tris NuPAGE Novex gel (Invitrogen). The eletrophoresed proteins were transferred onto a nitrocellulose membrane by electroblotting. The membrane was probed with 11D2 monoclonal antibody (MAb) specific for the E1 protein of WEEV [17] and 3F3 MAb specific for E2 [17], followed by the incubation with alkaline phosphatase-conjugated anti-mouse antibody. The E1 and E2 proteins were visualized by chromogenic substrate.

2.5. Immunization, serum collection, and WEEV challenge

Female BALB/c mice (17–20 g) were obtained from the pathogen-free mouse breeding colony at DRDC Suffield, with the original breeding pairs purchased from Charles River Canada (St. Constant, Quebec, Canada). The use of the mice was approved by Animal Care Committee at DRDC Suffield. The guidelines of the Canadian Council on Animal Care were followed for caring and handling the mice.

A total of 24 mice were divided into four groups of six mice each. Mice in Group 1 were each immunized intramuscularly (IM) with 10^7 plaque forming units (pfu) of Ad5-WEEV diluted in 100 μl of phosphate-buffered saline (PBS) and boosted 4 weeks later with the same dose of Ad5-WEEV. As controls, mice in Group 2 were each injected with 10^7 pfu of Ad5-EGFP and boosted 4 weeks later; mice in Group 3 were immunized three times (2 weeks apart) with 50 μl of inactivated WEEV vaccine [12]; and mice in Group 4 were each injected with PBS. Serum was collected from each mouse at day 11 after each immunization to monitor neutralizing antibody titers. Two weeks after the final injection, mice were challenged intranasally (IN) with 1.5×10^3 pfu of 71V-1658 as described previously [4]. The challenged mice were weighted and examined daily for 14 days. The severity of infection was measured using the following scoring system: 0, normal; 1, slightly ruffled hair, very active, no visible signs of infection; 2, very ruffled hair, definite signs of infection, not as active, but still fairly mobile; 3, very ruffled hair, hunched posture, reduced mobility; 4, very ruffled hair, hunched posture, little or no mobility, rapid breathing. Mice scored at the scale of 4 were considered terminally ill and were euthanized.

2.6. Plaque reduction neutralization assay

Pooled sera from each group of the mice given Ad5-WEEV, Ad5-EGFP, inactivated WEEV vaccine, or PBS were heat-inactivated at 56 °C for 30 min. The two-fold diluted sera were mixed with 71V-1658 and incubated at room temperature for 45 min. The serum–virus mixtures were inoculated to the confluent Vero cells cultured in 6-well plates and incubated at 37 °C for 45 min. The inoculated cells were then incubated at 37 °C for 72 h in a gum tragacanth overlay. Plaques were counted and the titers of WEEV-specific neutralization antibodies were determined as reciprocals of the highest serum dilution that resulted in a 50% reduction in the number of plaques.

2.7. Passive serum transfer and protection study

Pooled sera collected from naïve mice and the mice after the final immunization with Ad5-WEEV or Ad5-EGFP were heat-inactivated and 1:3 diluted in PBS. Three groups of six mice each were injected intraperitoneally (IP) with 200 μl of naïve mice serum, Ad5-WEEV-immune serum, or Ad5-EGFP-immune serum. Twenty-four hours later, serum was collected from each mouse to determine the titers of WEEV-specific neutralizing antibodies and the mice were challenged IN with 1.5×10^3 pfu of the 71V-1658 strain of WEEV.

2.8. Statistical analysis

Statistical analyses were done using PRISM® 4 software (GraphPad Software Inc., San Diego, CA). The significance of the difference in survival between the Ad5-WEEV-immunized group and controls was determined by a two-tailed Fisher's exact test. The significance of the difference in mean survival times between the Ad5-WEEV-immunization group and controls was analyzed by a two-tailed paired *t* test.

3. Results

3.1. Construction of HAd5 vector containing the E3-E2-6K-E1 gene

The recombinant HAd5 vector, Ad5-WEEV, expressing the E1 and E2 structural proteins of the 71V-1658 strain of WEEV was constructed by the insertion of the E3-E2-6K-E1 gene into the E1 region of the HAd5 genome using a published method [18,19]. The E3-E2-6K-E1 gene was placed between the immediate-early promoter of cytomegalovirus (CMV) and the polyadenylation signal of Simian virus 40 (SV40) (Fig. 1A). The insertion of the E3-E2-6K-E1 gene into the HAd5 genome displaced the E1 region of HAd5. Because the E1 is essential for HAd5 replication, the replacement of the E1 renders Ad5-WEEV replication-deficient, which increases the safety of the vector. The presence of the E3-E2-6K-E1 gene in Ad5-WEEV was detected by PCR using primers specific for the E3-E2-6K-E1 gene. As shown in Fig. 1B, a 2.9 kb amplicon that is consistent with the anticipated size of the E3-E2-6K-E1 gene of 71V-1658 [14] is present in the viral DNA of Ad5-WEEV (lane 4).

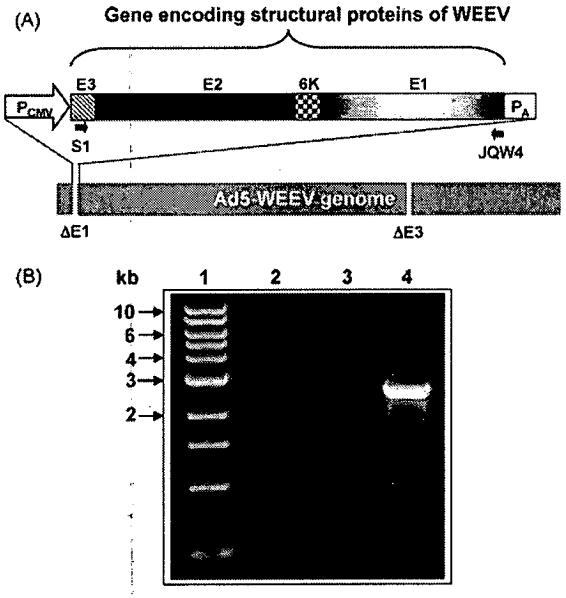

Fig. 1. The E3-E2-6K-E1 gene is present in Ad5-WEEV. (A) Schematic representation of the Ad5-WEEV genome. The diagram shows the gene encoding E3-E2-6K-E1 is flanked by the immediate-early promoter of cytomegalovirus (P_{CMV}) and the polyadenylation signal of Simian virus 40 (P_A). The expression cassette was cloned into the E1 coding region of HAd5. S1 and JQW4 are primers used for PCR detection of the E3-E2-6K-E1 gene. ΔE1 and ΔE3 illustrate deletions in the E1 and E3 coding regions of the HAd5 genome. (B) PCR detection of the E3-E2-6K-E1 gene of Ad5-WEEV. Lane 1, DNA ladder (NEB Ltd., Pickering, Ont., Canada); lane 2, no DNA-template control; lane 3, PCR product from the viral DNA of Ad5-EGFP and lane 4, PCR product from the viral DNA of Ad5-WEEV.

The 2.9 kb amplicon is not shown in the PCR product of the viral DNA from the Ad5-EGFP control vector (lane 3), suggesting that Ad5-WEEV contains the E3-E2-6K-E1 gene.

3.2. Ad5-WEEV expresses both E1 and E2 proteins

Having demonstrated that Ad5-WEEV contains the E3-E2-6K-E1 gene, we next examined the expression of E1 and E2 by Ad5-WEEV. E1 and E2 from Ad5-WEEV-infected 293 cells were probed by Western immunoblotting using 11D2 MAb specific for the E1 protein of WEEV and 3F3 MAb specific for E2 [17]. As shown in Fig. 2, both E1 and E2 proteins were detected in Ad5-WEEV-infected cells (lanes 4 and 8). E1 and E2 migrated at around 47 kDa, which is consistent with the calculated molecular mass [14]. The proteins are not shown in mock-infected (lanes 2 and 6) or Ad5-EGFP-infected cells (lanes 3 and 7), suggesting that Ad5-WEEV expresses E1 and E2.

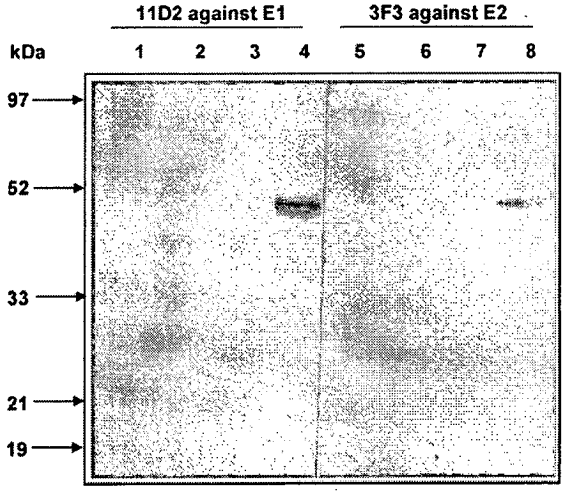

Fig. 2. The E1 and E2 proteins are produced from cells infected with Ad5-WEEV. Proteins extracted from mock-infected (lanes 2 and 6), Ad5-EGFP-infected (lanes 3 and 7), or Ad5-WEEV-infected (lanes 4 and 8) 293 cells were separated by 10% SDS-PAGE. The electrophoresed proteins were transferred to a nitrocellulose membrane and probed with 11D2 MAb specific for E1 and 3F3 MAb specific for E2. Lanes 1 and 5, MultiMark Multi-colored standard (Invitrogen).

3.3. Immunization of mice with Ad5-WEEV induces WEEV neutralizing antibodies and completely protects mice from a lethal dose challenge of WEEV

To determine the immunogenicity of Ad5-WEEV, we measured the neutralizing antibodies against the 71V-1658 strain in mice immunized with Ad5-WEEV using a plaque reduction neutralization assay [20]. As indicated in Fig. 3, BALB/c mice were immunized twice with Ad5-WEEV. As controls, three groups of mice were each inoculated with PBS, three doses of inactivated WEEV vaccine [21], or two doses of Ad5-EGFP. Sera from each group were collected at day 11 after each inoculation and were assessed for WEEV-

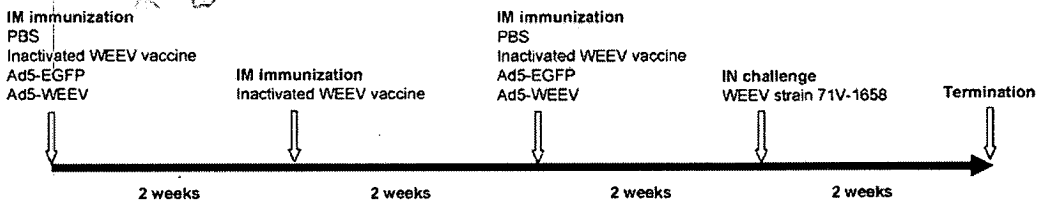

Fig. 3. Immunization protocol. Inactivated WEEV vaccine was given intramuscularly (IM) three times at 2 weeks apart. Ad5-WEEV and controls of Ad5-EGFP and PBS were given IM twice at 4 weeks apart. Two weeks after the final immunization, all the mice were challenged intranasally (IN) with the 71V-1658 strain of WEEV and monitored for 2 weeks for the severity of the disease.

Table 1
Induction of WEEV-neutralizing antibodies in mice immunized with Ad5-WEEV

Group	Before immunization	Day 11 after first immunization	Day 11 after final immunization
PBS control	<1:10	<1:10	<1:10
Inactivated WEEV vaccine	<1:10	<1:10	<1:10
Ad5-EGFP control	<1:10	<1:10	<1:10
Ad5-WEEV	<1:10	1:20	1:160

Table 2
Protection of mice against the challenge of WEEV after immunization with Ad5-WEEV

Group ($n=6$)	% survival on day 14 p.c (survivors/total)	Survival times (days) (mean ± S.D.)	Clinical score on day 9 p.c. (mean ± S.D.)	Body weight (mean g ± S.D.)	
				Pre-challenge	Day 9 p.c.
Ad5-WEEV	100 (6/6)[a]	14.0 ± 0.0[b,c]	0.0 ± 0.0	21.4 ± 0.2	22.1 ± 0.5
Inactivated WEEV vaccine	100 (6/6)	14.0 ± 0.0	1.2 ± 0.4	20.5 ± 1.6	22.3 ± 2.7
Ad5-EGFP	17 (1/6)[d,a]	10.7 ± 1.9[c,e]	2.7 ± 1.2	21.0 ± 0.6	14.5 ± 3.1
PBS control	0 (0/6)[d]	10.0 ± 1.3[b,e]	3.5 ± 0.5	22.5 ± 0.6	14.1 ± 0.8

[a] Ad5-WEEV vs. Ad5-EGFP, $P=0.015$, two-tailed Fisher's exact test.
[b] Ad5-WEEV vs. PBS control, $P=0.001$, two-tailed paired t test.
[c] Ad5-WEEV vs. Ad5-EGFP, $P=0.007$, two-tailed paired t test.
[d] Ad5-EGFP vs. PBS control, $P=1.000$, two-tailed Fisher's exact test.
[e] Ad5-EGFP vs. PBS control, $P=0.576$, two-tailed paired t test.

specific neutralizing antibodies. Table 1 shows that mice immunized with Ad5-WEEV developed WEEV-neutralizing antibodies (1:20) on day 11 after the first immunization. Following the second immunization, the titers of neutralizing antibodies were increased by eight-fold (1:160). No WEEV-specific neutralizing antibodies were detected in mice inoculated with PBS, inactivated WEEV vaccine, or Ad5-EGFP. These results demonstrate that humoral immune response can be induced in mice after immunization with Ad5-WEEV.

We next determined if the Ad5-WEEV-immunizated mice could survive a lethal dose challenge of the 71V-1658 strain of WEEV. The mice were challenged IN with 1.5×10^3 pfu of 71V-1658 two weeks after the final immunization and monitored for the change of body weight and the severity of the infection. All of the mice immunized with Ad5-WEEV survived and remained healthy during the 14-day observation, showing no signs of infection and no weight loss (Table 2). While the mice immunized with inactivated WEEV vaccine were all protected with no weight loss, these mice showed signs of infection on day 9 post-challenge (p.c.) (Table 2), followed by complete recovery on day 14 p.c. In contrast to the mice immunized with Ad5-WEEV or inactivated WEEV vaccine, only one out of six mice in the Ad5-EGFP vector control group and none in the PBS group survived on day 14 p.c. (Table 2). The mean surviving time for these two groups was about 10 days. Signs of infection in these mice started on day 5 p.c. and peaked on day 9 p.c. The progress of the disease in Ad5-EGFP and PBS control groups after the 71V-1658 challenge was similar to our previous observation [4]. Additionally, both control groups lost more than 30% of the original body weight on day 9 p.c. (Table 2). Taken together, Ad5-WEEV immunization confers complete protection against a lethal dose challenge of WEEV.

3.4. Serum from mice immunized with Ad5-WEEV provides passive protection

Antibodies play an important role in control of alphavirus infection [22,23]. To determine if neutralizing antibodies induced by Ad5-WEEV immunization could provide protection against WEEV infection, three groups of six mice each were injected IP with sera (1:3 diluted in PBS) from mice immunized with Ad5-WEEV or Ad5-EGFP or from naïve mice. At 24 h after the injection, sera from the mice were collected and analyzed for neutralizing antibodies to WEEV and mice were challenged IN with 1.5×10^3 pfu of 71V-1658. As shown in Fig. 4A, more than half (4/6) of mice that were injected with serum collected from the Ad5-WEEV-immunized mice survived the WEEV challenge and the majority of these mice contained WEEV-neutralizing antibodies (Table 3). However, these mice showed signs of infection on day 5 p.c. (Fig. 4B), suggesting that the protection is incomplete. None of the mice that were injected with serum from Ad5-EGFP-immunized mice or naïve mice survived the challenge (Fig. 4A) and these mice developed severe infection starting on day 5 p.c. (Fig. 4B). No measurable or very low level (1:8) WEEV-specific neutralizing antibodies were present in these mice (Table 3). These results

Table 3
Titers of WEEV-specific neutralizing antibodies in mice injected with serum from immunized mice

Treatment group ($n=6$)	Naïve	Ad5-EGFP	Ad5-WEEV
WEEV-neutralizing antibody titer in individual mouse	1:8	<1:8	1:32
	<1:8	<1:8	<1:8
	<1:8	<1:8	1:8
	<1:8	<1:8	<1:8
	1:8	1:8	1:32
	<1:8	1:8	1:16

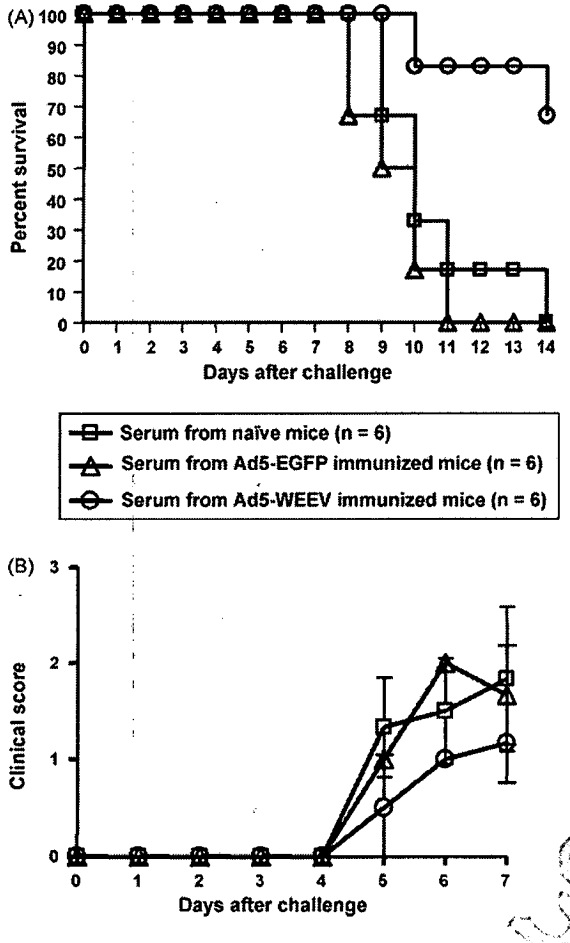

Fig. 4. Serum from Ad5-WEEV-immunized mice provides protection against a lethal dose challenge of WEEV. (A) Survival of the mice injected with serum from naïve mice or the mice immunized with Ad5-WEEV or Ad5-EGFP. Three groups of six mice each were injected intraperitoneally (IP) with serum from naïve mice, Ad5-WEEV- or Ad5-EGFP-immunized mice. At 24 h after the injection, mice were challenged intranasally (IN) with 71V-1658 and monitored for 14 days. (B) Disease severity in challenged mice. The mice were scored for the severity of WEEV infection using a scoring system described in Section 2. The means of severity score for six mice in each group are shown. Error bars represent the S.D. of the mean.

indicate that WEEV-neutralizing antibodies induced by Ad5-WEEV can provide a partial protection against a lethal dose challenge of WEEV.

4. Discussion

This study demonstrates that HAd5 vectors have a great potential for the development of a vaccine for WEEV. The HAd5-vectored WEEV vaccine expressing E1 and E2 completely protected mice from a lethal dose challenge of WEEV and immunization with the vaccine generated WEEV-neutralizing antibodies that contribute to the protective immunity.

Conventional approaches for WEEV vaccine development involve the chemical inactivation or the attenuation of WEEV. A formalin-inactivated WEEV vaccine has been licensed for veterinary use [24]. A similar inactivated WEEV vaccine under IND status is available for use by laboratory personnel at risk of contracting WEEV [21]; however, the immunogenicity of the vaccine is poor and short lived. Compared to the inactivated vaccine, live-attenuated vaccines often give a quick and long lasting protection because these vaccines induce immune response by mimicking a natural infection. Live-attenuated vaccine candidates for WEEV have been made by the deletion of the PE2 furin cleavage site followed by the selection of a second-site suppressor mutation in the E1 or E2 gene [10] and a single-dose immunization of these live WEEV vaccines completely protected animals from the challenge [10]. However, a major concern with the live-attenuated WEEV vaccines is their potential to revert to virulent wild-type viruses.

HAd5 vectors could be used as an alternative approach for WEEV vaccine development because of the following advantages [25]. First, vaccines based on HAd5 vectors are proved to be safe for human use [26]. Unlike live-attenuated vaccines, there is no risk of reverting to a virulent phenotype from the heterologous viral gene delivered by HAd5 vectors. Second, vaccines based on HAd5 vectors work by mimicking natural viral infections. Therefore, they induce swift and durable humoral and cellular immune responses. Indeed, a single-dose immunization of HAd5-vectored vaccines is sufficient to provide a rapid protective immunity against several viral and bacterial infections [27–32]. Third, vaccines based on HAd5 vectors induce mucosal immunity [33], which is important for preventing viral infections transmitted by the aerosol route. Thus, HAd5 vectors are ideal for the development of vaccines against bioterrorism agents that are mostly disseminated by the aerosol [25,34]. Finally, vaccines based on HAd5 vectors are relatively easy to grow in a large quantity in cell culture, which could speed up the production.

The E1 and E2 envelope proteins of alphaviruses are encoded by a subgenomic mRNA and are derived by proteolytic cleavage of the E3-E2-6K-E1 polypeptide [35]. E2 is initially synthesized as a precursor protein, PE2, which consists of E3 and E2, followed by cleavage of E3 from PE2 by furin-like protease activity. The E2 protein binds to E1 to form the spikes on the surface of the virion. E2 is related to the attachment of the virus to a cellular receptor and E1 is related to virus fusion with the cell membrane [36]. HAd5-vectored vaccine candidates for Venezuelan equine encephalitis virus (VEEV), a virus closely related to WEEV, have been developed in which the E3-E2-6K of VEEV was expressed from HAd5 vectors [37]. After intranasal immunization, these vaccine candidates provide good protection against the aerosol challenge of the epizootic VEEV but less protection against enzootic VEEV [37]. The HAd5-vectored VEEV vaccines also improve efficacy of a DNA vaccine in a prime-boost immunization regimen [38].

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We chose to express both E1 and E2 of WEEV because studies from other alphaviruses demonstrated that both of these proteins are the major determinants for the induction of immune protection [22,23,39]. To express E1 and E2, we constructed Ad5-WEEV in which the E3-E2-6K-E1 gene of the 71V-1658 strain of WEEV, flanked by the immediate-early promoter of CMV and the polyadenylation signal of SV40, was inserted in the E1 region of HAd5. Both E1 and E2 were processed and expressed from cells infected with Ad5-WEEV (Fig. 2) and the induction of WEEV-neutralizing antibodies by Ad5-WEEV suggests that E1 and E2 expressed from Ad5-WEEV contain native neutralizing epitopes. WEEV-specific neutralizing antibodies were detected after a single-dose immunization of mice with Ad5-WEEV and the titers were increased by eight-fold after the second immunization (Table 1). All the Ad5-WEEV-immunized mice survived following a lethal dose challenge of WEEV and did not show signs of infection (Table 2).

All the mice immunized with three doses of inactivated WEEV vaccine were survived after the challenge (Table 2) although they did not show measurable WEEV-neutralizing antibodies (Table 1). We also observed previously that the mice immunized with a DNA vaccine for WEEV were protected from a lethal dose challenge of WEEV without detectable neutralizing antibodies [12]. We found that cellular immunity induced by the DNA vaccine could be related to the protection [12]. Additionally, Schmaljohn et al. demonstrated that non-neutralizing antibodies can also prevent alphavirus infection [23]. It appears that WEEV-specific neutralizing antibodies are required for conferring complete protection because mice immunized with inactivated WEEV vaccine developed signs of infection (Table 2). The infection could be due to the limited replication of the virus after the challenge, which Cole and McKinney described that hamsters immunized with inactivated WEEV vaccine developed viremia after the WEEV challenge although they were 100% protected [41]. Because mice immunized with Ad5-WEEV generated WEEV-specific neutralizing antibodies (Table 1) and no signs of infection were shown in these mice (Table 2), it will be interesting to see if Ad5-WEEV could induce a sterile immunity. Taken together, these results indicate that HAd5-vectored WEEV vaccine can offer better protection than inactivated WEEV vaccine.

The passive protection study showed that more than half of the mice (4/6) receiving the serum from Ad5-WEEV-immunized mice survived the challenge (Fig. 4A). However, these mice were partially protected (Fig. 4B), which may be due to the amount of the neutralizing antibodies present in the serum is too low (Table 3) to offer complete protection or the neutralizing antibodies alone may not be sufficient for the complete control of WEEV infection. The latter has been demonstrated in studies from other alphaviruses, such as Sindbis virus, in which the complete control of infections requires both humoral and cellular immune responses [22,23,40]. Additionally, the lack of detectable WEEV-specific neutralizing antibodies in mice immunized with inactivated WEEV vaccine (Table 1) and partial protection of the mice against the challenge (Table 2) further suggest that both humoral and cellular immunities are required for complete protection.

We have shown that a DNA vaccine encoding the capsid and the E3-E2-6K-E1 of WEEV completely protected mice from challenge [12]. The complete protection conferred by Ad5-WEEV expressing only E3-E2-6K-E1 indicates that the capsid is not required for the protection when HAd5 vectors are used for vaccine delivery. It will be interesting to determine if the E2 or E1 of WEEV alone could offer protection against the virus, which has been demonstrated for VEEV in which HAd5 vectors expressing only the E3-E2-6K of VEEV protected mice from airborne challenge of the virus [37].

In conclusion, we have shown that two-dose immunization of HAd5 vectors expressing the structural proteins of WEEV elicits an antibody response and completely protects mice from a lethal dose challenge of WEEV. Further studies are needed to determine the efficacy of the HAd5-vectored WEEV vaccine for the cross-protection against different strains of WEEV and the impact of pre-existing neutralizing antibodies against HAd5 vectors on the efficacy of the vaccine. And it will be interesting to see if a single-dose immunization can offer the same protection as two-dose immunization.

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