Defence Research and Development Canada
Recherche et développement pour la défense Canada

DEFENCE R&D DÉFENSE

Interferon-gamma gene delivery partially protects mice from the lethal infection of western equine encephalitis virus

Josh Q.H. Wu, Nicole D. Barabé, Damon Chau, Aaron Sha, Andrew Marshall

Defence R&D Canada

Technical Report

DRDC Suffield TR 2008-224

December 2008

Canada

Interferon-gamma gene delivery partially protects mice from the lethal infection of western equine encephalitis virus

Josh Q.H. Wu, Nicole D. Barab□, Damon Chau, Aaron Sha, Andrew Marshall
DRDC Suffield

Defence R&D Canada □ Suffield

Technical Report
DRDC Suffield TR 2008-224
December 2008

Principal Author

Original signed by Dr Josh Q.H. Wu

Dr Josh Q.H. Wu

Approved by

Original signed by Dr Les P. Nagata

Dr Les P. Nagata

Head, Biotechnology Section

Approved for release by

Original signed by Dr Paul D'Agostino

Dr Paul D'Agostino

Chair, DRDC Suffield Document Review Panel

Abstract

Western equine encephalitis virus (WEEV) is a potential biological and bioterrorism agent. The aerosolized virus is easily transmitted to humans. Single-dose, fast-acting vaccines and therapeutics are urgently needed to provide rapid protection against WEEV in the event of bioterrorism attack or biowarfare. We found previously that a single dose injection of interferon alpha (IFN-α) expressed from human adenovirus type 5 (HAd5) vector rapidly and completely protects mice against WEEV. This result prompted us to investigate whether a single-dose injection of interferon-gamma (IFN-γ) expressed from HAd5 vector would also offer protection against WEEV in mice. A replication-deficient HAd5 vector (Ad5-mIFNγ) expressing mouse IFN-γ (mIFN-γ) was constructed. A single-dose injection of mice with Ad5-mIFNγ produced a high level of serum mIFN-γ. The production of mIFN-γ in mice was rapid, which was detected as early as 9 h after injection. A single-dose injection of Ad5-mIFNγ either before or immediately after WEEV challenge delayed the progression of WEEV infection and extended the time of survival. However, a single-dose injection of Ad5-mIFNγ did not improve the overall survival rate of the challenged mice. Thus, a single-dose injection of mIFN-γ expressed from HAd5 vector offers partial protection against the lethal infection of WEEV in mice. This work is part of an ongoing Technology Investment Fund (TIF) project of DRDC entitled "Development of adenoviral vector technology for post-exposure protection against viral threats."

R□sum□

Le virus de l□enc□phalite □quine de l□ouest (VEEO) a le potentiel d□agir comme un agent biologique et de bioterrorisme. Le virus en a□rosol peut se transmettre facilement aux humains. On a un besoin urgent de vaccins □dose unique et action rapide et de th□rapeutiques contre le VEEO en cas d□attaque bioterroriste ou de guerre biologique. On a d□j□trouv□ qu□une injection □dose unique d□interf□ron alpha (IFN-α) exprim□e □partir de vecteur d□ad□novirus humain du type 5 (HAd5) prot□ge rapidement et compl□tement les souris contre le VEEO. Ce r□sultat nous a incit□s □examiner si l□injection □dose unique d□interf□ron-gamma (IFN-γ) exprim□□partir du vecteur HAd5 prot□geait aussi contre le VEEO chez les souris. Une r□plication du vecteur d□ficient de HAd5 (Ad5-mIFNγ) exprimant une souris IFN-γ (mIFN-γ) a □t□construite. Une injection □dose unique sur une souris ayant Ad5-mIFNγ a produit un haut niveau de s□rum mIFN-γ. La production de mIFN-γ chez la souris a □t□rapide et d□tect□e 9 heures apr□s l□injection. Un injection □dose unique d□Ad5-mIFNγ soit avant ou imm□diatement apr□s le test de provocation au VEEO a retard□la progression de l□infection VEEO et a □tendu la dur□e de survie. Une injection □dose unique d□Ad5-mIFNγ n□a cependant pas suffi □am□liorer le taux g□n□ral de survie des souris soumises au test de provocation. Une injection □dose unique de mIFN-γ exprim□e □partir du vecteur HAd5 n□offre donc qu□une protection partielle contre l□infection l□tale du VEEO chez les souris. Ces travaux font partie d□un projet du Fonds d□investissement technologique (FIT) de RDDC appel□: □D□veloppement de la technologie des vecteurs d'ad□novirus pour la protection post-exposition contre les menaces virales. □

Executive Summary

Interferon-gamma gene delivery partially protects mice from the lethal infection of western equine encephalitis virus

Josh Q.H. Wu, Nicole D. Barab□, Damon Chau, Aaron Sha, Andrew Marshall;
DRDC Suffield TR 2008-224; Defence R&D Canada □ Suffield.

Introduction or background:

Western equine encephalitis virus (WEEV) is transmitted through mosquitoes and causes serious and often fatal infection of the central nervous system in humans. Besides its importance as a mosquito-borne pathogen for humans, WEEV is listed as a Category B bioterrorism agent by the Centers for Disease Control and Prevention and by the National Institute for Allergy and Infectious Diseases. Current medical countermeasures, however, are non-existent. Consequently, DRDC Suffield is pursuing development of single-dose, fast-acting therapeutics and vaccines which could provide rapid protection against WEEV in the event of bioterrorism attack or biowarfare. To this end, we have used an adenovirus, a harmless common cold virus, as a vector for the delivery of therapeutics and vaccines. Previously, we found that type I interferon, interferon alpha (IFN-α), delivered by adenovirus vector provides complete protection against lethal dose challenge of WEEV in mice. These results prompted us to evaluate the efficacy of adenovirus-mediated expression of interferon gamma (IFN-γ) against WEEV infection.

Results:

A human adenovirus vector expressing mouse interferon gamma (Ad5-mIFNγ) was made through recombinant DNA technology. Cells inoculated with Ad5-mIFN secreted mouse interferon gamma (mIFN-γ) and inhibited the replication of both WEEV and Venezuelan equine encephalitis virus. Mice given a single-dose injection of Ad5-mIFNγ produced a high level of serum mIFN-γ as early as 9 h after the injection. Mice given the Ad5-mIFN injection had extended survival time and less severe infection than mice given a control vector. However, a single-dose injection of Ad5-mIFNγ did not offer complete protection which has been shown for the adenovirus vector expressing mouse IFN-α.

Significance:

In conclusion, our study suggests that the IFN-γ gene delivery by adenovirus vector only provides partial protection against lethal infection of WEEV in mice.

Future plans:

We will focus our future experiments on testing multiple-dose injections and on using different injection routes such as intranasal administration to improve the efficacy of adenovirus-mediated IFN-γ gene delivery.

Sommaire

Interferon-gamma gene delivery partially protects mice from the lethal infection of western equine encephalitis virus

Josh Q.H. Wu, Nicole D. Barab□, Damon Chau, Aaron Sha, Andrew Marshall;
DRDC Suffield TR 2008-224; R & D pour la d□fense Canada □ Suffield.

Introduction ou ant□c□dents:

Le virus de l□enc□phalite □quine de l□ouest (VEEO) est transmis par les moustiques et cause une infection grave et souvent fatale du syst□me nerveux central chez les humains. Le VEEO est non seulement important chez les humains comme pathog□ne provenant de moustiques mais le virus est aussi list□comme un agent de bioterrorisme de la cat□gorie B par le Centers for Disease Control and Prevention et par le National Institute for Allergy and Infectious Diseases. Il n□existe actuellement aucune contremesure m□dicale. De ce fait, RDDC Suffield poursuit le d□veloppement de th□rapeutiques et de vaccins □dose unique et action rapide capables de prot□ger rapidement contre le VEEO en cas d□attaque bioterroriste ou de guerre biologique. On a, dans ce but, utilis□un ad□novirus comme vecteur, un virus grippal courant et inoffensif, pour administrer les th□rapeutiques et les vaccins. On avait trouv□auparavant que ce type I d□interf□ron alpha (IFN-α), conduit par le vecteur de l□ad□novirus fournit une protection compl□te contre la dose l□tale du test de provocation du VEEO chez les souris. Ces r□sultats nous ont incit□s □ □valu□l□efficacit□contre l□infection VEEO de l□expression d□interf□ron gamma (IFN-γ) exprim□e □partir d□ad□novirus.

R□sultats:

Un vecteur d□ad□novirus humain exprimant l□interf□ron gamma (Ad5-mIFNγ) de souris a □t□ cr□□au moyen de la technologie d□ADN recombinant. Des cellules inocul□es avec Ad5-mIFN ont secr□t□l□interf□ron gamma (mIFNγ) de souris et ont inhib□la r□plication du VEEO et du virus enc□phalomy□lite du cheval du Venezuela. Les souris qui ont re□u une injection □ dose unique d□Ad5-mIFNγ ont produit un haut niveau de s□rum mIFN-γ d□s 9 heures apr□s l□injection. Les souris ayant re□u l□injection Ad5-mIFN ont eu une dur□e de vie □tendue et une infection moins grave que les souris ayant re□u le vecteur t□moin. Une injection □dose unique d□Ad5-mIFNγ n□a cependant pas offert la protection compl□te montr□e par le vecteur d□ad□novirus exprimant la souris IFN-α.

Port□e des r□sultats:

En conclusion, notre □tude sugg□re que la livraison g□n□tique IFN-γ par le vecteur d□ad□novirus ne produit qu□une protection partielle contre l□infection l□tale du VEEO chez les souris.

Perspectives d□avenir:

Nous concentrerons nos exp□riences futures sur les tests d□injections □doses multiples et sur l□utilisation de diff□rentes routes d□injections telles que l□administration intranasale pour am□liorer l□efficacit□de la livraison du g□ne IFN-γ exprim□□partir d□ad□novirus.

Table of Contents

List of Figures

Acknowledgements

We thank animal caretakers at DRDC Suffield and our BSL-3 staff for their assistance with mouse experiments. We are also grateful to Erica Chan-Wong and Ying-Min Huang for help in the construction of Ad5-mIFNγ and Ad5-Empty. This project is supported by the Technology Investment Fund of DRDC.

Introduction

Western equine encephalitis virus (WEEV) is a single-stranded, positive-sense RNA virus which belongs to the *Alphavirus* genus of the *Togaviridae* family [1]. The virus was first isolated in 1930 from infected horses in the San Joaquin Valley of California [2] and later found to be distributed in the western two-thirds of North America and in Central and South America. In North America, WEEV is maintained in a cycle involving the mosquito vector of *Culex tarsalis* and the vertebrate hosts of domestic and passerine birds, such as house sparrows and finches. Humans and equines are infected by the virus through mosquito bites. Signs and symptoms of human WEEV infection range from mild flu-like illness to severe encephalitis with the overall case fatality rate of 3% [1].

Outbreaks of WEEV had serious impact on both human and animal health. During a WEEV outbreak in the 1930s, over 300,000 horses and mules were infected in the United States, while 52,500 infected horses - with 15,000 deaths- were reported in the Canadian prairie province Saskatchewan alone [3]. In 1952, there were 375 confirmed human cases with nine deaths reported in California [4]. In recent years, human and equine cases of WEEV infection have declined dramatically in western North America despite the consistent isolation of the virus from mosquitoes and wild birds [5]. The reason for the decline of WEEV infection in both humans and equine is not fully understood. A recent study suggests that the decrease in WEEV virulence since the 1950s could contribute to the decline of human and equine cases [6]. It could also be due to the reduction of horse population after the modernization of agriculture and transportation.

Besides its importance as a mosquito-borne pathogen for humans and equines, WEEV is a potential biological and bioterrorism agent [7] due to the ease of transmission via the aerosol route with high mortality [8, 9] and the ease of production in large quantity [10]. WEEV is listed as a Category B bioterrorism agent by the Centers for Disease Control and Prevention and by the National Institute for Allergy and Infectious Diseases.

Current medical countermeasures against the biothreat of WEEV are non-existent. No antiviral drugs are available for treatment of WEEV infection and no licensed vaccines are available for prevention. Several experimental vaccines have been reported to be effective against WEEV. A formalin-inactivated WEEV vaccine approved by the U.S. Food and Drug Administration as an Investigational New Drug is available only to the laboratory workers who are at risk of exposure to the virus during their work [11]. Live attenuated vaccine candidates for WEEV provide protection against WEEV challenge in a baby chicken model of WEEV infection [12]. A DNA vaccine for WEEV also offers protection against the lethal challenge of WEEV in mice [13].

We have been focusing on development of single-dose, fast-acting therapeutics and vaccines which could be used to provide rapid protection against WEEV in the event of bioterrorism attack or biowarfare. To this end, we have established a lethal, intranasal challenge model of WEEV in mice [14] and used adenovirus as a vector for the delivery of therapeutics and vaccines. Adenovirus is a nonenveloped, double-stranded DNA virus. Because of its high efficiency for cell entry and the ease to manipulate adenoviral genome, adenovirus has been extensively used as a vector for the delivery of foreign genes [15]. The most commonly used

adenovirus for vector development is human adenovirus type 5 (HAd5). HAd5 vector lacks the E1 and E3 regions, which makes the vector replication deficient. We have demonstrated that a HAd5-vectored WEEV vaccine rapidly prevents WEEV infection in mice after a single-dose vaccination [16, 17]. We and others have also explored the potential of interferons and their inducers for prevention and treatment of WEEV infection [18-20]. We found that type I interferon, interferon alpha (IFN-α), delivered by HAd5 vector provides complete protection against lethal dose challenge of WEEV in mice [18]. These results prompted us to evaluate the efficacy of HAd5-mediated expression of interferon gamma (IFN-γ) against WEEV infection.

IFN-γ is a type II interferon which is secreted by natural killer cells and activated T lymphocytes upon viral infection. IFN-γ directly inhibits the replication of a variety of viruses, including herpes simplex virus [21, 22], hepatitic C virus [23], foot-and-mouth disease virus [24], measles virus [25], and Japanese encephalitis virus [26]. Besides its direct antiviral activity, IFN-γ modulates immune responses through promoting CD4+ T helper cell type 1 (T_H1) lymphocytes to eliminate intracellular pathogens, inducing the expression of MHC class I and II on cell surfaces, stimulating antigen-presenting cells, and regulating IgG heavy chain switching [27]. IFN-γ regulates the expression of more than 100 of IFN-stimulated genes (ISGs) [28]. Among them, the genes encoding nitric oxide synthase (iNOS) and indoleamine 2,3-dioxygenase (IDO) appear to be the major contributors for the direct antiviral activity of IFN-γ [25, 26, 29, 30]. To regulate the expression of ISGs, IFN-γ first binds to its receptor on cell surface. The binding activates the Janus kinase (JAK) and leads to assemble the homodimers of signal transducer and activator of transcription 1 (STAT1) proteins. The STAT1 homodimers then translocate to the nucleus and bind to gamma-activated sequence elements within the promoter region of the ISGs [31].

The role of IFN-γ in control of alphaviral infection has been reported in several studies. IFN-γ has been shown to directly inhibit the replication of Sindbis virus, a model pathogen of the alphaviruses [32]. Mice lacking the receptors for IFN-α, -β, and -γ are significantly more susceptible to fatal Sindbis virus infection than mice lacking the receptors for IFN-α and -β only, suggesting that IFN-γ is directly involved in the innate immune response against alphaviral infection [33]. Treatment of the mice that lack the receptors for IFN-α and -β with recombinant IFN-γ significantly extended the survival of these mice [33]. IFN-γ has been found to mediate the noncytolytic clearance of Sindbis virus from neurons [34-36]. So far, little is known about the role of IFN-γ in control of WEEV infection. Therapeutic potential of adenovirus-mediated delivery of IFN-γ gene has been demonstrated in animal models for progressive pulmonary tuberculosis [37] and foot-and-mouth disease [24]. Recently, adenovirus-mediated delivery of IFN-γ gene has been proven safe after being tested in non-human primates [38]. Based on these findings, we investigated whether single-dose injection of HAd5 vector expressing mouse IFN-γ (mIFN-γ) would inhibit the replication of WEEV in cells and protect mice against lethal challenge with WEEV.

Materials and Methods

Cells and viruses

Vero, HEK293 and L929 cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, and antibiotics-antimycotics. A HAd5 vector expressing mouse IFN-α (mIFN-α) was constructed from a previous study [18]. The 71V-1658 strain of WEEV was provided by the Centers for Disease Control and Prevention and the Fleming strain of WEEV was purchased from the ATCC [14]. The TC-83 strain of Venezuelan equine encephalitis virus (VEEV) was obtained from Dr. George Ludwig (the United States Army Medical Research Institute of Infectious Disease, Frederick, MD). Seed stocks of WEEV and VEEV were made by a single cycle of amplification of the original viruses in Vero cells. The titers of the seed virus stocks were determined by plaque assay in Vero cells as described previously [16]. All of the experiments with WEEV were carried out in the Biosafety Level 3 laboratory at Defence Research and Development Canada Suffield (DRDC Suffield) in compliance with the guidelines of Health Canada and the Canadian Food Inspection Agency.

Construction of Ad5-mIFNγ and Ad5-Empty

Ad5-mIFNγ expressing mIFN-γ was constructed using AdEasy system [39]. To make Ad5-mIFNγ, mIFN-γ gene was isolated from plasmid pORF5-mIFNγ (InvivoGen) by *Eco*RI-*Nhe*I digestion. The *Eco*RI-*Nhe*I fragment containing mIFN-γ gene was cloned into the compatible sites of pCI (Promega) to yield pCI-mIFNγ. A *Sgr*AI-*Nhe*I fragment was isolated from pCI-mIFNγ and ligated into *Xma*I-*Xha*I-treated pUC19 to generate pUC-mIFNγ. A *Kpn*I-*Hind*III fragment was isolated from pUC-mIFNγ and cloned into the compatible sites of pShuttle-CMV (Qbiogene) to generate transfer plasmid pSCMV-mIFNγ. An infectious plasmid, pAd5-mIFNγ, was obtained by co-transformation of *Pme*I-linearized pSCMV-mIFNγ and pAdEasy-1 (Qbiogene) into *E. coli* BJ5183 strain (Qbiogene). Ad5-mIFNγ was generated by transfection of *Pac*I-digested pAd5-mIFNγ into HEK293 cells with Lipofectamine 2000 (Invitrogen). Ad5-Empty was constructed by co-transformation of pAdEasy-1 and *Pme*I-linearized pShuttle-CMV into BJ5183 to generate pAd5-Empty and the virus was generated by transfection of *Pac*I-digested pAd5-Empty into HEK293 cells. Ad5-mIFNγ and Ad5-Empty were amplified in HEK293 cells and purified by Adeno-X Mega Purification Kit (Clontech). The titers of the purified viruses were determined by the tissue culture infectious dose 50 (TCID50) assay [40].

PCR characterization of Ad5-mIFNγ

To verify the presence of mIFN-γ gene in Ad5-mIFNγ, DNA was extracted from purified Ad5-mIFNγ using DNeasy Tissue Kits (QIAGEN). mIFN-γ gene was amplified using two sets of primers: one containing forward primer f-IFN (5'-CAC CAT GGC CAA CGC TAC AC-3') and reverse primer r-IFN (5'-CGC TTC CTG AGG CTG GAT TC-3') and another

containing forward primer f-Emp (5′-GGC GTG GAT AGC GGT TTG AC-3′) and reverse primer r-Emp (5′-AAA CGA GTT GGT GCT CAT GG-3′). PCR was carried out by HotStarTaq DNA polymerase (QIAGEN) with 15 min of initial activation at 95 °C, 30 cycles of 45 s of denaturation at 94 °C, 45 s of annealing at 58 °C, and 1 min of extension at 72 °C. A final 10 min of extension at 72 °C was carried out after the 30-cycle amplification. The purified viral DNA from Ad5-Empty was also amplified using the same primer sets. A 5-µl aliquot of the PCR product was analyzed by electrophoresis in 0.8% agarose gel.

Western immunoblot

The expression of mIFN-γ in cells infected with Ad5-mIFNγ was detected by Western immunoblot. HEK293 cells seeded in T25 flasks were infected with Ad5-mIFNγ or Ad5-Empty at a multiplicity of infection (MOI) of 1 in a total of 4 ml of DMEM containing 2% FBS. A total of 4 ml of supernatant was collected from each infected flask at 24 h after infection. The supernatant was concentrated to about 250 µl by Amicon Ultra-15 (Millipore). The concentrated supernatant was mixed with sodium dodecyl sulfate (SDS) sample buffer, heated at 95 °C for 5 min, and loaded onto 10% Bis-Tris SDS-polyacrylamide gel along with the MultiMark multi-colored protein standard from Invitrogen. Proteins were electrophoresed at 100 V for 1.5 h and transferred to a nitrocellulose membrane at 30 V for 1 h. The membrane was incubated with a rabbit polyclonal antibody specific for mIFN-γ (PBL Biomedical Laboratores) for 1 h at room temperature and developed by WesternBreeze Kit (Invitrogen).

In vitro virus inhibition assay

L929 cells seeded in 12-well plates were inoculated in triplicate with purified Ad5-mIFNγ, Ad5-mIFNα, or Ad5-Empty at an MOI of 10. At 24 h after infection, cells were infected with the 71V-1658 strain of WEEV, the Fleming strain of WEEV, or the TC-83 strain of VEEV at an MOI of 0.1. After incubation at 37 °C for 48 h, supernatants from the infected cells were collected and titers of VEEV and WEEV in the supernatants were measured by plaque assay in Vero cells.

Measurement of serum mIFN-γ in mice given Ad5-mIFNγ

Female BALB/c mice (17-20 *g*) were obtained from the pathogen-free breeding colony at the animal care facility of DRDC Suffield. The original breeding pairs were purchased from Charles River Canada. Animal experiment protocols were approved by the DRDC Animal Care Committee. The guidelines of the Canadian Council on Animal Care were followed for animal care and experimentation. To determine the time course of mIFN-γ expression in mice given Ad5-mIFNγ, mice were each given intramuscular (IM) injection of a single-dose of 10^7 plaque forming units (PFU) of Ad5-mIFNγ. Control mice were given the same dose of Ad5-Empty. Serum was collected at 3, 9, 15, 24, 72, 120, and 168 h (3 mice for each time point) after injection. Serum collected from each mouse at each time point was pooled and serum concentration of mIFN-γ was determined by the Mouse Interferon Gamma ELISA Kit from PBL Biomedical Labs.

Mouse challenge and protection studies

We evaluated the efficacy of Ad5-mIFNγ for protection against lethal challenge of WEEV in mice. In the first experiment, female BALB/c mice (17-20 *g*) were each given a single-dose, IM injection of 10^8 PFU of Ad5-mIFNγ. Control mice were given 10^8 PFU of Ad5-Empty or 10^7 PFU of Ad5-mIFNα. At 24 h after injection, mice were each challenged intranasally with a 25 LD50 dose of the WEEV 71V-1658 strain. The challenged mice were monitored daily for 14 days for the survival and the severity of infection which was recorded using a 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; and 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. In the second experiment, each mouse was first challenged intranasally with a 25 LD50 dose of 71V-1658. Immediately after challenge, mice were each given a single-dose, IM injection of 10^8 PFU of Ad5-mIFNγ. Control mice were each given 10^8 PFU of Ad5-Empty or 10^7 PFU of Ad5-mIFNα. Mice were monitored daily for 14 days for the survival and the severity of infection using the same scoring system.

Statistic analysis

PRISM□ 4 program (GraphPad Software Inc.) was used for statistical analysis. A *P* value of less than 0.05 is considered to be significant. The yield of VEEV among multiple groups was compared by one-way analysis of variance and the yield of WEEV between two groups was compared by the two-tailed, unpaired *t* test. The *t* test was also used to compare the mean survival time between mice given Ad5-mIFNγ and mice given Ad5-Empty. Clinical scores between mice given Ad5-mIFNγ and mice given Ad5-Empty were compared by the Mann-Whitney test. The percentage of survival of the challenged mice was plotted by Kaplan-Meier survival curves.

Results

Construction and characterization of HAd5 vector expressing mIFN-γ

We constructed a recombinant HAd5, Ad5-mIFNγ, to deliver and express mIFN-γ gene (Fig. 1). The expression of mIFN-γ gene was driven by the immediate-early promoter of cytomegalovirus. We also constructed another recombinant HAd5, Ad5-Empty, which does not contain mIFN-γ gene. This virus was used as a control throughout the study (Fig. 1A). Both Ad5-mIFNγ and Ad5-Empty are replication-defective because of the deletion of the E1 region that is required for adenovirus replication. The gene encoding mIFN-γ was detected in Ad5-mIFNγ by PCR (Fig. 2, lanes 1 and 4). To determine whether mIFN-γ is secreted from cells inoculated with Ad5-mIFNγ, HEK293 cells were inoculated with Ad5-mIFNγ at an MOI of 10. Cell culture media were collected at 24 h after inoculation and mIFN-γ was probed by a specific polyclonal antibody against mIFN-γ. As shown in Fig. 3, mIFN-γ was detected in media from Ad5-mIFNγ-inoculated cells, but not from mock or Ad5-Empty-inoculated cells, confirming mIFN-γ was expressed from the cells inoculated with Ad5-mIFNγ.

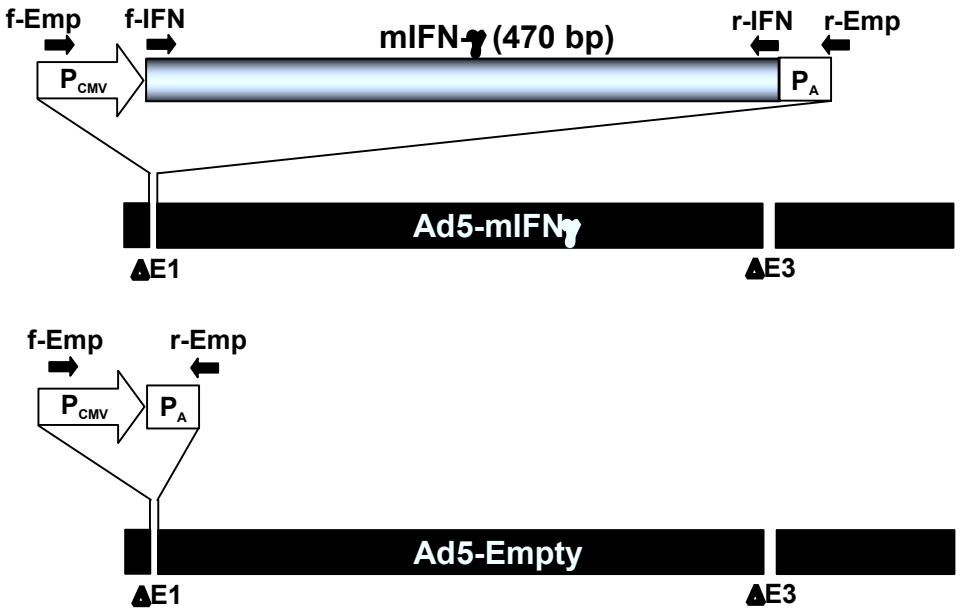

Figure 1. Schematic diagrams of Ad5-mIFNγ and Ad5-Empty. *ΔE1 and ΔE3 indicate the deletions of E1 and E3 coding regions of the adenoviral genome. PCR primers for the detection of mIFN-γ gene are shown as black arrows. P_{CMV} represents the immediate-early promoter of cytomegalovirus. P_A represents the polyadenylation signal of SV40.*

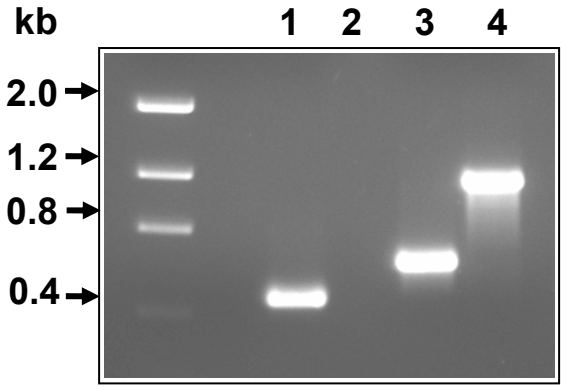

Figure 2. PCR detection of mIFN-γ gene in the Ad5-mIFNγ genome. *PCR was carried out for the viral DNA extracted from Ad5-mIFNγ (lanes 1 and 4) and Ad5-Empty (lanes 2 and 3). The PCR products were analyzed on 0.8% agarose gel. Lanes 1 and 2: PCR products amplified using primers f-IFN and r-IFN; Lanes 3 and 4: PCR products amplified using primers f-Emp and r-Emp.*

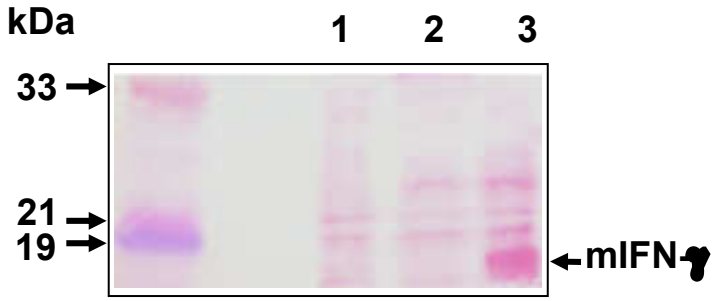

Figure 3. Secretion of mIFN-γ in cells inoculated with Ad5-mIFNγ. *Supernatants were collected from HEK293 cells at 24 h after inoculation with Ad5-Empty or Ad5-mIFNγ. The proteins in the supernatants were separated by 10% SDS-PAGE and probed with a rabbit polyclonal antibody against mIFN-γ. Lane 1: supernatant collected from mock-infected cells; Lane 2: supernatant collected from Ad5-Empty-infected cells; and Lane 3: supernatant collected from Ad5-mIFNγ infected cells.*

Inhibition of alphavirus replication in cells inoculated with Ad5-mIFNγ

We next examined whether cells inoculated with Ad5-mIFNγ could inhibit the replication of WEEV and another alphavirus, VEEV. L929 cells were inoculated with Ad5-mIFNγ at an MOI of 10. As controls, cells were inoculated with Ad5-Empty or Ad5-mIFNα. At 24 h after inoculation, cells were infected at an MOI of 0.1 with the 71V- 1658 strain of WEEV, the Fleming strain of WEEV, or the TC-83 strain of VEEV, a live-attenuated VEEV vaccine strain. The yield of the viruses in the supernatants collected at 48 h after infection was determined by plaque assay in Vero cells.

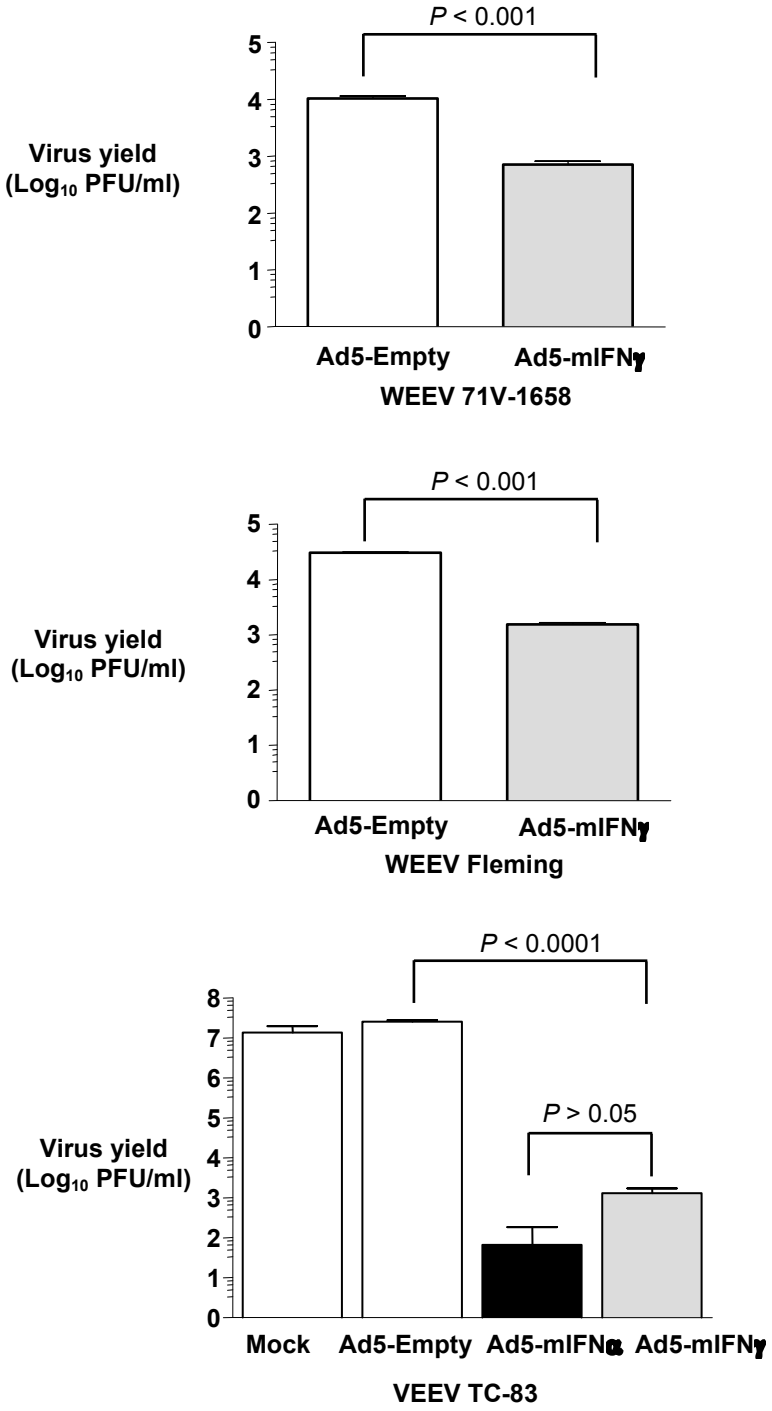

Figure 4. Inhibition of alphaviruses by Ad5-mIFNγ. *L929 cells were inoculated in triplicate with Ad5-mIFNγ, Ad5-mIFNα or Ad5-Empty at an MOI of 10. At 24 h after inoculation, cells were infected with WEEV or VEEV at an MOI of 0.1. The yield of viruses was determined by plaque assay in Vero cells. Error bars represent the SD of virus concentration.*

As shown in Fig. 4, moderate inhibition of WEEV was seen in cells inoculated with Ad5-mIFNγ. The yield of 71V-1658 or Fleming in the supernatants from Ad5-mIFNγ-treated cells is about 1-log less than that in the supernatants from Ad5-Empty-treated cells ($P < 0.001$). The yield of VEEV TC-83 in the supernatant collected from Ad5-mIFNγ-treated cells is 4-log less than that in the supernatant from Ad5-Empty-treated cells ($P < 0.0001$). The level of the inhibition of TC-83 by Ad5-mIFNγ is similar to that by Ad5-mIFNα ($P > 0.05$). The higher level inhibition of TC-83 by Ad5-mIFNγ may be due to the high sensitivity of TC-83 to IFNs [41].

Time course of mIFN-γ production in mice after a single-dose injection of Ad5-mIFNγ

To know how fast and how long mIFN-γ could be expressed in mice given a single-dose injection of Ad5-mIFNγ, we studied the time course of the mIFN-γ production in Ad5-mIFNγ-injected mice. Mice were each given an IM injection of 10^7 PFU of Ad5-mIFNγ or Ad5-Empty. Serum samples were collected at 3, 9, 15, 24, 72, 120, and 168 h after injection and measured for mIFN-γ by ELISA. Serum mIFN-γ was detected as early as 9 h after injection and reached a high level at 24 h after injection (Fig. 5). The level of serum mIFN-γ, however, dropped significantly with very little detectable mIFN-γ by 72 h after injection. The result suggests that the mIFN-γ production in mice given a single-dose Ad5-mIFNγ injection is rapid but does not last for a long period of time.

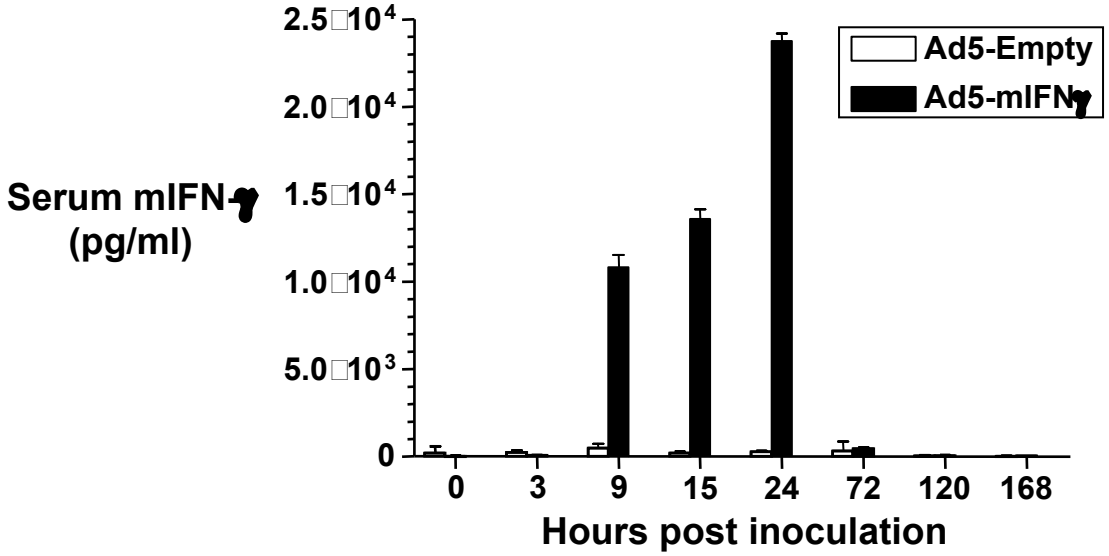

Figure 5. Time course of mIFN-γ production in mice inoculated with Ad5-mIFNγ. *BALB/c mice were each given a single-dose, intramuscular inoculation of 10^7 PFU of Ad5-mIFNγ or Ad5-Empty. Serum was collected at indicated time points (3 mice for each time point). The concentrations of mIFN-γ in pooled sera from each time point were determined in triplicate by ELISA. Error bars represent the SD of serum concentration of mIFN-γ.*

Efficacy of Ad5-mIFNγ against lethal challenge of WEEV in mice

Having demonstrated that Ad5-mIFNγ inhibited the replication of WEEV in cells and that Ad5-mIFNγ produced mIFN-γ in mice, we evaluated the efficacy of Ad5-mIFNγ against the lethal infection of WEEV in a mouse intranasal challenge model developed previously in our laboratory [14]. We chose the intranasal challenge model because it partially resembles the aerosol route infection of WEEV. To test the efficacy of Ad5-mIFNγ, mice were given a single-dose, IM injection of 10^8 PFU of Ad5-mIFNγ. Control mice were given a single-dose of either 10^8 PFU of Ad5-Empty or 10^7 PFU of Ad5-mIFNα. At 24 h after injection, mice were each challenged intranasally with a lethal dose (25 LD_{50}) of the 71V-1658 strain of WEEV. The challenged mice were monitored up to 14 days for survival and severity of the infection. In another experiment, mice were first challenged intranasally with a 25 LD_{50} dose of 71V-1658. Immediately after challenge, mice were given a single-dose, IM injection of 10^8 PFU of Ad5-mIFNγ, 10^8 PFU of Ad5-Empty or 10^7 PFU of Ad5-mIFNα.

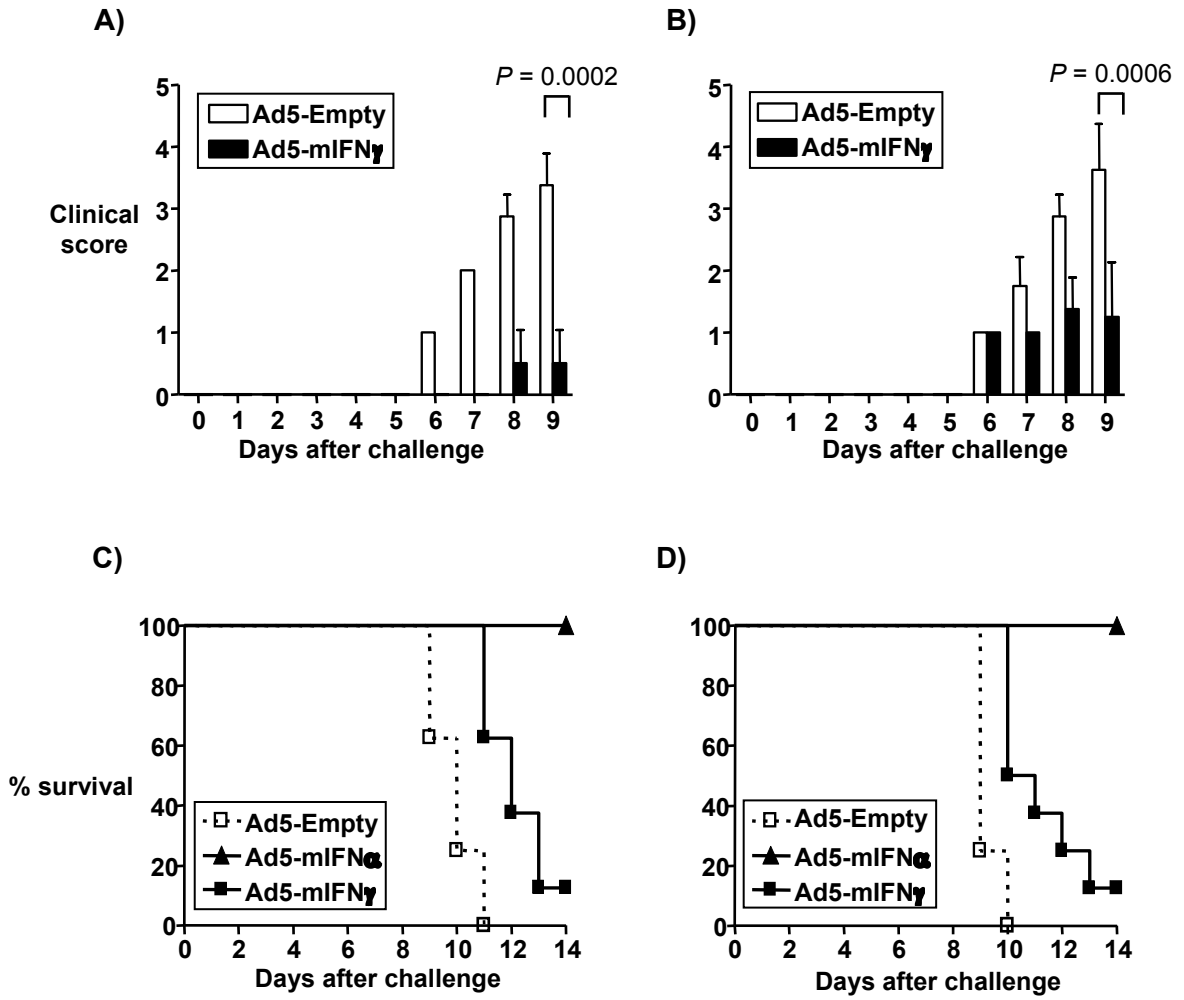

Figure 6. Efficacy of Ad5-mIFNγ against a lethal dose challenge of WEEV in mice. *A) and C) BALB/c mice (total of 8 per group) were each given a single-dose, intramuscular injection of 10^8 PFU of Ad5-mIFNγ, 10^8 PFU of Ad5-Empty, or 10^7 PFU of Ad5-mIFNα. At 24 h after injection, mice were challenged intranasally with a lethal dose (25 LD_{50}) of WEEV 71V-1658. B) and D) BALB/c mice (total of 8 per group) were first challenged with a 25 LD_{50} dose of 71V-1658. Immediately after challenge, mice were each given a single-dose injection of 10^8 PFU of Ad5-mIFNγ, 10^8 PFU of Ad5-Empty, or 10^7 PFU of Ad5-mIFNα. All of the challenged mice were monitored for 14 days for clinical signs of infection and survival.*

A single-dose injection of Ad5-mIFNγ before or immediately after challenge delayed the progression of WEEV infection. Mice given Ad5-mIFNγ at 24 h before challenge did not show any signs of infection until day 8 after the challenge whereas all of the mice given Ad5-Empty control had severe infection at this time (Fig. 6A). The mean clinical score on day 9 after challenge in the mice given Ad5-mIFNγ was much lower than that in the mice given Ad5-Empty (0.5 ± 0.5 compared to 3.4 ± 0.5; $P = 0.0002$). The lower mean clinical score on day 9 after challenge was also observed in mice given Ad5-mIFNγ immediately after challenge (1.3 ± 0.9 in Ad5-mIFNγ compared to 3.6 ± 0.7 in Ad5-Empty; $P = 0.0006$) (Fig. 6B).

A single-dose injection of Ad5-mIFNγ also extended the survival time of the challenged mice. The mean survival time in mice given Ad5-mIFNγ before challenge was significantly longer than that in mice given Ad5-Empty (12.1 ± 0.4 days compared to 9.9 ± 0.3 days; $P = 0.0005$). Similarly longer survival time was also seen in mice given a single-dose injection of Ad5-mIFNγ immediately after challenge (11.2 ± 0.6 days in Ad5-mIFNγ compared to 9.3 ± 0.2 days in Ad5-Empty; $P = 0.004$). It appears, however, that a single-dose injection of Ad5-mIFNγ did not offer complete protection which has been shown for Ad5-mIFNα [18]. As indicated in Figs. 6C and 6D, by day 14 after challenge, seven out of eight mice given Ad5-mIFNγ before or immediately after challenge died whereas all the control mice given a single-dose injection of Ad5-mIFNα survived and remained healthy. Taken together, these results suggest that a partial protection was achieved after a single-dose injection of Ad5-mIFNγ.

Discussion

In this report, we evaluated the efficacy of HAd5-mediated mIFN- γ gene delivery against WEEV infection in a mouse lethal challenge model. Three major findings were obtained from the study. First, the expression of mIFN-γ by HAd5 vector in cells inhibited the replication of WEEV and VEEV *in vitro*. Second, a single-dose injection of HAd5 vector expressing mIFN-γ in mice rapidly produced a high level of mIFN-γ. Finally, a single-dose injection of HAd5 vector expressing mIFN-γ partially protected mice against WEEV infection by slowing the progression of the infection and extending the survival time of the challenged mice.

The following several factors may likely contribute to the only partial protection being conferred by Ad5-mIFNγ after a single-dose administration. The first is the low sensitivity of WEEV to mIFN-γ treatment (Fig. 2). The second is the rapid depletion of mIFN-γ from mice after Ad5-mIFNγ injection. As shown in Fig. 3, the expression of mIFN-γ in mice after a single-dose injection of Ad5-mIFNγ was short lived, lasting less than 3 days. The short-term expression of mIFN-γ in mice after a single-dose injection of Ad5-mIFNγ could result from the inhibition of HAd5 vector by mIFN-γ. Previous studies showed that IFN-γ, but not IFN-α, inhibits the replication of adenovirus [42, 43]. Thus, the mIFN-γ expressed from Ad5-mIFNγ could eliminate Ad5-mIFNγ itself, which in turn leads to the reduced expression of mIFN-γ. A steady, high-level expression of mIFN-γ may be achieved by multiple-dose injections of Ad5-mIFNγ to improve the efficacy of Ad5-mIFNγ. Thirdly, because of the blood-brain barrier, the entry of HAd5 vector into the brain after IM injection is limited, which may result in a low brain concentration of mIFN-γ, one which is insufficient to provide complete inhibition of WEEV replication in the brain. Several approaches have been used to enhance the gene delivery to the brain by HAd5 vector, which include intranasal inoculation [44], *in situ* brain perfusion [45], and the modification of HAd5 vector by re-directing it to the receptor-mediated transcytosis pathway [46].

The partial protection conferred by Ad5-mIFNγ is in contrast to the complete protection provided by Ad5-mIFNα [18]. One explanation for this difference could be due to the major role of IFN-α played in the early control of replication and spread of the alphaviruses [47]. Mice lacking the IFN-α receptor, but not the IFN-γ receptor, are highly susceptible to the lethal infection of VEEV and Sindbis virus [33, 48]. Although IFN-α and IFN-γ both have direct antiviral activity, IFN-α appears to inhibit virus replication in nervous tissue cells more effectively than IFN-γ [49]. IFN-α stimulates the expression of over 300 genes [28], which amongst the genes encoding viperin, the zinc finger antiviral protein (ZAP), ISG15, ISG20, and p56 directly inhibit the replication of the alphaviruses [50]. Alphaviruses such as eastern equine encephalitis virus also encode proteins that determine the sensitivity of virus to IFN-α and the virulence of the viruses in mice [51], further demonstrating that the importance of IFN-α for the inhibition of alphaviral replication.

Another explanation for the difference in protection against WEEV between Ad5-mIFNγ and Ad5-mIFNα could be due to Ad5-mIFNα being able to maintain a high-level expression of mIFN-α in mice for a long period of time. We found that high-level production of mIFN-α in mice could last for at least 5 days after a single-dose, intramuscular injection of Ad5-mIFNα (J.Q.H. Wu, unpublished data). IFN-α in combination with ribavirin is used for treatment of patients with chronic hepatitis C virus infection [52].

The therapeutic potential of IFN-α has also been demonstrated in animal models for VEEV [53] and WEEV [18, 19]. Although IFN-γ is effective to protect against certain viral and bacterial diseases [22, 24, 37, 54], its clinical application is limited to the treatment of chronic granulomatous disorder and severe osteoporosis [55]. IFN-γ mainly involves the regulation of the adaptive immune response. IFN-γ expressed from a viral vector or a DNA plasmid has been shown to enhance the efficacy of viral vaccines [56, 57]. Because of the potent immunoregulatory function of IFN-γ, the delivery of IFN-γ gene by HAd5 vector could also be used as a novel approach to improve the efficacy of WEEV vaccine.

Conclusion

Our study indicates that the IFN-γ gene delivery by HAd5 vector has potential to provide partial protection against lethal infection of WEEV in mice. The efficacy of the IFN-γ gene delivery by HAd5 vector may be improved by giving multiple-dose injections and by using different injection routes such as intranasal administration. Additionally, the IFN-γ gene delivery could be used to enhance the immune response of WEEV vaccine because of the important role of IFN-γ played in the regulation of the adaptive immune response.

References

1. Griffin, D.E. (2007), Alphaviruses. In *Fields Virology*, Volume I, 5th Edition, D.M. Knipe and P.M. Howley, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 1023-1067.

2. Meyer, K.F., Haring, C.M., and Howitt, B. (1931), The Etiology of Epizootic Encephalomyelitis of Horses in the San Joaquin Valley, 1930, *Science,* 74, 227-228.

3. Calisher, C.H. (1994), Medically important arboviruses of the United States and Canada, *Clin Microbiol Rev*. 7, 89-116.

4. Reisen, W.K., and Monath, T.P. (1989), Western equine encephalomyelitis. In T.P. Monath, ed., *The Arboviruses: Epidemiology and Ecology*, Volume 5, (Boca Raton, Fla.: CRC Press), pp. 89-137.

5. Reisen, W.K., Fang, Y., and Brault, A.C. (2008), Limited interdecadal variation in mosquito (Diptera: Culicidae) and avian host competence for Western equine encephalomyelitis virus (Togaviridae: Alphavirus), *Am J Trop Med Hyg,,* 78, 681-686.

6. Forrester, N.L., Kenney, J.L., Deardorf, E.R., Wang, E., and Weaver, S.C. (2008), Virulence studies in extinct and extant lineages of western equine encephalitis virus, In *Abstract of 27th Annual Meeting of American Society for Virology* (Cornell University, Ithaca, New York: ASV), pp. 314-315.

7. Sidwell, R.W., and Smee, D.F. (2003), Viruses of the Bunya- and Togaviridae families: potential as bioterrorism agents and means of control, *Antiviral Res*, 57, 101-111.

8. Hanson, R.P., Sulkin, S.E., Beuscher, E.L., Hammon, W.M., McKinney, R.W., and Work, T.H. (1967), Arbovirus infections of laboratory workers. Extent of problem emphasizes the need for more effective measures to reduce hazards, *Science,* 158, 1283-1286.

9. Reed, D.S., Larsen, T., Sullivan, L.J., Lind, C.M., Lackemeyer, M.G., Pratt, W.D., and Parker, M.D. (2005), Aerosol exposure to western equine encephalitis virus causes fever and encephalitis in cynomolgus macaques, *J Infect Dis*, 192, 1173-1182.

10. Franz, D.R., Jahrling, P.B., Friedlander, A.M., McClain, D.J., Hoover, D.L., Bryne, W.R., Pavlin, J.A., Christopher, G.W., and Eitzen, E.M., Jr. (1997), Clinical recognition and management of patients exposed to biological warfare agents, *JAMA,* 278, 399-411.

11. Hoke, C.H., Jr. (2005), History of U.S. military contributions to the study of viral encephalitis, *Mil Med,* 170, 92-105.

12. Turell, M.J., O'Guinn, M.L., and Parker, M.D. (2003), Limited potential for mosquito transmission of genetically engineered, live-attenuated western equine encephalitis virus vaccine candidates, *Am J Trop Med Hyg,* 68, 218-221.

13. Nagata, L.P., Hu, W.G., Masri, S.A., Rayner, G.A., Schmaltz, F.L., Das, D., Wu, J., Long, M.C., Chan, C., Proll, D. (2005), Efficacy of DNA vaccination against western equine encephalitis virus infection, *Vaccine,* 23, 2280-2283.

14. Nagata, L.P., Hu, W.G., Parker, M., Chau, D., Rayner, G.A., Schmaltz, F.L., and Wong, J.P. (2006), Infectivity variation and genetic diversity among strains of Western equine encephalitis virus, *J Gen Virol,* 87, 2353-2361.

15. Kay, M.A., Glorioso, J.C., and Naldini, L. (2001), Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics, *Nat Med,* 7, 33-40.

16. Wu, J.Q., Barabe, N.D., Chau, D., Wong, C., Rayner, G.R., Hu, W.G., and Nagata, L.P. (2007), Complete protection of mice against a lethal dose challenge of western equine encephalitis virus after immunization with an adenovirus-vectored vaccine, *Vaccine,* 25, 4368-4375.

17. Barabe, N.D., Rayner, G.A., Christopher, M.E., Nagata, L.P., and Wu, J.Q. (2007), Single-dose, fast-acting vaccine candidate against western equine encephalitis virus completely protects mice from intranasal challenge with different strains of the virus, *Vaccine,* 25, 6271-6276.

18. Wu, J.Q., Barabe, N.D., Huang, Y.M., Rayner, G.A., Christopher, M.E., and Schmaltz, F.L. (2007), Pre- and post-exposure protection against Western equine encephalitis virus after single inoculation with adenovirus vector expressing interferon alpha, *Virology,* 369, 206-213.

19. Julander, J.G., Siddharthan, V., Blatt, L.M., Schafer, K., Sidwell, R.W., and Morrey, J.D. (2007), Effect of exogenous interferon and an interferon inducer on western equine encephalitis virus disease in a hamster model, *Virology,* 360, 454-460.

20. Wong, J.P., Nagata, L.P., Christopher, M.E., Salazar, A.M., and Dale, R.M. (2005), Prophylaxis of acute respiratory virus infections using nucleic acid-based drugs, *Vaccine,* 23, 2266-2268.

21. Peng, T., Zhu, J., Hwangbo, Y., Corey, L., and Bumgarner, R.E. (2008), Independent and cooperative antiviral actions of beta interferon and gamma interferon against herpes simplex virus replication in primary human fibroblasts, *J Virol,* 82, 1934-1945.

22. Sainz, B., Jr., and Halford, W.P. (2002), Alpha/Beta interferon and gamma interferon synergize to inhibit the replication of herpes simplex virus type 1, *J Virol,* 76, 11541-11550.

23. Frese, M., Schwarzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O., and Bartenschlager, R. (2002), Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs, *Hepatology,* 35, 694-703.

24. Moraes, M.P., de Los Santos, T., Koster, M., Turecek, T., Wang, H., Andreyev, V.G., and Grubman, M.J. (2007), Enhanced antiviral activity against foot-and-mouth disease virus by a combination of type I and II porcine interferons, *J Virol,* 81, 7124-7135.

25. Obojes, K., Andres, O., Kim, K.S., Daubener, W., and Schneider-Schaulies, J. (2005), Indoleamine 2,3-dioxygenase mediates cell type-specific anti-measles virus activity of gamma interferon, *J Virol,* 79, 7768-7776.

26. Lin, Y.L., Huang, Y.L., Ma, S.H., Yeh, C.T., Chiou, S.Y., Chen, L.K., and Liao, C.L. (1997), Inhibition of Japanese encephalitis virus infection by nitric oxide: antiviral effect of nitric oxide on RNA virus replication, *J Virol,* 71, 5227-5235.

27. Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004), Interferon-gamma: an overview of signals, mechanisms and functions, *J Leukoc Biol, 75,* 163-189.

28. Der, S.D., Zhou, A., Williams, B.R., and Silverman, R.H. (1998), Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays, *Proc Natl Acad Sci U S A,* 95, 15623-15628.

29. Karupiah, G., Xie, Q.W., Buller, R.M., Nathan, C., Duarte, C., and MacMicking, J.D. (1993), Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase, *Science,* 261, 1445-1448.

30. Biron, C.A., and Sen, G.C. (2007), Innate responses to viral infections In D.M. Knipe and P.M. Howley, eds., *Fields Virology*, Volume I, 5th Edition, (Philadelphia: Lippincott Williams & Wilkins), pp. 249-278.

31. van Boxel-Dezaire, A.H., and Stark, G.R. (2007), Cell type-specific signaling in response to interferon-gamma, *Curr Top Microbiol Immunol,* 316, 119-154.

32. Ryman, K.D., White, L.J., Johnston, R.E., and Klimstra, W.B. (2002), Effects of PKR/RNase L-dependent and alternative antiviral pathways on alphavirus replication and pathogenesis, *Viral Immunol,* 15, 53-76.

33. Ryman, K.D., Meier, K.C., Gardner, C.L., Adegboyega, P.A., and Klimstra, W.B. (2007), Non-pathogenic Sindbis virus causes hemorrhagic fever in the absence of alpha/beta and gamma interferons, *Virology,* 368, 273-285.

34. Binder, G.K., and Griffin, D.E. (2001), Interferon-gamma-mediated site-specific clearance of alphavirus from CNS neurons, *Science, 293,* 303-306.

35. Burdeinick-Kerr, R., and Griffin, D.E. (2005), Gamma interferon-dependent, noncytolytic clearance of sindbis virus infection from neurons *in vitro*, *J Virol,* 79, 5374-5385.

36. Burdeinick-Kerr, R., Wind, J., and Griffin, D.E. (2007), Synergistic roles of antibody and interferon in noncytolytic clearance of Sindbis virus from different regions of the central nervous system, *J Virol,* 81, 5628-5636.

37. Mata-Espinosa, D.A., Mendoza-Rodriguez, V., Aguilar-Leon, D., Rosales, R., Lopez-Casillas, F., and Hernandez-Pando, R. (2008), Therapeutic effect of recombinant adenovirus encoding interferon-gamma in a murine model of progressive pulmonary tuberculosis, *Mol Ther,* 16, 1065-1072.

38. Li, Y., Huang, W.L., Liu, R.Y., Zhou, L., Chai, L.P., Li, H.L., Han, H.Y., Huang, B.J., Zeng, M.S., Zhu, X.F. (2008), Evaluation of Long-term Toxicity of Ad/hIFN-gamma, an Adenoviral Vector Encoding Human IFNgamma Gene, in Non-human Primates, *Hum Gene Ther,* ahead of print. doi:10.1089/hgt.2007.180.

39. He, T.C., Zhou, S., da Costa, L.T., Yu, J., Kinzler, K.W., and Vogelstein, B. (1998), A simplified system for generating recombinant adenoviruses, *Proc Natl Acad Sci USA,* 95, 2509-2514.

40. Hierholzer, J.C., and Killington, R.A. (1996), Virus isolation and quantitation. In B.W.J. Mahy and H.O. Kangro, eds., *Virology Methods Manual,* (San Diego: Academic Press), pp. 25-46.

41. White, L.J., Wang, J.G., Davis, N.L., and Johnston, R.E. (2001), Role of alpha/beta interferon in Venezuelan equine encephalitis virus pathogenesis: effect of an attenuating mutation in the 5' untranslated region, *J Virol,* 75, 3706-3718.

42. Mistchenko, A.S., Diez, R.A., and Falcoff, R. (1987), Recombinant human interferon-gamma inhibits adenovirus multiplication without modifying viral penetration, *J Gen Virol,* 68 (Pt 10), 2675-2679.

43. Mistchenko, A.S., and Falcoff, R. (1987), Recombinant human interferon-gamma inhibits adenovirus multiplication *in vitro*, *J Gen Virol,* 68 (Pt 3), 941-944.

44. Lemiale, F., Kong, W.P., Akyurek, L.M., Ling, X., Huang, Y., Chakrabarti, B.K., Eckhaus, M., and Nabel, G.J. (2003), Enhanced mucosal immunoglobulin A response of intranasal adenoviral vector human immunodeficiency virus vaccine and localization in the central nervous system, *J Virol,* 77, 10078-10087.

45. Toyobuku, H., Sai, Y., Kagami, T., Tamai, I., and Tsuji, A. (2003), Delivery of peptide drugs to the brain by adenovirus-mediated heterologous expression of human oligopeptide transporter at the blood-brain barrier, *J Pharmacol Exp Ther,* 305, 40-47.

46. Tang, Y., Han, T., Everts, M., Zhu, Z.B., Gillespie, G.Y., Curiel, D.T., and Wu, H. (2007), Directing adenovirus across the blood-brain barrier via melanotransferrin (P97) transcytosis pathway in an *in vitro* model,. *Gene Ther,* 14, 523-532.

47. Griffin, D.E. (2003), Immune responses to RNA-virus infections of the CNS, *Nat Rev Immunol*, 3, 493-502.

48. Grieder, F.B., and Vogel, S.N. (1999), Role of interferon and interferon regulatory factors in early protection against Venezuelan equine encephalitis virus infection, *Virology,* 257, 106-118.

49. Schijns, V.E., Van der Neut, R., Haagmans, B.L., Bar, D.R., Schellekens, H., and Horzinek, M.C. (1991), Tumour necrosis factor-alpha, interferon-gamma and interferon-beta exert antiviral activity in nervous tissue cells, *J Gen Virol,* 72 (Pt 4), 809-815.

50. Zhang, Y., Burke, C.W., Ryman, K.D., and Klimstra, W.B. (2007), Identification and characterization of interferon-induced proteins that inhibit alphavirus replication, *J Virol,* 81, 11246-11255.

51. Aguilar, P.V., Adams, A.P., Wang, E., Kang, W., Carrara, A.S., Anishchenko, M., Frolov, I., and Weaver, S.C. (2008), Structural and nonstructural protein genome regions of eastern equine encephalitis virus are determinants of interferon sensitivity and murine virulence, *J Virol,* 82, 4920-4930.

52. Feld, J.J., and Hoofnagle, J.H. (2005), Mechanism of action of interferon and ribavirin in treatment of hepatitis C, *Nature,* 436, 967-972.

53. Lukaszewski, R.A., and Brooks, T.J. (2000), Pegylated alpha interferon is an effective treatment for virulent venezuelan equine encephalitis virus and has profound effects on the host immune response to infection, *J Virol,* 74, 5006-5015.

54. Segura, S., Gamazo, C., Irache, J.M., and Espuelas, S. (2007), Gamma interferon loaded onto albumin nanoparticles: *in vitro* and *in vivo* activities against *Brucella abortus*, *Antimicrob Agents Chemother,* 51, 1310-1314.

55. Young, H.A., and Bream, J.H. (2007), IFN-gamma: recent advances in understanding regulation of expression, biological functions, and clinical applications, *Curr Top Microbiol Immunol,* 316, 97-117.

56. Peng, Y., Lin, F.C., Verardi, P.H., Jones, L.A., McChesney, M.B., and Yilma, T.D. (2007), Pseudotyped single-cycle simian immunodeficiency viruses expressing gamma interferon augment T-cell priming responses *in vitro*, *J Virol,* 81, 2187-2195.

57. Wang, J., Gujar, S.A., Cova, L., and Michalak, T.I. (2007), Bicistronic woodchuck hepatitis virus core and gamma interferon DNA vaccine can protect from hepatitis but does not elicit sterilizing antiviral immunity, *J Virol,* 81, 903-916.

List of symbols/abbreviations/acronyms/initialisms

ATCC	American type culture collection
DMEM	Dulbecco's modified Eagle media
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
HAd5	Human adenovirus type 5
IDO	Indoleamine 2,3-dioxygenase
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
iNOS	Nitric oxide synthase
ISGs	IFN-stimulated genes
LD_{50}	Lethal dose 50
mIFN-γ	Mouse interferon-gamma
MOI	Multiplicity of infection
PFU	Plaque forming units
WEEV	Western equine encephalitis virus

DOCUMENT CONTROL DATA

(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1. ORIGINATOR (the name and address of the organization preparing the document. Organizations for who the document was prepared, e.g. Establishment sponsoring a contractor's report, or tasking agency, are entered in Section 8.)

Defence R&D Canada – Suffield
PO Box 4000, Station Main
Medicine Hat, AB T1A 8K6

2. SECURITY CLASSIFICATION
(overall security classification of the document, including special warning terms if applicable)

Unclassified

3. TITLE (the complete document title as indicated on the title page. Its classification should be indicated by the appropriate abbreviation (S, C or U) in parentheses after the title).

Interferon-gamma gene delivery partially protects mice from the lethal infection of western equine encephalitis virus

4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.)

Wu, Josh Q.H.; Barab, Nicole D.; Chau, Damon; Sha, Aaron; Marshall, Andrew

5. DATE OF PUBLICATION (month and year of publication of document)

December 2008

6a. NO. OF PAGES (total containing information, include Annexes, Appendices, etc) 31

6b. NO. OF REFS (total cited in document) 57

7. DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

Technical Report

8. SPONSORING ACTIVITY (the name of the department project office or laboratory sponsoring the research and development. Include the address.)

Defence R&D Canada – Suffield

9a. PROJECT OR GRANT NO. (If appropriate, the applicable research and development project or grant number under which the document was written. Please specify whether project or grant.)

WBE 16ua 01

9b. CONTRACT NO. (If appropriate, the applicable number under which the document was written.)

10a. ORIGINATOR'S DOCUMENT NUMBER (the official document number by which the document is identified by the originating activity. This number must be unique to this document.)

DRDC Suffield TR 2008-224

10b. OTHER DOCUMENT NOs. (Any other numbers which may be assigned this document either by the originator or by the sponsor.)

11. DOCUMENT AVAILABILITY (any limitations on further dissemination of the document, other than those imposed by security classification)

(x) Unlimited distribution
( ) Distribution limited to defence departments and defence contractors; further distribution only as approved
( ) Distribution limited to defence departments and Canadian defence contractors; further distribution only as approved
( ) Distribution limited to government departments and agencies; further distribution only as approved
( ) Distribution limited to defence departments; further distribution only as approved
( ) Other (please specify):

12. DOCUMENT ANNOUNCEMENT (any limitation to the bibliographic announcement of this document. This will normally corresponded to the Document Availability (11). However, where further distribution (beyond the audience specified in 11) is possible, a wider announcement audience may be selected).

13. ABSTRACT (a brief and factual summary of the document. It may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall begin with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (S), (C) or (U). It is not necessary to include here abstracts in both official languages unless the text is bilingual).

Western equine encephalitis virus (WEEV) is a potential biological and bioterrorism agent. The aerosolized virus is easily transmitted to humans. Single-dose, fast-acting vaccines and therapeutics are urgently needed to provide rapid protection against WEEV in the event of bioterrorism attack or biowarfare. We found previously that a single dose injection of interferon alpha (IFN-α) expressed from human adenovirus type 5 (HAd5) vector rapidly and completely protects mice against WEEV. This result prompted us to investigate whether a single-dose injection of interferon-gamma (IFN-γ) expressed from HAd5 vector would also offer protection against WEEV in mice. A replication-deficient HAd5 vector (Ad5-mIFNγ) expressing mouse IFN-γ (mIFN-γ) was constructed. A single-dose injection of mice with Ad5-mIFNγ produced a high level of serum mIFN-γ. The production of mIFN-γ in mice was rapid, which was detected as early as 9 h after injection. A single-dose injection of Ad5-mIFNγ either before or immediately after WEEV challenge delayed the progression of WEEV infection and extended the time of survival. However, a single-dose injection of Ad5-mIFNγ did not improve the overall survival rate of the challenged mice. Thus, a single-dose injection of mIFN-γ expressed from HAd5 vector offers partial protection against the lethal infection of WEEV in mice. This work is part of an ongoing Technology Investment Fund (TIF) project of DRDC entitled Development of adenoviral vector technology for post-exposure protection against viral threats.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS (technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifies, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus, e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus-identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)

Western equine encephalitis virus, Interferon gamma, adenovirus vector, gene delivery

Defence R&D Canada

Canada's Leader in Defence
and National Security
Science and Technology

R & D pour la défense Canada

Chef de file au Canada en matière
de science et de technologie pour
la défense et la sécurité nationale

www.drdc-rddc.gc.ca