Development of broadly neutralizing monoclonal antibodies against western equine encephalitis virus (WEEV) and eastern equine encephalitis virus (EEEV)

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Prepared for: Canadian Forces Health Services, Operational Medicine

Defence Research and Development Canada

Scientific Report
DRDC-RDDC-2017-R057
April 2017

IMPORTANT INFORMATIVE STATEMENTS

This research has been done in collaboration between Defence Research and Development Canada and Mapp Biopharmaceutical, Inc.

In conducting the research described in this report, the investigators adhered to the 'Guide to the Care and Use of Experimental Animals, Vol. I, 2nd Ed.' published by the Canadian Council on Animal Care.

Abstract

Defence Research and Development Canada – Suffield Research Centre is in collaboration with Mapp Biopharmaceutical, Inc. to develop broadly neutralizing monoclonal antibodies (NAbs) against western and eastern equine encephalitis viruses (WEEV and EEEV). In order to develop these NAbs, female Balb/c mice were immunized with immunogens of WEEV or EEEV in different formats with different strains. After three immunizations, the spleens were aseptically dissected from the immunized mice and the splenocytes then fused with mouse myeloma cells. Five thousand hybridoma clones from five cell fusions were screened for anti-WEEV and anti-EEEV NAbs respectively. As a result, five anti-WEEV and three anti-EEEV hybridoma clones were identified to secrete NAbs, which neutralized WEEV or EEEV infectivity in Vero cells. These NAbs were then produced and purified. Their neutralizing activities were confirmed in an *in vitro* neutralization assay. Two NAbs (G8-2-H9 and 12WA) with a 50% inhibitory concentration (IC_{50}) of 30 ng/mL against WEEV strain 71V-1658 or two NAbs (G1-2-H4 and G1-4-C3) with IC_{50} of 1.5 and 4.5 µg/mL against EEEV strain PE6 were further tested against various strains of WEEV or EEEV. Both G8-2-H9 and 12WA neutralized 6 of 8 tested strains, while both G1-2-H4 and G1-4-C3 neutralized all 4 tested EEEV strains, indicating these NAbs are likely to have broadly neutralizing activity against WEEV or EEEV infectivity. To prove the concept, G8-2-H9 was evaluated *in vivo* and demonstrated that a dose of 100 µg per mouse could protect 100% of the mice from a lethal intranasal WEEV challenge if given 24 hrs before exposure. These NAbs have the potential to be developed as broad-spectrum antibody-based medical countermeasures against WEEV and EEEV infections.

Significance to defence and security

The research in inhalational animal models and the occurrence of laboratory accidents demonstrate the risk to humans from aerosol exposure to the encephalitic alphaviruses in a biological attack. Currently, no medical countermeasures (MedCMs) exist to combat infections with these viruses. The development of effective MedCMs to the encephalitic alphaviruses will provide important protection in the event of biowarfare attack with these agents for both biodefence and public health.

Résumé

Le Centre de recherches de Suffield de Recherche et développement pour la défense Canada, en collaboration avec Mapp Biopharmaceutical Inc., travaille à la mise au point d'anticorps monoclonaux neutralisants à large spectre (AcmNL) contre les virus de l'encéphalite équine de l'Ouest (VEEO) et de l'Est (VEEE). Pour mettre au point ces anticorps neutralisants (AcN), nous avons vacciné des souris Balb/c femelles en utilisant des immunogènes sous différentes formes et provenant de souches diverses de VEEO et de VEEE. Après trois doses d'immunisation, nous avons prélevé, de manière aseptique, la rate des rongeurs et fusionné les splénocytes à des cellules myélomateuses de souris. Cinq mille clones d'hybridomes issus de cinq fusions cellulaires ont été analysés à la recherche d'AcN anti-VEEO et anti-VEEE. Nous avons trouvé cinq clones d'hybridomes anti-VEEO et trois d'anti-VEEE qui produisaient des Ac capables de neutraliser, respectivement, l'infectiosité du VEEO et du VEEE dans les cellules Vero. Nous avons produit et purifié ces AcN, et confirmé leur activité de neutralisation par un essai *in vitro*. Deux des AcN (G8-2-H9 et 12WA) pour lesquels la concentration inhibitrice 50 % (CI 50) était de 30 ng/mL contre la souche du VEEO 71V-1658 et deux AcN (G1-2-H4 et G1-4-C3) pour lesquels la CI 50 était respectivement de 1,5 et de 4,5 µg/mL contre la souche VEEE PE6 ont été testés contre différentes souches de VEEO et de VEEE. Les Ac G8-2-H9 et 12WA ont neutralisé six des huit souches testées, tandis que les Ac G1-2-H4 et G1-4-C3 ont neutralisé les quatre souches de VEEE testées, laissant supposer que ces anticorps pourraient avoir une activité neutralisante à large spectre contre l'infectiosité des VEEO et VEEE. Pour démontrer le concept, nous avons évalué l'Ac G8-2-H9 *in vivo* et montré qu'une dose de 100 µg par souris pouvait protéger 100 % des souris contre une administration intranasale létale de VEEO si les Ac étaient administrés 24 heures avant l'exposition au virus. Ces AcN pourraient servir de contre-mesure médicale à base d'anticorps à large spectre contre les infections au VEEO et au VEEE.

Importance pour la défense et la sécurité

La recherche à l'aide de modèles animaux d'inhalation et la survenue d'accidents de laboratoire montrent que l'exposition aux alphavirus à l'origine d'encéphalites, par des aérosols, présente un risque pour les humains en cas d'attaque biologique. À l'heure actuelle, il n'existe aucune contre-mesure médicale pour combattre les infections causées par ces virus. La mise au point de mesures de prévention efficaces contre les alphavirus causant une encéphalite permettra d'assurer une protection importante en cas d'attaque bioterroriste avec ces virus, tant sur le plan de la biodéfense que de la santé publique.

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The authors wish to acknowledge the technical assistance of Mr. Damon Chau as a member of the Project Team. The technical assistance from the containment level 3 and animal care support groups at Defence Research and Development Canada – Suffield Research Centre to the project is also greatly appreciated.

1 Introduction

The three encephalitic alphaviruses, Venezuelan, western, and eastern equine encephalitis viruses (VEEV, WEEV, and EEEV) are classified by the Centers for Disease Control and Prevention (CDC) as biological threat agents [1]. In nature, these viruses infect humans by bites from infectious mosquitoes and human infection typically results in an acute and highly incapacitating disease characterized by severe symptoms similar to influenza. However, fatal encephalitis can result from these viruses crossing the cerebral vascular endothelium or the olfactory epithelium [2].

Accidental laboratory infections with these viruses [3] and experimental studies in animals [4] have demonstrated that all three alphaviruses are highly infectious by the aerosol route. For example, in 1959, a freeze-dried vial containing VEEV was accidentally dropped by Soviet medical personnel in a stairwell and infected 20 laboratory staff [5]. Furthermore, alphavirus infections via the aerosol route seems to develop much faster with higher morbidity than the natural (mosquito bite) route since the aerosol route likely allows more virus to contact with olfactory neurons, thus facilitating the earlier viral invasion to the brain [6]. In addition, these alphaviruses could be easily obtained in high titers with large quantities by cell culture and are relatively stable (either liquid or dry) in the environment. As such, VEEV was weaponized as an offensive incapacitating agent by the pre-1992 Soviet and pre-1969 United States biological warfare programs [7]. Currently no licensed vaccines and therapeutics are available against these viruses for humans [8].

Although the three encephalitic alphaviruses share many common features, such as structure, epidemiology, transmission, and clinical manifestations, their infectivity and virulence differ from each other in humans [3].

VEEV is the most infective alphavirus and humans are extremely susceptible to VEEV, particularly from aerosol exposure. Only a few viral particles (10–100 organisms) are required to infect one person [5]. More accidental laboratory infections have occurred with VEEV than any other pathogens with a total of >150 cases by aerosol exposure [3]. Approximately 90 to 100% of exposed individuals become infected and symptomatic, but encephalitis only occurred in a small percentage of patients with the mortality rate of around 1% [3].

EEEV and WEEV are 10 to 20 times less infectious than VEEV, but cause more severe disease with a high incidence of encephalitis [9]. EEE is one of the most severe mosquito-transmitted diseases with approximately 50% to 70% mortality and significant brain damage in most survivors [3]. It is quite understandable that the virulence of WEEV is between VEEV and EEEV since WEEV is a natural recombinant virus, formed from a Sindbis virus (a much less virulent member of alphaviruses) and EEEV [10]. The mortality rate for WEEV is about 3–7% in humans [3]. What is more concerning is that out of 16 laboratory accidents, 4 deaths resulted.

Antibodies, naturally produced in the body as part of the immune response to infectious agents, can also be introduced artificially to treat infectious diseases. Neutralizing monoclonal antibodies (NAbs) can confer immediate and consistent protection against infectious agents when administered regardless of the recipient's immune status. NAbs can be used for both pre- and post-exposure protections; therefore, they have great value as effective MedCMs against infectious agents [11, 12].

In 2007, Defence Research and Development Canada – Suffield Research Centre (DRDC – Suffield Research Centre) developed a highly potent humanized anti-VEEV NAb, hu1A4A1 [13–15]. DRDC – Suffield Research Centre was approached by Mapp Biopharmaceutical, Inc (Mapp Bio) to compete and collaborate on a U.S. Defense Threat Reduction Agency (DTRA) project to develop a broad-spectrum monoclonal antibody (mAb) cocktail against the three encephalitic alphaviruses, VEEV, WEEV, and EEEV. In the first year of the DTRA project, DRDC – Suffield Research Centre finished a proof-of-concept study to characterize hu1A4A1, produced in plants by Mapp Bio with great success, but encountered a setback in development of anti-WEEV and anti-EEEV NAbs due to the unavailability of potent immunogens. These results were documented in a previous DRDC Scientific Report [16].

In the second year of the DTRA project, the development of anti-WEEV and anti-EEEV NAbs was focused on production of highly effective immunogens. The immunization protocol was amended. The new protocol included immunogens with different formats (formalin-inactivated viruses, plasmid DNA vaccine, adenovirus DNA vaccine, and recombinant antigens) from different strains. After three immunizations, the spleens were aseptically dissected from the immunized mice and the splenocytes then fused with mouse myeloma cells. Eight neutralizing hybridoma clones (5 anti-WEEV clones and 3 anti-EEEV clones) were isolated from screening approximately 10 thousand hybridoma clones, from which NAbs were produced and purified. Two anti-WEEV and two anti-EEEV NAbs were tested and shown to have broadly neutralizing activity against various strains of WEEV or EEEV. One of anti-WEEV NAbs, G8-2-H9 was further evaluated in an intranasal challenge mouse model for its pre-exposure prophylaxis efficacy against WEEV infection. A dose of 100 µg/mouse could provide 100% protection to the mice against WEEV Fleming (Flem) challenge if given 24 hrs pre-exposure.

2 Materials and methods

2.1 Reagent, cells, and viruses

Gibco cell culture reagents, including high glucose Dubecco's minimal essential medium (DMEM), Hank's balanced salts solution (HBSS), fetal bovine sera (FBS), and Synth-a-Freeze defined protein-free cryopreservation medium were purchased from Life Technologies (Fisher Scientific, Ottawa, ON). Melon Gel purification kits, Nunc cryovials, and Invitrogen cloning kits and reagents were purchased from Fisher Scientific. TiterMax Gold adjuvant was purchased from Cedarlane (Burlington, ON). Clonacell-HY Kit was from Stem Cell Technologies (Vancouver, BC). Isoflurane was obtained from Baxter (Mississauga, ON).

Vero (ATCC CCL-81) cells, Sp 2/0 mouse myeloma cells, and HEK 293 cells were from American Type Culture Collection (ATCC, Manassas, VA).

EEEV strains were kindly provided by Dr. George Ludwig, United States Army Medical Research Institute for Infectious Diseases (USAMRIID), Frederick, MD.

WEEV 71V-1658 (71V) was kindly provided by Dr. Nick Karabatsos, CDC; WEEV Fleming (Flem) and California were purchased from ATCC; WEEV B11 and CBA87 were kindly provided by Dr. George Ludwig, USAMRIID; WEEV McMillan, Mn548, and Mn520 were kindly provided by Drs. Mike Drebot and Harvey Artsorb, National Microbiology Laboratory (Winnipeg, MN).

2.2 Cells and viruses maintenance

Hybridoma cell lines were maintained in DMEM supplemented with 10% heat-inactivated FBS. Vero cell and HEK 293 cells were grown in DMEM supplemented with 5% FBS. Cell lines were grown and resuspended in Synth-a-Freeze defined protein-free cryopreservation medium, and 1 mL aliquots were transferred to NUNC cryovials (Fisher Scientific). Cells were frozen overnight at -70°C (insulated cell freezing container, prior to storage in liquid nitrogen (vapour phase storage).

All experiments with live viruses were carried out in DRDC – Suffield Research Centre Containment Level (CL)-3 facilities, in compliance with Health Canada and Canadian Food Inspection Agency Guidelines. Virus seed stocks were made by inoculation of Vero cells with virus suspensions at a multiplicity of infection of less than 0.1. The supernatants were clarified by centrifugation, aliquoted, and stored at -70°C in CL-3. Plaque assays were performed in 6 well multi-plates, and stained using an agarose neutral red overlay as described [17] using Vero cells.

2.3 Vaccines

A formalin-inactivated encephalomyelitis vaccine, consisting of EEEV and WEEV, and tetanus (Zoetis vaccine) for horses was purchased from Zoetis Canada, Inc. (Kirkland, QC).

A plasmid DNA vaccine, pVHX-6-WEEV 71V was made at DRDC – Suffield Research Centre [18], expressing the structural proteins (capsid, E3, E2, 6K and E1) of WEEV 71V.

An adenovirus DNA vaccine, pAd-EEEV PE6 was constructed at DRDC – Suffield Research Centre by cloning the structural proteins of EEEV strain PE6 to an adenovirus vector using AdEasy system (Qbiogene, Carlsbad, CA) according to the manufacturer's protocol. The recombinant adenoviral construct, pAd-EEEV PE6 was linearized with Pac I and transfected into HEK 293 cells cultured in DMEM with 5% FBS for amplification and then the amplified adenovirus was purified by a chromatographic method.

Recombinant E1 or E2 (rE1 or rE2) antigens of WEEV Flem was prepared at DRDC – Suffield Research Centre by cloning the E1 or E2 gene into a pCRT7 bacterial expression vector, expression in bacteria [19]. The C-terminal 6×His-tagged rE1 and rE2 proteins were expressed in bacteria and purified by the immobilized metal affinity chromatography.

2.4 Animals

Female Balb/c mice (4–6 week old) were obtained from Charles River Canada (St Constant, QC). All mouse experiments were performed in strict accordance with the guidelines set out by the Canadian Council on Animal Care. The animal care protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments of DRDC – Suffield Research Centre (protocol number: W1H-13-1-2-0). All efforts were made to minimize suffering.

2.5 Generation and screening of hybridomas

To develop anti-WEEV or anti-EEEV NAbs, female BALB/c mice were intramuscularly (i.m.) immunized with immunogens in different formats from different strains as shown in Figs.1 and 2.

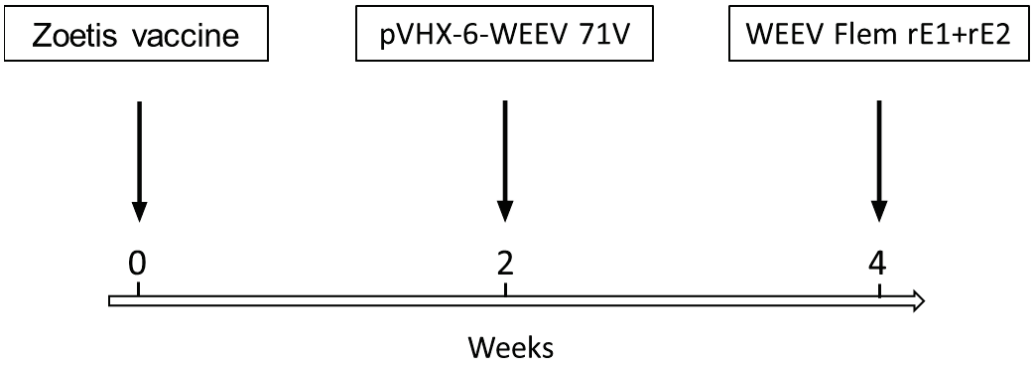

Figure 1: *WEEV immunization scheme. Female Balb/c mice were i.m. primed with 100 µl/mouse of Zoetis vaccine at week zero, and boosted with 100 µl/mouse of pVHX-6-WEEV 71V (100 µg) at week 2 and 100 µl/mouse of recombinant E2 (100 µg) and E1 (100 µg) of WEEV Flem, emulsified in 100 µl of TiterMax Gold adjuvant at week 4.*

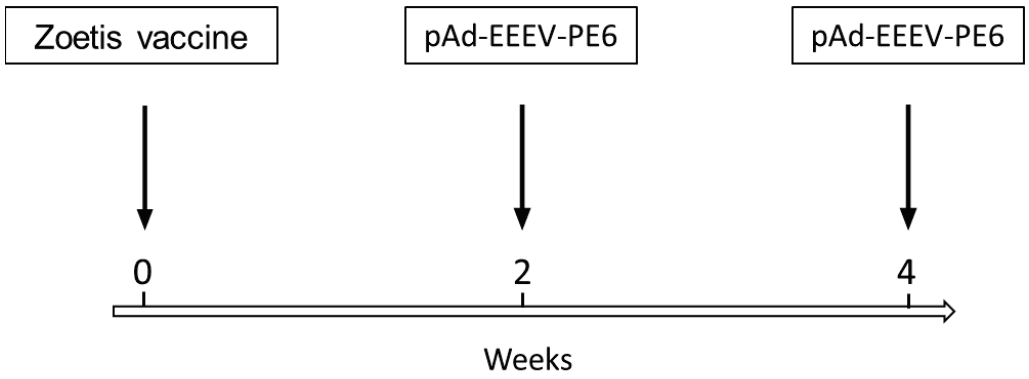

Figure 2: *EEEV immunization scheme. Female BALB/c mice were i.m. primed with 100 µl/mouse of Zoetis vaccine at week zero, and boosted with 100 µl of pAd-EEEV PE6 (100 µg) at week 2 and 4.*

Spleens were aseptically dissected from the immunized mice three days after the last booster and were ground gently with autoclaved frosted-glass slides in DMEM and then filtered through a wire mesh screen to prepare splenocytes. Hybridomas were produced by fusing the splenocytes with Sp 2/0 myeloma cells using a Clonacell-HY Kit (Stem Cell Technologies, Vancouver, BC). After 2 weeks in semisolid medium, individual hybridoma clones were picked and transferred to 96-well plate (VWR, Mississauga, ON) and grown for 7 days in Clonacell Medium E (Stem Cell Technologies, Vancouver, BC) as described [20].

The hybridoma clone culture supernatants were screened using a Vero cell-based neutralization assay in CL-3 laboratories. Briefly, 20 plaque forming units (pfu)/well of EEEV PE6 or 125 pfu/well of WEEV 71V, respectively, were incubated with hybridoma clone culture supernatant at 37°C for 1 hr. The mixtures were then added to Vero cells preplated in 96-well plates (10^4 cells/well). Two or three days later, cells were checked under microscope for cytopathic effect (CPE). The positive hybridoma clones without CPE were expanded.

2.6 NAb purification

NAbs were purified from the cell culture supernatant by a Melon Gel purification kit according to the manufacturer's protocol. The supernatant was dialyzed for two exchanges (1 hr each) in Melon Gel IgG Purification Buffer pH 7.0 and then was added to a column containing the Melon Gel resin. After 5 minute-incubation with end-over-end mixing, the purified IgG was collected in the flowthrough. All IgG purified samples were aliquoted and stored at -70°C.

2.7 Anti-alphavirus neutralization assay

As shown in Fig. 3, the alphavirus neutralization test (ANT) was carried out in 96-well plates. One hundred µg/ml NAb was started in first well and then three-fold dilutions were made in the next well, until the last well (the 12^{th} well). The volume of each well was 50 µl. Subsequently, 50 µl of virus, 100 x 50% tissue culture infective dose ($TCID_{50}$) was added to each well and then pre-incubated at 37°C for 1 hr to allow neutralization of the virus. Thereafter, 10,000 Vero cells per well were added in a volume of 50 µl. Plates were then incubated for 3 days at 37°C and under

5% CO_2. After 3 days, the plates were examined under microscope. The neutralizing antibody titer, expressed as 50% inhibition concentration (IC_{50}), was identified as the highest dilution that resulted in 50% inhibition of cytopathic effect (CPE).

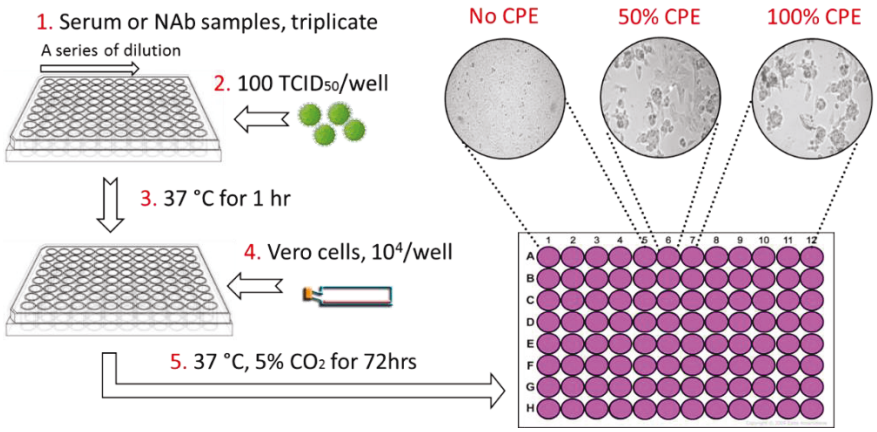

Figure 3: *Anti-alphavirus neutralization titration assay. A series of 3-fold dilutions was made for each purified antibody in 50 µl. Fifty µl of virus was added to each well and then pre-incubated at 37°C for 1 hr. Thereafter, 10,000 Vero cells per well were added in a volume of 50 µl. Plates were then incubated for 3 days at 37°C and under 5% CO_2. After 3 days, the plates were examined under microscope. The neutralizing antibody titer was identified as the highest dilution that resulted in 50% inhibition of CPE.*

2.8 *In vivo* protective efficacy

Experiments were carried out to evaluate the anti-WEEV efficacy of G8-2-H9 in a pre-exposure prophylaxis setting. Groups of 5 mice were given 100 µg purified G8-2-H9 or HBSS (negative control) i.m. at 24 hrs before challenge. Mice were put in the chamber of isoflurane aerosol unit to render mice unconscious. The unconscious mice were instilled intranasally (i.n.) with 5×10^3 pfu of WEEV Flem. They were examined daily for 14 days for body weight and signs of infection.

3 Results

3.1 Generation and selection of neutralizing hybridoma clones

Mice were immunized with WEEV or EEEV immunogens in different formats with different strains, including formalin-inactivated WEEV/EEEV viruses, pAd-EEEV PE6, pVHX-6-WEEV 71V, and rE1/rE2 of WEEV Flem. Following the last booster, five mice for either WEEV or EEEV with the highest neutralizing titers were sacrificed and splenocytes were prepared and fused with myeloma cells in a standard hybridoma fusion protocol. Five thousand clones were picked up from semisolid medium and transferred to 96-well plates for WEEV or EEEV respectively. A Vero cell-based neutralization assay was performed to screen for anti-WEEV or anti-EEEV neutralizing clones. Finally, a panel of 5 anti-WEEV and 3 anti-EEEV hybridoma clones were identified to secrete NAbs, neutralizing WEEV or EEEV infectivity in Vero cells.

3.2 *In vitro* neutralization assay

The results of ANT assay for anti-WEEV NAbs are summarized in Table 1. Five anti-WEEV NAbs showed positive results, neutralizing WEEV 71V infectivity in Vero cells with various 50% inhibition concentrations (IC_{50}) from 0.03 to 0.27 µg/mL. The two anti-WEEV NAbs with the lowest IC_{50} (0.03 µg/mL), G8-2-H9 and 12WA were further evaluated against other 7 WEEV strains and demonstrated to neutralize 5 of 7 strains. Whereas, three anti-EEEV NAbs showed positive results, neutralizing EEEV PE6 infectivity in Vero cells with various IC_{50} from 1.5 to 13.5 µg/mL as shown in Table 2. G1-2-H4 and G1-4-C3 were further evaluated against other 3 EEEV strains and demonstrated to neutralize all the tested strains.

Table 1: *Anti-WEEV NAbs.*

	IC_{50} (µg/mL)							
	Flem	71V	CBA87	B11	Mn548	Mn520	California	McMillan
G8-2-H9	0.03	+	+	+	+	+	-	-
12WA	0.03	+	+	+	+	+	-	-
G4-2-A4	0.09	N/A	N/A	N/A	N/A	N/A	N/A	N/A
G4-2-H1	0.27	N/A	N/A	N/A	N/A	N/A	N/A	N/A
G4-2-H7	0.27	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 2: *Anti-EEEV NAbs.*

	IC_{50} (µg/mL)			
	EEEV-PE6	FL93-969	Williams	435731
G1-2-H4	1.5	+	+	+
G1-4-C3	4.5	+	+	+
G1-4-A2	13.5	N/A	N/A	N/A

3.3 *In vivo* protective efficacy

Anti-WEEV NAbs, G8-2-H9 was further tested *in vivo* to investigate whether the NAb could protect mice against WEEV challenge in a pre-exposure prophylaxis setting. As shown in Fig. 4, G8-2-H9 could provide 100% protection to the mice against a lethal dose challenge of WEEV Flem without any infection signs (ruffled fur, hunched back, less movement, and weight loss), while all control mice died with 6 days post-exposure with serious infection signs.

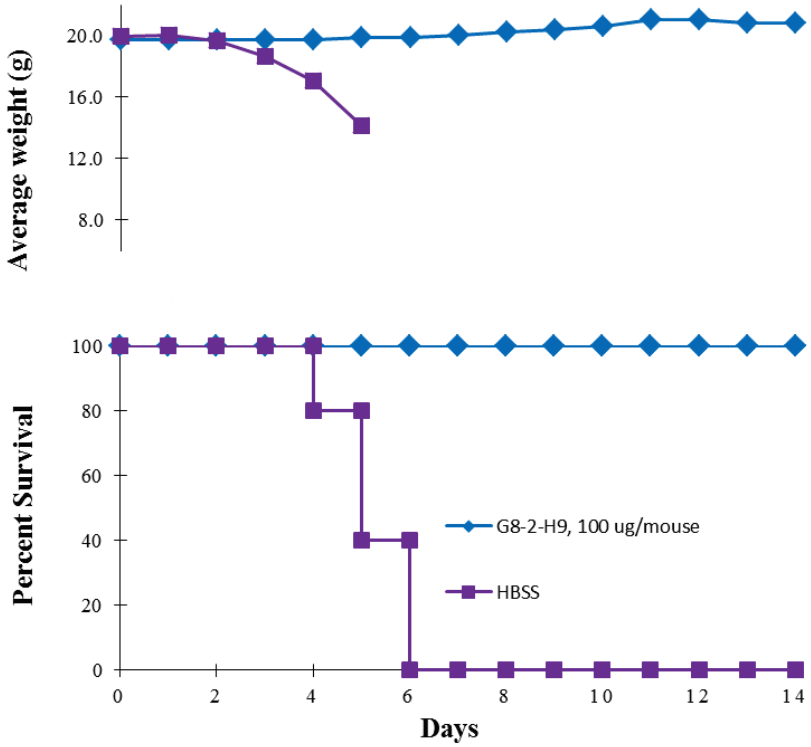

Figure 4: *Evaluation of G8-4-H9 anti-WEEV efficacy in a pre-exposure prophylaxis setting. A group of 5 mice were administered with 100 µg given i.m. per mouse G8-2-H9 24 hrs before intranasal challenge with WEEV Flem (5×10^3 pfu). The mice were monitored daily for signs of infection for 14 days.*

4 Discussion

The host antibody response plays a pivotal role in both prevention and treatment of viral infections. There are two types of antibodies, which will be produced against numerous epitopes on virus proteins when the host is attacked by a virus. These are NAbs and non-neutralizing antibodies. Only a small fraction of antibodies are NAbs, capable of blocking virus infection to the host cells by interfering with virion binding to receptors on cells, blocking uptake and preventing uncoating of the genomes in cells, or inducing aggregation of virus particles [21]. The vast majority of antibodies are non-neutralizing antibodies. Such antibodies bind specifically to virus particles, but do not neutralize viral infectivity. Sometimes they may enhance infectivity since antibodies can interact with receptors on macrophages. The virus-antibody complex might be engulfed into the macrophages by endocytosis through the antibody fragment crystallizable region receptor. Viral replication can then proceed because the antibody does not block infectivity. Since NAbs are minority in the pool of antibodies elicited by a microbe, it is difficult to isolate these NAbs from the immunized mice.

Another consideration is there are numerous different strains of WEEV and EEEV. Broad-spectrum anti-WEEV or anti-EEEV NAbs, which can neutralize more strains are much desirable as MedCMs against WEEV or EEEV infections. EEEV North American (NA) strains are more homogeneous than the WEEV strains [22].

In this study, various formats of immunogens including formalin-inactivated viruses, DNA vaccine, adenovirus vector-based vaccine, or recombinant viral envelop proteins from different stains were used not only to enhance the immune response in mice, but also to increase the odds of capturing broad-spectrum anti-WEEV or anti-EEEV hybridoma clones. As such, inactivated EEEV/WEEV viruses (a commercial vaccine for horses) were used to prime the mice. The strains of this vaccine are unknown due to commercial confidentiality. Afterwards, the two boosters were given to the mice using DNA plasmid vaccine, pVHX-6-WEEV 71V and recombinant rE1 and rE2 of WEEV Flem for development of anti-WEEV NAbs or adenovirus DNA vaccine, pAd-EEEV PE6 for development of anti-EEEV NAbs. In this way, only the B-cell clones recognizing the common epitopes shared by these different WEEV or EEEV strains would be boosted and grow.

In general, the conventional approach for isolating NAbs from the immunized mice is to identify the antigen-binding clones first by an immunoassay and then determine the neutralizing clones by an *in vitro* neutralization assay from these antigen-binding clones. Since alphaviruses are CL-3 agents, which cause serious and potentially lethal human diseases and therefore prohibited to be used in the immunoassay, conducted at CL-2 laboratories. Instead, either inactivated viruses or recombinant antigens should be used for the immunoassay. However, the conformation of inactivated virus or recombinant antigens might not be the exact same as naïve virus or antigens. Sometimes, the slight change might affect neutralizing epitopes to be recognized by NAbs and these NAb clones would be missed by the immunoassay. In order to overcome this hurdle, an *in vitro* Vero cell-based neutralization assay using live viruses was developed and applied to screen anti-WEEV or anti-EEEV neutralizing clones, conducted at CL-3 laboratories from screening 10 thousand hybridoma clones. Although the work load was dramatically increased, it led to success in the identification of 5 anti-WEEV and 3 anti-EEEV NAbs.

There are 8 WEEV strains stored at DRDC – Suffield Research Centre. Based on virulence and genetic diversity, the eight strains could be divided into two groups: a high virulence group consisting of strains California, Fleming, and McMillan; and a low virulence group including strains CBA-87, Mn548, B11, Mn520 and 71V-1658, as shown in Fig. 5 [17], EEEV strains can also be divided into two groups based on virulence and sequence, NA strains and South American (SA) strains [23]. NA strains are much more virulent than SA strains which are associated with mild clinical symptoms. Between the two groups, there are around 23% differences in sequence [24].

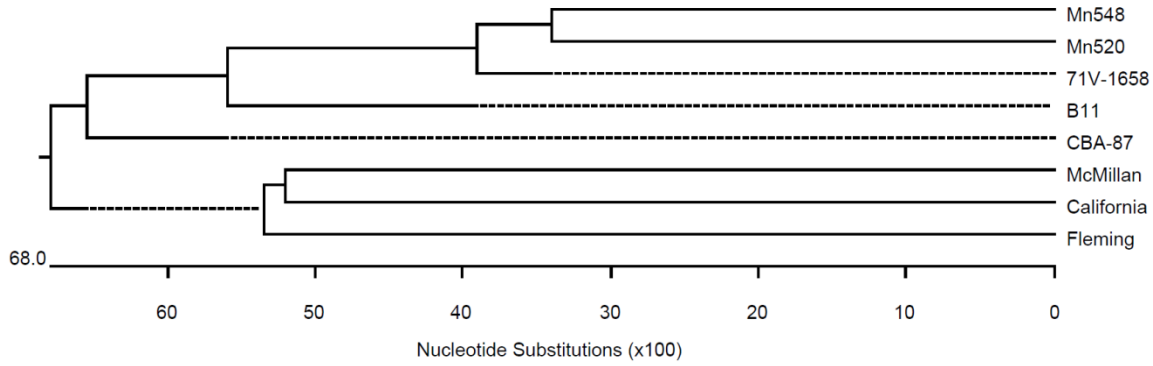

Figure 5: *A cluster diagram of WEEV structural proteins from 8 strains. Horizontal lines are proportional to the number of substitutions between branch points. The length of each pair of branches represents the distance between sequence pairs.*

To investigate whether these NAbs had broad spectrum activity against various strains of WEEV or EEEV infectivity, two anti-WEEV NAbs and two anti-EEEV NAbs were tested against multiple strains. What is interesting is that the California and McMillan strains share 4 amino acids in the E2 protein which are not present in the other strains of WEEV examined [17]. This could account for the lack of neutralization detected for these two viruses. WEEV 71V was used to initially screen the hybridomas, and could miss antibodies which neutralize these two strains of WEEV. On the other hand, anti-WEEV NAbs G8-2-H9 and 12WA were confirmed to neutralize 6 out of 8 tested strains. Whereas, anti-EEEV NAb G1-2-H4 and G1-4-C3 could neutralize all the four tested EEEV strains, including three NA strains and one SA strain. These results indicate that these NAbs recognize a common or conserved neutralizing epitope, shared by various strains of WEEV or EEEV, respectively. Screening of NAbs to WEEV McMillan and California ought to be considered going forward.

5 Conclusion

To date, there have not been any publications describing anti-EEEV NAbs. There is only one report describing 4 anti-WEEV NAbs, developed from macaques, which were shown to neutralize one WEEV strain in an *in vitro* neutralizing assay. No *in vivo* efficacy results were reported for these antibodies.

In our study, 5 anti-WEEV and 3 anti-EEEV NAbs have been developed. Anti-WEEV NAbs, G8-2-H9 and 12WA and anti-EEEV NAbs, G1-2-H4 and G1-4-C3 were further evaluated and exhibited broadly neutralizing activity when tested against diverse strains of WEEV or EEEV. To expand on the results, G8-2-H9 was investigated in a lethal WEEV i.n. challenge mouse model and demonstrated 100% protection to the mice if given 24 hrs pre-exposure. The two anti-WEEV NAbs (G8-2-H9, 12WA) and two anti-EEEV NAbs (G1-2-H4, G1-4-C3) had been shipped to one of our research collaborative partners, UK Defence Science and Technology Laboratory for further evaluation of anti-WEEV or anti-EEEV efficacy in a mouse inhalation challenge model after the CBR Equipment and Material Transfer Agreement was signed off. DNA sequences of heavy and light chain variable regions for the four NAbs will be determined and humanized. These NAbs have the potential to be developed as a broad-spectrum monoclonal antibody cocktail with anti-VEEV NAb for protection against the three encephalitic alphaviruses.

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References

[1] Moran GJ: **Threats in bioterrorism. II: CDC category B and C agents**. *Emergency medicine clinics of North America* 2002, **20**(2):311–330.

[2] Schafer A, Brooke CB, Whitmore AC, Johnston RE: **The role of the blood-brain barrier during Venezuelan equine encephalitis virus infection**. *Journal of virology* 2011, **85**(20):10682–10690.

[3] Zacks MA, Paessler S: **Encephalitic alphaviruses**. *Veterinary microbiology* 2010, **140**(3–4):281–286.

[4] Steele KE, Twenhafel NA: **REVIEW PAPER: pathology of animal models of alphavirus encephalitis**. *Veterinary pathology* 2010, **47**(5):790–805.

[5] **Weapons of Mass Destruction (WMD)—Venezuelan equine encephalitis virus.** http://www.globalsecurity.org/wmd/intro/bio_vee.htm (Access date: 2017).

[6] Hanson RP, Sulkin SE, Beuscher EL, Hammon WM, McKinney RW, Work TH: **Arbovirus infections of laboratory workers. Extent of problem emphasizes the need for more effective measures to reduce hazards**. *Science* 1967, **158**(3806):1283–1286.

[7] Sidwell RW, Smee DF: **Viruses of the Bunya- and Togaviridae families: potential as bioterrorism agents and means of control**. *Antiviral research* 2003, **57**(1–2):101–111.

[8] Nagata LP, Wong JP, Hu WG, Wu JQ: **Vaccines and therapeutics for the encephalitic alphaviruses**. *Future Virology* 2013, **8**.

[9] Reed DS, Lackemeyer MG, Garza NL, Norris S, Gamble S, Sullivan LJ, Lind CM, Raymond JL: **Severe encephalitis in cynomolgus macaques exposed to aerosolized Eastern equine encephalitis virus**. *The Journal of infectious diseases* 2007, **196**(3):441–450.

[10] Hahn CS, Lustig S, Strauss EG, Strauss JH: **Western equine encephalitis virus is a recombinant virus**. *Proceedings of the National Academy of Sciences of the United States of America* 1988, **85**(16):5997–6001.

[11] Hu WG, Nagata LP: **Opportunities and Challenges of Therapeutic Monoclonal Antibodies as Medical Countermeasures for Biodefense**. *Journal of Bioterrorism & Biodefense* 2016, **7**(3).

[12] Hu WG, Nagata LP, Vallerand A: **Novel Technology Platform of Therapeutic Antibody Development against Biothreat Agents: Cutting Edge Innovation for National Defence and Public Security**. Defence Research and Development Canada, *DRDC Scientific Letter*, DRDC-RDDC-2015-L189, 2015.

[13] Hu WG, Chau D, Wu J, Jager S, Nagata LP: **Humanization and mammalian expression of a murine monoclonal antibody against Venezuelan equine encephalitis virus**. *Vaccine* 2007, **25**(16):3210–3214.

[14] Hu WG, Phelps AL, Jager S, Chau D, Hu CC, O'Brien LM, Perkins SD, Gates AJ, Phillpotts RJ, Nagata LP: **A recombinant humanized monoclonal antibody completely protects mice against lethal challenge with Venezuelan equine encephalitis virus**. *Vaccine* 2010, **28**(34):5558–5564.

[15] Hu WG, Nagata LP, Vallerand A: **Development of Fast-Acting, Long-Lasting, and Cost-Effective Medical Countermeasure against Venezuelan Equine Encephalitis Virus**. Defence Research and Development Canada, *DRDC Scientific Letter*, DRDC-RDDC-2016-L013, 2016.

[16] Nagata LP, Hu WG: **Development of a Broad-Spectrum Monoclonal Cocktail for Prevention of VEEV, WEEV, and EEEV**. Defence Research and Development Canada, *DRDC Scientific Report*, DRDC-RDDC-2015-R195, 2015.

[17] Nagata LP, Hu WG, Parker M, Chau D, Rayner GA, Schmaltz FL, Wong JP: **Infectivity variation and genetic diversity among strains of Western equine encephalitis virus**. *The Journal of general virology* 2006, **87**(Pt 8):2353–2361.

[18] Nagata LP, Hu WG, Masri SA, Rayner GA, Schmaltz FL, Das D, Wu J, Long MC, Chan C, Proll D *et al*: **Efficacy of DNA vaccination against western equine encephalitis virus infection**. *Vaccine* 2005, **23**(17–18):2280–2283.

[19] Hu WG, Chau D, Wong C, Masri SA, Fulton RE, Nagata LP: **Cloning, expression and purification of envelope proteins E1 and E2 of western equine encephalitis virus and potential use of them as antigens in immunoassays**. *Veterinary microbiology* 2008, **128**(3–4):374–379.

[20] Hu WG, Yin J, Jager S, Wong C, Fulton C, Rayner GA, Aw C, Fisher GR, Dai X, Nagata LP: **A novel approach to development of monoclonal antibodies using native antigen for immunization and recombinant antigen for screening**. *Hybridoma* 2008, **27**(4):307–311.

[21] Klasse PJ, Sattentau QJ: **Occupancy and mechanism in antibody-mediated neutralization of animal viruses**. *The Journal of general virology* 2002, **83**(Pt 9):2091–2108.

[22] Brault AC, Powers AM, Chavez CL, Lopez RN, Cachon MF, Gutierrez LF, Kang W, Tesh RB, Shope RE, Weaver SC: **Genetic and antigenic diversity among eastern equine encephalitis viruses from North, Central, and South America**. *The American journal of tropical medicine and hygiene* 1999, **61**(4):579–586.

[23] Casals J: **Antigenic Variants of Eastern Equine Encephalitis Virus**. *The Journal of experimental medicine* 1964, **119**:547–565.

[24] Arrigo NC, Adams AP, Weaver SC: **Evolutionary patterns of eastern equine encephalitis virus in North versus South America suggest ecological differences and taxonomic revision**. *Journal of virology* 2010, **84**(2):1014–1025.

List of symbols/abbreviations/acronyms/initialisms

ANT	Alphavirus Neutralizing Antibody
ATCC	American Type Culture Collection
CDC	Centers for Disease Control
CL	Containment Level
CPE	Cytopathic Effect
DMEM	Dubecco's Minimal Essential Medium
DRDC	Defence Research and Development Canada
DTRA	Defence Threat Reduction Agency
EEEV	Eastern equine encephalitis virus
FBS	Fetal bovine serum
HBSS	Hank's Balanced Salts Solution
IC_{50}	Inhibition concentration 50%
i.m.	Intramuscular
i.n.	Intranasal
mAb	Monoclonal Antibody
Mapp Bio	Mapp Biopharmaceutical Inc.
MedCM	Medical Countermeasures
NAbs	Neutralizing Monoclonal Antibodies
pfu	Plaque-Forming Unit
$TCID_{50}$	Tissue Culture Infective Dose 50%
USAMRIID	US Army Medical Research Institute of Infectious Diseases
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus

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Development of broadly neutralizing monoclonal antibodies against western equine encephalitis virus (WEEV) and eastern equine encephalitis virus (EEEV)

4. AUTHORS (last name, followed by initials – ranks, titles, etc., not to be used)

Hu, W.-G.; Nagata, L.P.; Ennis, J. Zeitlin, L.

5. DATE OF PUBLICATION (Month and year of publication of document.)	6a. NO. OF PAGES (Total containing information, including Annexes, Appendices, etc.)	6b. NO. OF REFS (Total cited in document.)
April 2017	27	24

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.)

Scientific Report

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

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

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Defence Research and Development Canada – Suffield Research Centre is in collaboration with Mapp Biopharmaceutical, Inc. to develop broadly neutralizing monoclonal antibodies (NAbs) against western and eastern equine encephalitis viruses (WEEV and EEEV). In order to develop these NAbs, female Balb/c mice were immunized with immunogens of WEEV or EEEV in different formats with different strains. After three immunizations, the spleens were aseptically dissected from the immunized mice and the splenocytes then fused with mouse myeloma cells. Five thousand hybridoma clones from five cell fusions were screened for anti-WEEV and anti-EEEV NAbs respectively. As a result, five anti-WEEV and three anti-EEEV hybridoma clones were identified to secrete NAbs, which neutralized WEEV or EEEV infectivity in Vero cells. These NAbs were then produced and purified. Their neutralizing activities were confirmed in an *in vitro* neutralization assay. Two NAbs (G8-2-H9 and 12WA) with a 50% inhibitory concentration (IC_{50}) of 30 ng/mL against WEEV strain 71V-1658 or two NAbs (G1-2-H4 and G1-4-C3) with IC_{50} of 1.5 and 4.5 µg/mL against EEEV strain PE6 were further tested against various strains of WEEV or EEEV. Both G8-2-H9 and 12WA neutralized 6 of 8 tested strains, while both G1-2-H4 and G1-4-C3 neutralized all 4 tested EEEV strains, indicating these NAbs are likely to have broadly neutralizing activity against WEEV or EEEV infectivity. To prove the concept, G8-2-H9 was evaluated *in vivo* and demonstrated that a dose of 100 µg per mouse could protect 100% of the mice from a lethal intranasal WEEV challenge if given 24 hrs before exposure. These NAbs have the potential to be developed as broad-spectrum antibody-based medical countermeasures against WEEV and EEEV infections.

Le Centre de recherches de Suffield de Recherche et développement pour la défense Canada, en collaboration avec Mapp Biopharmaceutical Inc., travaille à la mise au point d'anticorps monoclonaux neutralisants à large spectre (AcmNL) contre les virus de l'encéphalite équine de l'Ouest (VEEO) et de l'Est (VEEE). Pour mettre au point ces anticorps neutralisants (AcN), nous avons vacciné des souris Balb/c femelles en utilisant des immunogènes sous différentes formes et provenant de souches diverses de VEEO et de VEEE. Après trois doses d'immunisation, nous avons prélevé, de manière aseptique, la rate des rongeurs et fusionné les splénocytes à des cellules myélomateuses de souris. Cinq mille clones d'hybridomes issus de cinq fusions cellulaires ont été analysés à la recherche d'AcN anti-VEEO et anti-VEEE. Nous avons trouvé cinq clones d'hybridomes anti-VEEO et trois d'anti-VEEE qui produisaient des Ac capables de neutraliser, respectivement, l'infectiosité du VEEO et du VEEE dans les cellules Vero. Nous avons produit et purifié ces AcN, et confirmé leur activité de neutralisation par un essai *in vitro*. Deux des AcN (G8-2-H9 et 12WA) pour lesquels la concentration inhibitrice 50 % (CI 50) était de 30 ng/mL contre la souche du VEEO 71V-1658 et deux AcN (G1-2-H4 et G1-4-C3) pour lesquels la CI 50 était respectivement de 1,5 et de 4,5 µg/mL contre la souche VEEE PE6 ont été testés contre différentes souches de VEEO et de VEEE. Les Ac G8-2-H9 et 12WA ont neutralisé six des huit souches testées, tandis que les Ac G1-2-H4 et G1-4-C3 ont neutralisé les quatre souches de VEEE testées, laissant supposer que ces anticorps pourraient avoir une activité neutralisante à large spectre contre l'infectiosité des VEEO et VEEE. Pour démontrer le concept, nous avons évalué l'Ac G8-2-H9 *in vivo* et montré qu'une dose de 100 µg par souris pouvait protéger 100 % des souris contre une administration intranasale létale de

VEEO si les Ac étaient administrés 24 heures avant l'exposition au virus. Ces AcN pourraient servir de contre-mesure médicale à base d'anticorps à large spectre contre les infections au VEEO et au VEEE.

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Vaccines; encephalitic alphaviruses; efficacy