

VACCINES AND THERAPEUTICS TO THE ENCEPHALITIC ALPHAVIRUSES

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SUMMARY

This article is a review of vaccines and therapeutics in development to the encephalitic alphaviruses which includes eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV). The encephalitic alphaviruses are endemic within regions in North and South America. Hosts are normally exposed after being bitten by infectious mosquitoes and infection can develop into encephalitis in equines and humans with severe rates of morbidity and mortality. These viruses are also potential biological threat agents, being highly infectious via an aerosol route of exposure. In humans, EEEV and WEEV are neurotropic viruses targeting the CNS and causing encephalitis. Mortality rates are 50% and 10%, respectively for these viruses. On the other hand, VEEV produces a systemic flu-like illness with pathogenesis in the lungs and lymphoid tissue in adults and older children. The incidence of encephalitis is less than 5% in younger children with a case mortality rate of 1%. The host response to virus infectivity is briefly discussed, along with a number of promising therapeutic and prophylactic approaches. These approaches can be broadly classified as virus specific and include vaccines, antibody therapy and gene-silencing oligonucleotides; or broad-spectrum, including interferon and activation of host's innate immunity.

Keywords:

Alphaviruses, vaccines, antivirals, immune modulators, antibodies, eastern equine encephalitis, western equine encephalitis and Venezuelan equine encephalitis

INTRODUCTION

The subject of this article is a review of vaccines and therapeutics being developed to combat the encephalitic alphaviruses, which include eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV). The encephalitic alphaviruses are New World alphaviruses, endemic within regions in North and South America. Humans and equines are normally exposed to these viruses after being bitten by infectious mosquitoes; however, these viruses are also highly infectious via respiratory exposure and can cause encephalitis in humans with severe morbidity and mortality rates. Due to their high titres attained in cell culture and their ease of transmission via aerosol exposure, these three alphaviruses are potential biological threat agents. In fact, VEEV was produced in multi-ton quantities and stockpiled in the former USSR and the USA as an incapacitating agent [1].

The alphaviruses comprise a group of about 28 enveloped viruses with a positive sense, non-segmented single-stranded RNA genome of around 11 - 12 kb [2-4]. They form enveloped virions with icosahedral symmetry and are 60–70 nm in diameter. All alphaviruses share basic structural, sequence and functional similarities, including a genome with two polyprotein gene clusters [3,5]. In-depth reviews of the molecular biology [5] and pathogenesis [6] of the alphaviruses have been written for further details in these areas.

Geographically, the Old World alphaviruses include Sindbis virus (SINV), Chikungunya virus and Ross River virus, which can be associated with rash and arthritis. SINV, along with Semliki Forest virus (causes systemic febrile illness), have been extensively studied and are the best

characterized alphavirus [5,6]. The New World alphaviruses include the encephalitic alphaviruses which are naturally transmitted by mosquitoes to horses and humans, with wild birds, rodents and other animals serving as the reservoir. In humans, EEEV and WEEV are neurotropic viruses which produce limited viremia, followed by CNS infection across the cerebral vascular endothelium or the olfactory epithelium. EEE was identified as a disease of horses in 1831, with epizootics recorded between 1845 and 1912 [7]. EEEV North American strains are more virulent than the South American EEEV strains, with a case mortality rate averaging 50%. Young children are more susceptible than adults to developing encephalitis, and have higher case mortality rates [2,8]. WEEV was determined to be a natural recombinant virus, formed from a Sindbis-like virus and EEEV [9]. SINV is a relatively nonpathogenic member of the WEEV antigenic complex of alphaviruses [4], however, well characterized SIN mouse models of encephalitis have been developed [6]. WEE is endemic in western North America and in South America. While very few cases of human infection have been reported in recent years, major epidemics of WEE have been recorded in the past. The most extensive epidemic occurred in the western United States and Canada in 1941 and included 3,336 recognized human cases and 300,000 cases of encephalitis in horses and mules, [10]. The case fatality rate for WEE has been estimated to be 10% for humans and 20% for equines, and similar to EEE, severe disease is more likely in young children. Sixteen cases of laboratory acquired WEE infections have been reported with 4 fatalities [11,12].

VEE is found in Central and South America, and in the extreme Southern United States. It forms an antigenic complex composed of 6 subtypes [4]. VEE is characterized by a systemic febrile illness with pathogenesis in the lungs and lymphoid tissue [6] with a flu-like illness observed in

adults and older children. As with other alphavirus, younger children may develop more severe illness, with the incidence of encephalitis less than 5% and a case mortality rate of around 1% [13]. VEEV is highly infectious and a frequent cause of laboratory acquired infections [14,15], with as little as a few viral particles able to cause an infection. Accidental laboratory aerosol infections of young adults causes a flu-like febrile illness with abrupt onset of symptoms appearing 1-5 days after exposure, and with no fatalities reported [14-16]. The potential use of these viruses as biological threat agents requires vaccines and therapeutics that are able to protect against an aerosol route of exposure.

HOST IMMUNE RESPONSE

Based primarily on mouse studies to SINV and VEEV infection, the host's immune response to alphavirus infection has been elucidated. As previously mentioned, WEEV and EEEV are neurotropic viruses producing little or no viremia, where VEEV produces a systemic febrile illness. However, in general, the SIN and VEE mouse models of acute encephalitis are comparable amongst the alphaviruses. The host initially activates an innate immune response to alphavirus infection through induction of interferon (IFN) [17]. The replication of the virus and formation of a double-stranded RNA is a critical step for INF induction. IFN offers a measure of protection early in infection and may serve to limit virus replication until the adaptive immune system responds [6]. Excess cytokines induced by IFNs may cause damage due to cytokine-storm effects, as mice deficient in Interleukin 1 β have reduced mortality when infected with neurovirulent strains of SINV [18]. Within 4-5 days of infection, an adaptive immune response is stimulated, with both the cellular and humoral immune responses contributing to virus clearance

[19,20]. The humoral antibody response is detected with 3-4 days post-infection in the form of IgM and IgA. IgG appears at 7-14 days post-infection and is maintained at relatively high levels for years. This antibody response is important for viral clearance and recovery [20]. An equally important virus-specific T cell response also facilitates recovery from alphavirus infection by directly killing infected cells, producing antiviral cytokines and enhancing the humoral response [6]. CD8 T cells rapidly clear SINV in the brain via removal of virus-infected cells, while CD4 T cells produce IFN- γ which has been demonstrated to clear virus from the CNS in a VEEV infectivity model in B cell-deficient mice [21]. Lastly, mucosal immunity and T cells may play a role in providing protection from the aerosol infectivity of alphaviruses [22], a property useful in biological defense applications.

THERAPEUTICS

We are currently lacking licensed vaccines and therapeutics against VEEV, WEEV and EEEV. The following review of medical countermeasures to the alphaviruses covers promising potential candidates in which proof-of-principle studies have been conducted or the products are in pre-clinical or clinical evaluation.

Antibodies

The importance of antibodies has been well documented in the host response to alphaviruses [19,20]. Neutralizing and non-neutralizing antibodies to E1 and E2 administered to mice, before or after infection with virus, were protective against SINV [20]. Antibodies act through two

mechanisms to inhibit viral infections. They can directly bind to the virus through neutralization [23] or interference with replication. The second is indirect effects [24], which result from the crosslinking of the antibody fragment crystallizable (Fc) receptors, and mediate antibody-dependent cell-mediated cytotoxicity, complement dependent cytotoxicity and antibody dependent cellular phagocytosis to eliminate pathogens and infected cells. In addition, with a serum half-life of around 20 days, passive antibody therapy can provide protection for several weeks, during which time other countermeasures may be initiated such as vaccination [25, 26] .

In 1951, WEEV hyperimmune sera prepared in rabbits was administered passively to protect mice from subsequent WEEV infection [27]. Studies have confirmed that hyperimmune sera administration before or at the time of challenge with VEEV, WEEV, or EEEV was efficacious in providing protection to animals from fatal encephalitis [28-30]. Since serum contains only a small fraction of antigen specific antibodies, at the present time only purified antibodies from human plasma can be approved for clinical applications for the treatment of infectious diseases, including rabies and hepatitis [31]. Despite unquestioned efficacy in the fight against infectious diseases, human plasma-derived antibody therapy suffers from a number of clear drawbacks, such as the limited availability of donor blood, batch-to-batch variation, risk of adventitial disease transmission and the high cost of production.

The major benefit afforded by mouse hybridoma technology is that it is possible, in principle, to develop an antibody against any target of choice and to produce it in unlimited amounts. The alphavirus envelope is composed of heterodimers of the envelope proteins E1 and E2. The E2 protein has been found to be responsible for binding to the host cell, while E1 protein mediates

fusion of the virion envelope with the membranes of acidified endosomes, allowing release of the nucleocapsid into the cytoplasm and the onset of viral replication [5]. The development of protective antibodies against VEEV has focused on anti-E2 monoclonal antibodies (mAbs), since these may have the capability to block viral entry into a host cell. A variety of neutralizing or protective mouse mAbs against VEEV have been reported [32-35]. Diligent mapping of neutralization epitopes led to the determination that the region between residues 182-207 on the E2 made up a major neutralization domain [33,36]. The various mAbs (3B4C4, 1A4A1, 1A3A9, and 1A3B7) against this region have been proven to possess both neutralizing activity *in vitro* and protective effect *in vivo* against VEEV infection [33]. In addition, another neutralization epitope on VEEV E2 has recently been located within residues 115-119 [37].

Although, anti-E1 mAbs do not typically neutralize virus infectivity *in vitro*, some were still able to protect against lethal VEEV challenge *in vivo* [38]. For example, mAb 3B2A9 or antibody single chain fragment (scFv) ToR67-3B4 against VEEV E1 were able to protect mice against VEEV challenge [39,40]. E3 protein is a small glycoprotein (~65 residues) and functions as a signal sequence for translocation of E2 precursor, PE2 consisting of E2 and E3, into the endoplasmic reticulum. The furin-mediated cleavage of E3 from E2 is essential for virus maturation and occurs between Golgi body and the cell surface. An anti-VEEV E3 mAb, 13D4, was found to inhibit the production of virus in VEEV-infected cells and provided protection against lethal VEEV challenge in mice [41].

Murine mAbs, however, have serious disadvantages as therapeutic agents in humans [34]. They induce a human anti-mouse antibody (HAMA) response. Repeat administration of murine mAbs

may result in rapid clearance of the murine mAbs and induction of anaphylaxis, which can sometimes be fatal [42]. To overcome this hurdle, humanization of murine mAbs has been developed, by which murine antibody frameworks were replaced by human antibody versions in order to reduce the HAMA response [43]. To date, three anti-VEEV E2 antibodies (3B4C4, 1A4A1 and 1A3B7) developed against Trinidad donkey (TrD) (subtype IA/B) have been successfully humanized (Hy4 IgG, hu1A4A1IgG1-2A and Hu1A3B7, respectively) [44,-46]. All of these antibodies could provide solid protection when administered 24 hr before virus challenge. When the administration of Hy4 IgG was delayed to 24 hrs post-exposure to 100 mean morbidity dose 50 (MD₅₀), 75% of the mice could still be rescued [44]. Administration of 50 µg per mouse of hu1A4A1IgG1-2A provided 100% of protection to mice 24 hrs after a subcutaneous (s.c.) challenge with 100×lethal dose 50 (LD₅₀) of TrD strain [47]. Lastly, administration of 100 µg of Hu1A3b7 per mouse intraperitoneal (i.p.) 24 hrs post-exposure with 10 or 100 × LD₅₀ of TrD strain intranasal (i.n.), could protect 100% or 90% of mice, respectively. Hu1A3B7 was further tested for its therapeutic efficacy when antibody administration was delayed to 48 hrs after VEEV challenge; but unfortunately, the efficacy was dropped (40%) [48]. This result is understandable since VEEV penetrates into brain across the blood-brain barrier (BBB) at day two of infection [49]. VEEV may enter neuron cells immediately after they cross the BBB and at this point, antibodies appears to be unable to prevent the development of encephalitis in the mouse model.

A human antibody, F5 nIgG was isolated from a phage display library of human bone marrow donors, and was found to bind to the residues 115-119 of VEEV E2, a newly recognized neutralization epitope [37]. In a therapeutic setting, a dose of 500 µg per mouse, administered

(i.p.) 24 hrs post challenge with TrD strain of $100 \times MD_{50}$ s.c. could protect 90% or 100% of the mice respectively [50]. Another product, ToR67-3B4, is a single chain fragment variable (scFv) recombinant antibody, developed from an antibody gene library of the non-human primate, macaque that was immunized with a veterinary vaccine against EEEV, WEEV, and VEEV, and boosted with VEEV vaccine strain TC83 [40]. It is a human-like antibody fragment since macaques share a high degree of antibody sequence similarity with humans [51], and is VEEV E1 specific. To increase the serum half-life and add Fc effector sites, the scFv was reconstructed into a bivalent scFv with human IgG1 Fc (scFv-Fc). Administration of the scFv-Fc at dose of 100 μ g per mouse (i.p.) 6 hrs post-exposure after a lethal aerosol dose of TrD (subtype IA/B) or Mena II (subtype IC), provided 80 to 100% protection of the mice, respectively [40].

Taken together, administration of a dose averaging 100 μ g per mouse of these humanized or human antibodies 24 hrs before or after peripheral or aerosol challenge of virulent VEEV strains (subtype 1) could provide 80% to 100% protection of the mice.

Interferons

As previously mentioned, interferons (IFNs) are secreted cytokines which play a key role in innate immune response against viral infections. Based on their differences in amino acid sequence and cellular receptors they bind to, IFNs can be grouped into three types [52]. Type I includes 13 IFN α subtypes, IFN β , IFN κ , IFN ϵ , IFN ω , IFN τ and IFN δ . Type II contains the single member IFN γ . Type III consists of IFN- λ 1, - λ 2, and - λ 3, also known as interleukin (IL)-29, IL-28A, and IL-28B. IFN α has been well characterized for antiviral activity and is approved

in combination with Ribavirin for treatment of chronic hepatitis C [53]. The antiviral activity of IFN α is mainly through inducing host cells to produce several antiviral proteins.

Lukaszewski and Brooks conducted an efficacy study of IFN α against a virulent strain of VEEV [54]. Mice given pegylated IFN α as single daily injection from 2 days before challenge to 5 days after the challenge were more than 75% protected against a lethal s.c. challenge and 67% protected against an aerosol low-dose challenge. Only 36% of mice, however, survived after an aerosol high-dose challenge. Interferon alfacon-1 is a synthetic IFN α derived from a consensus sequence of several IFN α subtypes and was approved for the treatment of hairy cell leukemia, malignant melanoma and AIDS-related Kaposi's sarcoma. Julander et al. tested interferon alfacon-1 for pre-exposure prophylaxis against WEEV in a hamster model [55]. Hamsters were injected daily with interferon alfacon-1 starting 4 h before challenge until 8 days post challenge. The treated hamsters were completely protected against lethal challenge of WEEV given either i.p. or i.n. route [55].

Although IFN α has proven to be effective for pre-exposure protection against VEEV and WEEV, it requires multiple daily injections due to short half-life of IFN α . This makes it impractical to be used extensively as an antiviral prophylaxis or therapy against various viral infections, along with its current high costs per dose of IFN α . To overcome the problem of short half-life of IFN α , a human adenovirus vector expressing IFN α was evaluated for pre- and post-exposure protection against WEEV in a mouse lethal challenge model [56]. Adenovirus-vectored expression of IFN α provided 100% protection against several strains of WEEV after a single dose, intramuscular (i.m.) inoculation given at 24 h, 48 h or 1 week prior to virus

challenge. When given 6 h post virus challenge as a single i.m. inoculation, it delayed the onset of disease progression for all the treated mice and protected 60% of mice from death. Efficacy study on adenovirus-vectored expression of IFN α against VEEV demonstrated that it provides 100% protection against 10 LD₅₀ when it was given 24 h before s.c. challenge; however, it is less effective against a high dose challenge. It appears that adenovirus-vectored expression of IFN α did not confer post-exposure protection against 10 LD₅₀ s.c. challenge of VEEV [57]. These proof-of-concept studies demonstrate that IFN α expressed from the human adenovirus vector can be used as a single dose prophylaxis for pre -exposure protection against WEEV and VEEV, and may have limited potential for post-exposure protection.

Specific Antiviral Therapy Using Antisense Oligonucleotides and SiRNA

Rapid advances in molecular biology, genomics and rational drug design have paved the way for novel development of safe and efficacious antiviral therapeutics. Viral genome sequences from various alphaviruses are readily available, thus providing unique opportunities to design and develop novel nucleic acid-based antiviral therapeutics which specifically target viral genes. Among these novel classes of antiviral therapeutics which have potential for the prevention and treatment of alphaviral infections are gene silencing approaches using antisense (AS) oligonucleotides and small interfering RNA (siRNA). AS are single stranded oligonucleotides which can inhibit the production of viral proteins by binding directly to viral mRNA expressing virus proteins, or mediating the catalytic degradation of target viral mRNA. siRNA, on the other hand, are double stranded oligonucleotides that catalyses the degradation of complimentary mRNAs via a natural cellular process known as RNA interference.

Targeting alphaviral RNA sequences with AS and siRNA is an appealing strategy for prevention and treatment of alphaviral infections. This is because silencing of one key viral protein can effectively disrupt the whole viral replication cycle, and siRNA can also potentially be designed to have specific antiviral activity by gene silencing but also possibly stimulate the innate antiviral defence mechanisms, resulting production of antiviral cytokines (including type I interferons) which confer a broad antiviral state in the host effective against a number of alphaviruses [58].

Unmodified AS molecules are either unstable and are readily degraded by serum nucleases, or can produce unwanted side effects in the body. To overcome these drawbacks, Paessler, et al., designed a third generation AS drug product, called peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs, where the ribose ring of the nucleotide backbone was replaced by a morpholine ring), which showed enhanced stability and efficacy against VEEV infection in both cell culture and mouse model [59]. In this cutting edge work, PPMOs targeting the 5'-terminal and AUG translation start sites region of VEEV were found to block the production of infectious VEEV particles in cell culture and to be efficacious against multiple strains of VEEV. When groups of mice were treated with PPMOs before VEEV challenge (2 doses at -24 hr and -4 hr) they were found to be fully protected (100% survival) from the otherwise lethal infection, while mice receiving only post-infection treatment (5 doses daily on day 1 through 5) were partially protected (63% survival). In the VEEV infected groups of mice, there were no survivors in the control groups treated with random sequence of PPMO (0% survival). Furthermore, the PPMOs were found to be well tolerated in mice and did not produce any apparent ill effects [59]. In recent years, two clinical phase II studies were completed evaluating

the phosphorodiamidate morpholino oligomers against hepatitis C and West Nile viral infections [60]. These studies demonstrated the safety of antisense drug candidates. It is therefore envisaged that when fully developed, these modified AS drug candidates will be very valuable as therapeutic agents against VEEV and other alphaviruses, addressing current deficiency on the lack of approved antivirals against these viruses.

The development of siRNAs against alphaviruses is in the early research phase, and there is much pre-clinical and clinical work which needs to be completed before concrete conclusions can be made about the full potential for siRNA therapeutics against alphaviruses. O'Brien et al. performed a landmark study in which they designed multiple siRNAs that target the highly conserved areas of various gene sequences between divergent strains of VEEV [61]. Their concept of optimizing therapeutic effectiveness was by using multiple siRNAs that target the conserved areas of the virus genome. It offers therapeutic advantage by increasing chances of antiviral activity against various VEEV strains and also helps to prevent the emergence of escape mutants. Using this approach, they tested the ability of using 4 individual or pooled siRNAs to inhibit the viral replication of six human pathogenic strains of VEEV *in vitro* in baby hamster kidney 21 cells. The results showed that all of the individual siRNAs inhibited one strain of VEEV replication by 75-91 %, and levels of inhibition were statistically significant compared to the control. When the antiviral activity of the pooled VEEV specific siRNAs were evaluated, it was found that they inhibited all six human strains of VEEV tested by 96-97%, compared to the viral replication in cells transfected with a pool of non-targeting siRNAs [61].

It is envisaged that these siRNAs will be tested for *in vivo* antiviral activity in the mouse or non-human primate models of VEEV infection. However, in order to optimize antiviral efficacy *in vivo*, an effective siRNA delivery system, such as liposomes, nanoparticles or adenoviral vector, will be required to deliver these charged macromolecules into the intracellular site of infection (primarily the cytoplasm) of infected cells.

Human/humanized antibodies have shown to be safe and effective therapeutics against VEEV in a mouse model, however, the 24 hr window for treatment needs to be improved. The ability to deliver an antibody across the BBB may extend the therapeutic window for antibodies and other products. The AS PPMOs against VEEV also demonstrate promise for post-exposure protection, and further effort in this area will be eagerly awaited. The question will remain, can a therapeutic be effective if given after the initial appearance of symptoms (fever).

PROPHYLAXIS

Broad-Spectrum Antiviral Role of Toll-Like Receptor Agonists

All viruses are obligate intracellular pathogens and they replicate intracellularly by interfering with normal gene functions of infected cells to reproduce viral genetic and protein components. During the viral replication cycle in the host, both ss and ds RNAs of viral origin accumulate intracellularly in infected cells. The presence of these viral RNAs are readily detected and recognized by toll-like receptors (TLRs). TLRs are transmembrane signalling proteins that are expressed by cells of the innate immune system, including monocytes, macrophages and

dendritic cells. TLRs which recognize RNAs (TLR-3, -7, -8 and -9) are strategically located in endosomal membranes of these cells while non-nucleic acid recognizing TLRs are located on the plasma membrane.

Activation of TLRs which recognize and bind to viral RNAs serves as an integral part of the host's innate system to mount antiviral and inflammatory responses to combat viral infections. When these TLRs are activated, a number of immunological and cellular cascade events are induced and these include the production of type I IFNs, activation of the interferon-inducible protein kinase, stimulation of 2'-5' oligoadenylate synthetase, activation of natural killer cells and macrophages, among numerous other effects. Due to other broad-effects on the host's innate and adaptive immune responses, TLR and TLR signalling pathways have become a promising area for the design of novel antiviral therapeutics in recent years.

Immune modulators are a class of compounds that are able to provide broad spectrum protection to a wide range of viruses, through nonspecific stimulation of the immune system. A broad range of compounds have been identified, and include examples such as CpG and polysaccharides. In a WEEV mouse infectivity model, cationic liposome–DNA complexes (cationic lipids mixed with CpG oligomers) given to mice 24 hr pre-exposure provided 80% protection after s.c. challenge; however, no protection of i.n. or aerosol challenged animals was obtained [62].

The use of TLR-3 agonists as potential antiviral agents effective against alphavirus was described by Wong et al., using a synthetic ds RNA called Poly ICLC. Poly ICLC is a synthetic,

double-stranded polyribonucleic-polyribocytidylic acid stabilized with poly-L-lysine carboxymethyl cellulose. It elicits broad antiviral innate immune responses through recognition and interaction with TLR-3, and induces the production of interferons- α , - β and - γ *in vivo* [63]. Poly ICLC is an experimental drug in clinical development with broad-spectrum antiviral activity against VEEV [64]. Liposomal Encapsulated (LE) Poly ICLC has been developed with reduced toxicity and longer therapeutic efficacy [63]. In mouse studies, LE Poly ICLC has been shown to be promising in protecting mice against intranasal administered WEEV with 100% protection obtained after 2 doses two days apart [65]. Similarly, Ampligen® (poly-I:poly-C 12U) protects hamsters against WEEV with 2 doses, 4 hr prior to infection and 2 days postinfection [55].

There are two primary distinct advantages of using TLR agonists to stimulate the host's innate immunity to combat alphaviruses. The first is that these drugs can provide a broad-spectrum antiviral state which can provide broad protection against a wide number of alphaviruses, and the second being that these drugs are robust and less likely to develop drug resistance. This is because these drugs target the host's innate immune cells rather than virus proteins, the latter being the normal target of action of conventional antiviral drugs. Therefore if the genes expressing the virus proteins mutate, these TLR agonists can still offer robust protection against the virus mutants.

Poly ICLC had successfully gone through phase I clinical trial in the US, affirming its safety and dose tolerability, in normal human volunteers. Its antiviral efficacy against WEEV, VEEV and

against respiratory viruses including SARS-coronavirus, pandemic and seasonal influenza, and respiratory syncytial virus are well documented [63].

Vaccines

A commercial, trivalent inactivated vaccine is available for immunization of horses against VEEV, EEEV, and WEEV [66]; however, no licensed human vaccines are available for these viruses. Currently, personnel at risk of exposure to VEEV are recommended by the Centers for Disease Control, to be immunized with a live, attenuated vaccine TC-83 as an Investigational New Drug (IND) followed by booster vaccination with formalin-inactivated C-84 if required [67]. Formalin-inactivated EEEV (TSI-GSD 104) and WEEV vaccines (CM 4884) were also developed and can be used as INDs for at-risk personnel [68]. The major drawback of TC-83 is its high reactogenicity rate (about 20% in vaccinees) [67], which is unacceptable for being licensed as a human vaccine. The formalin-inactivated vaccines for VEEV, EEEV, and WEEV have better safety profile, however, the immunogenicity is low for these vaccines, requiring three doses, two weeks apart for immunization and annual boosters to maintain protective immune response.

Several approaches have been used to develop safer and more effective vaccines for VEE, EEE, and WEE [69, 70]. The first is reverse genetics to introduce mutations to the viral sequence. Using this approach, Davis et al. developed a live-attenuated VEEV vaccine candidate designated as V3526 from a full-length cDNA clone of the TrD strain by deleting a furin cleavage site from the PE2 glycoprotein and inserting a single amino acid mutation in the E1 glycoprotein [71]. V3526 is

protective against aerosol or subcutaneous challenge of various subtypes of VEEV in rodents, horses, and nonhuman primates [72-74]. In addition, study of neurovirulence of V3526 in mice demonstrated that it is less virulent than TC-83 [75]. Further study in nonhuman primates found that V3526 is essentially nonneurovirulent when injected through intrathalamic/intraspinal or subcutaneous route [76]. The success of pre-clinical studies made V3526 the lead candidate VEE vaccine to move forward to the safety and immunogenicity study in a phase 1 clinical trial. Unfortunately, V3526 caused headache, fever, malaise and sore throat in a significant number of vaccinees, although the vaccine was able to induce strong immune responses [77]. These adverse effects prompted the discontinuation of the clinical trial for V3526 as a live attenuated vaccine for VEE. The subsequent studies suggest that gamma-irradiated or formalin-inactivated V3526 has a potential to replace the existing formalin-inactivated vaccine C-84 for VEE [78]. However, it is envisioned that an effective adjuvant would be required to reduce the number of doses previously required for formalin-inactivated vaccines.

A second approach for overcoming the problems of the traditional live attenuated VEEV vaccines is through the construction of chimeric SINV (one of the least pathogenic alphaviruses in humans) expressing structural proteins of VEEV, EEEV or WEEV. To construct chimeric SIN/VEE viruses, the genes encoding the replicative enzymes and the *cis*-acting RNA elements of Sindbis virus were ligated with the genes encoding the structural proteins of VEEV TC-83 strain. Mouse studies showed the chimeric virus was highly attenuated and immunogenic [79, 80]. A similar approach was used to make chimeric SIN/EEE viruses encoding structural proteins from the North American strain of EEEV. The chimeric virus is highly attenuated in mice and confers complete protection against i.p. challenge of a homologous strain of EEEV [81]. Study in

a nonhuman primate EEEV model demonstrated that the chimeric virus conferred 82% protection against aerosol challenge of a virulent North American strain of EEEV after a single dose vaccination. The chimeric vaccine appears safe in animals, which did not cause encephalitis-related histopathologic changes [82]. A chimeric SIN/WEE virus was also developed and completely protected mice against intranasal lethal, high dose challenge of virulent WEEV strains [83]. This approach looks to be very promising, but further studies on formulating a trivalent vaccine, and examining problems with potential interference amongst the three components needs to be investigated. And as a live attenuated vaccine, potential problems with reversion must also be studied.

A third approach is DNA vaccination, which injects a DNA plasmid encoding protein antigens to produce immune protection. Since its early introduction in the 1950's, DNA vaccination has revolutionized the field of vaccinology due to its excellent safety profile, stability and ease of manufacture. Indeed, a world first veterinary DNA vaccine protecting horses against West Nile virus was licensed by the U.S. Department of Agriculture in 2005 [84]. DNA vaccination has been proposed to develop an ideal biodefense vaccine against alphaviruses [85]. Such a vaccine should be safe and easily formulated against known and emerging pathogens. So far, great effort has been made to develop a DNA vaccine against VEEV. In a proof-of-the-concept study, a DNA vaccine encoding the E2 glycoprotein of the TC-83 strain of VEEV was constructed. When the vaccine was injected by particle-mediated bombardment using the Helios™ gene gun, it induced a high level of IgG in mice [86]. Further study showed a VEEV DNA vaccine expressing the structure proteins of C-E3-E2-6K-E1 of the virulent TrD strain of VEEV fully protects mice and Guinea pigs against s.c. homologous virus challenge after gene gun delivery

[87]. The DNA vaccine also provided 80% protection in mice against aerosol challenge of homologous VEEV. Subsequently, a pilot study was conducted in three nonhuman primates to test the DNA vaccine against the homologous challenge of VEEV via aerosol challenge [88]. Although complete protection was not achieved, all cynomolgus macaques given the VEEV DNA vaccine through gene gun had less severe infection compared to the negative control macaque and two out of three vaccinated macaques showed no detectable serum viremia. Dupuy et al. demonstrated that codon optimization and intramuscular electroporation delivery improves immunogenicity and efficacy of a VEEV DNA vaccine [89]. The vaccine contains codon-optimized E3-E2-6K-E1 envelope glycoprotein of VEEV TrD strain. Mice injected with the vaccine by intramuscular electroporation (3 doses 3 weeks apart) generated a similar high level of VEEV-neutralizing antibody comparable to that observed in mice administered the live-attenuated VEEV vaccine TC-83. The vaccine confers complete protection in mice against a lethal dose challenge of aerosolized VEEV. Strong neutralizing antibody responses were also observed in cynomolgus macaques given the vaccine (2 doses 56 days apart). The vaccinated macaques did not show viremia and had reduced clinical signs of infection after an aerosol challenge. These results make this VEEV DNA vaccine an attractive candidate for clinical development. Limited effort was made to develop a DNA vaccine for WEEV. We constructed a DNA vaccine encoding the structural proteins of C-E3-E2-6K-E1 of the 71V-1658 strain of WEEV [90]. Mice given the vaccine through the gene gun (3 doses 2 weeks apart) reached 100% protection against intranasal challenge of a homologous WEEV strain and 50% protection against heterologous challenge. Further study indicated that the 6K-E1 structural protein is sufficient in conferring protection [91].

Viruses, such as vaccinia virus and adenovirus, can be modified into molecular vehicles to deliver genes encoding protective antigens of VEEV, EEEV or WEEV. Several studies from our group demonstrated that an adenovirus-vectored vaccine for WEEV has a great potential for further pre-clinical development [92-94]. The adenovirus-vectored WEEV vaccine encoding E3-E2-6K-E1 structural proteins of the 71V-1658 strain of WEEV confers rapid and complete protection against both homologous and heterologous strains of WEEV in a mouse lethal intranasal challenge model. The vaccine candidate is effective after a single dose vaccination through intramuscular, intranasal, or oral route. For VEE, a virus-vectored vaccine was first made based on a vaccinia virus vector expressing structural proteins of the TrD strain of VEEV [95]. The vaccine provided protection of mice against peripheral challenge of various subtypes of VEEV. However, only partial protection was achieved against aerosol challenge. An adenovirus vector expressing the E3-E2-6K structural proteins of VEEV was constructed [96]. Intranasal immunization with the vaccine protects mice against aerosol challenge of a homologous strain of VEEV but was less protective against the heterologous strains.

Finally, production of virus-like particles (VLPs) through molecular engineering could become a novel approach for developing safe and effective vaccines against VEEV, EEEV, and WEEV. VLPs were first reported by Bayer et al., who found that the surface antigen of hepatitis B can form particles which lack the viral genome [97]. The finding opened a new door to develop safer vaccines for infectious diseases. Currently, several VLP-based vaccines have been licensed for human use, which include two hepatitis B vaccines: Engerix-B® (GlaxoSmithKline) and Recombivax HB® (Merck & Co.) and two human papillomavirus vaccines: Cervarix® (GlaxoSmithKline) and Gardasil® (Merck & Co.) for preventing cervical cancer [98]. Although

there has been no reports on construction of VLP-based vaccines for VEE, EEE, and WEE, Akahata et al. developed a VLP vaccine against an Old World alphavirus, Chikungunya virus, which shares many similarities with VEEV, EEEV, and WEEV in terms of viral structure [99]. Cells transfected with a plasmid expressing C-E3-E3-6K-E1 structural proteins of Chikungunya virus produced VLPs which mimic the conformation of the wild-type virus. These VLPs induced high titer of neutralizing antibodies in both mice and nonhuman primates after two-dose, intramuscular vaccination. The VLP vaccine also protected animals against a high-dose challenge of Chikungunya virus.

In conclusion, tremendous effort has been made to develop live attenuated vaccines for VEE, EEE, and WEE since these vaccines mimic natural infection and induce robust immune responses. However, there are severe side effects of the live attenuated VEE vaccine TC-83 in humans and safety issues for the live attenuated VEEV vaccine candidate V3526 during Phase I human clinical trial. With high morbidity and mortality of the infections caused by VEEV, EEEV and WEEV, ensuring the lack of reversion of the live attenuated vaccines to more virulent phenotype after vaccination and that there is no virus shedding, are paramount. Therefore, major challenges could face the licensure of live attenuated or chimeric vaccines for VEE, EEE, and WEE for human use. Plasmid DNA vaccination through intramuscular electroporation is safe and effective in animal studies. It will be interesting to see if these results will translate well to human safety and immunogenicity studies. Usually, the low immunogenicity of plasmid DNA vaccines is a major hurdle for human use. We found that the adenovirus-vector platform is suitable for making a single-dose, fast acting and needle-free vaccine for WEE. An adenovirus-vectored HIV vaccine demonstrated a good safety profile and high immunogenicity after a single

dose vaccination in humans [100]. The major hurdle for using adenovirus-vectored vaccines is the concern over pre-existing immunity against the vector in human population, which could reduce the efficacy of the vaccines. However, it appears this problem can be solved by intranasal delivery of the vaccine [101]. The emerging VLP-based vaccine platform could be an alternative approach for making safe vaccines for VEE, EEE and WEE. The success of development and animal testing of a VLP-based vaccine for Chikungunya virus paves the way to construct similar VLP-based vaccines for VEE, EEE, and WEE. In addition, the availability of a plant-based manufacturing technology could solve the problem of cost-effective, large scale production of VLP-based vaccines [102].

FUTURE PRESPECTIVES

The encephalitic alphaviruses, EEEV, WEEV and VEEV are potential biothreat agents and can infect humans via an aerosol route of exposure with severe consequences as demonstrated by the number of laboratory acquired infections. A recent study with related neurovirulent SINV in mice indicates that virus in the CNS is initially rapidly cleared (by day 8 post infection) by CD8 T cells and IgM antibody-secreting cells, followed by gradual clearing of the virus over a period of several weeks. CD4 T cells (IFN- γ production and helper function), and cells secreting IgG and IgA accumulate and remain in the CNS. Lastly, B memory and plasma cells maintain low levels of viral RNA and prevent establishment of persistent viral infection [103]. Taken together, these properties are what we may want a vaccine or immune modulator to mimic alone or in combination, for effective pre- or post-exposure prophylaxis/therapy of alphaviruses.

Humanized monoclonal antibodies to VEEV have been demonstrated to protect mice and can be used therapeutically to treat mice 24 hr post-infection. Furthermore, antibodies can be used synergistically with interferon alpha for treatment of alphavirus infections [6]. AS and siRNA products are promising therapeutics, and in the case of siRNA, need to demonstrate their therapeutic potential *in vivo*. With the short therapeutic window demonstrated in animal models of infectivity (24 hr), it will be important to determine whether this will translate to the human clinical situation, especially from a biothreat perspective. Further research in understanding the pathogenesis of respiratory exposure in animal models may give us better insight in understanding the clinical disease and host response of these alphaviruses in humans. IFNs are very expensive and have not demonstrated therapeutic efficacy, however, adenovirus-vectored IFN α and immune modulators could be candidates for proceeding to clinical development, as they could be developed for multiple indications. Broad-spectrum protection afforded by Poly ICLC and adenovirus-vectored IFN α (2-3 weeks and 1 week, respectively), may allow development of products against public health-related infectious diseases (influenza, dengue) that could also be useful against potential biothreats such as the alphaviruses. Use of these products may provide sufficient protection for first responders in mitigation of a biothreat incident. Furthermore, the potential antiviral applications of host specific inhibitors have showed promising therapeutic effectiveness against alphaviruses. In one recent study, the use of small molecule inhibitors of GSK-3 β (a host protein known to modulate pro-inflammatory cytokine genes) have been shown to reduce VEEV-induced mortality, inhibit VEEV cell death, and modulate pro- and anti-apoptotic genes, all of which contribute to enhanced survival in mice against VEEV infection [104]. Lastly, many promising recombinant vaccine options are in late stage research, many of which can provide single dose protection against an aerosol exposure. A

combination of vaccines and therapeutics may be what is needed to protect military and civilian responders, and to treat casualties of a natural outbreak or a bioterrorist incident.

EXECUTIVE SUMMARY

INTRODUCTION

- The encephalitic alphaviruses include eastern (E-), western (W-) and Venezuelan equine encephalitis viruses (VEEV) which can cause severe morbidity and mortality as mosquito transmitted diseases.
- They are potential biological threat agents, being easily grown and aerosol transmitted.

HOST RESPONSE

- Innate immunity is activated in initial response to an alphavirus infection
- Adaptive immunity is stimulated within 4-5 days of infection

THERAPEUTICS

- There are currently no licensed vaccines or therapeutics to treat alphaviral infections

Antibodies

- Human or humanized antibodies demonstrate 24 hrs post-exposure protection in mouse infectivity models

Interferon

- IFN α has demonstrated short-term prophylaxis.
- Adenovirus-vectored IFN α may provide a low cost alternative to IFN α , and limited post-exposure potential.

Antisense Oligonucleotides and siRNA

- Antisense peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) are stable, well tolerated and effective post-infection.
- siRNA strategies have demonstrated *in vitro* antiviral activity, but require *in vivo* efficacy testing.

Broad-Spectrum Antiviral Role of Toll-Like Receptor Agonists

- CpG delivered in liposomes provides pre-exposure protection against s.c. challenge, but not i.n. or aerosol challenge with WEEV.
- Poly ICLC can provide short-term pre-exposure protection against intranasal challenge with WEEV and VEEV.

Vaccines

- VEEV V3526 live attenuated vaccine failed phase 1 clinical trials due to reactogenicity
- Chimeric Sindbis/VEEV/EEEV/WEEV vaccines demonstrate good efficacy in mouse infectivity models using a single dose.
- An adenovirus vectored vaccine to WEEV demonstrates single dose, rapid protection, but concerns on pre-existing immunity to adenovirus must be addressed.
- Virus-like particle vaccines could be promising safe and effective vaccines.

FUTURE PERSPECTIVES

- The host's response for elimination of a virus may dictate what therapeutic or prophylactic strategies are effective against the alphaviruses.
- As a potential biological threat agent, pathogenesis via an aerosol route of exposure is important.
- A number of promising solutions are developing, including broad spectrum approaches based on immune modulation and interferon, effective vaccine strategies and potential therapeutics (human/humanized mAbs, AS and siRNA).

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Table 1. Prophylaxis and therapeutic candidates under development against Venezuelan, eastern and western equine encephalitis viruses

Prophylaxis & Therapeutics	Summary & Notes
Monoclonal antibodies	VEEV - Humanized mAbs - protective for both pre- (100% at -24 hr) and post-exposure (80-100% at 24 hr, 40% at 48 hr) in mouse aerosol challenge model [39, 46, 48].
Interferon alpha	<p>VEEV - Pegylated interferon alpha, pre-exposure protection (36-75%, daily) against s.c. and aerosol challenge [54].</p> <p>- Adenovirus-vectored expression of IFNα, pre-exposure protection (100% at -24 hrs) against s.c. challenge in mice after a single dose injection. Post-exposure protection not demonstrated [57].</p> <p>WEEV- Interferon alfacon-1, pre-exposure protection (100%, daily) in a hamster i.p. or i.n. challenge model [55].</p> <p>- Adenovirus-vectored expression of IFNα, pre- (100% to -7 days) and post-exposure protection (60% at 6 hrs) against i.n. challenge in mice after single dose [56].</p>
Antisense oligonucleotides	VEEV - Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs), protective for pre- (100%) and post-exposure (63% at 24 hr, then daily) in mouse i.n. challenge model [59]
Si RNA	VEEV - Multiple siRNA targeting conserved areas of viral genome, pool inhibited (96-97%) multiple strains of VEEV in cell testing [61].
Broad-spectrum Toll-like receptor agonists	<p>CpG</p> <p>WEEV - pre-exposure protection (80% at -24 hrs) in mouse s.c. challenge model [62].</p> <p>Poly ICLC</p> <p>VEEV - Pre- and post-exposure protection in rhesus monkey challenge model [64]</p> <p>WEEV - Pre-exposure protection (100%) in mouse intranasal challenge model [65]</p> <p>Ampligen®</p> <p>WEEV - Pre-exposure protection (100%) in hamster intranasal challenge model [55]</p>
Vaccines	<p>Inactivated</p> <p>VEEV - Formalin-inactivated vaccine (C-84) under Investigational New drug (IND) status, 3 doses two weeks apart, boosters [66].</p> <p>EEEV - Formalin-inactivated vaccine (TSI-GSD 104) under IND status, 3 doses two weeks apart, boosters [68].</p>

	<p>WEEV - Formalin-inactivated vaccine (CM 4884) under IND status, 3 doses two weeks apart, boosters [68].</p> <p>Live attenuated</p> <p>VEEV - TC-83 under IND status (single dose) with high reactogenicity rate in humans [68]</p> <p>- V3526, protective (single dose) against aerosol or s.c. challenge in rodents, horses, and nonhuman primates [72-74]. Significant side effect in phase I human safety trial [77].</p> <p>- Chimeric SIN/VEE vaccine, highly attenuated and immunogenic (single dose) in mice [79,80].</p> <p>EEEV - Chimeric SIN/EEE vaccine, highly attenuated and protective (single dose) in a mouse i.p. challenge model [81].</p> <p>WEEV - Chimeric SIN/WEE vaccine, protective (single dose) against i.n. challenge in mice [83].</p> <p>DNA vaccine</p> <p>VEEV - Protective (3 doses 28 days apart) against aerosol challenge in mice and cynomolgus macaques after intramuscular electroporation delivery of the vaccine [89].</p> <p>WEEV - Protective (3 doses 14 days apart) against intranasal challenge in mice [90, 91].</p> <p>Viral-vectored</p> <p>VEEV – Adenovirus- (3 doses at 0, 7 and 21 days) or Vaccinia-vectored vaccine (single dose), protective against aerosol challenge in mouse model [95, 96].</p> <p>WEEV - Adenovirus-vectored vaccine, protective against intranasal challenge in mice after a single dose vaccination [92-94]</p>
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