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SUPPLEMENT ARTICLE

Developing a Prototype Pathogen Plan and Research Priorities for the Alphaviruses

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The *Togaviridae* family, genus, *Alphavirus*, includes several mosquito-borne human pathogens with the potential to spread to near pandemic proportions. Most of these are zoonotic, with spillover infections of humans and domestic animals, but a few such as chikungunya virus (CHIKV) have the ability to use humans as amplification hosts for transmission in urban settings and explosive outbreaks. Most alphaviruses cause nonspecific acute febrile illness, with pathogenesis sometimes leading to either encephalitis or arthralgic manifestations with severe and chronic morbidity and occasional mortality. The development of countermeasures, especially against CHIKV and Venezuelan equine encephalitis virus that are major threats, has included vaccines and antibody-based therapeutics that are likely to also be successful for rapid responses with other members of the family. However, further work with these prototypes and other alphavirus pathogens should target better understanding of human tropism and pathogenesis, more comprehensive identification of cellular receptors and entry, and better understanding of structural mechanisms of neutralization.

Keywords. *Togaviridae*; *Alphavirus*; prototype pathogen.

INTRODUCTION TO THE *TOGAVIRIDAE* FAMILY

Taxonomy

The *Togaviridae* family consists of positive-sense, single-stranded ribonucleic acid (RNA) viruses with only 1 genus, *Alphavirus*. The International Committee on the Taxonomy of Viruses (https://talk.ictvonline.org/ictv-reports/ictv_9th_report/positive-sense-rna-viruses-2011/w/posrna_viruses/275/togaviridae) recognizes 32 species of alphaviruses; the majority are mosquito-borne and cause disease in humans and/or domesticated animals, whereas a few are important pathogens of fish. One species, *Eilat virus*, is considered an insect-specific alphavirus that is completely defective for replication in vertebrates and appears to only infect mosquitoes in nature [1]. Although most alphaviruses cause acute febrile disease in humans, infection with the Old World members is often accompanied by severe arthralgia, whereas the New World viruses sometimes cause central nervous system disease, which can be fatal [2]. An important exception is Mayaro virus (MAYV), a New World arthritogenic alphavirus that is genetically related to the Old World viruses.

Ecology and Epidemiology

The mosquito-borne alphaviruses are zoonotic and use a wide range of amplifying hosts during enzootic transmission cycles, including rodents, birds, and nonhuman primates [2]. Human infection generally occurs via spillover, where enzootic or bridge vectors with an appropriate host range feed first on an infected zoonotic host, then later a human. Only 1 alphavirus, chikungunya virus (CHIKV), has shown sustained amplification in humans after emergence from nonhuman primate (NHP)-amplified enzootic cycles in sub-Saharan Africa. Sustained human-human transmission is mediated by peridomestic *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus* mosquitoes, leading to major, explosive epidemics that travel globally via infected people [3]. Several other alphaviruses, including MAYV [4] and *Venezuelan equine encephalitis virus* (VEEV) [5, 6], are also capable of generating human viremia levels sufficient to infect *A aegypti*, suggesting their potential for emergence to near-pandemic proportions such as CHIKV. Ross River virus (RRV) is probably also transmitted through human amplification in sustained cycles, although the vectors in this case are likely *Aedes vigilax*, *Aedes camptorhynchus*, and *Culex annulirostris*, which are not highly peridomestic like *A*

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aegypti and have much narrower geographic distributions [7]. Other alphaviruses, including *Eastern equine encephalitis* (EEEV), *Western equine encephalitis* (WEEV), and *Madariaga virus* generate little human viremia despite being among the most virulent members of the family. This tendency for humans to be "dead-end" hosts (insufficient viremia to serve as amplifying hosts) is a major factor in limiting the pandemic potential for many alphaviruses.

Replication

The human-pathogenic alphaviruses generally enter cells via receptor-mediated endocytosis, with the receptors recently identified for several (Figure 1) [8–10]. These receptors bind to the E2 glycoprotein that, along with E1, forms heterotrimeric spikes on the surface of enveloped virions [11]. Upon E1-mediated endosomal fusion, the nucleocapsid disassembles to release the genomic RNA, leading to translation of the nonstructural polyprotein open reading frame (ORF1). The nonstructural proteins, along with host components, form replication complexes on the surface of cytoplasmic vesicles, leading to the production of minus-strand genomic and plus-strand subgenomic (SG) RNAs; the latter encodes an ORF for the structural polyprotein (ORF2). Minus strands are then copied into plus-strand genomic and SG RNAs for further translation, and encapsidation signals near the 5′ end of the genomic RNA [12] combine with capsid proteins to form cytoplasmic nucleocapsids. The envelope glycoproteins are inserted into the endoplasmic reticulum and processed through the secretory pathway to be embedded as E2/E1 trimers in the plasma membrane. These combine with nucleocapsids via a capsid-cytoplasmic E2-tail interaction to initiate budding of virions from the cell surface.

Pathogenesis

Alphavirus infections are frequently asymptomatic, or they manifest as general flu-like illness with rash [2]. However, alphaviruses are often broadly categorized into 2 groups based on their associated pathologies, which manifest in severe infections. The arthritogenic (predominantly Old World) alphaviruses cause systemic infection characterized by joint pain with swelling and myalgia, whereas the encephalitic (New World) alphaviruses are associated with infection of the central nervous system (CNS) and encephalitic disease. Although alphaviruses typically cause acute infections that resolve within weeks of symptoms, long-term joint (arthritogenic viruses) and neurological (encephalitic viruses) sequelae have been described for many of these viruses. Details for individual alphaviruses and pathogenic categories are found below.

GAPS IN THE KNOWLEDGE BASE

To develop countermeasures for prototype alphaviruses, which could also be rapidly adapted for any member of the family, a few important gaps in basic virology remain to be addressed. These include sampling the genetic and antigenic diversity of key members including CHIKV and the VEE complex viruses, as well as viruses yet to be discovered. Additional gaps include (1) the lack of receptor identification or confirmation for many human-pathogenic members and (2) high-resolution imaging of receptor-E2 interactions for most. The role of receptor interactions in determining the tropism and pathogenesis of these viruses is still far from understood. Structural intermediates that occur between receptor binding, endosomal fusion, and budding are also lacking. Much progress has been made in understanding epitopes involved in attachment of antibodies (including those that neutralize), the mechanisms of neutralization, and to a lesser extent identification of T cell epitopes; however, most of this work has been performed on only a small number of alphaviruses.

Although several antiviral host factors and their mechanisms of action for controlling alphavirus replication have been elucidated (eg, PKR, IFIT1, ZAP, ISG20), there is still much to be discovered regarding the role of innate immune factors in alphavirus restriction [2]. In particular, variability in the resistance or susceptibility of different family members to antiviral factors, and the molecular mechanisms that underlie these differences, is lacking for many of the host factors described. As with virus-receptor interactions described above, understanding of how intracellular host factors (both antiviral and non-antiviral genes) contribute to cellular tropism is not well understood, and it has only been explored for a limited number of viruses. Likewise, host factors and responses that determine viral tropism and pathogenesis for distinct niches in the host have been explored in more detail for some (eg, the brain and mechanisms of neuroinvasion and blood-brain barrier disruption) but less so for others (eg, the liver).

MODELS OF DISEASE

Cell culture and animal models are critical (1) for the interrogation of disease mechanisms driven by viral infection and (2) for testing safety and efficacy of therapeutics before their approval for use by the US Food and Drug Administration (FDA). The evaluation of the preclinical efficacy in a model depends on well defined end points such as (1) species or cell line selection, (2) challenge strain and dose, (3) route of exposure, (4) clinical endpoints that mimic human disease, and (5) route and timing of countermeasure administration.

Animal models for the study of arthritogenic and encephalitic alphavirus infection include mice, hamsters, guinea pigs, birds, and/or NHPs [13–18]. For alphaviruses, mice and NHP stand as the 2 most widely accepted models for proof-of-concept or preclinical evaluation of the efficacy of therapeutics and vaccines. For diseases with low incidence in

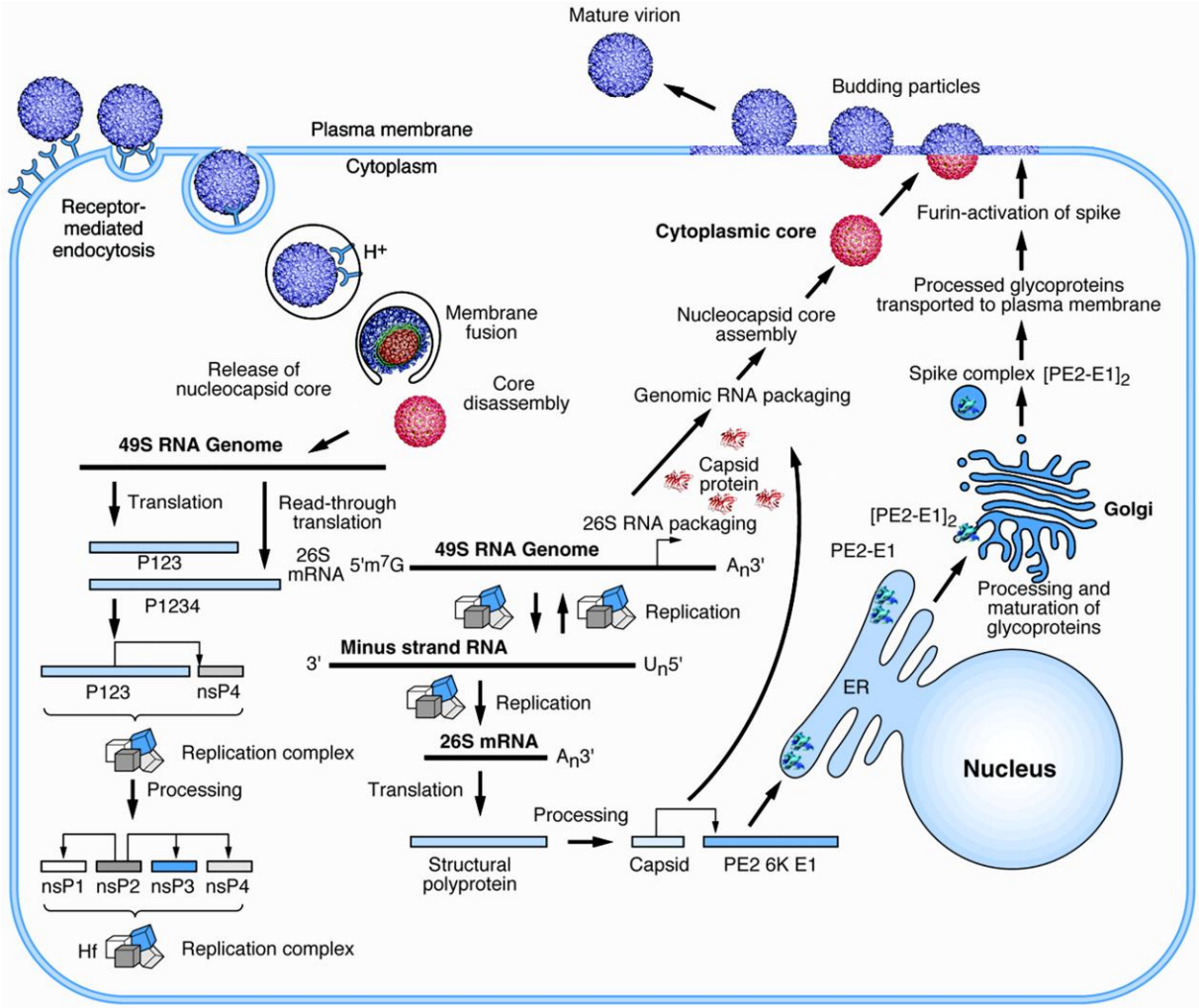

Figure 1. Replication cycle of an alphavirus. The start of the cycle is shown on the left with the attachment of a virion to a cellular receptor. After fusion of the viral envelope, disassembly of the core, and release of the genomic ribonucleic acid (RNA), replication proteins are translated and processed (bottom left). These proteins enable the replication of the input genomic RNA (bottom center) and translation of the subgenomic messenger RNA (mRNA) into structural proteins. Cytoplasmic assembly of genomic RNA and capsid proteins produces the nucleocapsid core that associates with processed envelope glycoproteins (right) at the plasma membrane resulting in budding of infectious virions. Scale varies. Courtesy of Richard Kuhn with permission from the publisher [9]. ER, endoplasmic reticulum.

the human population, such as the neurotropic alphaviruses, the path to FDA approval for licensure may require validated animal models for Phase II and III clinical trials. Hence, although several elements are known about the progression of the host response and disease in animal models of alphaviruses, well characterized, validated models are not available for most of these viruses and represents an important gap in the field. Successful implementation of animal models in preclinical or clinical trials will also require validated in vitro assays of immune and clinical correlates to measure outcomes. More importantly, the correlates of protection must bridge the animal model to the human experience. These assays should be readily transferable across different organizations engaged in the efforts.

In general, validation of an animal model requires investigation and justification of the viral dose administered, the viral strain, the route of virus administration, the animal, and the clinical signs and optimal endpoints in the animal model chosen. Validation of viruses and cells demands historical tracking of origin and passage history (ie, authenticating the origin of the cells and viruses used). The evaluation of each viral seed stock for its 50% infectious or lethal dose and validation of the viral genome by sequencing are critical for validated animal studies. Current recommendations in the field are to use viral seed stocks amplified from infectious clones to minimize seed stock variation and avoid selection of genotypes that impact phenotype (eg, glycosylation, receptor binding, and virulence). Cells used in preclinical and clinical studies require routine

testing for contamination (eg, cell, virus, and mycoplasma), morphology, and functionality.

Arthritogenic Alphavirus Models

The global distribution of arthritogenic alphaviruses present a continued threat to public health [19, 20]. The most notable of these include CHIKV, o'nyong-nyong virus, MAYV, and RRVs. To be able to generate relevant animal models, it is critical to understand the patterns of infection and pathogenesis of each type of alphavirus. Although the timeline of incubation (3–13 days) and illness vary after transmission to a human, most infections present with fever and have a short viremia of a few days followed by acute and subacute phases that may lead to chronic illness. The chronic phase, defined as ongoing pain longer than 12 weeks, can last for years and can include inflammatory rheumatism, musculoskeletal pain, asthenia, and headache. Chronic conditions are generally associated with illness caused by CHIKV, MAYV, and RRV infections [20–22]. Chronic illness is associated with the inflammatory responses elicited from the persistence of viral replication in synovial tissues. Human illness caused by arthritogenic alphaviruses is nonlethal and typically self-limiting, albeit in some cases, symptoms may last for years.

After transmission via mosquito bite, arthritogenic alphaviruses replicate in tissue-resident myeloid cells and fibroblasts, then they traffic to the proximal draining lymph node [2]. Here, virus replicates and further disseminates via the blood to other peripheral organs, including the liver, spleen, and joints. In the joints, CHIKV replicates in fibroblasts (connective tissue), myofibers (muscle cells), and macrophages. Joint pathology is driven by immune cell infiltration (mononuclear cells) into the site of infection (synovia) with robust proinflammatory responses in the joint. Infection of the joints also leads to bone destruction, resulting from perturbed osteoclast/osteoblast homeostasis. Mechanistically, this process results from production of interleukin 6 that stimulates production of receptor activator of nuclear factor-κB ligand (RANKL) from osteoblasts, which inhibit osteoprotegerin, leading to increased osteoclastogenesis and bone resorption [23]. Aside from acute infection, arthritogenic alphaviruses, CHIKV in particular, has been associated with recurring and chronic arthralgia that can last from months to years. Although the precise mechanism of chronic arthralgia is unknown (persistent viral replication vs immunopathology in the absence of virus), studies suggest that prolonged inflammatory and antibody (AB) responses likely contribute.

As would be expected, animal models for the arthritogenic alphaviruses are not lethal; however, the virulence varies across strains. Although mouse models are not ideal for preclinical efficacy due to potential lethality, lack or involvement of neurological symptoms, and limitations in arthritis at sites of infection, they provide useful tools for proof-of-concept studies [24–27]. A key endpoint in evaluation of therapeutics and vaccines for the arthritogenic alphaviruses is joint swelling, which is evaluated and measured in ankles, wrists, and gastrocnemius muscles. Clinical signs for CHIKV and MAYV include acute biphasic swelling response in the ipsilateral foot and ankle that peaks on days 6–8 postinfection. In addition, severe inflammatory synovitis and myositis occur in the joints and skeletal muscle around the foot and are evaluated by histopathological scoring of hematoxylin and eosin-stained hind limb tissues [25, 28]. Immune-deficient mouse models of arthritogenic alphaviruses would not be appropriate for preclinical or clinical testing for obvious challenges of translation of outcomes to healthy individuals.

The most advanced NHP model for vaccine testing is for CHIKV and has been in development since the 1950s [14]. The pathogenesis of CHIKV in both rhesus and cynomolgus macaques mirrors human disease, although how the route of viral infection impacts pathogenesis is less understood. Disease severity correlates with viral infection dose. Nonhuman primates show viremia, fever, rash, lymphopenia, and immunoglobulin (Ig)M antibody response during the first week of infection. Of these clinical signs, viremia, fever, and lymphopenia provide excellent endpoints for efficacy testing [29, 30]. In addition, CHIKV persists in the spleen in rhesus and cynomolgus macaques with the later having more severe disease and greater duration of viral persistence [25, 31]. Limitations of NHP models include the lack of neurological signs observed in humans.

Encephalitic Alphavirus Models

VEEV, WEEV, and EEEV are significant pathogens of both medical and veterinary importance. Human disease is highlighted by fatal encephalitis and permanent neurological sequelae in survivors. Of the 3 viruses, EEEV causes the most severe disease with human case-fatality rates of 30%–90% in those with neurological disease [32]. The survivors suffer from debilitating and permanent long-term neurological sequelae at rates of 35%–80% [32, 33]. Despite the discovery of these viruses more than 80 years ago, the mechanism(s) that underlie the pathogenesis are not well understood. The vast majority of infections are diagnosed at late stages, and the virus-induced pathology and/or host inflammatory response are presumably responsible for the fatal outcome.

Similar to the arthritogenic alphaviruses during the acute phase of infection, the encephalitic alphaviruses replicate in tissue resident cells in the periphery that traffic to the draining lymph node where virus replicates further and disseminates to peripheral organs including the liver, spleen, and CNS. Differences in cellular tropism among encephalitic alphaviruses relate to the distinct pathogenic mechanisms of these related viruses [34]. Although VEEV predominantly infects myeloid cells in the periphery and lymph node leading to

robust production of interferon (IFN), EEEV replicates poorly in myeloid cells, thus circumventing robust activation of innate immunity [35]. Indeed, robust activation of innate immunity and production of IFN is thought to cause significant prodrome observed after VEEV infection, but which is absent in EEEV infections. Although several routes of CNS infection may be involved for different encephalitic alphaviruses, neuroinvasion after natural routes of infection appears to predominantly involve the circumventricular organs of the brain (eg, pineal body) and the nerves innervating the olfactory neuroepithelium [36]. Long-term neurological sequelae are particularly prevalent in EEEV and WEEV cases, and this may be an underappreciated consequence of VEEV infection due to the higher prevalence of asymptomatic and undiagnosed VEEV infections relative to EEEV and WEEV [37].

Interferons play a key role in early restriction of alphavirus replication, and as with many other viruses, deficiencies in IFN signaling results in greater disease severity [38]. Natural infection and immunization typically produce robust long-term protective humoral immunity including neutralizing antibodies (NAbs) that are important for resolution of acute infection. T cells have also been shown to play both protective and pathogenic roles during alphavirus infections, although different T cell subsets seem to be protective in different models [39, 40]. Protective T cell responses, in particular, are important during infection with encephalitic alphaviruses, which invade the CNS before the onset of robust IgG responses.

Two recent studies with EEEV in cynomolgus macaques provide insights into the potential underlying mechanism(s) of pathogenesis [41, 42]. After introduction into the brain via the aerosol route, many critical physiological parameters under the control of the autonomic nervous system (ANS) such as respiration, activity, temperature, heart rate, blood pressure, food/fluid intake, circadian rhythm, sleep, and electrical activity of the heart and the brain were rapidly and profoundly changed leading to the NHPs meeting the euthanasia criteria. We were surprised to find that one of the NHPs met the euthanasia criteria by exhibiting a sudden cardiac event. A follow-up pathology study on the organs and tissues of the NHPs at the time of euthanasia demonstrated rapid virus dissemination throughout the brain and spinal cord including the ANS control centers [43]. The virus likely spread by hijacking the axonal transport system, which is an essential neuronal homeostatic process responsible for movement of RNA, proteins, and organelles within the neuron. Thirty-five virions were observed in a single axon of a neuron in a 160 nm section [43]. Consequently, this mechanism has the potential to rapidly transport a tremendous amount of virus throughout the CNS. However, despite the extensive dissemination, most brain and spinal cord tissues exhibited minimal or no microscopic lesions with the cellular architecture remaining intact. In addition, minimal or no host inflammatory infiltrate was observed in majority of the tissues. This strongly suggests that EEEV infection causes local and global neuronal dysfunction leading to dysregulation of critical physiological parameters. This neuronal dysfunction likely contributes to or exacerbates viral and host-induced pathology to produce the fatal outcome. Whether these mechanisms also underlie VEEV and/or WEEV pathogenesis remains to be determined.

LANDSCAPE OF MEDICAL COUNTERMEASURES

There is a lack of approved human vaccines and antiviral drugs for public use against alphavirus infections [44]. Further research is needed to expand current knowledge of alphavirus immunity to identify safe, immunogenic, and protective medical countermeasures for alphavirus outbreaks including vaccines and antibodies.

Vaccination Strategies to Prevent Alphavirus Infection or Disease

Numerous approaches to identify vaccine candidates have been tried or are currently being tested in ongoing clinical trials (Table 1). Strategies used include the use of live-attenuated viruses, generation of chimeric viruses, and formalin inactivation of virus particles [44–50]. These vaccine candidates have been shown in some experiments and trials to be immunogenic and protective for several alphaviruses [48, 51–54]. However, some vaccine candidates are reactogenic, require frequent boosting, or their immunogenicity is disrupted by the inactivation methods used [44, 47, 55–57].

Another candidate vaccination approach involves using virus-like particles (VLPs), which are noninfectious molecules that structurally resemble intact virions [58, 59]. Monovalent or trivalent VLP vaccines elicited immunogenic responses in nonhuman primates for protection and were safe and tolerable in Phase I clinical trials [59–61]. Yet another strategy is to use deoxyribonucleic acid (DNA)- or messenger RNA (mRNA)-based antigen delivery methods, which may enhance the speed of candidate vaccine generation. The DNA and mRNA vaccines encoding some alphavirus structural proteins are immunogenic in animals [62–64].

Antibody-Mediated Mechanisms of Action Against Alphaviruses

In addition to vaccines, antibodies (Abs) provide an alternate route to medical countermeasures. Furthermore, understanding the Ab response can help inform rational vaccine design. Antibody responses are important in the protection, treatment, clearance, and maintenance of alphaviruses [65–68]. Passive transfer studies of immune animal serum or purified IgG from plasma samples of immune individuals highlight the ability of Abs to protect mice against alphavirus infection [69, 70]. In addition, mRNA vectors discussed above also can express Abs in recipients. Expression of a potent CHIKV monoclonal Ab (mAb) as a lipid-encapsulated mRNA protected against

Table 1. Clinical Trials of Alphavirus Vaccine Candidates[a]

NCT Number	Title of Clinical Trial	Type	Biologicals	Phases	Study Design	Sponsor/ Collaborators
NCT03879603	VRC 313: A Trivalent Virus-like Particle (VLP) Encephalitis Vaccine (WEVEE) in Healthy Adults	VLP	VRC-WEVVLP073-00-VP	Phase 1	Prevention	National Institute of Allergy and Infectious Diseases (NIAID)
NCT03776994	Venezuelan Equine Encephalitis Monovalent Virus-Like Particle Vaccine	VLP	VEE VLP			SRI International; US Army Medical Research Institute of Infectious Diseases
NCT03829384	Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of mRNA-1944 in Healthy Adults	mRNA	mRNA-1944			ModernaTX, Inc.
NCT04603131	Clinical Trial to Evaluate the Immunogenicity of Chikungunya Vaccine	Formalin-inactivated	BBV87			Bharat Biotech International Limited
NCT03382964	Study to Assess the Safety and Immunogenicity of a Chikungunya Virus Vaccine Candidate (VLA1553) in Healthy Volunteers	Live-attenuated	VLA1553			Valneva Austria GmbH
NCT03325075	Safety, Tolerability, and Immunogenicity of VAL-181388 in Healthy Subjects	mRNA	VAL-181388			ModernaTX, Inc.; Defense Advanced Research Projects Agency
NCT01489358	Chikungunya Virus Vaccine Trial in Healthy Adults	VLP	VRC-CHKVLP059-00-VP			National Institute of Allergy and Infectious Diseases (NIAID); National Institutes of Health Clinical Center (CC)
NCT03028441	Phase I Trial of Measles Vectored Chikungunya Vaccine	VLP	VRC-CHKVLP059-00-VP			National Institute of Allergy and Infectious Diseases (NIAID)
NCT01984983	Study of a DNA-based Venezuelan Equine Encephalitis Virus DNA Vaccine Administered by Electroporation in Healthy Volunteers	DNA	VEE DNA Vaccine Candidate			Ichor Medical Systems Incorporated; US Army Medical Research Institute of Infectious Diseases
NCT04440774	Research Study to Assess New Chikungunya and Zika Vaccines in Healthy Adults in Mexico	Adenovirus	ChAdOx1 Chik			University of Oxford
NCT04131595	Vaccination Trial of a Recombinant MVA-BN-WEV Vaccine in Healthy Adult Subjects	Vaccinia virus	MVA-BN-WEV			Bavarian Nordic; JPM CBRN Medical
NCT01159561	Western Equine Encephalitis Vaccine, Inactivated	Inactivated	WEE: TSI-GSD 210			US Army Medical Research and Development Command
NCT00582088	Safety and Immunogenicity of Venezuelan Equine Encephalomyelitis Vaccine (VEE C-84) as a Booster to VEE TC-83	Formalin-inactivated	VEE C-84	Phase 2		US Army Medical Research and Development Command
NCT03992872	Phase 2 Open-label Study of Alum-adjuvanted Chikungunya Virus-like Particle Vaccine (PXVX0317)	VLP	WRAIR			Emergent BioSolutions; Walter Reed Army Institute of Research (WRAIR)
NCT03807843	Chikungunya Vaccine Study in Previously Exposed Adults (V184-006)	VLP	MV-CHIK			Themis Bioscience GmbH; Walter Reed Army Institute of Research (WRAIR)
NCT02861586	Phase II Study to Evaluate Safety and Immunogenicity of a Chikungunya Vaccine	VLP	MV-CHIK			Themis Bioscience GmbH
NCT03635086	Safety, Tolerability and Long-term Immunogenicity of Different Formulations of a Chikungunya Vaccine (V184-005)	VLP	MV-CHIK			Themis Bioscience GmbH
NCT03483961	Trial of a Chikungunya Vaccine, PXVX0317 CHIKV-VLP, in Healthy Adults	VLP	CHIKV VLP			Emergent BioSolutions
NCT03101111	Study of a Live Attenuated Chikungunya Vaccine in a Previously Epidemic Area	VLP	MV-CHIK			Themis Bioscience GmbH; Walter Reed Army Institute of Research (WRAIR)
NCT02562482	Trial for Safety and Immunogenicity of a Chikungunya Vaccine, VRC-CHKVLP059-00-VP, in Healthy Adults	VLP	VRC-CHKVLP059-00-VP			National Institute of Allergy and Infectious Diseases (NIAID)

Table 1. Continued

NCT Number	Title of Clinical Trial	Type	Biologicals	Phases	Study Design	Sponsor/ Collaborators
NCT05065983	A Study to Assess the Safety and Immunogenicity of PXVX0317 Chikungunya Virus-Like Particle Vaccine	VLP	CHIKV VLP			Emergent BioSolutions
NCT03531242	Safety and Immunogenicity Study of Venezuelan Equine Encephalomyelitis (VEE) Vaccine as Booster Vaccine in Adults	Inactivated, Dried	VEE: C-84, TSI-GSD 205			US Army Medical Research and Development Command
NCT03051386	Safety and Immunogenicity of Venezuelan Equine Encephalomyelitis Vaccine in Healthy Adults	Live-attenuated	VEE TC-83			US Army Medical Research and Development Command; US Army Medical Research Institute of Infectious Diseases
NCT02654509	Safety and Immunogenicity Study of the Eastern Equine Encephalitis (EEE) Vaccine	Formalin-inactivated	EEE: TSI-GSD 104			US Army Medical Research and Development Command
NCT02466750	Safety and Immunogenicity Study of the Western Equine Encephalitis (WEE) Vaccine	Inactivated	WEE: TSI-GSD 210			US Army Medical Research and Development Command
NCT00582504	Safety and Immunogenicity Study of the Venezuelan Equine Encephalomyelitis Vaccine	Live-attenuated	VEE TC-83			US Army Medical Research and Development Command
NCT00584805	Safety and Immunogenicity Study of Eastern Equine Encephalitis (EEE) Vaccine	Formalin-inactivated	EEE: TSI-GSD 104			US Army Medical Research and Development Command
NCT04566484	Seamless Controlled Trial To Evaluate Safety And Immunogenicity of Chikungunya Vaccine in Latin America and Asia	Formalin-inactivated	BBV87	Phases 2 and 3		International Vaccine Institute
NCT01604746	Additional 6-Month Safety Follow-up After Completion of Precursor Study 880801	Inactivated	Ross River Virus (RRV) Vaccine	Phase 3		Ology Bioservices
NCT01242670	Ross River Virus (RRV) Vaccine Study	Inactivated	Ross River Virus (RRV) Vaccine			Ology Bioservices
NCT04786444	Study to Demonstrate Consistency of Three Lots of a Live-attenuated Chikungunya Virus Vaccine Candidate in Healthy Adults	Live-attenuated	VLA1553			Valneva Austria GmbH
NCT04546724	Pivotal Study to Evaluate Safety and Immunogenicity of a Live-Attenuated Chikungunya Virus Vaccine Candidate in Adults	Live-attenuated	VLA1553			Valneva Austria GmbH
NCT05072080	A Phase 3 Trial of the VLP-Based Chikungunya Vaccine PXVX0317	VLP	CHIKV VLP/adjuvant			Emergent BioSolutions
NCT04838444	Antibody Persistence And Long Term Safety Of A Chikungunya Virus Vaccine Candidate (VLA1553)	Live-attenuated	VLA1553			Valneva Austria GmbH
NCT04650399	A Multicenter Study to Evaluate Safety and Immunogenicity of a Live-attenuated Chikungunya Vaccine in Adolescents	Live-attenuated	VLA1553			Butantan Institute; Valneva Austria GmbH
NCT04441905	Phase 1 Study of SAR440894 vs Placebo	mAb	SAR440894	Phase 1	Treatment	National Institute of Allergy and Infectious Diseases (NIAID)
NCT03590392	Safety and Immunogenicity of a Candidate CHIKV Vaccine (CHIK001)	Adenovirus	ChAdOx1 Chik			University of Oxford
NCT02230163	Clinical Evaluation of Anti-CHIKV Hyperimmune Intravenous Immunoglobulins	Ig	anti-CHIKV hyperimmune immunoglobulins	Phases 1 and 2		Centre Hospitalier Universitaire de Pointe-a-Pitre

Abbreviations: CHIKV, chikungunya virus; DNA, deoxyribonucleic acid; Ig, immunoglobulin; mRNA, messenger ribonucleic acid; NCT, ClinicalTrials.gov Identifier;

[a]Adapted from ClinicalTrials.gov.

infection in mice, expressed well in nonhuman primates [71], and was safe, tolerable, and expressed in Phase I clinical trials [72].

Neutralizing E2-Specific Antibody Response

The E2 glycoprotein is a target for many neutralizing anti-alphavirus mAbs. In general, neutralization activity corresponds with protection [73], and virus-specific humoral responses from immunized mice or immune individuals are well characterized for several alphaviruses. Numerous Ab-binding epitopes have been identified within the E2 glycoprotein [74, 75], and cross-neutralizing E2-specific mAbs have been identified against the arthritogenic alphaviruses [76–78]. In contrast, a cross-neutralizing E2-specific mAb against the encephalitic alphaviruses has yet to be identified [79, 80]. Potently neutralizing E2-targeting Abs can interfere with different steps in the virus replication cycle, including virus entry, viral egress, and cell-to-cell spread. Blockade of virus entry can occur through multiple mechanisms, including virus aggregation [81], direct blockade of attachment to host receptors (such as Mxra8 or LDLRAD3), or indirect blockade through steric hindrance [8, 9, 82]. After attachment, mAbs can inhibit viral entry by blocking structural transitions [83] or inhibit viral fusion by structurally stabilizing the E2 glycoprotein [78, 84–86].

Protective E1-Specific Antibody Response

The E1 glycoprotein is another target for protective anti-alphavirus mAbs [73, 87–91]. In contrast to E2-specific mAbs, E1-specific mAbs are generally nonneutralizing or weakly neutralize virus in standard focus-forming assays [82, 90, 91]. This may be due to obstruction by the E2 glycoprotein, because exposure of cryptic E1 epitopes requires presentation of different conformational states [92–94] or pretreatment with altered conditions [88, 95–99]. Weakly neutralizing antibodies (NAbs) target Domain III, likely due to its greater exposure on mature virions [82, 100].

Several mAbs recognize the highly conserved fusion loop region and exhibit broad binding to alphaviruses. The ability of nonneutralizing mAbs to inhibit virus egress corresponds with protective in vivo efficacy against homologous and heterologous alphaviruses [90, 91]. During the diagnostic assessment of infection, cross-protective anti-alphavirus mAbs could serve as pan-alphavirus medical countermeasure candidates to limit viral replication and increase the therapeutic window for potent virus-specific treatments. Understanding the conserved epitopes recognized by these Abs can also aid in rational, structure-based, pan-alphavirus vaccine design.

Fc-Mediated Antibody Functions

Because protective capability does not necessarily correlate with neutralization potency of anti-alphavirus mAbs, Fc-mediated effector functions likely play a substantial role in protection against alphaviruses [66, 73, 88]. In mouse models, optimal clearance of infection and reduction of joint swelling for CHIKV- or MAYV-induced musculoskeletal disease required Fc-FcγR interactions, primarily on monocytes [77, 101]. In some cases, reduced efficacy in $Fc\gamma R^{-/-}$ mice was observed, and protection depended on mAb isotype and N297 glycosylation, which modulates effector function [77, 91]. Further assessment is needed to identify non-NAb-based medical countermeasures that are efficacious against alphaviruses.

PROTOTYPE PATHOGENS

Considerations for prototype pathogen assignments included importance as human pathogens, representative pathogenesis patterns, the availability of animal models that recapitulate human disease, current knowledge of replication and pathogenesis, and the status of countermeasure development. Chikungunya virus is by far the most important cause of human disease, with recent outbreaks spreading to near-pandemic proportions due to its propensity for human amplification and peridomestic vector transmission [3]. It is also one of the more heavily studied alphaviruses, has good murine and excellent NHP models, and has vaccines in late stages of clinical trials [102, 103] as well as promising monoclonal antibody therapies [72, 101]. Among the other arthritogenic alphaviruses, RRV is also well studied with some vaccine development reported but has not shown the potential for widespread epidemics beyond Australia and some Oceanic islands.

The second prototype selected was VEEV, for many of the same reasons as CHIKV. It is also relatively well studied for structure and replication, and it is well understood epidemiologically with extensive human disease and some potential for widespread outbreaks (equine-amplified to date, but with potential for human amplification), a long history of vaccine development, but with limited clinical trials due in part to an underappreciated disease burden [104], and some therapeutic monoclonal antibody development [91]. Compared to the arthritogenic alphaviruses, EEEV and WEEV are more virulent but cause less human disease and seem to have less pandemic potential, due to their lack of equine or human amplification [2]. They also have limited vaccine or therapeutic antibody development.

There are important disadvantages in selecting CHIKV and VEEV as prototypes, most obviously their recommended biosafety level 3 (BSL3) containment. However, reliable methods for alphavirus attenuation including chimerization [105–107], genomic deletions [108], and rearrangements that alter levels of gene expression [109] have facilitated generation of viruses that are structurally identical to these and other BSL3 alphaviruses [107] but that can be safely handled at BSL2.

SUMMARY AND FUTURE DIRECTIONS

Preparing for an alphaviral pandemic requires a focus on 2 primary pathogen types: arthritogenic and encephalitic. Although

the developmental algorithm is similar for both, there are specific elements that must be considered for each. Key among these elements are the need for appropriate models, an understanding of the various routes of pathogenesis and host immune response, and data regarding the modes of action for the wide array of vaccines and therapeutics.

Critical to future development of any countermeasures is the need for better and appropriate testing models. For alphaviruses, although many distinct in vitro and animal models exist, they are not well standardized and must be refined to incorporate variables such as age, microbiomes, and long-term sequelae or chronic conditions that are not currently considered. Cell culture models are extremely limited in that they do not simulate entire systems with complex interactions such as synovial joint tissues or brain parenchyma, minimizing the understanding of specific cell types involved in infection. In addition, cell culture models of neuroinvasion do not provide information on delivery across the blood-brain barrier. Thus, until appropriate cell models can be developed, relevant animal systems are critical.

Although animal models do give the most complete profile of pathogenesis, there remains a general lack of knowledge regarding both early infection events and the chronic conditions that exist for many alphaviruses. Receptors are not typically identified, but there is hope that CRISPR technology could facilitate this process. In addition, for the encephalitic alphaviruses, particularly VEEV, animal models also need to address the immunodeficiency that follows infection. Finally, because most alphavirus countermeasure development has focused on the bioweapon property of being infectious by aerosol, there is a strong need for re-evaluation of models to focus on natural route of infection (via mosquito bite).

A final challenge that limits extensive research on the alphavirus infection processes is that many key human pathogens are Risk Group 3 (RG3) and require BSL3 laboratory practices. Because these facilities are not always readily available and due to the risk of working with these agents, there are concerns over how to protect laboratorians performing the critical research.

Although several obstacles do exist to for development of prototype alphavirus countermeasures, much work has already provided a wealth of valuable information that will be critical. First, relatively consistent correlates of protection (NAbs) have been identified for several alphaviruses, which could accelerate vaccine development across the genera. Second, and most importantly, a range of vaccine platforms exist or are currently under development that could be rapidly applied to different alphaviruses. This baseline knowledge provides the foundation to develop the alphavirus prototype pathogen profile for increased preparedness to respond to this group of viruses.

Notes

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