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Alphavirus virus-based chimeric vaccines against encephalitic alphaviruses

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Expert Review

Encephalitic viruses in the Family *Togaviridae*, genus *Alphavirus* are zoonotic pathogens that are transmitted via hematophagous arthropods and have a widespread distribution in North, Central and South America and include Venezuelan equine encephalitis virus (VEEV), Western equine encephalitis virus (WEEV), Eastern equine encephalitis virus (EEEV). The deficit in specific antiviral drugs or vaccines for effective treatment or prevention of infection and disease in humans has prompted the development of recombinant live attenuated vaccines utilizing Sindbis virus (SIN), a relatively nonpathogenic alphavirus in humans, as a means for expression all of the structural proteins of the virulent alphaviruses. The safety and efficacy of these chimeric SIN/VEE viruses have been extensively evaluated in animal models, including immunodeficient mice. The epidemiological distribution of these viruses and the disease manifestations are reviewed briefly. Progress in the evaluation of the safety, immunogenicity and efficacy of the SIN/VEEV and SIN/EEEV candidate vaccines against VEEV and EEEV, respectively, as well as chimeric SIN/RVFV vaccine candidates and the potential for elucidation of the mechanism of efficacy employing mice with selective immunodeficiencies is discussed.

Alfavirusna kimerična cjepiva protiv alfavirusnih uzročnika encefalitisa

Riječ stručnjaka

Virusi encefalitisa iz porodice *Togaviridae*, roda *Alphavirus* su zoonozni uzročnicikoje prenose hematofagni člankonošci rasprostranjeni u Sjevernoj, Centralnoj i Južnoj Americi te uključuju virus venezuelskog konjskog encefalitisa (engl. *Venezuelan equine encephalitis virus*, VEEV), virus zapadnog konjskog encefalitisa (engl. *Western equine encephalitis virus*, WEEV) i virus istočnog konjskog encefalitisa (engl. *Eastern equine encephalitis virus*, EEEV). Manjak specifičnih antivirusnih lijekova ili cjepiva za učinkovito liječenje ili sprječavanje infekcija i bolesti u ljudi potaklo je razvoj rekombiniranih živih atenuiranih cjepiva koja sadrže Sindbis virus (SIN), relativno bezopasan alfavirus za ljude, kao ekspresijski vektor svih strukturalnih proteina virulentnih alfavirusa. Sigurnost i učnkovitost ovih kimeričnih SIN/VEE virusa opsežno je proučavana na životinjskim modelima, uključujući imunodeficijentne miševe. Epidemiološka rasprostranjenost ovih virusa i manifestacije bolesti ukratko su opisane. Raspravlja se o postignutom napretku u ocjenjivanju sigurnosti, imunogeničnosti i učinkovitosti SIN/VEEV i SIN/EEEV potencijalnih cjepiva protiv VEEV odnosno EEEV, kao i potencijalnih kimeričnih SIN/RVFV cjepiva te moguće razjašnjenje mehanizma djelotvornosti koristeći miševe selektivne imunodeficijentnosti.

Epidemiology of encephalitic alphaviruses

Geographic distribution

Encephalitic viruses in the Family *Togaviridae*, genus *Alphavirus* are zoonotic pathogens that are transmitted via hematophagous arthropods and have a widespread distribution in North, Central and South America (reviewed in [1]). These viruses also are highly infectious via the aerosol route, thus have been responsible for numerous laboratory accidents (>150 documented cases without an associated perforating injury) and have been developed as a biological weapon in the U.S and in the former Soviet Union. These alphaviruses were first isolated in the 1930s from diseased horses in California, in Virginia and New

Jersey, and from an infected child in Caracas, Venezuela, and were subsequently named based on their region of isolation as Western equine encephalomyelitis virus (WEEV), Eastern equine encephalomyelitis virus (EEEV) and Venezuelan equine encephalomyelitis virus (VEEV), respectively. Subsequently, additional isolates have been obtained from infected mosquitoes, horses, humans, and other vertebrate species, mainly birds and rodents. Western equine encephalitis virus is distributed from the mid-Western states of Michigan and Illinois to the West coast and clinical cases have been reported in 21 states while EEEV is distributed from Texas to Florida along the Gulf coast and from Georgia to New Hampshire along the Atlantic Coast, with cases reported in 19 states, including the mid-Western states of Wisconsin, Illinois and Michigan. The transmission cycle and detailed distribution are described in [2], and are not covered in this review.

Disease

WEEV, EEEV and VEEV can be neuroinvasive and cause a range of mild to severe neurological symptoms. The alphaviruses WEEV, EEEV, VEEV and, more rarely, Ross River virus, Chikungunya virus and Highlands J virus (HJV) can cause encephalitis in equines or humans [1].

Venezuelan equine encephalitis virus (VEEV)

VEEV has an incubation period of 2–10 days, which results typically in non-specific flu-like symptoms, as with EEEV and WEEV. Severe encephalitis is a less common outcome of VEEV infection in comparison to EEEV and WEEV infection, although VEEV-associated encephalitis is a more common outcome in children. Neurological disease, including disorientation, ataxia, mental depression, and convulsions can be detected in up to 14 % of infected individuals, especially children, although the human case-fatality rate is low (< 1 %). Neurological sequelae of encephalitis are common. The predominant pathological findings in fatal human VEE cases include edema, congestion, hemorrhages, vasculitis, meningitis and encephalitis in the CNS; interstitial pneumonia, alveolar hemorrhage, congestion and edema in the lungs; follicular necrosis and lymphocyte depletion in lymphoid tissue; and diffuse hepatocellular degeneration [1].

Eastern equine encephalitis virus (EEEV)

EEEV has an incubation period of 4–10 days and the infection may lead to sudden onset of fever, general muscle pains, and headache of increasing severity. In human cases of encephalitis, fever, headache, vomiting, respiratory symptoms, leucocytosis, hematuria, seizures and coma may occur. EEEV is considered to be the most virulent of the encephalitic alphaviruses, with a case-fatality rate estimated at > 33 %. Clinical imaging studies of serologically-confirmed human EEEV infection detect changes in the basal ganglia and thalami, suggesting brain edema, ischemia and hypoperfusion in the early stage of disease. Gross pathological examination of fatal human cases demonstrated brain edema with necrosis, facial or generalized edema, vascular congestion and hemorrhage in the brain and visceral organs. Histopathological observations of the brain include vasculitis, hemorrhage and encephalitis [1].

Western equine encephalitis virus (WEEV)

WEEV has an incubation period of 2–7 days, and the infection may result in mild disease with non-specific symptoms, e.g., sudden onset of fever, headache, nausea, vomiting, anorexia and malaise, and in some cases, more severe neurological signs. Encephalitis or meningo-encephalomyelitis occurs in a minority of infected individuals, resulting in neck stiffness, confusion, tonic-clonic seizures, somnolence, coma and death. Neurological sequelae of 5–30 % has been reported in survivors of encephalitis, in particular among children < 1 year old. Serological studies suggest an age-associated relationship between inapparent to apparent infections, with significantly increased ratio for children over age 14. The overall case-fatality rate is about 3 %. Encephalitis due to WEEV is characterized by a vasculitis and focal hemorrhages mainly affecting the basal ganglia and the nucleus of the thalamus, which may be mistaken for resolved infarcts in elderly patients [1].

Sindbis virus-based chimeric vaccine approach

Recombinant live-attenuated vaccines and, in particular, an alphavirus-based approach, represent a viable approach to the production of safe, immunogenic and efficacious vaccines against the encephalitis alphaviruses [3–7]. Alphavirus genomes are relatively easy to manipulate, and infectious clones are available for construction and in vitro transcription of recombinant viruses for vaccine production by cell transfection [8]. By utilizing as a vector the genome of Sindbis virus (SINV), a relatively nonpathogenic alphavirus in humans, chimeric SIN/VEE virus(es) can be designed to express all of the structural proteins of the virulent alphavirus. These constructs contain the cis-acting RNA elements and non-structural protein genes of the SINV genome, which are required for replication and transcription of the subgenomic RNA, e.g., 5' untranslated region (UTR), 3' UTR, and the subgenomic promoter. The promoter element, located upstream of the subgenomic RNA transcription start, and the four 5' terminal nucleotides of the subgenomic RNA encompass the end of nsP4 and the termination codon of the nsP-coding open reading frame (schematically depicted in [7]).

For VEEV, this approach has been used to evaluate a variety of representatives among the different VEEV serotypes (epidemic and endemic strains). For EEEV, vaccine candidates were developed using structural protein genes from a North American (NA) EEEV strain and from a naturally attenuated Brazilian (SA) EEEV isolate [3].

Following electroporation of the in vitro transcribed RNA, these chimeric SIN/VEE and SIN/EEE viruses replicate to high titer in the commonly used and readily available mammalian fibroblast cell line, baby hamster kidney cells (BHK) [3, 4, 7].

Safety in animal model

The mouse encephalitis model is well established for several alphaviruses, although it generally lacks the ability to reproduce the vascular component of the disease that is typical for EEE in humans. A murine model for VEEV-induced encephalitis and lymphotropism is well established [4, 5, 9–12]. Experimental studies have demonstrated that VEEV infection results in biphasic disease, which starts with productive infection of lymphoid tissue and ends in the destruction of the CNS caused by very efficient viral replication and a »toxic« neuroinflammatory response. By the time encephalitis has developed, infectious virus is usually absent from peripheral organs and blood. However, virus replicates to high titers in the brain and encephalitic mice die within 5–7 days of infection. This classical mouse model is useful to study the mechanisms of pathogenesis in mice and to evaluate vaccine (and antiviral drug) efficacy because CNS infection consistently occurs in naive mice and is uniformly fatal [4, 12]. Recently, this experimental approach has been used in mice with selective immunodeficiencies to facilitate studies of VEEV clearance from the brain and also provides additional safety data for these vaccines [6].

Safety in immunocompetent mice

VEEV vaccines

The safety of several chimeric SIN/VEE virus vaccine candidates have been tested in mice of different genetic background and age, and via several routes of infection. All were evaluated in parallel with the investigational vaccine, TC83, which is currently used for vaccination of research and military personnel, but which is documented to have significant adverse effects. SIN83 chimeric virus at doses in range of 2×10^4 to 2×10^6 PFU did not cause any detectable clinical disease in either adult or weanling mice when monitored up to 21 days after either s.c. or i.c. inoculation [4]. When tested at a higher s.c. dose of 5×10^6 PFU, none of the chimeric SIN/VEE virus, e.g., SIN83, SIN/ZPC, or SIN/TRD resulted in morbidity or mortality [5]. This is in contrast to mortality of 10 % and 100 % for TC83, which were both fatal to newborn outbred (NIH Swiss) mice at comparable doses of 2×10^5 and 5×10^6, respectively [4, 7]. Another VEEV vaccine candidate undergoing preclinical testing, V3526, was 100% fatal to these mice at the lower dose of 1×10^5 [7]. Further, TC83 was 100 % fatal by i.c. inoculation at the higher doses (2×10^5 and 2×10^6); although at the lower dose, TC83 was not lethal for majority of the mice, it caused neurological sequelae (ataxia, paralysis) and weight loss in 33–44 % of the mice [4]. Similarly, in adult inbred (C57BL/6) mice, no deaths were observed in mice that received two doses of 5×10^5 PFU approximately one month apart and monitored for a total of 42 days [6].

EEEV vaccines

The safety of the chimeric SIN/EEEV vaccines were evaluated in 8-week-old, female NIH Swiss mice inoculated subcutaneously with doses in the range of 3.7–5.8 $\log_{10}$ PFU per animal. Both of the chimeric SIN/EEE virus strains (SIN/NAEEEV and SIN/SAEEEV) were highly attenuated in mice, evidenced by the lack of neurological disease, febrile response, or significant weight loss, although intracraneal inoculation of weanling (6-day-old) NIH Swiss mice with these vaccines in the same dose range (5 $\log_{10}$ PFU) resulted in higher neurovirulence than the s.c. route, but with delayed kinetics for SIN/SAEEEV in comparison to the parental SAEEEV [3].

Safety in immunodeficient mice

VEEV vaccines have been evaluated for safety in immunodeficient mice developed on the C57BL/6 background. We selected the chimeric SIN/ZPC virus as a promising vaccine candidate for further examination of its safety in inbred mice with selective immunodeficiencies in the T cell compartments ($\alpha\beta$ T cell receptor (TCR)-deficient or $\gamma\delta$ TCR-deficient mice), in the B cell compartment (μMT-deficient mice), or in their ability to respond to the cytokine interferon gamma (IFN-γR-deficient mice). Vaccines were delivered via two s.c. inoculations (5×10^5 PFU/animal) and survival evaluated for 14 days following the booster dose [6]. All alpha-beta ($\alpha\beta$) TCR- and $\gamma\delta$ TCR--deficient, and μMT-deficient mice survived vaccination and none of these animals showed any signs of disease. 93 % of IFN-γR-deficient mice survived vaccination. The deaths of 2/30 animals occurred suddenly without any previous signs typical of VEE (anorexia and/or paralysis), no infectious virus could be isolated from their brains of the two animals, thus it is suggested that the overall poor health status of these animals was a contributing factor [6].

Immunogenicity & efficacy

VEEV vaccines

To evaluate the immunogenicity and efficacy of the chimeric SIN/VEE virus, we have utilized as challenge virus the ZPC738 strain, a 1997 sentinel hamster isolate from Venezuela [13] that is characterized as an enzootic subtype ID strain, [5], which is uniformly lethal in naive outbred (NIH Swiss) mice by 7 days after intranasal (i.n.) inoculation of 2×10^5 PFU/animal [5]. Immunodeficient mouse strains were similar in their susceptibility to disease development and death following VEEV infection, with disease observed between 6 and 8 days following infection with the parental VEEV strain (ZPC738) infection, as observed for the parental (C57BL/6) immunocompetent mice

[14]. Therefore, these strains also represent a valid alternative vaccination/challenge model for chimeric SIN/VEE virus and have enabled us to examine different immune effector mechanisms.

Immunogenicity

Immunocompetent newborn [4] and adult NIH Swiss [5] as well as adult C57BL/6 [6] mice consistently developed neutralizing antibody ($PRNT_{80}$ titer > 20), although the titers varied, depending upon the number of vaccinations [5], the vaccine dose [4, 5], the time post-vaccination [5], and the chimeric virus evaluated. In contrast, immunodeficient (C57BL/6) mice vary considerably in their ability to develop VEEV-specific neutralizing antibody, and antibody titers did not correlate with disease development and survival outcomes [6], but are likely to depend upon the selective nature of the immunodeficiency related to absence of mature B cells (e.g., in μMT mice) or to priming deficiencies (e.g., lack of T and B cell interactions in $\alpha\beta$ TCR- and to a lesser extent, in IFN-γR – and $\gamma\delta$ TCR-deficient mice).

Efficacy

The chimeric SIN/VEE vaccines were highly efficacious in newborn and adult NIH Swiss as well as in C57BL/6 mice challenged with ZPC738, as described above, irrespective of the challenge method (i.c., i.n., or s.c.) [4–6]. Efficacy studies were first performed using the chimeric SIN83 vaccine in 6-day-old NIH Swiss mice. SIN83 provided 100 % protection against lethal s.c. challenge and did not develop clinical symptoms of disease at any time during the 2 month observation period, regardless of the dose of SIN83 used for immunization (in the range of 10^3–10^6 PFU) [4], although mice were only partially protected against i.n. and i.c. challenges with high doses of ZPC738 [5].

Protection studies using the chimeric SIN/ZPC vaccine were performed in adult immunocompetent C57BL/6 (wild type, WT) mice and their immunodeficient counterparts, as described for safety studies. Several replicate experiments with observation over a 28 day period following challenge with ZPC738 were carried out. None of the $\alpha\beta$ TCR-deficient mice survived challenge mock-vaccinated WT mice (0 of 18; 2 replicates; 0 %). Vaccinated $\gamma\delta$ TCR--deficient mice were protected, with survival of 98 %, which was significantly greater (Fisher's exact test; p = = 0.0001) than for vaccinated WT mice (93%). Survival of IFN-γR KO mice was reduced in comparison to vaccinated WT and $\gamma\delta$ TCR KO mice, although the survival time was also extended in comparison to mock-vaccinated and vaccinated $\alpha\beta$ TCR-deficient mice. The majority of μMT-deficient mice were not protected from challenge (13% survival) and this was not significantly different from mock vaccinated WT mice (Fisher's exact test; $p = 0.2748$) [6]. In addition to survival analysis, virus titers in the brain and peripheral organs of vaccinated, challenged immunodeficient mice were evaluated as a marker of protection. At day 3 post-challenge, viral titers in the brain and peripheral tissues of wild type vaccinated mice were uniformly low and at day 7 post-challenge, were undetectable, in contrast to mock-vaccinated wild type mice. For vaccinated immunodeficient mice, high levels of infectious virus were detected in all tissues at either day 3 or day 7, with the highest titers in the brain at day 7 post-challenge [6].

EEEV vaccines

EEEV vaccine candidates were evaluated in outbred (NIH Swiss) mice using as challenge virus the NA strain FL93–939 (10^6 PFU/animal via intraperitoneal route, i.p.), which causes clinical encephalitis by day 3–4 and is uniformly lethal to mice by day 4–5 [3]. Mice were vaccinated with doses in the range of 3.7–5.7 or 3.8–5.8 $\log_{10}$ PFU 10 for NA and SA based chimeric virus (respectively) via subcutaneously route and challenged (10^6 PFU/i.p.). The chimeric SIN/NAEEEV and SIN/SAEEEV vaccines provided 100 % protection for both vaccines/all doses tested, with the exception of the low dose SIN/SAEEEV, which protected 80 % of the mice. These survival outcomes correlated with the development of detectable IgG antibodies in vaccinated mice. The antibody titer, as determined via plaque reduction neutralization test as well as ELISA, showed a dose response to SIN/NAEEEV, with the highest mean titers resulting from immunization with 5.7 $\log_{10}$ PFU and the SIN/NAEEEV (5.7 log dose) elicited cross-reactive neutralizing antibodies against the SA EEEV strain, albeit at a relatively lower titer. At the lowest SIN/NAEEEV dose (3.7 $\log_{10}$ PFU), 70 % of the mice had detectable PRNT titer against homologous virus, but among those that were negative (3/10), one had a positive IgG titer by ELISA.

Future directions

Ongoing VEEV studies enable us to dissect the features of the immune response and its relationship to the pathogenesis of the virulent VEEV as well as to understand the basis of the protection elicited by chimeric SIN/VEE viruses. This approach also has application to other neurovirulent viruses for which little or no treatment or vaccination options are available. Currently, we are developing and evaluating chimeric SINV-based vaccines against Rift Valley Fever virus (RVFV) [15] and highly pathogenic avian influenza A/H5N1 (Paessler, unpublished).

Mechanisms of protection against VEE

Protection in immunodeficient mice provide insight into the mechanism of protection, which appears to be mediated predominantly via selective T cell population [6]. This is suggested by reconstition of protection in $\alpha\beta$ TCR-deficient mice by transfer of bulk $CD3^+$ T cells using an adoptive transfer approach performed using VEEV-specific T

cells isolated from the spleens of SIN/ZPC-vaccinated donor C57BL/6 mice. Transfer of these cells into $\alpha\beta$ TCR--deficient mice resulted in restoration of protection from lethal encephalitis that was not observed in the mock-transfered mice.

Chimeric SIN/RVFV vaccines

Several recombinant SIN/RVFV viruses were designed to express large fragments containing the segments of heterologous RVFV proteins, e.g., E2-RVFV envelope glycoprotein Gn fusion proteins, on the surface of chimeric, infectious viral particles or secreted from infected cells [15]. Gn was efficiently expressed by these chimeric SIN/RVFV constructs, could be produced in cell culture, albeit at a reduced efficiency compared to constructs expressing other heterologous genes, and were subsequently evaluated in mice for their ability to induce protection against RVFV (ZH501strain) challenge. Mice were immunized once or twice with 5×10^6 PFU of the recombinant viruses that expressed either a secreted form of RVFV Gn or E2-Gn fragments, and challenged via s.c. with 5×10^3 PFU of RVFV ZH501. All of the constructs induced at least partial protection against the challenging virus, and a second booster dose increased protection. Chimeric viruses containing VEEV or SINV-E2 fused in the amino terminus with the 318-a.a.-long fragment of RVFV Gn were the most efficacious among the constructs tested, and mice developed no clinical signs of the disease. Ongoing studies focus on the improved production of these viruses.

Conclusions

Chimeric alphavirus-based vaccines represent an important advance in vaccine development for a number of infectious pathogens, as Sindbis replicon particles have been shown to be safe and effective for the induction of both cell--mediated and humoral immune responses [16]. As summarized in this review, SIN/VEE and SIN/EEE vaccines have proven to be safe in all animal models tested to date, e.g., newborn and adult outbred mice, adult inbred immunocompetent and immunodeficient mice, as well as hamsters. Significantly, the alphavirus vaccines are immunogenic and efficacious in outbred and inbred immunocompetent and immunodeficient animals [3]. Other preclinical studies of SIN-based influenza A/H5N1 vaccines have demonstrated efficacy in the Balb/C mouse model (Paessler, unpublished). This approach is an intense focus of investigation for the development of vaccines against specific pathogens as well as the optimization of delivery methods and adjuvant augmentation of immune responses [17–22]. For VEEV studies, this vaccine approach has led to the development of a unique model for studying the persistence of attenuated variants of VEEV in the brains of chimeric SIN/VEE vaccinated, VEEV-challenged $\gamma\delta$ TCR deficient mice, which survive and do not develop clinical disease [6].

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