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Vaccines for Venezuelan equine encephalitis

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Abstract

Arboviruses are capable of causing encephalitis in animals and human population when transmitted by the vector or potentially via infectious aerosol. Recent re-emergence of Venezuelan equine encephalitis virus (VEEV) in South America emphasizes the importance of this pathogen to public health and veterinary medicine. Despite its importance no antivirals or vaccines against VEEV are currently available in the USA. Here we review some of the older and newer approaches aimed at generating a safe and immunogenic vaccine as well as most recent data about the mechanistic of protection in animal models of infection.

Venezuelan equine encephalitis virus and its replication

Venezuelan equine encephalitis virus (VEEV, *Alphavirus* in the *Togaviridae* family) is an enveloped virus with a non-segmented, positive-sense RNA genome of approximately 11.4 kb (Fig. 1). The 5' two-thirds of the genome encodes four nonstructural proteins (nsP1 to nsP4) that form an enzyme complex required for viral replication [1–3]. The full-length RNA then serves as a template for the synthesis of positive-sense genomic RNA and for transcription of a subgenomic 26S RNA [1]. The approximately 4-kb-long, subgenomic RNA corresponds to the 3’ one-third of the viral genome and is translated into a structural polyprotein that is proteolytically cleaved into the capsid and the envelope glycoproteins E2 and E1 [4].

Epidemiology of encephalitic alphaviruses

Most of the encephalitic viruses in the Family *Togaviridae*, genus *Alphavirus* are zoonotic pathogens that are transmitted via hematophagous arthropods. These pathogens have a widespread distribution in North, Central and South America (reviewed in [5]). Some of them are highly infectious via the aerosol route, thus have been responsible for numerous laboratory accidents (>150 documented cases without an associated perforating injury) and/or have been developed as a biological weapon in the U.S and in the former Soviet Union. First virus isolations were reported 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.

Disease in humans

VEEV infection has an incubation period of 2–10 days, which results typically in non-specific flu-like symptoms. 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%).

Mouse model for VEEV infection

The murine model for VEEV-induced disease is established and typically utilizes subcutaneous inoculation [6–9]. Previous studies have demonstrated that the murine model is characterized by biphasic disease, which starts with the productive infection of lymphoid tissue and culminates in the destruction of the CNS by viral replication and a “toxic” neuroinflammatory response that is uniformly lethal [10–16]. By the time the acute encephalitis has developed in an infected mouse, the virus is usually absent from the peripheral organs and blood [10–16]. The mouse model is useful for testing of vaccine and drug efficacy.

Humoral immunity

Protection from peripheral inoculation or natural alphavirus infection depends mostly on the production of neutralizing antibodies [17,18]. While virus neutralizing antibody is important for the protection against natural (peripheral) challenge mediated by mosquito-borne transmission, more recent studies demonstrate that even relatively high serum titers of polyclonal neutralizing antibody achieved via passive transfer (not achievable with any vaccination known to authors) do not protect mice from intranasal (i.n.) challenge in the mouse model of infection [19,20]. These data supports the conclusion that virus neutralizing antibody plays a significant role in preventing the penetration of the CNS after peripheral challenge with VEEV, while it is relatively ineffective in controlling the rapid onset of CNS disease following i.n. infection [20,21].

Alpha Beta (αβ)T cell response

The αβ T cells represent the major proportion of T cells that respond to various pathogens and are subdivided into $CD4^+$ helper and $CD8^+$ cytotoxic cells. These “conventional” T cells have been well characterized functionally. Prior studies in mice vaccinated with TC83 suggest that Th1-type responses predominate [22]. However, in mice vaccinated parenterally with TC83, cytotoxic T cell activity could not be detected in the spleen or draining lymph node [23]. It was previously demonstrated that host factors contribute to mortality in neurovirulent Sindbis virus (NSV)-induced encephalitis model in mice [24]. Animals deficient in αβ-, but not γδ-T cells had lower mortality rates when infected with neuroadapted Sindbis virus (NSV), indicating their different contribution to the outcome of the brain infection [25]. $CD4^+$ T cell effector functions in encephalitis may include a combination of Th1, Th2 and regulatory T cells ($CD25^+$, forkhead family transcription factor, $Foxp3^+$) activities [26–28]. In other models of acute encephalitis, such as experimental autoimmune encephalomyelitis (EAE) as well as in more chronic encephalitis described for toxoplasmic encephalitis (TE), the myelin basic protein specific $CD4^+$ T cells are crucial for the development of the encephalitis. $CD8^+$ T cells contribute to both pathogenesis of and recovery from encephalitic flavivirus infections [29, 30] but their role in VEEV-induced encephalitis, immunopathology and protection is less clear. In general, it has been hypothesized that cell-mediated cytotoxicity is less critical for control of cytopathic viruses such as VEEV in comparison to noncytopathic viruses [31,32]. This can be extended to the CNS where elimination of virus from neurons is thought to be via non-lytic mechanisms due to the poor regenerative capacity of this cell type [24,32–37].

Gamma delta (γδ) T cell response

Recent studies suggest an important role for another well studied T cell subpopulation, γδ T cells, in disease development and lethal outcomes of VEEV infection [19]. Specifically, qualitative and quantitative changes in the inflammatory cellular infiltrates in vaccinated and challenged mice suggest a regulatory role in the secondary response to virus. However, direct evaluation of their role in pathology vs. protection is limited by the lack of feasible methods for isolating sufficient quantities for adoptive transfer. This cell population constitutes a relatively minor subset of T cells in lymphoid tissues despite being well represented in other sites including the peripheral blood and in the epithelial and mucosal layers in the respiratory and gastrointestinal (GI) tract [38,39]. The role of γδ T cells in the CNS has not been functionally well-defined, despite studies showing their potential importance in diminishing the neurovirulence of West Nile Virus (WNV) [40] and modulating the progression of neurocysticercosis in TCR-δ chain-deficient mice [41]. In the case of viral infections, γδ T cells can substitute for αβ T cells in a virus model of demyelination [42]. In the neurovirulent Sindbis virus model, animals deficient in αβ, but not γδ T cells have lower mortality rates, indicating the differential contribution of these cell types to the outcome of the brain infection [25].

Live-attenuated VEE vaccines

Following upon the success of the 17D yellow fever vaccine by Theiler [43], VEEV was attenuated by 83 serial passages in guinea pig heart cells to produce the TC-83 strain [44]. TC-83 was first tested extensively in equids during the 1971 Texas VEE epizootic/epidemic, where it may have contributed to limiting the spread northward. Although the vaccine produces viremia, fever and leucopenia in horses, robust neutralizing antibodies are generated as well as protection from VEEV challenge [45]. The TC-83 strain continues to be manufactured in Mexico and Colombia for use as a live vaccine in equids, but is currently only marketed in inactivated form in the U.S.

The history of serious laboratory infections by VEEV as well as its development as a biological weapon in the U.S. and former USSR prompted the use of TC-83 in humans as an investigational new drug (IND) product. TC-83 produces seroconversion in about 80% of humans (neutralizing antibody titers ≥20) but mild to moderate flu-like symptoms in about 205 of volunteers. Persons who fail to seroconvert in the U.S. Army Special Immunizations program are given boosters of C-84 (inactivated TC-83) [46]. Unlike TC-83, C-84 produces only occasional, mild, reactions. Some rodent studies indicate that TC-83 protects mice better against aerosol challenge than C-84 [47], although neither completely protects nonhuman primates against aerosol exposure [48].

The reactogenicity and limited immunogenicity of TC-83 may be the result of only 2 attenuating mutations among the 12 mutations that accompanied in vitro passage of the Trinidad donkey strain [49]. Presumably, these mutations are subject to reversion in vaccinees, which also presents a risk of epizootic amplification. To produce a more stably attenuated VEEV vaccine candidate, the Trinidad donkey genome was cloned in cDNA form and attenuation was achieved by inserting either point mutations or a PE2 cleavage-signal mutation combined with an E1 gene resuscitating mutation. The latter strain, called V3526, is safe and immunogenic for mice and nonhuman primates, and appears superior to TC-83 in rodents [50–52]. V3526 also appears to have a lower risk for environmental transmission and distribution [53,54]. Although V3526 elicits neutralizing antibodies in nonhuman primates only against homologous IAB VEEV strains, it protects against aerosol challenges with both IAB and IE strains [55]. Furthermore, it is not neurovirulent when administered intracranially to juvenile rhesus macaques [56].

Because TC-83 can be transmitted by mosquito vectors [57] and was isolated in mosquitoes collected in Louisiana during the 1971 VEE epizootic/epidemic [57], efforts were made to improve it safety by preventing its ability to infect insects. Translation of the viral structural proteins and, ultimately, viral replication was made dependent on an internal ribosome entry site of encephalomyelocarditis virus, and the subgenomic promoter was inactivated with 13 synonymous mutations (Fig. 2)[58]. This recombinant virus further attenuated TC-83, but was completely unable to infect mosquito cells or mosquitoes in vivo. Thus, the manipulation of structural protein gene expression may be useful not only as a safety feature of live vaccine strains but also as an attenuation mechanism.

Inactivated VEE vaccines

Due to the economic devastation caused by VEE epizootics in regions of Latin American that relied on equids for agriculture and transportation, vaccines were first produced soon after VEEV was isolated in 1938 [59,60]. Formalin-inactivated preparations were initially made from mouse brain and other animal tissues following infection with wild-type, subtype IAB strains isolated during epizootics [61]. These vaccines were probably efficacious in most animals, but the equid-amplification potential of the parent viruses presented a high risk if residual live virus remained in vaccine lots. Sequencing studies showing conservation among all IAB strains isolated from 1938–1973 [62,63], as well as the isolation of live virus from at least one human vaccine preparation [64], indicated that this “escape” of VEEV from incompletely inactivated vaccines probably occurred on several occasions. The disappearance of subtype IAB VEEV since 1973, when inactivated vaccines were replaced by the live-attenuated TC-83 strain, supports this conclusion.

Currently, commercial equine vaccines marketed in the U.S. consist of inactivated TC-83, often combined with the other principal alphaviral encephalitides, eastern and western equine encephalitis viruses.

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 [19,21,65–67]. 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 (Fig. 3).

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 PFUdid 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 [66]. 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 [21]. 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 [66,67]. Another VEEV vaccine candidate undergoing preclinical testing, V3526, was 100% fatal to these mice at the lower dose of 1×10^5 [67]. Additional safety studies were performed in inbred mice with selective immunodeficiencies in the T cell compartments (αβ T cell receptor (TCR)-deficient or γδ 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 [19].All alpha-beta (αβ) TCR- and γδ TCR- deficient, and μMT-deficient mice survived vaccination and none of these animals showed any signs of disease.

The chimeric SIN/VEE vaccines were highly efficacious in 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.) [19,21,66], and in immunocompetent C57BL/6 (wild type, WT) mice and their immunodeficient counterparts. Vaccinated γδ TCR-deficient mice and WT 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 γδ TCR KO mice, although the survival time was also extended in comparison to mock-vaccinated and vaccinated αβ 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; $p = 0.2748$) [19].

Recent results strongly suggest that VEEV-specific $CD4^+$ T cells are the primary cell population responsible for protection from lethal encephalitis elicited by vaccination with this chimeric live-attenuated vaccine [20]. A minor proportion of μMT mice survive lethal challenge infection following vaccination suggesting that antibody is not absolutely required for protection from VEEV-mediated lethal encephalitis. Passive transfer of hyperimmune serum reactive with VEEV into immunocompetent mice results in a statistically significant increase in survival time from a median of 8 days for the mice treated with hyperimmune serum in comparison to 5.5 days for the placebo (PBS-treated) controls. However, all hyperimmune serum-treated animals succumbed to infection within 12 days (0% survival, 0/8 mice), indicating that antibody alone is not sufficient for protection from infection.

Alphavirus replicons

Because only the nonstructural proteins and cis-acting RNA sequences are required for alphavirus genome replication, the structural protein genes can be replaced and foreign antigens expressed at high levels [68,69]. These replicon genomes can be packaged into virus-like particles by capsid and envelope proteins provided in trans from a second genetic construct (Fig. 4). To reduce the probability of recombination between replicon and "helper" RNAs, two separate helpers can be used to encode the structural proteins [70]. Packaging cell lines that constitutively express the structural proteins can also be used to produce replicon particles [71]. Even in packaged form, the replicon genomes cannot produce structural proteins, so they express their foreign genes during only one round of replication and cannot spread to other cells. This immunogenicity advantage of antigenic gene replication with the safety advantage of limited spread has resulted in the use of alphavirus replicon systems to express many immunogens from other viruses or proteins [72,73]. However, these replicons can also be used to immunize against homologous or heterologous alphaviruses without the safety concerns of a live replicating virus. Furthermore, alphavirus replicon particles appear to have intrinsic systemic and mucosal adjuvant activity due to their RNA replication activity [74].

DNA vaccines

Recent studies employed directed molecular evolution to generate cross-reactivity Venezuelan equine encephalitis virus (VEEV) envelope glycoproteins. Dupuy et al. [75] generated libraries of chimeric genes expressing variant envelope proteins and used selected variants to vaccinate mice. Their results indicate that it is possible to improve the immunogenicity and protective efficacy of alphavirus DNA vaccines using directed molecular evolution. Previously, others

have shown that vaccinia virus recombinants as well as adenovirus-based approach can be successfully utilized to generate protective immunity against VEEV in the mouse model [17, 76]

Future directions

Development of a vaccine that would induce rapid protection in people and animals remains an important issue. New generation of safe chimeric viruses that are unable to infect mosquitoes is currently in development and preclinical studies are ongoing. This would be a major step toward production of live-attenuated vaccine that is safer for the environment and would minimize the risk for vaccine-born outbreaks as reported in 1970"s for TC83 vaccine. However, additional efficacy trials in non-human primates are required to confirm the protective response for some of the candidates and for initiation of potential clinical trials. Some of the recent experimental data indicates that the immune response against VEEV needed to control the brain infection may differ from the one required to control the peripheral infection. Therefore, vaccines that protect against the natural infection may differ in the future from those that are supposed to protect against high-dose air-borne challenge and may be more attenuated. Our group is also trying to use the most recent pathogenesis data to design a therapeutic vaccine that would reverse already established encephalitis and help clear the virus from the brain.

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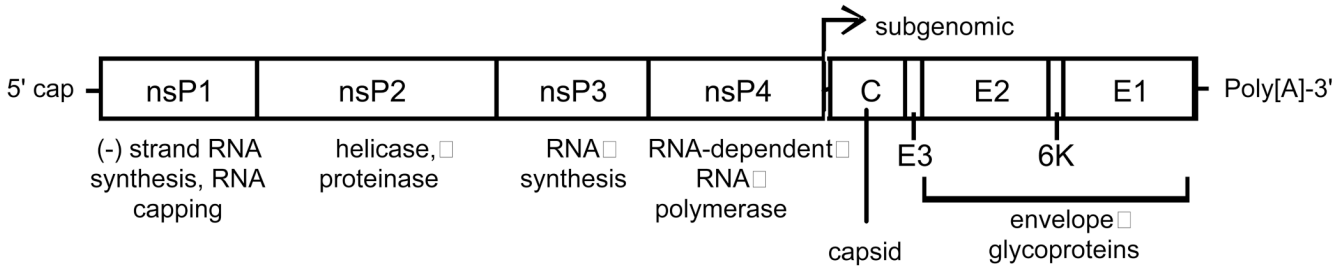

Figure 1.
Genomic organization of Venezuelan equine encephalitis virus.

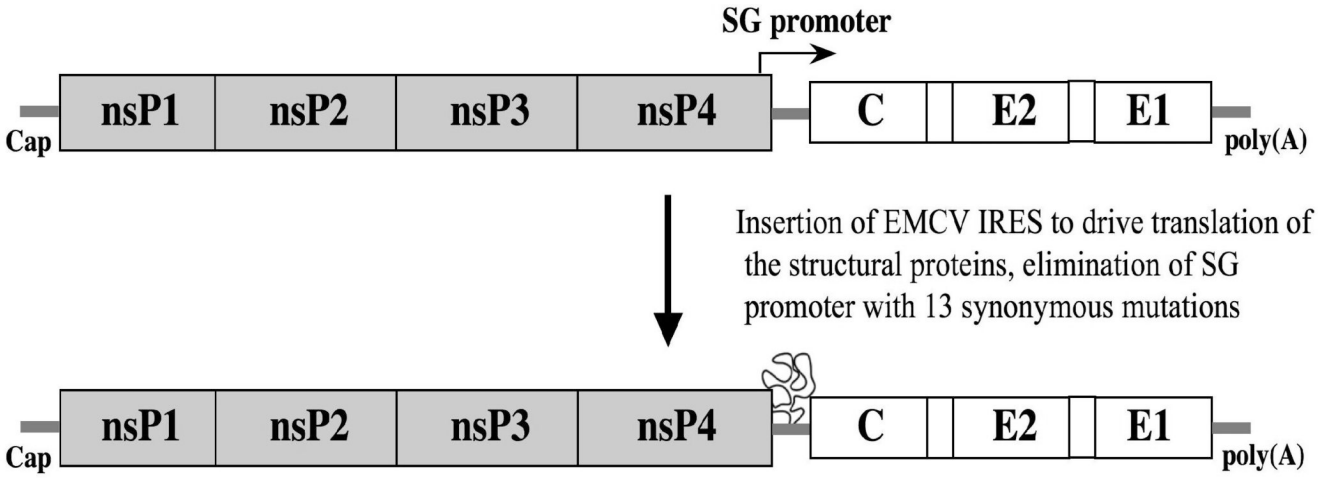

Figure 2.
Mutant strain of TC-83 with the subgenomic promoter inactivated and the translation of the structural protein genes placed under the contol of an encephalomyelocarditis virus internal ribosome entry site (IRES). From reference [58].

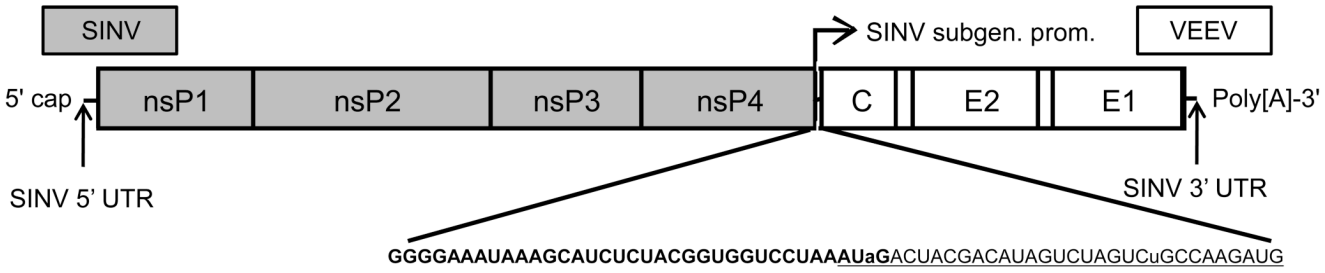

Figure 3.

Genetic organization of chimeric Sindbis/Venezuelan equine encephalitis virus vaccine candidates described in references [21,66]. The cis-acting sequences were derived from Sindbis virus strain Toto1101 or AR339, along with the nonstructural protein genes, while the structural protein genes were derived from Venezuelan equine encephalitis virus strain TC-83 or ZPC738. Bold letters represent SINV-specific sequence, and underlined letters represent VEEV-specific sequence. Lowercase letters indicate mutations introduced into the VEEV sequence to make the junction more SINV-like and to preserve the putative secondary structure of the 5' UTR in the VEEV subgenomic RNA.

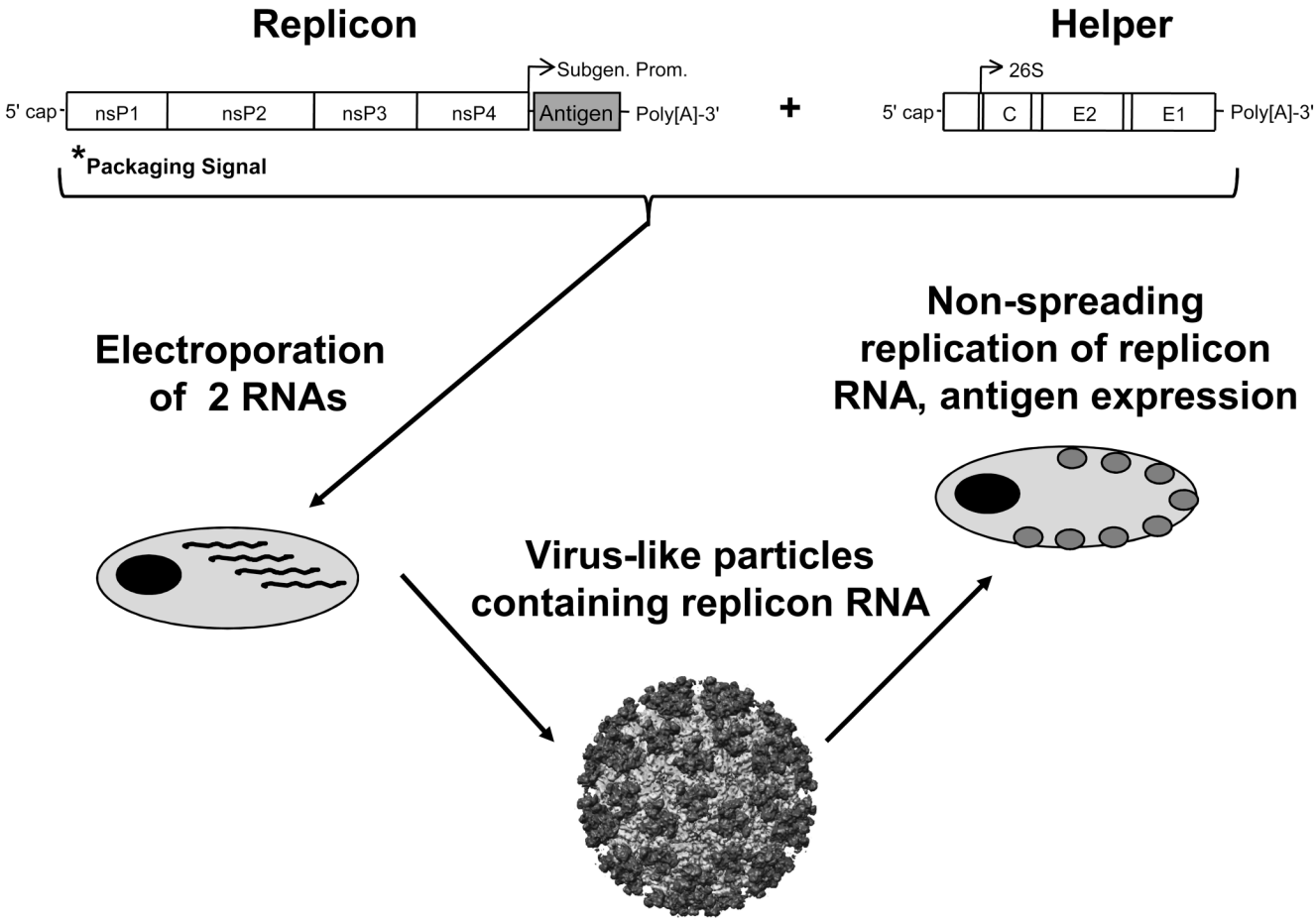

Figure 4.
Genetic organization of alphavirus replicons and helpers used to package them into virus-like particles. In some cases, the helper construct is divided into the capsid and E2/E1 protein to minimize the change of RNA recombination that can produce complete genomes and live virus.