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Alphavirus replicon vaccines

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Abstract

The alphavirus replicon technology has been utilized for many years to develop vaccines for both veterinary and human applications. Many developments have been made to the replicon platform recently, resulting in improved safety and efficacy of replicon particle (RP) vaccines. This review provides a broad overview of the replicon technology and safety features of the system and discusses the current literature on RP and replicon-based vaccines.

Keywords: alphavirus biology, dendritic cell tropism, safety, DIVA

Introduction

Traditionally, veterinary vaccines consisted of either modified live or inactivated preparations. Modified live vaccines (MLV) have the possibility of reverting to virulence with subsequent spread among surrounding animals (Botner *et al.*, 1997; Madsen *et al.*, 1998). Inactivated vaccines often generate insufficient cell-mediated immunity required for protection and so must be combined with adjuvants that are able to induce the required immune response (Minke *et al.*, 2004; Meeusen *et al.*, 2007). Thus, there has been a focus on 'second-generation' vaccines, some of which have already been licensed for commercial veterinary use (Meeusen *et al.*, 2007).

Alphavirus replicon-based vaccines represent a viable option for next-generation vaccine development. To date, alphavirus replicon-based vaccines have not been approved by any government regulatory agency for use in animals or humans. Alphavirus replicon particles (RPs) are single-cycle, propagation-defective particles that are not able to spread beyond the initial infected cells. RPs are produced by removing the alphavirus structural genes from the replicon RNA vector and replacing them with a heterologous gene or genes of interest. Helper genes can be provided *in trans* along with replicon RNA and co-transfected into permissible cells, resulting in the packaging of the replicon RNA. RP vaccines have been evaluated in many different species of animals as well as humans with a proven record of safety and efficacy. These vaccines are capable of inducing robust and balanced immune responses and offer many other advantages that ideal vaccines possess.

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Alphavirus biology

The *Alphavirus* genus belongs to the Togaviridae family and contains 28 virus species (Griffin, 2007). Alphaviruses are positive-sense, single-stranded RNA viruses with a genome of approximately 11.5 kb in length. The positive-sense genome contains two open reading frames (ORFs) and encodes four non-structural proteins and five structural proteins (Strauss and Strauss, 1994). The 5′ ORF encodes four non-structural proteins (nsp1–4) and the 3′ ORF encodes the virus structural proteins (capsid and glycoproteins (E3, E2, 6 K and E1)) (Strauss and Strauss, 1994; Rayner *et al.*, 2002). The non-structural proteins are translated from the positive-sense genomic

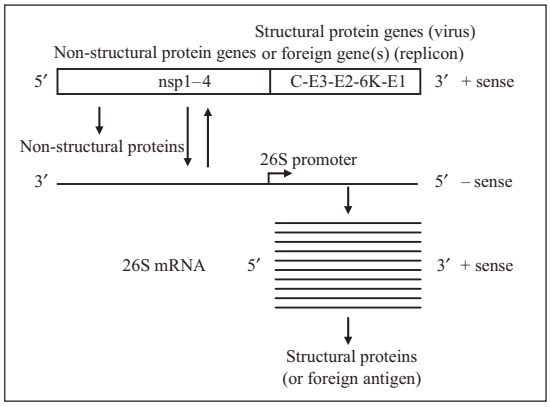

Fig. 1. Alphavirus genome organization and replication strategy.

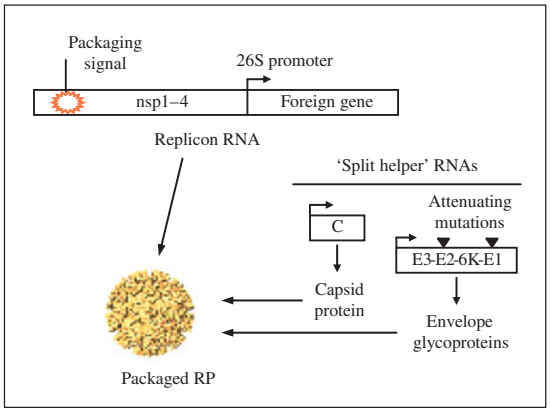

Fig. 2. Alphavirus RP vaccine and packaging system. Portions of this image courtesy of AlphVax Inc. (Research Triangle Park, NC, USA).

RNA and function to transcribe full-length negative-sense RNA (Fig. 1). Translation of the nsp1–3 polyprotein is terminated by an opal stop codon located between nsp3 and nsp4; the polyprotein nsp1–4 is produced when translational read through occurs at the nsp3–4 junction (Kinney *et al.*, 1988), although there are notable exceptions where the opal stop codon is replaced by a sense codon, as is the case for strains of Semliki Forest virus (SFV), O'nyong-nyong virus (ONNV) and Sindbis virus (SIN) (Takkinen, 1986; Levinson *et al.*, 1990; Strauss and Strauss, 1994).

The negative-sense RNA is a template for both additional genomic RNA as well as 26S subgenomic mRNA. The 26S promoter is located between the two ORFs on the negative-sense RNA and is recognized by the non-structural proteins for transcription of a sub-genomic mRNA, from which the structural proteins are translated. This 26S mRNA is produced in 10-fold molar excess when compared to genomic RNA (Strauss and Strauss, 1994). The structural proteins are translated from the sub-genomic 26S mRNA as a polyprotein that is subsequently co-translationally and post-translationally cleaved to release the capsid protein and the two mature envelope glycoproteins (E1 and E2) (Jose *et al.*, 2009). Foreign genes of interest can be inserted in the place of alphavirus structural genes in cDNA clones, generating a self-replicating RNA (replicon) capable of expressing the foreign gene when introduced into cells. The self-amplifying replicon RNA directs the translation of large amounts of heterologous protein in transfected cells, reaching levels as high as 15–20% of total cell protein (Pushko *et al.*, 1997). The replicon RNA can be packaged into RP by supplying the structural genes *in trans* in the form of capsid and glycoprotein helper RNAs (Fig. 2) (Pushko *et al.*, 1997; Kamrud *et al.*, 2010b). When the helper and replicon RNAs are co-transfected into permissible cells, the replicon RNA is efficiently packaged into single-cycle, propagation-defective RP that are morphologically indistinguishable from native alphaviruses (our unpublished observation). Importantly, only the replicon RNA is packaged into RP, as the helper RNAs lack the packaging sequence required for encapsidation (Volkova *et al.*, 2006). Therefore, the resulting RP are propagation-defective and are incapable of producing progeny particles or virus.

Several features of alphavirus RP make them attractive for vaccine development: (1) a proven record of safety; (2) high expression levels of heterologous genes; (3) dendritic cell (DC) tropism; (4) protective and balanced immune responses; (5) multivalent vaccine construction; (6) resistance to anti-vector immunity; (7) commercial vaccine production; and (8) differentiating infected from vaccinated animals (DIVA).

Safety

Replicon vectors have been developed from several different alphaviruses, including Venezuelan equine encephalitis virus (VEEV), SIN and SFV (Liljestrom and Garoff, 1991; Bredenbeek *et al.*, 1993; Pushko *et al.*, 1997). Replicon vectors lack the alphavirus structural protein genes but retain the non-structural genes and the 26S sub-genomic promoter. Early production of RP was hampered by recombination events that resulted in the generation of replication-competent virus (RCV) (Weiss and Schlesinger, 1991; Bredenbeek *et al.*, 1993; Rayner *et al.*, 2002); first-generation helper RNAs encoded the capsid and glycoprotein genes on the same RNA molecule, and thus only required one recombination event to create RCV (Geigenmuller-Gnirke *et al.*, 1991; Berglund *et al.*, 1993; Pushko *et al.*, 1997). The probability of this event occurring was greatly reduced by separating the helper RNAs onto two separate RNAs ('split helper' system). Pushko *et al.* were able to demonstrate increased safety of the system by decreasing recombination events leading to the production of infectious virus when utilizing the split helper system (Pushko *et al.*, 1997). When both helper genes (capsid and glycoprotein) were

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present on a single RNA and co-electroporated into cells with replicon RNA expressing a heterologous gene, infectious virus was typically recovered, even to levels as high as 2×10^5 PFU/ml (Pushko *et al.*, 1997). However, when the helper RNAs were split into two separate RNA molecules, there was no recovery of infectious virus by plaque assay, blind passaging, or intracerebral inoculation of mice (Pushko *et al.*, 1997). Similar split helper systems have also been developed for SIN and SFV helper RNAs, resulting in no recovery of infectious virus (Frolov *et al.*, 1997; Smerdou and Liljestrom, 1999).

The split helper system greatly reduces the occurrence of RCV, as separation of the helper RNAs requires two independent recombination events to occur for generation of RCV (Pushko *et al.*, 1997; Smerdou and Liljestrom, 1999). Initially, helper RNAs were designed to contain a 26S promoter downstream of the 5′-untranslated region because of the significant impact the promoter has on the production of high levels of subgenomic transcripts. However, a recent study demonstrated that the 26S promoter is not required for functional helper RNAs (Kamrud *et al.*, 2010a). Removal of the 26S promoter results in helper RNAs that are not independent transcriptional units, and further reduces the probability of functional recombinations between the replicon and helper RNAs. In the same study, a stop codon was introduced at the 3′-end of the capsid gene in place of the chymotrypsin-like cleavage site (Kamrud *et al.*, 2010a, b). This mutation negates the cleavage activity of the capsid protein, adding another safeguard against functional recombination. Thus, helper RNAs lacking 26S promoters that have been manipulated to include a capsid stop codon have a reduced probability of functional recombination compared with the standard split helper RNA system (Kamrud *et al.*, 2010a). The introduction of the split helper system and subsequent modifications were significant advances in replicon technology that have facilitated RP vaccine evaluation *in vivo* without risk of reversion to virulence.

Human preclinical evaluations of cytomegalovirus (CMV) and influenza RP vaccines have been completed with no adverse effects reported in toxicology studies. An HA/NA influenza RP vaccine was administered to white rabbits four times with no toxic side effects and no relevant clinical parameter differences observed between RP and placebo vaccinated animals (Hubby *et al.*, 2007). This CMV vaccine has recently been evaluated in a Phase I human study where the vaccine was well tolerated with only mild to moderate local reactogenicity and minimal systemic reactogenicity even after three doses (Bernstein *et al.*, 2010).

Other studies have used the mouse model of intracranial (IC) injection to demonstrate the safety of RP. IC injection of VEEV RP resulted in only transient pathology (1–2 days) and weight loss (1 day) with a rapid return to pre-injection status (Kowalski *et al.*, 2007). The neurovirulence of both SIN and SFV RP expressing LacZ has also been evaluated following IC inoculation. Beta-galactosidase activity was detectable in brains for 14–28 days, but no morbidity, neuropathology, or loss of motor skills was observed in either study, indicating a lack of reversion to the parental virus strains (Altman-Hamamdzic *et al.*, 1997; Lundstrom *et al.*, 1999). A biodistribution study done with the same SFV RP system demonstrated that, following IC injection, there was no evidence of RP RNA co-localization to either the liver or the brain after 15 days, and no abnormalities were observed during histopathological examination (Morris-Downes *et al.*, 2001). These results correlate with safety studies we have completed in pigs and mice following injection with a VEEV RP vaccine expressing an H3 influenza gene, indicating a lack of shedding and spread of RP RNA and a lack of reversion to virulence following vaccination (Vander Veen *et al.*, 2012).

The current molecular features of the RP system result in safe vaccines. However, if in such a rare circumstance the perfect pair of recombination events did occur in the exact order and positions needed (Kamrud *et al.*, 2010a), the resulting RCV would theoretically be no more virulent than the parent strain being used as the vaccine vector. The attenuated VEEV strain TC-83 (commonly used in replicon vector development and production) has been used extensively to vaccinate military personnel and lab workers against VEEV infection (Alevizatos *et al.*, 1967; Burke and Ramsburg, 1977). A retrospective study of hundreds of humans who received the vaccine from 1976 to 1990 indicated that TC-83 vaccine caused some transient reactions, but no serious sequelae were reported (Pittman *et al.*, 1996). This strongly suggests that if multiple improbable functional recombination events were to occur during RP production, the resulting recombinant virus would be no more virulent than the infectious parent strain and thus would not have serious public health consequences.

High expression levels of heterologous genes

Pushko *et al.* demonstrated that transfection of several cell lines with RP expressing the Lassa virus N protein resulted in expression levels of nearly 20% of total cell protein (Pushko *et al.*, 1997). Kamrud *et al.* engineered the VEEV replicon to allow further optimization of protein yield and replicon packaging efficiency (Kamrud *et al.*, 2007). Internal ribosome entry site (IRES) elements were inserted into the replicon vector downstream of the 26S promoter to allow for cap-independent translation of heterologous genes. In addition, random nucleotide sequences of varying lengths were inserted between the 26S promoter and the IRES element. When compared to null replicons (no IRES) the spacer-IRES replicons expressed protein in some instances at > 50-fold increases (Kamrud *et al.*, 2007). Thus, by varying the length of the spacer sequences used in conjunction with IRES elements,

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replicons expressing the highest levels of heterologous protein or resulting in the highest RP yield can be readily identified for further vaccine evaluation.

DC tropism

A robust immune response is dependent on accurate and rapid presentation of the antigen to immune effector cells. DC are considered professional antigen presenting cells, thus vaccines that target these cells should induce robust and balanced immune responses. Inoculation of mice with VEEV RP revealed Langerhans cells (DC located in the skin) as the initial cell set to be infected (MacDonald and Johnston, 2000). In humans, VEEV RP have a selective myeloid DC tropism and these DC retain the capacity to acquire the mature phenotype upon migration to the local draining lymph node (Nishimoto *et al.*, 2007). SFV has also been shown to infect Langerhans cells and subsequently migrate to the local lymph node (Johnston *et al.*, 2000). A single amino acid substitution in the E2 glycoprotein of the SIN replicon vector significantly increased the affinity of the particle for human DC, resulting in an increase of major histocompatibility complex (MHC) class II molecules, CD86, and IFN-γ secreting cells (Gardner *et al.*, 2000). This natural or enhanced DC tropism of alphavirus RP results in a balanced and protective immune response following RP vaccination.

Protective immunity

The alphavirus replicon system has been used to develop efficacious RP vaccines for both human and veterinary applications. Influenza RP vaccines have been evaluated in chickens, pigs and humans. Complete protection against lethal H5N1 avian influenza challenge was demonstrated in 2-week-old chickens that received a single dose of RP vaccine expressing the homologous HA gene (Schultz-Cherry *et al.*, 2000). Recent reports also demonstrate that protective HI responses are elicited in young pigs following HA RP vaccination (Erdman *et al.*, 2010; Bosworth *et al.*, 2010; Vander Veen *et al.*, 2012). Following homologous challenge, there was a significant decrease in nasal shedding, viral load, rectal body temperatures and pulmonary pathology in HA RP vaccinated animals compared to placebo controls (Bosworth *et al.*, 2010; Vander Veen *et al.*, 2012).

The alphavirus replicon system was also used to rapidly produce a recombinant HA protein vaccine in response to the pandemic H1N1 influenza outbreak in 2009 with similar protection observed following homologous challenge (Vander Veen *et al.*, 2009). Other influenza gene candidates have also been evaluated in the RP system including neuraminidase (NA) and nucleoprotein (NP), with varying results (Sylte *et al.*, 2007; Hubby *et al.*, 2007; Bosworth *et al.*, 2010). Preclinical evaluation of HA and NA RP vaccines have been completed for human trials with no adverse effects observed following toxicology and safety testing, and robust humoral and cellular responses were elicited in mice, rabbits and rhesus macaques (Hubby *et al.*, 2007). In addition to evaluation as influenza vaccine candidates, RP not expressing any heterologous genes (null RP) were able to act as adjuvants and enhance the immunogenicity and efficacy of a trivalent inactivated influenza vaccine in rhesus macaques (Carrol *et al.*, 2011).

In addition to influenza, RP vaccines have been evaluated against several other animal diseases of veterinary importance. Balasuriya *et al.* used VEEV RP expressing the G_L and M proteins of equine arteritis virus (EAV) and demonstrated that these two major envelope proteins are necessary as a heterodimer for the induction of EAV neutralizing antibodies in mice (Balasuriya *et al.*, 2000). Further, horses vaccinated with the G_L/M RP vaccine were protected against virulent challenge, while horses receiving RP expressing only the G_L or M monomers were not protected from EAV (Balasuriya *et al.*, 2002). Similar research has been completed evaluating a related *Arterivirus*, porcine reproductive and respiratory syndrome virus (PRRSV), where the GP5/M heterodimer has also been shown to be important for induction of neutralizing antibody (Jiang *et al.*, 2006a, b, c). RP vaccines expressing the PRRSV GP5/M heterodimer have been evaluated in swine with a reduction in viremia observed post-challenge (Mogler, 2009; Mogler *et al.*, 2010). A recent study evaluated RP vaccines that encoded either the glycoproteins of Hendra virus or the glycoproteins of Nipah viruses (Defang *et al.*, 2011), agents of diseases of both veterinary and public health importance. These vaccines were able to induce cross-reactive neutralizing antibodies to both viruses, suggesting that a single vaccine against both viruses may be possible (Defang *et al.*, 2011).

In addition to alphavirus-based RP vaccines, alphavirus vectors can be designed to launch a self-replicating replicon RNA from a DNA plasmid *in vivo*. Replicon DNA vaccines have been developed for SIN, SFV and VEEV (Dubensky Jr *et al.*, 1996; Kohno *et al.*, 1998; Berglund *et al.*, 1998; Ljungberg *et al.*, 2007). The first step of expression involves RNA polymerase II-initiated transcription of replicon RNA from plasmid DNA in the transfected cells. Currently, the CMV immediate–early promoter is most frequently used (Ljungberg *et al.*, 2007). The second step of expression occurs when the replicon RNA enters the cytoplasm and the heterologous gene of interest is amplified from the native alphavirus 26S subgenomic RNA promoter. Previous SFV- and SIN-based replicon DNA vaccines have been shown to be immunogenic in small animal models (Berglund *et al.*, 1998; Hariharan *et al.*, 1998). A VEEV replicon-based DNA plasmid consistently expressed 3- to 15-fold more protein *in vitro* and increased humoral responses by several

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orders of magnitude over a conventional DNA vaccine (Ljungberg *et al.*, 2007). As the transcribed replicon RNA is self-amplifying, increases in cellular and humoral responses were also observed when 100-fold lower doses of replicon DNA were used compared to conventional DNA. Additionally, when the replicon DNA vaccine was used as a prime and VEEV RP expressing the same heterologous gene was given as the boost dose, both humoral and cellular immunity were increased significantly compared to VEEV DNA alone (Ljungberg *et al.*, 2007).

Similar replicon-based DNA vaccines have recently been evaluated in mice and pigs. An SFV replicon-based DNA vaccine has been produced, expressing the E2 glycoprotein of classical swine fever virus (CSFV). Pigs vaccinated with this DNA vaccine elicited low levels of neutralizing antibodies 1 week post-booster vaccination but exhibited decreased clinical symptoms and reduced viremia following homologous challenge when compared to control pigs (Li *et al.*, 2007). When evaluated in a mouse model, this vaccine elicited a specific lymphoproliferative response and an increase in IFN-γ and IL-4 CSFV-specific secretion (Zhao *et al.*, 2009a). When the CSFV DNA vaccine was used in a prime/boost regimen with a recombinant adenovirus expressing the homologous E2 glycoprotein, a significant increase in pre-challenge neutralizing antibody titers was observed with subsequent protection against heterologous CSFV challenge (Zhao *et al.*, 2009b). An SFV replicon-based DNA vaccine expressing the 1BCD gene of swine vesicular disease virus has also been shown to elicit neutralizing antibodies and lymphocyte proliferation in both guinea pigs and swine (Sun *et al.*, 2007). A SIN virus replicon-based DNA vaccine encoding the rabies virus glycoprotein G induced higher levels of humoral and cell-mediated immunity in mice than the conventional rabies DNA vaccine and comparable to the inactivated commercial rabies vaccine (Saxena *et al.*, 2008). All of the replicon DNA and inactivated virus vaccinated mice were completely protected against lethal challenge while some mice receiving the conventional DNA vaccine did not survive (Saxena *et al.*, 2008). These results indicate that the alphavirus replicon technology is flexible and that replicon RNA can be delivered to the host by several different methods. In addition, replicon technology can be used in conjunction with other recombinant systems to produce more efficacious vaccine regimens.

Multivalent vaccines

One of the advantages of the alphavirus replicon system is that the vector can be genetically modified to express several different genes from either the same or different pathogens. This can be accomplished via several methods, but the most common method is insertion of additional 26S promoter sites downstream of the non-structural genes. However, not all proteins are expressed at equimolar levels in this design and production levels can depend on the number of genes or gene position in the replicon, so each replicon must be empirically evaluated to determine optimum protein expression (Reap *et al.*, 2007a). Mice that received RP vaccines expressing different combinations of pp65, IE1 and gB proteins under control of three different 26S promoters developed high titers of neutralizing antibody and antigen-specific T-cell responses against CMV (Reap *et al.*, 2007b). Balasuriya *et al.* produced an RP vaccine co-expressing the G_L/M heterodimer that was required for protection against EAV (Balasuriya *et al.*, 2002).

RP vaccines expressing genes from two different pathogens are also able to induce protection against subsequent challenge. An RP vaccine co-expressing the glycoprotein genes of both Ebola and Lassa viruses protected guinea pigs from challenge with both viruses (Pushko *et al.*, 2001). The results obtained were the same as those achieved with RP vaccines expressing only one of the viral glycoproteins, indicating that protective immune responses can be induced against multiple and individual vaccine antigens at similar levels. In other studies, the authors have simply mixed different monovalent RP vaccines together prior to injection rather than produce the vaccine utilizing the multivalent approach of additional 26S promoters (Lee *et al.*, 2006; Hooper *et al.*, 2009). Similar to the previous results, a specific immune response was elicited in mice to each individual RP antigen including Marburg virus, anthrax and botulinum neurotoxin and protection was demonstrated following challenge with *Bacillus anthracis* and botulinum neurotoxins A and C (Lee *et al.*, 2006). The level of protection against *B. anthracis* induced by this multivalent vaccine formulation was similar to the protection demonstrated by vaccination with a monovalent anthrax RP vaccine (Lee *et al.*, 2003). Hooper *et al.* combined individual RP expressing four different smallpox virus antigens and demonstrated protection from lethal monkeypox virus challenge in cynomolgus macaques (Hooper *et al.*, 2009). Taken together, these results indicate that multivalent RP vaccines, regardless of the production method, are able to induce balanced antigen-specific immune responses and can elicit protection against multi-agent challenge. These results have important implications in decreasing the number of injections required for protection against multiple diseases or serotypes of a single disease agent and also for decreasing the cost of RP vaccine production.

Resistance to anti-vector immunity

As the alphavirus structural genes are not packaged and thus not expressed following vaccination, anti-vector immune responses are minimal. This lack of anti-vector immunity allows for multiple vaccinations of the same individual with either the same RP vaccine or different RP

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vaccines against multiple pathogens. Pushko *et al.* first demonstrated that RP could be used for sequential immunization by vaccinating mice with two doses of RP expressing the N protein of Lassa virus and then sequentially vaccinating the same mice with two doses of HA RP (Pushko *et al.*, 1997). These mice developed positive serum antibody responses against both antigens. Additionally, vaccinated mice were protected against influenza challenge, indicating that the Lassa N immune response did not interfere with subsequent influenza vaccination and the influenza-specific immune response. Similar results have been achieved following vaccination with RP expressing the HA protein from two different subtypes of influenza (our unpublished results). Ferrets were first immunized with an H3 RP vaccine and subsequently protected against homologous H3N2 influenza challenge. These protected ferrets (pre-immunized) were then vaccinated with H1 RP along with a group of naïve (non-H3 RP vaccinated) animals. Both the pre-immunized and naïve ferrets that received the H1 RP vaccine developed equivalent H1N1 hemagglutination inhibition (HI) serum antibody titers. These studies confirm that RP vaccines can be used sequentially without an inhibitory effect on vaccine efficacy. Other recombinant vaccines are often hindered by anti-vector immunity and it has been suggested that recombinant adenovirus vaccination regimens should include two heterologous vectors to avoid anti-vector immunity (Thorner *et al.*, 2006).

Commercial vaccine production

Many diseases that have not had efficacious vaccines developed against them are in the NIH Risk Group 3 (such as highly pathogenic avian influenza H5N1 and human immunodeficiency virus (HIV) types 1 and 2) or on the APHIS/CDC Select Agent List (such as botulinum neurotoxins, Ebola and Marburg viruses, *B. anthracis*, Hendra and Nipah viruses, foot-and-mouth disease virus (FMDV) and CSFV). Thus, all of these pathogens require either BSL3 or BSL4 production facilities for traditional vaccine production. This requirement has almost certainly been an impediment in the development of some of these vaccines. In addition, the alphavirus VEEV is listed as a select agent, except for the TC-83 attenuated vaccine strain. Thus, the TC-83 infectious clone can be utilized in the replicon system expressing the protective genes of the aforementioned select agents at low biocontainment levels for research and production. Since replicon-based vaccine production does not require growth of the pathogenic organism, select agent replicon-based vaccine development and production can occur in low biocontainment production facilities with no special biosecurity required. The capability to produce select agent vaccines in such production facilities is a huge advantage of the replicon system and has aided the development of these crucial vaccines for both humans and animals.

The attenuating determinants of TC-83 virus attenuation have been well defined (Kinney *et al.*, 1988, 1993). Because of the safety profile of attenuated TC-83, it has been recently developed as a replicon vector (Kamrud *et al.*, 2008; Hooper *et al.*, 2009; Bosworth *et al.*, 2010; Erdman *et al.*, 2010; Vander Veen *et al.*, 2012). A recent study compared a TC-83-based RP vaccine against a different RP vaccine (V3014-based vaccine produced in BSL3 facilities) in pigs. Both RP vaccines expressing H3N2 HA elicited the same antibody response in pigs, demonstrating that there is no difference in immunogenicity between RP vaccines produced using these two VEEV replicon systems (Erdman *et al.*, 2010).

Another advantage of RP vaccines is that many different cell lines are permissive to alphavirus infection, allowing a variety of cells to be evaluated for maximum yields. Vero and baby hamster kidney (BHK) cells are most commonly used for RP production, but Chinese hamster ovary (CHO), primary chicken embryo fibroblasts (CEF) and duck embryo fibroblasts (DEF), human embryonic kidney 293 and 293 T cell lines have also been utilized for RP production (our unpublished results; Pushko *et al.*, 1997). This long list of cells is in contrast to that for pathogenic virus growth which usually occurs optimally in one cell line. As in traditional vaccine production, these cells can be grown in large quantities using large-scale bioreactor microcarrier or suspension systems allowing efficient scale-up possibilities for RP production. There is also a report of stably transfected cell lines that have been developed to constitutively express the helper RNAs needed for RP production (Polo *et al.*, 1999). Similar to the split helper RNA system described above, only cell lines containing the structural protein genes on separate RNAs resulted in no recovery of RCV (Polo *et al.*, 1999).

DIVA capability

One of the attributes of a good next-generation vaccine is the capability to permit DIVA. The DIVA concept relies on the principle that a vaccinated animal will have a different immune response than an animal that is infected with the wild-type pathogen and that this immune response is readily detectable by some immunoassay. These DIVA vaccines become increasingly important when considering diseases that are not currently present in disease-free status countries (such as FMDV and CSFV) or for disease eradication and intense surveillance programs. In the case of FMDV, the current control policy has been primarily one of slaughtering the infected and contact animals (Uttenthal *et al.*, 2010). This policy could have a huge detrimental impact on domestic livestock production and potential export ramifications. Following the 2001 FMDV outbreak in the UK, there has been a growing demand for FMDV vaccination following an outbreak in order to reduce the large-scale slaughter of animals for control of the virus (Uttenthal *et al.*, 2010). Current FMDV vaccine

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research is focused on development of DIVA vaccines expressing the capsid proteins, including FMDV replicon-based vaccine research (our unpublished results; Yu *et al.*, 2006; Meeusen *et al.*, 2007).

Vaccines that have DIVA capability are also important for the control and eventual eradication of current endemic infectious diseases. PRRSV is endemic to the United States swine population and continues to have a huge economic impact (Neumann *et al.*, 2005). Current diagnostic assays for PRRSV target antibodies directed toward the nucleocapsid (N) protein. Antibodies to the N protein have been shown to be non-neutralizing (Dea *et al.*, 2000), therefore a vaccine need not include this protein to be efficacious. Thus, RP vaccines expressing any combination of PRRSV structural glycoproteins would therefore permit the differentiation of vaccinated from naturally infected animals. Similarly, a current ELISA for detecting influenza in pigs is based on NP antibody detection (Ciacci-Zanella *et al.*, 2010). RP vaccines expressing only the HA protein would therefore not induce a detectable immune response when used in conjunction with this diagnostic assay. Therefore, alphavirus replicon-based vaccines offer DIVA capabilities that can be important in different disease situations.

Conclusions

Research into the potential of alphavirus replicon-based vaccines has been ongoing for more than 20 years. Significant advancements have been made since these vectors were first used for the expression of heterologous genes. Improvements in both safety and the replicon vector design have significantly advanced the field of replicon-based vaccines. Both RP and replicon DNA vaccines have demonstrated robust and balanced immune responses with subsequent protection against a variety of diseases that have implications for both veterinary and human health. Thus, the alphavirus replicon technology offers great potential for the next generation of animal and human vaccines.

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