EXPERT REVIEWS

Self-replicating alphavirus RNA vaccines

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Recombinant nucleic acids are considered as promising next-generation vaccines. These vaccines express the native antigen upon delivery into tissue, thus mimicking live attenuated vaccines without having the risk of reversion to pathogenicity. They also stimulate the innate immune system, thus potentiating responses. Nucleic acid vaccines are easy to produce at reasonable cost and are stable. During the past years, focus has been on the use of plasmid DNA for vaccination. Now mRNA and replicon vaccines have come into focus as promising technology platforms for vaccine development. This review discusses self-replicating RNA vaccines developed from alphavirus expression vectors. These replicon vaccines can be delivered as RNA, DNA or as recombinant virus particles. All three platforms have been pre-clinically evaluated as vaccines against a number of infectious diseases and cancer. Results have been very encouraging and propelled the first human clinical trials, the results of which have been promising.

Keywords: adjuvant • alphavirus • electroporation • prime-boost • replicon • RNA vaccine • self-replicating RNA • vaccine

Naked nucleic acids as a possible vaccine technology came into focus in the early 1990s when it was discovered that delivery of naked nucleic acid into tissue could generate specific immune responses against the encoded protein antigen [1–3]. As early as 1990, Wolff *et al.* demonstrated that injection of RNA or DNA into mouse skeletal muscle readily expressed encoded proteins for long periods of time and that no special delivery system was required [4]. Soon it was discovered that naked nucleic acid delivery was also able to generate quite good immune responses and the terms DNA and genetic immunization were coined [5–8]. Vaccine research using plasmid DNA (pDNA) then exploded and numerous studies demonstrated that this vaccine platform could be employed to induce humoral and cellular immune responses against a large variety of antigens [9–17]. However, while pDNA has been commercially licensed for veterinary purposes [18–21], the technology is yet to be licensed for human use. pDNA has been in many clinical trials where the potency of humoral and cellular immune responses has been less than satisfactory. The reasons for this shortcoming is not clear but may be related to inefficient delivery into non-dividing cells, requirement to enter the cell nucleus for expression and insufficient stimulation of the innate immune system. Nevertheless, pDNA has been shown to be an excellent priming technology in combination with other vectors. Significantly enhanced immunogenicity of pDNA has also been achieved by the use of electroporation (EP) for delivery and by the use of immune stimulatory molecules (genetic adjuvants) [9,10,22,23].

Early studies using naked mRNA for immunization indicated that transgene expression and immunogenicity was comparable to that of pDNA [4,24]. Several subsequent studies demonstrated that indeed immunization with mRNA could be considered [25–36]. However, mRNA remained in the shade compared with pDNA vaccination probably due to perceived difficulties in production technology and cost, instability in long-term storage and after *in vivo* delivery. Later came the realization that these considerations may not be totally correct and that it could be advantageous to develop the mRNA platform for vaccines against infectious disease and cancer [9,23,27,31,37–40]. Indeed, it was recently shown that stable mRNA vaccines against infectious disease and cancer can be manufactured at GMP and that they are highly immunogenic targeting both the humoral and cellular arms of the immune

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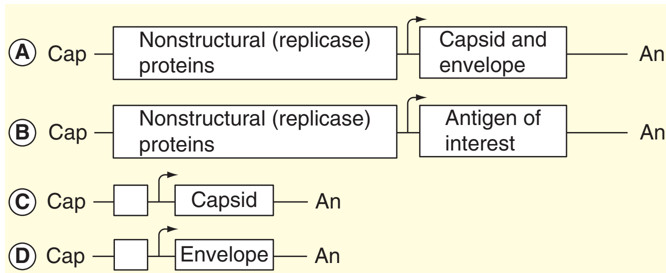

Figure 1. Schematic illustration of alphavirus replicons. (A) An infectious clone of an alphavirus. The cDNA copy of the alphavirus genome is placed on a plasmid under the SP6 or T7 promoter at the 5′ end. *In vitro* transcription is used to produce large amounts of full-length RNA that is then transfected, preferably by electroporation [46], into cells in culture to initiate virus replication. The complete replicon is self-replicating as the initial translation of the replicase will drive the amplification of the RNA in the cytoplasm. **(B)** An alphavirus RNA replicon vaccine vector designed to express only a foreign (antigen of interest) gene placed under the subgenomic promoter (arrow). The sequence encoding the structural proteins of the virus is replaced by one coding for the gene of interest. An alternative to this strategy is to keep the genomic sequences and duplicate the subgenomic promoter. In such case two subgenomic RNAs are made and new virions will be formed. Such constructs have, however, proven to be rather unstable. When vaccine constructs of type B are delivered as naked DNA, the replicon construct is placed under the CMV promoter on a plasmid. Vaccines of this type are referred to as DNA replicon. **(C & D)** When vaccines of type B are to be delivered (administered) by infection recombinant virus particles called virus replicons are produced in a cell culture system by the use of a split helper system. *In vitro* made RNA from B, C and D are co-transfected into cells. The helper constructs **(C & D)** encode for the capsid protein or the envelope proteins, respectively, but cannot replicate themselves as most of the replicase sequences have been removed. Replication (amplification) of both vaccine vector RNA and helper RNAs is accomplished by the activity of the replicase complex encoded by the vaccine vector. As a result, virus particles are produced that carry the vaccine RNA **(B)** within the nucleocapsids. Helper RNAs are not packaged as the packaging sequence (signal) recognized by the capsid protein and resident in the replicase region is absent.

system [9,23,37,38,41–45]. Clearly, the development of mRNA-based vaccines will face rapid technological advances and new areas of application.

Alphavirus infectious clones

The development of self-replicating RNA vaccines was dependent on the fact that alphaviruses have positive-stranded RNA genomes, which allowed for the construction of infectious cDNA clones. Accordingly, full-length clones of Semliki Forest (SFV) [46], Sindbis (SINV) [47] and Venezuelan encephalitis (VEEV) [48] viruses were further developed into expression vectors. Placing the genomic cDNA sequences under the SP6 or T7 promoters allowed *in vitro* production of full-length RNA that could be transfected into cells. Due to the self-replicating capacity of the alphaviral RNA, the virus life cycle was initiated and new virions formed by budding out of the cell. The alphavirus genomic organization is depicted in Figure 1. The genomic RNA has a 5′ cap structure (m^7G) and a poly-A sequence at the 3′ end. The first open reading frame encodes for the four non-structural proteins, designated nsP1–4 that constitute the RNA replicase. These proteins are made from a precursor polyprotein that self-cleaves into the individual polypeptides that form the replicase complex. The replicase drives the replication/amplification, from genomic length negative-strand intermediates, of the entire genomic RNA in the infected cell cytoplasm. Later in the infection cycle, the replicase switches to produce subgenomic RNA by initiating RNA synthesis at the subgenomic promoter. The subgenomic RNA encodes for the structural proteins of the virus, that is, the capsid protein and two envelope glycoproteins E1 and E2. The capsid protein is cytoplasmic and encapsidates new genomic RNAs. The two envelope proteins migrate to the cell surface where interactions between newly formed nucleocapsids and envelope spike proteins lead to the formation of new virions that bud from the cell plasma membrane [49–51].

Alphavirus expression vectors

Self-replication of the infectious alphavirus RNAs and also the alphavirus expression vectors requires three elements: the 5′ terminal region; an RNA packaging signal region in nsP2 (this is not needed for RNA replicon [RREP] and DNA replicon [DREP], see below) and the 3′ terminal untranslated region [52–59]. The first alphavirus vector expressed the CAT gene but needed supply of wild-type SINV for propagation [60]. Three general alphavirus vectors were subsequently developed for SFV [61], SINV [52] and VEEV [62]. These will be denoted RREP, DREP and virus replicon (VREP), emphasizing the type of molecules the vector consists of at delivery (Figure 1). The simplest vector is RREP and is a naked *in vitro* SP6 or T7 RNA transcript of an alphavirus recombinant cDNA that encodes the alphavirus replicase and a foreign gene insert (Figure 1B). When this RNA is transfected into cells, normal amplification (by virtue of the replicase) of the RNA occurs but no virus particles are formed as the construct lacks the necessary structural protein components of the virus. When the SP6 or T7 promoter at the 5′ end of the replicon is swapped for a eukaryotic promoter such as typically pCMV, a DREP vector is obtained. When DREP pDNA is transfected into cells, the DNA is transported into the nucleus where the cellular RNA polymerase drives, from the pCMV, initial transcription of the replicon. The recombinant RNA transcript is then transported into the cytoplasm, where translation of the alphavirus replicase occurs. Subsequently, the replicase drives the replication/amplification of the replicon in the same manner as occurs in the case of RREP.

When VREP particles (i.e., alphavirus virions carrying an RREP type of molecule as genome) are produced, there is a need for helper RNAs (Figure 1C & 1D) since RREP molecules lack the sequences encoding for the structural protein of the virus. The genes need to be provided in a production process for complementation in trans where the helper RNAs are co-transfected into cells together with the RREP vector. The

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present helpers are divided into two parts: one encoding the capsid protein and the other encoding the envelope spike proteins. Both helpers lack the region encoding for the replicase but retain the 5′ and 3′ elements required for replication. The first-generation helpers were in fact a single molecule encoding both capsid and envelope [61,63], however, they were prone to recombination with the vector generating wild-type replicating virus. Therefore, the present so-called split helper system was developed to eliminate this problem [62,64–66]. Efforts have also been made to produce stable packaging cell lines expressing both helpers from the genome. Such cell lines could be used for scale-up production of VREPs circumventing multiple EP and use of seed stocks to initiate the production process [67]. However, the titers obtained with this strategy were not as high as one would have hoped and the standard method still uses split helpers with EP, even in the case of GMP production. However, the conceptual benefit of using a stable packaging cell line still warrants further investigation.

Recently, an alternative or twist to the helper packaging system has been developed to deliver RREPs for immunization. In this system termed self-amplifying messenger RNA (SAM), *in vitro* made RREP is formulated with Zwitterionic, cationic and PEGylated lipids as well as cholesterol-forming synthetic lipid nanoparticles (LNPs) [68]. Expression of a reporter protein *in vivo* showed that 1 μg of SAM-LNP matched the expression levels of 10^6 IU of VREP. While expression in tissue ceased within 28 days for VREP, it continued for up to 63 days for SAM-LNP [68].

Table 1. RNA replicon and DNA replicon vaccine candidates.

Agent/disease	Antigen	Host	Vector	Ref.
Bovine viral diarrhea virus	NS3 (p80)	Mouse	SFV – DREP	[174]
Chikungunya virus	E1E2	Mouse	SFV – DREP	[77]
Classical swine fever virus	CS-E2	Mouse	SFV – DREP	[95]
Clostridium botulinum	AHc	Mouse	SFV – DREP	[175]
CT26 tumor	LacZ	Mouse	SFV – RREP	[176]
Hepatitis C virus	C & E2	Mouse	SFV – DREP	[177]
HIV-1	gp160, HIVconvs	Mouse	SFV – RREP, SFV – DREP	[78,178,179]
Infectious bursal disease virus	VP2	Chicken	SFV – DREP	[180]
Influenza	HA, NP	Mouse	SFV – RREP, SFV – DREP	[69,73]
Louping ill virus	prME	Mouse	SFV – RREP	[181]
Mycobacterium tuberculosis	Hsp70	Mouse	SFV – DREP	[182]
Plasmodium falciparum	Pf332	Mouse	SFV – RREP	[183]
Respiratory syncytial virus	F, G	Mouse	SFV – RREP, SFV – DREP	[181,184]
B16 tumor	Gp100, IL-18	Mouse	SINV – DREP	[185]
Breast cancer	HER2/neu$^+$	Mouse	SINV – DREP	[186,187]
Herpes simplex virus 1	gpB	Mouse	SINV – DREP	[76]
HIV-1	gp140	Mouse	SINV – RREP (SAM)	[68]
Influenza virus	HA	Mouse	SINV – RREP (SAM)	[79]
Measles virus	H, FUd	Mouse	SINV – DREP	[188,189]
Melanoma	MUC18, Trp-1	Mouse	SINV – DREP	[144,190]
M. tuberculosis	Ag85A	Mouse	SINV – DREP	[191]
Rabies virus	G	Mouse	SIN – DREP	[192]
Respiratory syncytial virus	F	Mouse	SINV – RREP (SAM)	[68]
SEOUL virus	M/S	Mouse	SINV – DREP	[194]
Prostate cancer	PSCA	Mouse	Venezuelan encephalitis – DREP	[193]
Rift Valley fever	Gn-Cd3	Mouse	Venezuelan encephalitis – DREP	[97]

DREP: DNA replicon; RREP: RNA replicon; SAM: Self-amplifying messenger RNA; SFV: Semliki Forest; SINV: Sindbis.

Self-replicating RNA vaccines – preclinical

The RREP vectors were among the first self-replicating alphavirus vaccines tested in animal models. In an early study, naked RNA expressing influenza antigens was delivered intramuscularly (im.) to mice generating strong humoral and cellular immune responses that were protective against an influenza virus challenge [69]. Other studies followed demonstrating that naked RREP RNA can be used for effective immunization against viral diseases, parasites and cancer (Table 1). The early studies were all performed as needle injections of RNA into the muscle. With the advent of EP technology, new studies were

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Table 2. Virus replicon particle vaccine candidates based on Semliki Forest.

Agent/disease	Antigen	Host	Ref.
Brucella abortus	IF3	Mouse	[195]
Chlamydophila pneumoniae	OMP-2	Mouse	[99]
Colon/mammary carcinoma	VEGFR-2	Mouse	[196]
Escherichia coli, *Salmonella typhimurium*	LacZ, Flagellin	Mouse	[142]
Glioma	IL-12, IL-18. Endostatin, LacZ	Mouse	[197–200]
Hepatitis C virus	C & E2, nsP, NS3	Mouse, macaque	[90,177,201,202]
HIV-1	gp160, Gag, Pol, HIVA, HIVconsv	Mouse, macaque	[87–89,178,203,204]
Human papilloma virus	E6, E7, IL-12	Mouse	[94,205–208]
Influenza	NP, HA	Mouse	[73,117,209]
K-BALB and CT-25 tumor	Virus replicon	Mouse	[210]
Louping ill virus	prME	Mouse, sheep	[211–213]
Murray Valley encephalitis virus	prME	Mouse	[214]
P815	P1A	Mouse	[92,215,216]
Plasmodium falciparum	PfEMP1	Rabbit, rat	[217]
Prion	PRNP	Mouse	[218]
Respiratory syncytial virus	F, G	Mouse	[125]
Retrovirus transduced tumor	LacZ	Mouse	[176]
SIV	Env, Gag, Pol, Rev, Nef, Tat	Macaque	[80–84,219–221]
TC-1	IL-12, IFN-α	Mouse	[222,223]
Tumor	Polyepitope	Mouse	[100]

conducted to compare whether the potency of RREPs could be improved by this method of delivery. Indeed, it was found that using RNA amounts in the 1–10 µg level, humoral and cellular immunity was induced at significantly higher levels as compared with administration by needle [70–72]. It should be noted that the method of EP used in these studies employed settings optimized for pDNA. Therefore, it may be possible to further increase the efficacy of RREP vaccines by changing EP settings.

In light of the successes in the pDNA vaccine field and considering the potency that the early alphavirus replicon vectors had shown in a variety of animal vaccine models, it became of interest to investigate whether recombinant replicons could be launched from a pDNA by direct injection of naked DNA. Therefore, SFV [73], SINV [74], VEEV [75] and DREP vectors were constructed by placing them under the pCMV promoter. It turned out that these vectors were much more potent as compared with conventional DNA and required less than 100-fold amounts of DNA to achieve the same immune responses [73,76]. Later the method of delivering DREP with EP was employed and showed that the dose can be lowered almost 1000-fold without loosing immune response compared with conventional pDNA [77,78]. DREP vectors have been extensively studied in pre-clinical studies in a variety of models for viral, non-viral diseases and cancer (Table 1).

The VREP platform is the one that has been most utilized for developing alphavirus vector vaccines candidates. Thus, the large majority of data stem from these studies, which have involved antigen of viral, non-viral and cancer origin (Tables 2,3,4). In general, immune responses have been robust both on the humoral and cellular side. Animal experiments have included mice, rabbits, cotton rats, pigs, calves, sheep, non-human primates and even fish. Where it has been possible, challenge experiments have been performed quite successfully. In fact, all have been successful except in the case of HIV/SIV, where large challenge doses have been used. It remains to be seen whether better protection could be achieved under challenge conditions that are more realistic.

The new lipid formulated RREP platform SAM-LNP has also been tested in animals. Administration im. of SAM expressing the respiratory syncytial virus (RSV) F envelope protein of gp140 of HIV-1 showed that robust humoral and cellular responses were obtained. Responses were clearly stronger than administration of corresponding unformulated naked RREP. The best route of administration seemed to be im., whereas intradermal (i.d.) was less efficient and subcutaneous (sc.) even less so [68]. The responses were somewhat stronger than delivery of conventional pDNA by EP and clearly stronger than delivery of pDNA by im. injection. Overall immune responses seem to be in parity with those obtained by administration of VREP or DREP by EP, although the latter was not directly compared in the study. It is difficult to directly compare strengths of immune responses as they often are related to dose (apple and oranges dilemma). The SAM technology was also tested in an H7N9 avian influenza model, where good IgG, hemagglutination inhibition and neutralization titers were

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obtained in mice. The responses against 1.0 μg of SAM-FLU were not as strong as for 0.1 μg of a influenza subunit vaccine formulated with MF59 adjuvant; however, again it is a difficult comparison to make [79].

Replicons combined with other vectors

Both VREP and DREP vaccines have been tested together with other virus vector vaccines in prime-boost combinations. Replicon vaccines have been used both for priming and boosting with a variety of antigens including VREP prime and Modified Vaccinia Ankara (MVA) boost [80–84] or VREP prime and protein (+ adjuvant) boost [85,86] for SIV, VREP prime and MVA [87,88] or NYVAC [87] boost or DREP prime and MVA boost [78] or DREP prime and VREP boost [75] MVA prime and VREP boost [89] for HIV. Other studies include VREP prime and MVA or Adeno5 boost [90] and protein (+ adjuvant) prime and VREP boost [91] for HCV. Cancer studies include VREP prime and Adeno5 [92,93], ALVAC [92] or virosome [94] boost. For studying vaccination against classical swine fever strategies using DREP prime and Adeno5 boost [95,96] and for Rift Valley fever, conventional DNA prime VREP boost [97] were employed. For *Plasmodium knowlesi*, a VREP prime and Pox or Adeno5 boosts were used [98], whereas a conventional DNA prime followed by VREP boost was employed for *Chlamydia* [99]. Finally, to study prime-boost effects on dominance a tumor poly-epitope construct was used with VREP prime and MVA boost [100]. Comparative studies for prime boost have also been done [101]. For prime-boost strategies, most studies have used alphavirus vectors for priming and only in a few cases for boosting. Thus, it is difficult to draw any general conclusion regarding the suitability of these vectors for prime or boost. The outcome may well depend on the situation at hand. In the large majority of cases, the prime-boost effect has clearly been synergistic.

Quite different approaches have involved use of chimeric virus vectors. Recently, a recombinant vaccinia poxvirus vector carrying a SFV replicon consisting of the replicase region and a reporter/antigen gene under the subgenomic promoter was constructed (VV/SFV). Another vector was also made which expressed the structural proteins of SFV from another vaccinia promoter within the same chimeric virus genome [102]. The replicon is expressed under the control of a vaccinia virus promoter such that infection with the hybrid virus led to expression of the alphavirus genome and even production of virions from the cell. Whether use of this hybrid vaccine can show enhancement of immune responses or modulation in the quality of induced immune responses remains to be seen.

In yet another chimeric approach, a recombinant adenovirus was made carrying a complete SFV replicon, where the structural genes of SFV were replaced by the E2 gene of classical swine fever virus (CSFV) (rAdV5-SFV-E2). Immunization of rabbits and pigs showed development of robust humoral and cellular immune responses that were protective against a lethal challenge by CSFV. In contrast, neither corresponding rSFV nor rAdeno5 expressing the CSFV E2 gene protected the

Table 3. Virus replicon particle vaccine candidates based on Sindbis.

Agent/ disease	Antigen	Host	Ref.
Bacillus anthracis	PA	Mouse	[224]
HCV	E1, E2, NS3	Mouse	[91]
HIV-1	gp140ΔV2	Macaque	[85,86]
HIV-1	Env, Gag	Mouse, Macaque	[225–227]
Human papilloma virus	E6, E7	Mouse	[228,229]
Influenza	NP	Mouse	[230]
Japanese encephalitis virus	prM, E, NS1, NS2a	Mouse	[231]
Measles virus	H, F, FUd	Mouse, Cotton rat	[188,232,233]
Parainfluenza virus 3	HA, NA	Mouse, Hamster	[234,235]
Plasmodium yoelii	Circumsporozoite protein	Mouse	[230]
Rift Valley fever	Gn, Gc, nsM	Mouse	[236]
Seoul virus	M, S	Syrian hamster	[194]

animals from CSFV infection [103]. A subsequent study showed that the potency of this chimeric vaccine allowed the use of quite low doses (10^6 IU), which were sufficient to induce protective immunity. This also meant that interfering anti-vector immunity was not induced while maternally derived antibodies had no inhibitory effects on the efficacy of the vaccine. The level of immunity and protection induced by rAdV5-SFV-E2 was comparable to that provided by the currently used live attenuated vaccine [104].

Clinical studies – vaccines against alphaviral disease

There have been several clinical trials aiming at inducing protective immunity to diseases caused by alphaviruses. For these studies, live attenuated viruses (VEEV, CHIKV) have been used (clinicaltrials.gov; [105–107]). It is important to follow these trials as they provide information of potency of alphavirus vaccines in humans to guide development of alphavirus-vectored vaccines.

The US Army Medical Research Institute of Infectious Diseases has developed an attenuated strain of VEEV referred to as TC-83. The attenuating mutations were later localized to only two sites: one nucleotide change (G-to-A) in the 5′ non-coding region and one amino acid change (Thr to Arg) in the E2 protein [108]. TC-83 showed improved safety as compared with previous live attenuated VEEV candidate vaccines and

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Table 4. Virus replicon particle vaccine candidates based on Venezuelan encephalitis.

Agent/disease	Antigen	Host	Ref.
Venezuelan encephalitis			
Borrelia burgdorferi	OspA	Mouse	[237]
Bovine viral diarrhea virus	E2	Calf	[238]
Cytomegalovirus	gB, pp65/IE1	Human	[121]
Cowpox virus	CPXV-B5, A33, B5 and A27	Mouse	[101,239]
Dengue virus	Prm, E	Mouse, macaque	[240–243]
Ebola virus	GP, NP, VP24, VP30, VP35, VP40	Mouse, macaque	[244–247]
Equine arteritis virus	G(L), M	Horse	[248]
Hendra and Nipah virus	Glycoprotein	Mouse	[249]
Influenza virus	HA	Mouse	[62]
HIV-1	MA/CA	Mouse, human	[119,126,227]
Human metapneumovirus	F, G	Mouse	[250]
Human papilloma virus	E7	Mouse	[251]
Influenza	HA, NA	Mouse, chicken, rabbit, macaque	[62,129,252–254]
Lassa virus	N	Mouse	[62]
Mammary tumor	neu	Mouse, rat	[255,256]
Marburg virus	GP, NP, VP40, VP35, VP30, VP24	Guinea pig, macaque	[257,258]
Melanoma	MDA-7 Trp-2	Mouse	[259]
Metastatic tumor	CEA	Human	[124]
Norwalk virus	NV1	Mouse	[127]
Plasmodium knowlesii	CSP, SSP2, AMA1, MSP1	Macaque	[98]
Prostate cancer	PSMA, STEAP	Mouse, rabbit, human	[123,193,260]
Respiratory syncytial virus	F, G	Mouse, cotton rat	[130]
SARS-CoV	Glycoprotein	Mouse	[261]
SIV	MA/CA, gp160, gp140	Macaque	[262–264]
Staphylococcus	SEB	Mouse	[265]
Sudan virus	gP	Macaque	[244]
Swine influenza	HA	Mouse, pig	[252,266]
Vaccinia virus	A33R, B5R, A27L, L1R	Mouse, macaque	[267]

elicited long-lasting antibody responses in volunteers [109], although virulence could in theory be restored by only a few reversion mutations. In another long-term follow-up after a primary immunization TC-83 and booster with a formalin-inactivated virus, it was shown that the duration of neutralizing antibodies measured as 80% plaque reduction neutralization titer >1:20 was 5.5–8 years [107]. It is noteworthy that 23% of those who received the TC-83 reported self-limited adverse events such as malaise, fever and headache within the first 10 days of vaccination, whereas the safety profile of the inactivated vaccine was substantially better.

Despite the increasing global incidence, there is currently no licensed vaccine against CHIKV infection. Early attempts to develop a CHIKV vaccine in the 1960s included formalin-inactivated virus preparations as well as attenuated strains [110,111]. An attenuated vaccine candidate was developed by the US Army Medical Research Institute of Infectious Diseases by passaging a clinical isolate in human MRC-5 cells [112,113]. The resulting attenuated strain (TSI-GSD-218 or 181/clone25) induced neutralizing antibody responses that lasted for more than 1 year in 57 of the 58 study subjects, and although it induced transient arthralgia in five vaccinees, it was considered safe [105,106]. Its further development was discontinued partly because of the reported side effects, but also because of uncertainties about the production process [113]. Nevertheless, it is still considered for development as a product by Indian Immunological Ltd. Of note, a recent study showed that 181/25 is only attenuated by two point mutations suggesting that reversions may occur [114] raising considerable safety concerns for this vaccine. An attenuated CHIKV vaccine candidate was engineered by substituting the subgenomic promoter for an internal response sequence element between the non-structural and the structural genes to attenuate the virus and to prevent it from replicating in the transmitting Aedes mosquito host. This vaccine was recently tested in non-human primates showing strong immunogenicity without signs of

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disease and prevented viremia upon challenge with wild-type CHIKV [115]. The CHIK-internal response sequence vaccine is now projected for Phase I clinical trials by Takeda Inc.

Interestingly, vaccinees that had previously been subjected to VEE TC-83 vaccination had a seroconversion rate of only 36%, suggesting that previous exposure to alphavirus infections may interfere with the *de novo* antibody response to a closely related alphavirus [106]. Similarly, vaccination with the CHIK virus vaccine also interfered with the development of anti-VEE neutralizing antibodies after a subsequent TC-83 immunization. Thus, a secondary heterologous immunization did not boost the neutralizing responses to the primary antigen. However, binding antibody responses demonstrated cross-reactivity that could be boosted by a heterologous alphavirus. Similarly, simultaneous immunization with inactivated WEE and EEE viruses led to immune interference and decreased response rates to both vaccines as compared with vaccinees that were vaccinated at different time points [116]. This has potential implications for designing immunization regimens also with VREP-vectored vaccines. This finding is of importance as pre-clinical studies have shown that alphavirus vector vaccines allow boosting several times without loss of immunogenicity [117,118]. Thus, it will be crucial to study whether there is a difference between alphavirus vector vaccines versus alphavirus vaccines that can result in productive infection upon administration.

Clinical studies – alphavirus-vectored vaccines

In addition to the alphavirus vaccine trials, there have also been a number of trials with alphavirus-vectored vaccines, both against infectious diseases such as influenza and HIV-1 as well as malignant tumors such as prostate cancer and colorectal carcinoma. To date, there are five reported studies with a VEEV-based VREP in the clinicaltrials.gov database all of which have been completed. These trials have focused on three viral infectious against HIV-1, influenza and CMV.

The safety and immunogenicity of a VEEV replicon expressing the HIV-1 subtype C gag gene was tested in healthy volunteers in the USA, Botswana and South Africa in a randomized, placebo-controlled, double-blind dose-escalation trial with 132 study subjects. The gag gene myristoylation site was deleted, thus preventing formation of virus-like particles. Although the vectored vaccine was considered safe, the immune responses were limited [119] with detectable cell-mediated immune responses in only 40% of the vaccinees in the highest dose tested. However, a modest but detectable binding antibody response was induced in the study subjects vaccinated with the highest doses. It has been speculated that a myristoylated gag gene would have been more immunogenic.

Alphavirus replicon vaccines targeting influenza have also been tested in clinical trials, although the results of these trials still are pending publication [120]. The vaccine encoded the hemagglutinin surface antigen of a seasonal H3N2 strain and was tested in a randomized, placebo-controlled, double-blind study comprising 216 healthy volunteers. Two different doses administered either sc. or im. were tested. In a separate trial, this vaccine was also tested in 28 elderly subjects (over 65 years of age). In both studies, the vaccine was well tolerated and immunogenic inducing both protective levels of antibodies as determined by hemagglutination inhibition, and T-cell responses.

In a third clinical trial, a CMV vaccine encoding three different CMV antigens was tested. This vaccine was shown to be immunogenic, inducing neutralizing antibodies as well as reasonable and polyfunctional T-cell responses [121]. Two different doses and two immunization routes were tested in 40 healthy volunteers that all received three immunizations. The responses were dose-dependent, and the sc. immunization route was reported to be more immunogenic than the im. route. Moreover, the vaccine displayed low reactogenicity and was considered safe. The immune responses obtained were in parity with protective responses obtained in VEEV and CHIKV clinical trials described above. Recently, when wild-type, attenuated and vectored CHIKV vaccine candidates were compared in a mouse challenge model, there was no difference in immunogenicity or protection [77,122]. Although not tested in humans, the results may indicate that alphaviruses as a platform are sufficiently potent.

As immune therapy, alphavirus replicons have been employed as vaccines against prostate cancer, colon cancer and will be tested against Her2 cancers such as breast cancer. In one study, 12 patients undergoing treatment for prostate cancer were enrolled and immunized with a PSMA-encoding VEE-based VREP. This study showed safety, but low antibody levels and undetectable T-cell responses and no clinical benefit of vaccination [123]. The study concluded that the lack of a robust immune response to vaccination was likely due to suboptimal dosing of the vaccine.

More encouraging are the results from a Phase I/II study, where 28 patients with advanced or metastatic stages of carcinoembryonic antigen (CEA)-expressing tumors were vaccinated repeatedly with a VREP-CEA vaccine. It was shown that the vaccine could break tolerance to CEA despite the induction of neutralizing antibody against VREP and elevated Treg numbers present in cancer patients [124]. In the sub-cohort displaying the strongest immune responses to vaccination, there was an increased long-term survival and evidence of resolving metastases in one patient. These data should be interpreted with some caution since the numbers of strong responders were small, but are nevertheless promising. The authors speculate that higher vaccine doses might have a positive impact on the immune response, but such studies remain to be conducted. The study center is recruiting for a follow-up study with the CEA-encoding vaccine. In addition, a VREP-Her2 replicon will also be tested in a separate trial.

Immunobiology of self-replicating RNA vaccines

The numerous studies that have been conducted have shown that alphavirus self-replicating RNA vaccines are capable of inducing potent humoral and $CD4^{+}$ and $CD8^{+}$ T-cell responses. They can do so on their own or in combination with other vaccine modalities in prime-boost combinations.

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Responses have for the most part been Th1 biased or in some cases Th1/Th2 balanced. The VREP vaccine can be delivered virtually by any route and im., sc., i.d. and i.n. routes have been successfully used. Apart from systemic responses, studies have also shown strong induction of mucosal immunity [125–132]. For the RREP and DREP vaccines, the im. and i.d. (by EP) routes have been used and for RREP-SAM the im. route.

When these vaccines are given as a prime, the interval between prime and following boost should be at least 3 weeks in a murine system. Thus, in a human situation the time period is expected to be longer. The reason for this is that the T-cell responses peak at day 7 and then rapidly contract until 21, where after a plateau of T-cell memory is reached. If a booster immunization is given before day 21, the risk is that the T cells have not contracted sufficiently and the expected booster effect will not be obtained [133]. In a recent study employing the RREP-SAM technology, it was found that a prime-boost interval of 8 weeks was superior to 3 weeks [79]. This could be due to much slower contraction period when the RNA is delivered by the SAM-LNP system. Indeed, it was shown that RREP-SAM expresses the antigen as long as up to 63 days post-immunization, which probably prolongs the initial response [68]. Proper boosting with alphavirus vectors will result in long-term T-cell memory with $CD8^{+}$ T cell having high recall capacity and increasing the dose promotes induction of T effector memory over T central memory development [133]. These immunization intervals as tested apply to prophylactic settings and may differ if these vectored vaccines are to be used therapeutically.

Immunization with alphavirus RNA vaccines results in strong upregulation of type I interferon (IFN) responses resulting in induction of an antiviral state [134–137]. Thus, while the IFN response aids in induction of the specific immune response, it at the same time hampers the response by inhibiting virus replication via alteration of mRNA translation rates, by increasing RNA degradation via RNaseL and by upregulation and activation of pro-apoptotic pathways [138] as well as inhibition by viperin [139,140]. Interestingly, viperin in itself further promotes this effect through upregulation of IFN in plasmacytoid dendritic cells [141]. The negative effect of IFN on replicon immunization can readily be seen when mice defective in the type I IFN receptor are used. In such cases, the T-cell responses clearly increase [77,133,142,143].

Vaccination with the RREP, DREP and VREP vaccine vectors mimic an infection of an RNA virus. Therefore, the produced ssRNAs and dsRNAs (and the RNA:DNA hybrid in the case of DREP) act as a self-adjuvant inducing all the necessary innate immune signals. These will include chemotactic factor for stimulating migration of antigen presenting cells to the replication site, promotion of cross-priming through the action of apoptosis and cell death, activation of RNA-activated protein kinase, activation of pattern recognition receptors such as Toll-like receptors (TLRs) TLR3, TLR7 and TLR9 as well as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5) [44,45,144–160]. Collectively, it is the signaling through TLRs and RIG-I-like receptors that ultimately results in the activation of the IFN response and subsequently upregulation of the IFN-stimulated genes.

The fact that the alphavirus RNAs stimulate such strong innate responses in a self-adjuvanting manner can also be used for stimulating the immune responses to antigen not expressed by the vectors themselves. There are several studies showing significant enhancement of immune responses when an antigen is co-delivered (co-mixed) with VREP particles. This adjuvant effect is IFN type I dependent [129,132,135,161–165]. In addition, it is possible to construct replicons expressing TLR agonists to further the immune responses [142].

An advantage of replicon vaccines is that they can be constructed rapidly with incorporating any mutations or variations one wishes to make. This is important considering emerging infections or for designing vaccines targeting new variants of pathogens, for example, influenza. Replicons can be made completely synthetically and can easily be produced using generic production platforms and in the case of RREP and DREP will not stand the risk of having cellular contaminants. While the immune systems sees them as viral infections, they are devoid of most safety issues connected to live (attenuated) vaccines. Alphavirus vectors can accommodate quite large pieces of inserts and should not constitute an issue for vaccine developers. Considering the risk of immunodominance, very large inserts would probably have to be avoided anyway.

Replicon vaccines can be used at lower doses than pDNA and they do not stand the risk of integrating into the genomic DNA. Using RREP or DREP replicon vaccines, there will be no neutralizing immunity induced against the vector allowing several booster immunizations with the same vaccine or with the same backbone for different vaccines. In the case of VREP the same seems to hold true. Studies have shown that pre-immunization with one vaccine based on the VREP backbone does not hamper immune responses against another vaccine with the same VREP backbone. Administration up to four-times with the same VREP vaccine did eventually induce binding antibodies as shown by ELISA, however, these antibodies were non-neutralizing [117,118]. Cellular immunity against the replicase has not been seen either. The reason for this is not totally clear but could depend on the fact that replicon RNA is amplified in lipid vesicles that might shield the replicase proteins from being exposed to the immune system. Lack of anti-vector immunity is central for the efficacy of a viral vector vaccine and very few people are infected by alphaviruses.

Expert commentary

Development of replicon vaccines has come of age as clearly demonstrated by the past 20 years of pre-clinical research. Overwhelming amounts of data using a vast number of animal models have shown that replicon vaccines induce potent immune responses. Where it has been possible to properly test, these vaccines have provided protective immunity. While the technology is still evolving, it is nevertheless time to move these vaccines into the clinic and compare them with existing vaccine

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platforms. Indeed, the results from three clinical trials involving VREP vaccines have been encouraging.

Most of the pre-clinical work has involved the use of VREP vaccines. While VREP particles are probably the most efficient way of administrating replicon vaccines (on a per RNA molecule basis), a major hurdle for the clinical development so far has been the production of particles, which involves use of packaging cell technology (using helper vectors or stable cell lines). Not only is this costly, but the titers are still too low for reasonable industrial scale, preparations need to be purified from possible cellular contaminants and virus stocks are not as stable as are naked nucleic acid preparation. While anti-vector immunity does not seem to be a major problem in research settings, it is certainly an issue that needs to be addressed if replicon vaccines based on VREP would be widely used for vaccination against a variety of diseases.

While the VREP technology is well established in the research field, the same is not true for self-replicating RREP and DREP vaccines, which still are in their infancy. These naked nucleic acid replicon technologies will, as is the case for other types of (m)RNA vaccines, face rapid advances within the next few years. This will involve both the design of the RNA molecules themselves as well as development of new ways of formulation and delivery. This may involve new use of nanotechnologies. Once we get a broader understanding of the interactions between replicons and the host immune systems, there will also be the possibility to modify the vaccines to modulate these interactions thus controlling the immune responses to achieve required effect.

Replicons and RNA vaccines will be the technologies of choice for development of vaccines against emerging diseases and against pathogens with rapid escape patterns. The synthetic construction and rapid production using generic production platforms put RNA vaccines in an advantageous position. Replicon will also be combined with other vaccine platforms in prime-boost regimens to address the expanding need for new vaccine interventions.

Five-year view

Today, we have a large number of vaccines available to protect us from infectious diseases and virus-induced cancers. At the same time, much effort is being made to develop strategies against many diseases for which we do not yet have vaccines or against emerging diseases. However, it may well turn out that those vaccines that we already have are particularly those that were easy to make and to develop vaccines against diseases such as HIV, tuberculosis, dengue, RSV and malaria, to name a few, we may have to come up with totally new solutions. These have to address important questions such as choice of antigen, antigenic variation, how to generate long-term immunological memory of correct quality and how to achieve potency in all population groups including the young and the elderly. First and foremost, we still have very limited understanding about immunological correlates of protection even for the established vaccines, let alone for those few in the clinical pipeline. In recent years, there has been tremendous development in a number of areas that will prove instrumental where vaccine research and development is concerned. These include reverse vaccinology [166], computational [167], structural [168,169], systems [170,171] and synthetic biology [79]. We must learn to better understand the interplay between innate and adaptive immunity, knowledge that will be crucial in guiding vaccinologists to design vaccines and vectors, immunization strategies and use of adjuvant signals. As a result of these efforts, replicon vaccines will probably play a central part as they are versatile synthetic molecules allowing new design to be tested rapidly. Therefore, for self-replication RNA replicon vaccines the following years will see rapid development on design, formulation and delivery technologies. While moving forward, it will be important to employ small iterative clinical trial for benchmarking and for testing new knowledge coming from basic immunology and other areas. The resent idea of creating a human immunome database and creating a human vaccines project [172,173] can be one way forward and will help replicon vaccine developers in their task.

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Key issues

- Recombinant self-replicating RNAs (replicons) can be used as vaccines and delivered as RNA, as lipid-formulated RNA, as DNA or as recombinant virus particles.
- Replicon vaccines will express the antigen of interest in tissue, thus mimicking live attenuated vaccines.
- Being derived from RNA viruses, replicons are seen by the immune system as a virus infection, which includes innate stimulatory signals enhancing the potency of these vaccines.
- Replicon vaccines are safe and easy to produce using generic production platforms.
- Numerous pre-clinical studies have underscored the potency of these vaccines against infectious diseases and cancer.
- Replicon vaccines have now entered human clinical trials with very encouraging results.

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