Systematic Review of Viral mRNA Vaccine Formulations, Modifications, and Adjuvants for Enhanced Safety and Efficacy

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Abstract

Traditional viral vaccines and adjuvants are associated with safety and efficacy concerns and have proven to be an ineffective tool in immediate response to outbreaks and pandemics. Validating studies and clinical trials have shown mRNA to be a nonimmunogenic, safe, and effective therapy for immunization. mRNA vaccines are inexpensive, easy to mass produce, and can potentially be used in response to uncontained viral outbreaks. Encoded in the mRNA is the selected epitope sequence to be translated and presented by antigen presenting cells. It is reported that adjuvating activation molecules such as cytokines may also be encoded in the mRNA strand. The mRNA strand itself must be designed in a way that eukaryotic cells can translate it. As such, more success has been reported with the inclusion of a proper 5' cap, 3' and 5' UTRs, and a poly-A tail. Furthermore, select base modifications, particularly 1-methylpseudouridine, have shown increased expression and as a result, lower doses may present the same benefits. Finally, various delivery methods have been described. While naked mRNA has shown the ability to be internalized and expressed, various nanoparticle technologies are reported to expedite and facilitate this process by reducing immunogenicity and protecting the mRNA from degradation. With initial reports of cellular and humoral responses equal to or greater than traditional vaccines along with low numbers of adverse events and traditional vaccine associated pathologies, mRNA vaccines present a new era of inoculation.

Introduction

Traditional vaccine technology has successfully eradicated viral diseases such as smallpox and rinderpest (1, 2). Fifteen vaccines have been licensed for use in humans, but developed of new vaccines since 2006 has been limited (3). Additionally, live animal, egg and cell culture, and molecular biology techniques are time-consuming and expensive in response to outbreaks and pandemics (3). This is highlighted by the difficulties containing outbreaks and high illness and mortality rates observed. Coronaviruses are associated with respiratory illness in humans ranging from the common cold to severe acute respiratory syndrome and Middle East respiratory syndrome (4). The most recent coronavirus disease-2019 outbreak has over 2 000 000 confirmed cases and 140 000 confirmed deaths (5). Influenza A, coronavirus, and hanta virus present respiratory viral outbreak and pandemic threats (6). These respiratory viruses, norovirus, hepatitis C virus (HCV), Ebola virus, and many others regularly emerge and reemerge as threats to human health and prove challenging to contain. Improvements in surveillance and outbreak response protocols will help but that does not negate the need for improvements in vaccinations (7, 8).

In addition to the time-consuming, expensive, and impractical nature in current vaccine manufacturing techniques for outbreak response, issues regarding efficacy, safety, and immunogenicity need to be addressed. Current vaccines have been associated with pathologies and adverse reactions to the vaccines, adjuvants, and injection site adverse reactions. One study on herpes zoster vaccinations in older adults found that vaccinated groups had higher incidences of adverse reactions and injections site adverse reactions (9). The authors noted that although the adverse events were rated mild to moderate, subjects receiving the vaccination were at higher risk of not returning for following doses compared to placebo. Respiratory syncytial virus is one of the leading causes of respiratory illness in toddlers and young children, but live attenuated vaccines have been associated with vaccine enhanced respiratory disease, which is worse than the illness caused by the virus. It is therefore a danger that protein-based vaccinations carry this possibility as well (10).

As mentioned before, there is a very limited number of available viral vaccines. Dengue, cytomegalovirus (CMV), human immunodeficiency virus (HIV), HCV, Ebola virus, and RSV are among just a few viruses with no available vaccinations using current platforms, yet they recurrently pose threats to human health.

Viral vaccine research has seen limited advancement in the last century. While better formulations of subunit-based, attenuated, and inactivated vaccines and improvements in adjuvant technology have occurred, they have not been enough to overcome the stated challenges. A novel platform is needed for a more precise design that can produced less expensively, much quicker, and will also address safety and efficacy concerns of protein based vaccines. Nucleic acid therapy provides this opportunity. Although DNA had not yet been identified, natural translation of exogenous genetic material in non-expressing organisms was first proposed in 1928 as non-virulent bacteria were made virulent after exposure to heat-killed virulent strains termed the "transforming principle" (11). Transformation in a laboratory using electroporation was done in the 1980s (12). Attempts to introduce DNA for therapeutic application such as protein replacement in protein deficiency diseases was met with great difficulties. The vectors used were inefficient and dangerous and genomic DNA integration had dangers of its own along with ethical considerations. mRNA and viral RNAs were translated in frog oocytes, but the idea of RNA therapeutics was not initially well entertained due to its unstable structure (13, 14). Despite this, experiments showed mRNA's transient nature did not pose much of a barrier and in fact provided advantages over DNA therapy as delivery is much easier and it does not pose risks of genomic integration, and envelopment in lipid nanoparticles (LNPs) was a simple and

effective solution to protect it from degradation and facilitate intracellular delivery (15).

With the validation of mRNA as a strong vaccine platform candidate, a lot of research has been done to make the technology better. To date, it has been shown that structural elements mimicking human mRNA such as 5' caps, 3' and 5' untranslated regions (UTRs), and poly-A tails, reduce immunogenicity and improve translation. Base pair modifications not commonly seen in endogenous mRNA have also show similar benefits. As previously mentioned, LNP delivery methods allowed for more efficient cellular uptake, but alternative nanoparticle technologies, such as polymer nanoparticles, and formulations, such as antigen-coated nanoparticles, may be more effective than LNP technologies. Most importantly, the coding sequence can be precisely chosen to match not only the correct epitope but can be mutated to match epitope conformations easier for cells and antibodies to recognize and be constructed to contain costimulatory molecules and cytokines for an improved immune response. This paper explores research done to date and elucidate what combinations may provide the best benefits.

Methods

Databases including PubMed, Cochrane Library, Ovid, Wiley Online Library, and SAGE Journals were searched for articles mentioning mRNA vaccinations. Various iterations of the search terms antiviral vaccine, mRNA vaccine, mRNA vaccine adjuvants, and mRNA vaccine design were searched. The articles collected were vetted by title, abstract, and discussion to identify relevant articles. Generally, the articles included were regarding research towards viral mRNA vaccines in animal models and humans. Studies aimed at validating exogenous mRNA translation in vivo, protein replacement therapies, and cancer research were generally excluded unless aspects of the study were relevant to the development of viral vaccines, such as adjuvants and modifications that are applicable in both cases.

Discussion

Internalization and presentation

Extracellular nucleic acids have been postulated to be internalized using scavenger receptor A (16). Evidence for scavenger receptor mediated internalization of naked mRNA through caveolae/lipid raft rich membrane domains has also been found in smooth muscle cells, keratinocytes, fibroblasts, and dendritic cells (17). The non-APCs most likely presented antigens on class I MHCs, but the mRNA translation mechanism after internalization has not been fully characterized. A study of the same year found that naked mRNA injected into lymph nodes was selectively up-taken through micropinocytosis by dendritic cells (DCs) (18). As will be discussed later, LNP and CNP capsules have been used as vehicles for delivery and their internalization mechanism has not been described but can be assumed to be through vesicle fusion and an unknown endosome escape mechanism (19, 10).

Escape and sorting mechanisms within cellular compartments have not been fully elucidated for APCs either, but the mRNA is presumably translated on free ribosomes in the cytosol. The intracellular origin of the antigen initially hints at class I MHC presentation in APCs as well, and while possible, APCs generally present on class II MHCs (20). Understanding the escape, translation, and presentation mechanisms in non-APCs and APCs will allow better formulations with appropriate signaling and costimulatory molecules to be designed.

Vaccine design

As the translating and presenting system is a eukaryotic cell, viral genetic material, whether positive or negative sense, single- or double-stranded, must first be transcribed to a single-stranded mRNA molecule. Eukaryotic translation is a tightly regulated process with various stringent components. The mRNA molecule must be designed to resemble eukaryotic mRNA in order to facilitate the translation process. This includes the incorporation of a modified guanosine 5' cap, 5' and 3' untranslated regions (UTRs), and a 200 nucleotide long poly-A tail (21). Shorter poly-A tails of 120 nucleotides have also been reported to independently enhanced mRNA stability (22). There has been debate regarding the UTR sequences. Tanguay and Gallie reported that shorter UTRs (19 bases) yielded higher expression than longer UTRs (156 bases) and this difference in expression was length and not sequence dependent (23). However, a 13 nucleotide sequence deletion in the 3' UTR of the β- globin gene in two individuals with β -thalassemia suggest UTR sequence is important for proper expression of mRNA as well (24). α and β- globin 5' and 3' UTRs are well accepted sequences in mRNA design as β -globin 5' and 3' UTRs improve translation and α- globin 3' UTRs improve stability (25). There is less debate on optimal 5' sequence than 3' sequence. Three independent studies agreed on 5' UTR sequence, but one used a much shorter 3' UTR and the other two had 3' UTRs of the same length but differed in sequence (shown below).

H9N2 immunization in chickens (19):

5' UTR: GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAUA UAAGAGCCACC

3` UTR: UGAUAAUAGGCUGGAGCCUCGGUGGCCAU

POWV immunization in mice (26):

5′ UTR: GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAA UAAGAGCCACC

3′ UTR: GCUGGAGCCUCGGUGGCCUAGCUUCUUGCCCCUG GCCUCCCCCCAGCCCCUCCUCCCCUUCCUGCACCCGUACC CCCGUGGUCUUUGAAUAAAGUCUGAGUGGGCGGC

ZIKV immunization in mice (27):

5' UTR: GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAU AUAAGAGCCACC

3' UTR: UGAUAAUAGGCUGGAGCCUCGGUGGCCAUGCUUC UUGCCCCUUGGGCC UCCCCCCAGCCCCUCCUCCCCUUCC UGCACCCGUACCCCCGUGGUCUUUGAAUAAAGUCUGA

These sequences all enhanced stability and translation independently, but no studies comparing these sequences directly were found. Characterization of a more universally accepted 3' UTR could be of clinical use to identify the most potent sequence and better regulate production for more efficacious vaccines.

Various base modifications have been proposed to reduce immunogenicity and improve stability and translation as well. Replacement of uridine with 5-methyluridine showed a nearly 2-fold increase in translation (21), postulated to occur by inhibition of protein kinase R mediated translation inhibition which generally occurs during viral attack (26). Inclusion of a 5-methylcytidine modification also showed a promising 4-fold translation enhancement in cultured mouse cells (21). Combined 1-methylpseudouridine/5-methylcytidine modifications was more recently found to increase protein luciferase expression up to 44 times greater than combined pseudouridine/5-methylcytidine (27). The animal and human studies examined used unmodified mRNA or single 1-methylpseudouridine modifications. Considering the above mentioned results, characterization of double modified mRNA induced antigen responses may be of interest.

The most foundational element in mRNA vaccine design is the antigen coding sequence. Viruses have relatively few genes coding for non-structural and structural elements from polymerases to spike and fusion proteins. Chosen antigen sequences vary by virus, but are usually surface protein subunits of naturally targeted epitopes. Common targets for influenza strains, RSV, and ZIKV include hemagglutinin (HA) and matrix protein 2 (M2e), fusion protein (F), and pre-membrane (prM) and envelope protein (E) respectively (19, 28, 10, 29). Mutations can also be introduced to stabilize more favorable antigen conformations for immune recognition, particularly for antigens that undergo major conformational changes, such as the RSV F protein, but not so much for certain glycoproteins. Espeseth et al. highlighted this by creating a pre-fusion stabilized F antigen encoding mRNA that elicited higher responses than other conformational stabilized variants (10).

mRNA vaccines may include costimulatory molecules such as traditional adjuvants or mRNA encoded molecules such as cytokines. TriMix encodes CD40L (T cell proliferation), TLR-4 variant (T-cell survival), and CD70 (induction of dendritic cell maturation). This is the most extensively used cytokine and allows for a more robust and specific cell-mediated response (30).

Finally, various nanoparticle technologies have been proposed for mRNA delivery including lipids and lipid derivatives, polymers, and others. While naked mRNA has shown the ability for internalization and translation, various limitations have hindered advancement in naked mRNA immunization including immunogenicity and rapid degradation, as studies employing naked mRNA, especially in human trials, have shown increased AEs and less robust immune responses (18). Lipid nanoparticles (LNPs) are the most extensively used vehicles as they are the simplest to produce, are non-immunogenic, and facilitate endocytosis. Various polymer nanoparticles have also been studied in animal models (Table 1), but regulatory guidelines have greatly limited research in humans and no human applications of polymer nanoparticles were found. This is certainly an area that warrants further development as chitosan nanoparticles (CNPs) have provided greatly enhanced immune response and decreased AEs and a diverse range of drug delivering nanoparticles are being developed (19).

Safety

The abovementioned considerations of mRNA vaccine design allow for protection against degradation and facilitate internalization and translation processes, but also help further regulate inherent immunogenicity and allow for safer vaccinations with less AEs. Animal studies regularly report successful immunizations without reporting AEs at injection sites, mRNA related reactions, adjuvant related reactions, or nanoparticle induced toxicities (10, 19, 36, 29). Animal models

Nanoparticle	Formulation	Virus	Payload	Dosage and model	Route of Administration	Reference
Lipids and Lipid Derivatives	1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)/1,2-diasteroyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) (50:50:1) or DOTAP, DOPE and DSPE-PEG-Mannose (50:50:1)	H1N1	HA mRNA	24 µg in C57BL/6 mice	Intranasal	(31)
	Ionizable cationic lipid/phosphatidyl choline/cholesterol/PEG-lipid (50:10:38.5:1.5)	HIV-1	Anti-HIV antibody VRC01 mRNA	1.4 mg/kg in BALB/c mice	Intravenous	(32)
	Ionizable cationic lipid/phosphatidyl choline/cholesterol/PEG-lipid (50:10:38.5:1.5)	ZIKV	prM-E glycoprotein mRNA and polycytidylic acid (poly(C)RNA)	50 µg in *Macaca mulatta*	Intradermal	(33)
Polymers	Chitosan surface coated with HA1/HA2 and M2e	H9N2	HA1/HA2 and M2e mRNA	4 µg in chickens	Intranasal	(19)
	Polyethyleneimine-stearic acid	HIV-1	HIV-1 gag mRNA	N.A. in BALB/c mice	Subcutaneous	(34)
	2-tridecyloxirana/1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (11.5:1)	ZIKV	prM-E glycoprotein mRNA	80 µg in C57BL/6 mice	Intramuscular	(35)

Table 1. List of various lipid and polymer based nanoparticle delivery methods in animal models.

have also reported no incidences of vaccine exacerbated pathologies. RSV vaccinations were discontinued because they could result in more serious infections, such as vaccine enhanced respiratory disease, instead of attenuating the virus, but this phenomenon was not observed in RSV mRNA vaccinated cotton rats (10). Investigations in non-human primates monitoring for injection site reactions, body weight, rectal temperature, hematologic variables, and clinical chemistry variables did not report adverse effects in response to self-amplifying HIV-1 mRNA intramuscular injections (36). These studies provided preclinical evidence validating the safety of mRNA vaccines for advancement to clinical trials.

Few mRNA vaccine clinical trials have been completed to assess safety and AEs, but initial results have shown mRNA vaccines to be well tolerated. A randomized placebo-controlled double-blind phase 1 trial using a chemically-modified LNP-encapsulated H10N8 HA mRNA vaccine on a two-dose schedule 3 weeks apart (intramuscular; 25, 50, 75, 100, and 400 µg and intradermal; 25 and 50 µg) and H7N9 HA mRNA vaccine on a two-dose schedule six months apart (intramuscular; 10, 25, and 50 µg) did not report any vaccine related severe AEs (N=357) (28). Two grade three solicited AEs (injection site erythema and headache) were reported at the 400 µg intradermal dose and this treatment arm was discontinued. A randomized placebo-controlled double-blind clinical trial delivering intradermal immunization with HIV-1 Gag and Nef mRNA transfected DCs reported no grade three or four AEs (N=10) (37). A phase 1 HIV-1 naked mRNA vaccine (iHIVRNA) study in 21 males delivering intranodal TriMix-300 g with 300, 600, and 900 g T-cell immunogen (HTI), an mRNA sequence targeting more vulnerable sites than traditional Gag and Nef targeting sequences, showed 31 grade 1/2 adverse effects, of which two had a definite relationship to the vaccine, and one grade 3 AE (30). A phase 2a randomized placebo-controlled double-blind study further evaluating internodal naked iHIVRNA vaccination in 40 HIV patients is currently being conducted (38).

Based on the large sample size and low AE rates of the influenza investigation, NP mRNA delivery appears to be correlated with lower rates of AEs. The HIV studies all had much smaller sample sizes but more reports of AEs. Due to the small sample sizes (N<30), this observation may not be significant. Additionally, although HIV participants were included on the basis of successful ART and acceptable immune system function parameters, there may be a predisposition to AEs based on a compromised immune system. Animal investigations more readily employ nanoparticle delivery techniques, which may not be as easy in human trials due to regulatory concerns. Regardless, a phase I clinical trial assessing the safety of the LNP delivered mRNA-1273 vaccine, encoding the SARS-CoV-2 spike protein and showed success and is being advanced to stage II and III clinical trials. Nanoparticle delivery has shown a large degree of preclinical success and may warrant advancement of this research to clinical phases.

Efficacy

In animals, the efficacy of mRNA vaccines has been greatly supported. An LNP-encapsulated base-modified Powassan virus (POWV) mRNA vaccine encoding prM and E proteins was given to mice in one or two intramuscular doses of 10 µg. After the first and second immunization, the 50% maximal effective inhibitory concentration titers were approximately 2-fold and 3-fold higher than placebo, respectively. In response to challenge, 100% of placebo-treated mice died, and all POWV immunized mice survived (39). HA2 and M2e peptide antigen-coated CNPs delivering H9N2 HA2 and M2e 1-methylpseudouridine-modified mRNA (CNP+RNA+Pr), empty antigen coated CNPs (CNP+Pr), or uncoated empty CNP were intranasally delivered to chickens and IgG, IgA, CD4+, and CD8+responses (19). HA2 specific IgG antibodies were significantly higher in CNP+RNA+Pr treated mice than CNP+Pr and CNP treated mice while M2e specific IgG antibodies were not statistically different between CNP+RNA+Pr and CNP+Pr. Both HA2 and M2e specific IgA antibodies were significantly higher for CNP+RNA+Pr than CNP+Pr treated mice. Virus neutralization titers were also significantly higher in CNP+RNA+Pr treated mice than CNP+Pr treated mice. CD4+ responses were reported at a higher degree than CD8+ responses in all mice, but CNP+RNA+Pr showed a significantly greater amount of CD4+ and CD8+ cell responses. The viral load in CNP and CNP+Pr mice was approximately 27 and 3 times higher than CNP+RNA+Pr mice respectively. LNP encapsulated 1-methylpsudouridine modified RSV F protein coding mRNA was intramuscularly delivered to mice in two 10 µg doses 3 weeks apart and compared to the RSV DS-Cav1 protein antigen with an aluminum phosphate-based adjuvant (10). Various mRNA sequences corresponding to conformation stabilized versions of F protein were included in the study to identify the most immunogenic conformation. They all elicited comparable or greater serum neutralization titers than the control. Control mice did not have detectable CD4+ or CD8+ responses while mRNA treated mice had significantly higher CD4+ and CD8+ responses as observed by increased IL-2, IFN- γ, and TNF- α. HIV-1 gp140 encoding self-replicating mRNA formulated with cationic nanoemulsion was used to intramuscularly immunize rhesus macaques with 50 µg mRNA. mRNA gave anti-gp140 titers comparable to gp140 protein immunized controls and antigen-specific B-cells levels of 1,500 per 106 peripheral blood monocytes versus 1,400 per 106 PBMCs observed in controls. mRNA immunization also provided greater cellular immune response as seen by IFN-g levels approximately 3 times greater than in protein immunized mice. Animal models have repeatedly shown comparable or greater specific systemic and mucosal humoral and cellular immune responses to mRNA produced antigens as observed by IgG, IgA, neutralizing antibodies, and IFN- γ, TNF- α, and interleukin producing CD4+ and CD8+ cells.

Based on the safety and efficacy demonstrated in animal models, clinical trials evaluating safety as primary endpoints and efficacy as secondary/exploratory endpoints are currently underway. Completed and current clinical trials for viral mRNA vaccines are mostly at phase 1, one study found was at phase 2, and none found were at phase 3. The phase 2 study is in progress and aims to evaluate the SARS-CoV-2 mRNA-1273 vaccine by Moderna and is expected to be completed August 2021 (40). Exploratory endpoints for all trials have revealed mixed results. As seen in Table 2, the clinical trials included induced some degree of humoral response but failed to elicit protective cell-mediated responses. Naked delivery of HIV targeting mRNA to DCs in vitro and in vivo did not elicit

Virus	Payload	Route of Administration	Immune Responses	Reference
H10N8 and H7N9	LNP-enveloped full-length HA encoding modified mRNA	Intramuscular or intradermal	High hemagglutination inhibition titers High microneutralization titers No significant IFN-γ detection	(28)
HIV-1	Naked Gag and Nef mRNA transfected DCs	Intradermal	Transient CD4+ and CD8+ responses No significant IFN-γ detection	(37)
HIV-1	Naked HIVACAT T-cell immunogen sequence encoding TriMix	Intranodal	Moderate increase in HTI targeting T-cells No impact on proviral reservoir	(30)
Rabies	Rabies glycoprotein CV7201	Intramuscular or intradermal	Produced WHO specified antibody titer of 0.5 U/mL in 71% of intramuscular and 46% of intradermal needle free participants but failed in needle syringe participants	(40)

Table 2. Completed clinical trials evaluating safety as the primary endpoint and assessing efficacy as a secondary/exploratory endpoint.

strong immune responses. As previously stated, naked mRNA delivery has limitations that can be overcome with nanoparticle delivery. In addition, Gag is a particularly difficult immunization target due to high mutation rates which constantly change the epitope. Needle-free administration of rabies vaccination did elicit humoral responses, but syringe administration did not. This highlights another variable to consider in the development of mRNA vaccines.

Conclusion

mRNA vaccines can elicit robust humoral and cell-mediated immune responses as validated in countless animal models. Strong mRNA vaccine candidates include classical eukaryotic mRNA structures including a 5' cap, 5' and 3' UTRs, and a poly-A tail. Several UTR sequences of varying lengths have independently enhanced translation. Unlike most eukaryotic mRNA however, select base modifications improve stability and translation and decrease immunogenicity of the mRNA molecule. 1-methylpseudouridine and 5-mythylcytidine have provided the most promising results but further investigation into the combined use of these is warranted. Co-administration with T-cell and DC proliferation and survival cytokines (TriMix) has supported stronger immune responses. While naked mRNA injections have been validated to produce protein products, LNPs and protein coated CNPs have shown enhanced uptake for translation and presentation.

Safety in clinical trials has been supported as low numbers of AEs are reported. Analysis of various biomarkers in preclinical investigations, including IFN-g, TNF-a, IgG, IgA, CD4+, and CD8+, have shown efficacy equal to or greater than traditional subunit or whole virus-based vaccines in animal models, but clinical trials have not shown protective cell-mediated immune responses. Continued research into nanoparticle delivery and characterizing the most efficacious 5' and 3' UTRs and epitope sequences will provide the greatest advancements to this platform. Although initial clinical trials have not met efficacy expectations set by animal models, initial successes of developing immunity in animals against viruses with no current vaccinations still makes mRNA vaccines a thrilling prospect and the available technology makes these challenges exciting to face and overcome.

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