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Developing methods for mRNA vaccines: A Review

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ABSTRACT: mRNA vaccines have a good immunological profile, a good safety profile, and the flexibility that genetic vaccines lack. Based on in situ protein production, mRNA vaccines may induce a balanced immune response that includes both cellular and humoral immunity while not being limited by MHC haplotypes. Furthermore, since it is a minimum and only temporary carrier of information that does not interact with the genome, mRNA is an inherently safe vector. Because any protein may be produced from mRNA without changing the manufacturing method, mRNA vaccines provide the most development flexibility. When taken as a whole, mRNA seems to be a viable vector that has the potential to provide the foundation for a game-changing vaccine technology platform. The present state of knowledge about several issues that should be addressed while creating an mRNA-based vaccination technology is outlined below. Because RNA is notoriously fragile, using it for medicinal purposes is a risky proposition. Despite the molecule's susceptibility to the almost ubiquitous ribonucleases (RNases),1 mRNA was initially pushed as a therapy in 1989, after the discovery of a widely applicable in vitro transfection method.

KEYWORDS: adjuvant, formulation, mRNA, mRNA production, mRNA design, mRNA uptake, protein expression

1. INTRODUCTION

Because RNA is notoriously fragile, using it for medicinal purposes is a risky proposition. Despite the molecule's susceptibility to the almost ubiquitous ribonucleases (RNases), mRNA was initially pushed as a therapy in 1989, after the discovery of a widely applicable in vitro transfection method. Only a few years later, mRNA was promoted as a vaccine platform, perhaps because it combines the immunological benefits of live attenuated vaccines, such as endogenous antigen expression and T cell induction, with the advantages of dead or subunit vaccines, such as specified composition and safety. When compared to DNA as a treatment or, more particularly, as a vaccination, mRNA has significant safety benefits. It contains just the components immediately needed for the encoded protein's production as a minimum genetic construct. Furthermore, although single-stranded RNA molecules may recombine in rare cases, mRNA does not interact with the genome. As a result, possibly harmful genetic integration is ruled out. Finally, since mRNA is non-replicative and metabolically decays after a few days8, it is only a transitory bearer of information due to its lack of chromosomal integration. mRNA, as the technical foundation for medicines and vaccines, has a lot of flexibility in terms of manufacturing and use. Because any protein may be transcribed and produced by mRNA, preventive and therapeutic vaccinations for illnesses as varied as infections and cancer, as well as protein replacement treatments, are theoretically possible. Because changes to the encoded protein only modify the sequence of the RNA molecule, its physico-chemical properties are mostly unchanged, a variety of products may be produced using the same existing manufacturing method, saving time and money compared to alternative vaccination platforms. In terms of effectiveness, mRNA-based therapies benefit from the fact that, unlike DNA, they do not need crossing the nuclear membrane. Unlike peptide vaccinations, mRNA vaccines are not restricted by MHC haplotype. Furthermore, since mRNA attaches to pattern recognition receptors, mRNA vaccines may be engineered to be self-adjuvating, a feature that peptide and protein-based vaccinations do not have. Overall, mRNA

represents a promising, though difficult, class of therapeutic molecules with the potential to serve as the foundation for a "revolutionary technology." We will discuss what has to be taken into account while creating an mRNA-vaccine technology, including mRNA production and quality, mRNA format and formulation, antigen/protein expression, and the immunological characteristics of mRNA-vaccines[1].

1.1 mRNA synthesis:

In vitro transcription of a cDNA template, usually plasmid DNA (pDNA), employing a bacteriophage RNA polymerase may yield functional synthetic mRNA. As a result, the first step in the synthesis of mRNA is the generation of pDNA. As a result, mRNA production may seem to take longer than pDNA production. Unpolished pDNA, on the other hand, includes remnants of bacterial genomic DNA as well as three different types of pDNA (supercoiled, relaxed circular, and linear) in varying amounts. As a result, preparing pure and invariant pDNA in a repeatable manner, as needed for a vaccination, is difficult. If linearized pDNA is transcribed using bacteriophage RNA polymerase, however, bacterial DNA remains and pDNA heterogeneity are not a problem since all DNA is eliminated during subsequent processing stages (see below). Synthetic mRNA has a protein-encoding open reading frame (ORF) bordered at the 3′ end by a poly(A) tail and a “cap,” which is a 7-methylguanosine residue linked to the 5′ end through a 5′-5′ triphosphate. A pDNA template for in vitro transcription must thus include at least a bacteriophage promoter, an ORF, a poly(d(A/T) sequence translated into poly(A), and a unique restriction site for plasmid linearization to guarantee specified transcription termination (the cap is not encoded by the template). In a combination comprising recombinant RNA polymerase (T7, T3, or SP6) and nucleoside triphosphates, the linearized pDNA template is transcribed into mRNA. Capped mRNA may be obtained via transcription. A cap analog, such as the dinucleotide m7G(5′)-ppp-(5′)G (referred to as "normal cap analog" in the following), may be used to do this. If the cap analog is greater than GTP, transcription begins using the cap analog instead of GTP, resulting in capped mRNA. The cap may also be inserted enzymatically after transcription. If the pDNA template does not have one, a poly(A) tail may be inserted after transcription. Following transcription, DNase is used to digest the pDNA template as well as any contaminating bacterial DNA[2].

1.2 mRNA purification:

At this stage, the sample includes the required mRNA transcript among a complicated combination of nucleotides, oligodeoxynucleotides, short abortive transcripts from initiation abortive cycling,16 and protein. A combination of precipitation and extraction procedures may be used to eliminate these pollutants from the sample. However, the sample contains other contaminating RNA species that are difficult to distinguish from the proper transcript using conventional methods: Premature termination during elongation results in transcripts that are shorter than expected. Template DNA linearized with an enzyme that leaves a 3′-overhang or remnants of nonlinearized template DNA produce transcripts that are longer than specified. The RNA-dependent RNA polymerase activity of bacteriophage polymerases also produces undesirable transcripts. As a result, mRNA will need to be processed further to eliminate such contaminated transcripts before it can be utilized as a medicinal ingredient. Shorter and longer transcripts were eliminated in a single chromatographic phase that separated mRNA by size, producing a pure single mRNA product. The use of such chromatographic purification in a GMP mRNA manufacturing process improved the activity of mRNA molecules in terms of

protein expression in vivo by several fold. When transcripts coding for luciferase or erythropoietin were purified by HPLC, increased protein expression was found as a consequence of rigorous mRNA purification. Because the erroneous transcript was removed, the rise in protein expression was considerably greater than anticipated. The scientists showed that enhanced protein expression after HPLC purification was attributable to the elimination of contaminating RNA, such as double-stranded RNA, which activates innate immune sensors and reduces protein production[3].

2. REVIEW OF LITERATURE

S Sorrentino in his study discloses about the structural, catalytic, and/or biological characteristics, human extracellular ribonucleases (RNase) and other members of the mammalian RNase A superfamily that may be divided into four distinct RNase families. Their incidence and distinguishing characteristics have been reported, and the data on their catalytic properties has been analyzed and compared to those of other animal RNases. It has been proposed that while pancreatic-type (pt) RNases could be defined as single-strand/pyrimidine 'preferring' ribonucleases, mammalian nonpancreatic-type (npt) RNases could be defined as single-strand/pyrimidine'specific' ribonucleases based on some results obtained with various single- and double-stranded polyribonucleotides[4].

R W Malone in his study focuses on N-[1-(2,3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA), a synthetic cationic lipid, that was integrated into a liposome to provide an effective and repeatable technique for RNA transfection (lipofectin). A linear response of luciferase activity is obtained by transfecting 10 ng to 5 micrograms of Photinus pyralis luciferase mRNA produced in vitro into NIH 3T3 mouse cells. The method may be used to transfect RNA into human, rat, mouse, Xenopus, and Drosophila cells with high efficiency. We investigated the effect of capping and beta-globin 5' and 3' untranslated regions on the translation efficiency of luciferase RNA produced in vitro using the RNA/lipofectin transfection method. Capped mRNAs containing beta-globin untranslated sequences generated at least 1000-fold more luciferase protein than mRNAs without these features after transfection of NIH 3T3 cells[5].

M R Hilleman in his study discuses about the Nearly two centuries of vaccinology and immunology research that will drive the creation of recombinant vector vaccines. Experimental vector vaccines may be viral, bacterial, or genetic in nature, and their acceptability will be determined by the user, the developer, and the licensing authority's perceptions of safety, effectiveness, and practicality. Non-propagable agents, early childhood vaccination, reproductive control, and new infectious pathogens may all benefit from recombinant vector vaccines, which will have to compete with other vaccine methods. The vector vaccination method may, at least in part, meet the needs of poor countries, such as cheap cost, temperature stability, and simplicity of administration[6].

3. DISCUSSION

3.1 m-RNA Design:

Synthetic mRNA for therapeutic purposes is often created using the same blueprint as eukaryotic mRNA. Because they are needed for effective translation, the cap and poly(A) tail are important components. 2,15,21 Cap and poly(A) tail, which are located at the 5′ and 3′ ends

of mRNA, are also needed to stabilize mRNA in the cytosol, where degradation is mostly mediated by exonucleases. However, mRNA needs 5′ and 3′ untranslated regions (UTRs) to border the ORF in order to enhance both translation and stability. Because 24-27 UTRs may affect translation and mRNA stability, they must be carefully selected. Specific cis-acting destabilizing sequences like as AU-rich elements and miRNA binding sites, for example, are typically located in UTRs, although they may also be present in ORFs. Such destabilizing messages must be avoided at all costs. Following these concerns, researchers have worked to discover useful mRNA components in order to enhance the translation and stability of synthetic mRNA molecules inside the cell. Improved mRNA formats will almost certainly result in better mRNA vaccines, since the effectiveness of an mRNA vaccine is generally believed to improve when protein expression is enhanced and maintained[7].

3.1.1 Cap:

By adding a cap analog in the process, mRNA may be capped during transcription. However, it has been shown that the normal cap analog is often integrated in the opposite direction, resulting in the m7G nucleotide serving as the first transcribed nucleotide rather than the cap. As a consequence, approximately a third of mRNA molecules do not get their caps methylation. This kind of mRNA isn't translated since the cap base isn't methylated. To prevent the formation of an unmethylated cap due to reverse orientation, mRNA may be transcribed without a cap analog and then capped using the vaccinia virus capping complex. A natural cap is added to the 5′-triphosphate of an RNA molecule by this complex, which contains triphosphatase, guanylyltransferase, and (guanine-7-) methyltransferase activity. However, an extra enzymatic step may make manufacturing more difficult, especially on a large scale. Alternatively, “anti-reverse” cap analogs may be used to produce a cap that is only in the proper orientation (ARCAs). The most frequent ARCA permits only the insertion of a nucleotide at the non-methylated guanosine after 3′-O-methylation of the base-methylated guanosine. In rabbit reticulocyte lysate, ARCA-capped mRNA translated with more than twice the efficiency of mRNA capped by conventional cap analog. Furthermore, mRNA transcribed in vitro using ARCA has been demonstrated to have a longer half-life in cultivated cells. ARCA-capped mRNA has been shown to enhance and extend protein expression in cultured cells in an independent research. Enzymatic 2'-O-methylation of the first transcribed nucleotide may boost protein production from in vitro transcribed, enzymatically capped mRNA, resulting in protein expression similar to that of mRNA capped with ARCA co-transcriptionally. The triphosphate linkage of ARCAs has been changed to prevent decapping of the matching mRNA and enhance binding of eukaryotic initiation factor 4E, which is important in ribosome recruitment. Modifications either replaced a bridging oxygen (e.g., (methylenebis)phosphonate and imidodiphosphate) or a non-bridging oxygen (e.g., (methylenebis)phosphonate and imidodiphosphate) for a bridging oxygen (e.g., (methylenebis)phosphonate and imidodiphosphate (e.g., phosphorothioate, phosphoroselenoate and boranophosphate). When compared to ARCA, phosphothioate-modified ARCAs produced mRNA with higher translation efficiency and a longer half-life in cultivated cells. Phosphorothioate-modified ARCAs, on the other hand, are synthesized as a mixture of two diastereomers that must be separated after synthesis due to their differences in biological activity[8].

3.1.2 Poly (A) tail:

When the poly(A) tail was first revealed to aid translation start, it was shown that when the length of the poly(A) tail was extended up to 68 residues, the effectiveness of polysome

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formation improved. By increasing the length of the poly(A) tail from 54 to 98 residues, translation of in vitro produced mRNA transfected into cultured cells increased somewhat. This research was expanded upon by looking at the impact of increasingly longer poly(A) tails on protein expression. When the poly(A) tail was extended from 64 to 150 residues, the peak protein level achieved one day following electroporation of mRNA into cells was doubled. Additional enzymatic polyadenylation of the poly(A) tail resulted in a modest increase in peak expression. Protein levels 16 hours after transfection of UMR-106 cells, on the other hand, rose with increasing poly(A) tail length only up to 60 residues, then decreased with increasing poly(A) tail length. In practice, it's worth noting that maintaining lengthy poly(d(A/T) sequences is difficult and highly reliant on the bacterial strain[9].

3.1.3 UTRs:

In vitro produced mRNA included 5'- and 3'-UTRs, particularly those of the Xenopus globin gene, from the start. In the mouse NIH 3T3 fibroblast cell line, both the Xenopus globin 5′- and 3′-UTRs were shown to confer significantly higher translational efficiency on heterologous mRNA. The creation of a library from amplified tumor-derived cRNA for use as vaccines against metastatic melanomas utilized a combination of the globin 5′-UTR, which improves translation, and the globin 3′-UTR, which is known to stabilize mRNA. Globin UTRs are still widely used in in vitro produced mRNA, including immunotherapy RNA. In vitro generated mRNAs with UTRs from non-globin genes have also been utilized to investigate the therapeutic potential of mRNA. The 5′-UTR of the tobacco etch virus improves in vitro transcribed mRNA translation in mammalian cells and has been found in mRNA producing erythropoietin in several cell types and animals. Furthermore, a structure of the human heat shock protein 70 5′-UTR improved mRNA translation in mammalian cells and was anticipated to be useful in genetic vaccination. Incorporating an internal ribosome entry site (IRES) into in vitro transcribed mRNA may be a different and/or complementary way to have therapeutic proteins expressed. The EMCV IRES, for example, was found in mRNAs coding for four transcription factors involved in the conversion of fibroblasts to pluripotent stem cells. Even mRNA without a cap has been effectively directed to protein production using the EMCV IRES. Dendritic cells transfected with such IRES-containing, cap-less mRNA protected animals against melanoma cell metastases after intravenous injection. Screening entire transcriptomes for sequence regions that enhance translation or mRNA stability may yield completely new UTRs[10].

3.1.4 ORF:

In many species, codon use is also thought to have an impact on translation efficiency. In humans, however, codon use bias is unrelated to tRNA levels or gene expression. To summarize, codon optimization is unlikely to enhance mRNA translation in humans (in general), especially if the ORF is already of human (or even mammalian) origin. The start codon should obviously be part of a Kozak sequence, and the sequence surrounding the stop codon may be improved. Furthermore, there should be no upstream start codons in the mRNA before the proper start codon[11].

3.2 m-RNA Uptake:

An mRNA vaccine must reach the cytoplasm of target cells in order to be translated and trigger an antigen-specific immune response. RNA vaccines, on the other hand, unlike DNA vaccines,

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just need to penetrate the plasma membrane, not the nuclear envelope, which may increase the chances of successful in vivo transfection. The absorption of mRNA by mouse muscle cells after simple injection, i.e. without the need of specific delivery methods, was shown as early as 1990. Numerous investigations afterwards showed that naked mRNA given locally is picked up by cells in target areas. Initially, the method by which naked mRNA reaches cells was unknown. However, identifying and clarifying the absorption pathway is critical for the creation of more effective mRNA vaccines. A slew of research have looked at how nucleic acids get into cells. The majority of them investigated the absorption pathways of pDNA, DNA oligonucleotides, siRNA, and long dsRNA, and discovered a complicated picture. The molecules entered cells via diffusion-controlled processes or a variety of endocytic routes, which were typically highly dependent on the cell type or species, and often exhibited vesicular localization, or trapping in endocytic or lysosomal compartments. However, owing to its unique mix of physico-chemical and structural characteristics, mRNA varies from these kinds of molecules. mRNA, unlike DNA, has uridine rather than deoxythymidine, prefers a C3′-endo conformation, and is hydroxylated at the 2′-position of the ribose. Because mRNA is single-stranded, it can fold into complex secondary and tertiary structures that double-stranded DNA and RNA molecules cannot. Finally, mRNA is distinguished from other single-stranded RNAs such as antisense RNA and aptamers by its length, which ranges from a few hundred to several thousand nucleotides. A mouse research exploring intradermal injection provided the first insight into the absorption process of naked mRNA. Local entrance into dermal cells that were not primarily professional antigen presenting cells (pAPCs) was shown to be saturable, improved by calcium, and linked to vesicle mobility. More in-depth in vitro research showed that naked mRNA absorption is a common occurrence in primary cells and cell lines of many kinds. Saturability of uptake was verified, as was the fact that it is temperature and dosage dependent. The majority of the mRNA seemed to enter cells through caveolae/lipid rafts, most likely mediated by (a) scavenger-receptor(s), which are known to accumulate in caveolae and selectively identify and promote internalization of negatively charged macromolecules. Macropinocytosis appears to have a modest role in the absorption of mRNA by several primary cells and cell lines. Macropinocytosis, on the other hand, seems to prevail mRNA absorption by dendritic cells after intranodal injection. When it comes to prepared mRNA vaccines, the situation gets even more complex. For example, a newly produced two-component vaccine including naked and protamine-complexed mRNA shows distinct absorption pathways and kinetics for the two components, despite the fact that both are taken up through an endosomal pathway. In vivo, mRNA uptake and expression are very effective (far more efficient than spontaneous absorption by cells in vitro) and even equivalent to cells transfected in vitro under ideal circumstances. In the case of local injections, hydrodynamic pressure may play a role in target cell transfection, just as it does with intravenous delivery. The relationship between pressure and transfection efficiency/protein expression, on the other hand, may not be linear, but it does indicate an optimum. In any case, it seems that a significant portion of the mRNA is trapped in endosomal vesicles. As a result, methods that increase the percentage of mRNA that enters the cytosol may be very beneficial to mRNA vaccines[6].

3.3 m-RNA Based Vaccines:

The idea of utilizing mRNA as a foundation for vaccinations was explored very soon once in vivo injection of mRNA was shown to be feasible. Subcutaneous injection of liposome-encapsulated mRNA encoding the nucleoprotein (NP) of the influenza virus elicited NP-specific cytotoxic T lymphocytes for the first time in 1993. (CTLs). 87 In this situation, naked mRNA, on the other hand, failed to activate particular CTLs. Following that, in response to a heterologous prime-boost schedule, the use of naked mRNA stimulated the production of

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antigen-specific antibodies (repeated intramuscular RNA vaccination, challenge with tumor cells). However, none of the animals were immune to tumor infection. The first demonstration of an antigen-specific antibody response generated simply by mRNA was made in the mouse epidermis utilizing particle-mediated mRNA delivery. In the year 2000, the field of mRNA vaccines was advanced by the introduction of a novel vaccination technique that allowed naked mRNA to be administered through intradermal injection. This simple vaccination strategy required no transfection chemicals, special equipment, or heterologous boost, yet it was able to elicit a full adaptive immune response, including antigen-specific antibodies and T cells with lytic activity against the model antigen -galactosidase. Following that, intradermal injection of total RNA extracted from the S1509 tumor cell line was demonstrated to produce immunity against a second tumor challenge. Tumor growth suppression was also accomplished by injecting in vitro transcribed and lipid-complexed mRNA encoding the model antigen ovalbumin intradermally and intravenously (OVA). An similar vaccine with mRNA coding for a model tumor/self-antigen, on the other hand, was unable to destroy TRAMP mice's tolerance to this self-antigen. MART1 mRNA may not only inhibit B16 melanoma development but also metastasis, according to a study that used histidylated lipopolyplexes for systemic injection. In a study comparing various delivery methods for naked mRNA vaccines, excellent immunogenicity against ovalbumin and influenza was found. After repeated and frequent injections into the lymph node, a viral hemagglutinin could be seen. 45 The scientists modified the antigen by adding an MHC class I molecule trafficking signal for enhanced antigen presentation in order to improve the vaccine's effectiveness. Perinodal, subcutaneous, and intradermal injections, unlike intranodal injections, did not elicit the same immune responses. 45 Recently, an alternate, simpler method to intradermal immunization was developed. In preventive and therapeutic contexts in mice, combining naked mRNA with protamine-formulated mRNA resulted in a two-component vaccine capable of eliciting robust immune responses and tumor protection. The two components in this vaccine serve complimentary roles: the naked mRNA ensures optimum antigen expression, while the protamine-complexed mRNA provides significant immunostimulatory effects. In particular, for tumor treatment, this novel kind of mRNA vaccination may be coupled with other, more traditional treatments like chemotherapy, resulting in better results than either treatment alone. An immunological response may also be generated by vaccination using pAPCs transfected with mRNA ex vivo as an alternative to direct injection of mRNA. In the EG.7-OVA and B16 melanoma models, mRNA-transfected murine dendritic cells (DCs) were demonstrated to activate anti-tumor immunity. Tumor development was also substantially slowed when epidermal cells enriched for Langerhans cells, which belong to the pAPC group, were transfected with total RNA obtained from tumor cells and injected. In vitro, transfection of human DCs with mRNA expressing CEA or the E6 antigen of the human papillomavirus type 16 elicited a primary CTL response. Ex vivo mRNA transfection of pAPCs is now the most used method for mRNA vaccination in clinics. A clinical study using telomerase mRNA-transfected DCs, for example, showed that such applications may trigger antigen-specific cellular immune responses. The underlying process, however, is time-consuming and labor-intensive, and it requires patient-specific (autologous) cell preparations.

Only a few clinical trials using mRNA-based vaccinations given directly have been reported. Autologous mRNA libraries generated from melanoma lesions were used in the first experiment, whereas a cocktail of protamine-complexed mRNAs encoding six distinct antigens was administered intradermally in a subsequent research utilizing an escalated treatment regimen. In a separate clinical study with patients with stage IV renal cell cancer, GM-CSF was given as an adjuvant 24 hours after immunization with six antigens, a strategy that will be described in more depth in the next section[5].

4. CONCLUSION

mRNA-based vaccines promise to be a game-changing vaccine technology platform for therapeutic and preventive purposes, almost two decades after the first successful injection of mRNA in vivo. The scientific community is now anticipating the release of the first clinical efficacy results. However, there is still a lot of room for mRNA-based vaccine research and enhancement. As previously mentioned, mRNA format and absorption are important factors for effective antigen expression, and they may be modified by new RNA designs as well as mRNA formulation and delivery. Any modifications to these parameters, however, may have a significant impact on mRNA synthesis and/or interactions with RNA-sensors, and should be carefully addressed from the start. In addition to the nucleotide changes described earlier, new delivery mechanisms may have a significant impact on vaccination adjuvanticity. While direct delivery into the cytosol would definitely increase antigen expression, the absence of contact with endosomal RNA receptors may significantly reduce vaccination immunostimulant, and this problem would very probably need to be addressed. Incorporating auxiliary mRNA molecules into an mRNA vaccination may be a promising way to get optimum results in the event of especially difficult treatments. Furthermore, combining it with other anti-tumor treatments is likely to give the best results. However, this would increase the vaccine's and/or treatment regimen's complexity, making development more difficult. In view of its flexibility, effectiveness, and safety, mRNA seems to be a potential vaccination vector. As a result, it may become a "disruptive technique" not just for cancer immunotherapy, but also for infectious disease vaccination, whether preventive or therapeutic.

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