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APPLICATION OF REVERSE GENETICS IN RNA VIRUS VACCINE DEVELOPMENT: A BRIEF REVIEW

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Abstract— RNA viruses can quickly spread and cause serious, even fatal, illnesses in both humans and animals. Platforms for creating and refining viral mutants for vaccine development have been made available by the introduction of reverse genetics methods for manipulating and studying the genomes of RNA viruses. In this article, we review the effects of RNA virus reverse genetics systems on previous and ongoing initiatives to develop efficient and secure viral treatments and vaccines.

Keywords— RNA virus, Vaccine, cDNA, Reverse Genetics

I. INTRODUCTION

One of the greatest achievements of human creativity, scientific research, and the joint international efforts of the public health community is the development of vaccines. When compared to pre-vaccination rates, the incidence and fatality rates of RNA virus infections including polio, measles, mumps, and rubella have decreased by more than 95% (1). Conventional methods for developing RNA virus vaccines, notwithstanding their historical effectiveness, such as live-attenuation through passaging (forward genetics) or inactivation, may be less effective at producing high-quality candidates than rational targeted mutagenesis (reverse genetics). The generation of vaccines is made easier by targeted alterations and directed attenuation by advancements in recombinant DNA technology and virus reverse genetics, which has revealed important insights into the replication and pathogenicity of RNA viruses. By enabling the investigation of specific genetic alterations in virus genomes, reverse genetics and molecular engineering of viruses have revolutionized the field of virology. The first infectious RNA viral clone to produce poliovirus was discovered in 1981 using cDNA (2). Since then, reverse genetic clones representing all significant virus families have been produced using reverse genetics technology and recombinant virus design. Additionally, these methods and strategies are now the main focus of new initiatives to develop vaccines that combine particular adjustments to the virus- or component-based systems to produce long-lasting immunity in the host without harmful side effects or hazards. Since the conception of successful live-

attenuated vaccines, the field of vaccine discovery and development has been shaped by new vaccine preparations utilizing suitable vectors, expression of particular viral proteins or components (subunit vaccines), and the creation of virus-like particles (VLPs). The difficulty of developing a secure, immunogenic platform that promotes long-lasting immunity in the presence of numerous viral systems has led to a remarkable amount of inventiveness and variation in the methods used. High immunogenicity, cheaper costs, and ease of transport and administration are all advantages of using replicating viruses in vaccines, such as live-attenuated or chimeric vector-based platforms. But in immunocompromised hosts, these viruses may not be sufficiently attenuated and may revert to more dangerous characteristics. Subunit, attenuated, or killed microorganism vaccines, on the other hand, offer the advantages of being typically safer and having the ability to provide the most immunogenic components. The expenses, length of development, and lower induced immune responses, however, bring their own difficulties in terms of planning and execution. We explain RNA virus reverse genetics systems and give a summary of current initiatives to apply reverse genetics technology in the creation of secure and reliable vaccines in this review.

II. BIOLOGY OF RNA VIRUS & REVERSE GENETICS DESIGN

In RNA virus reverse genetics, First, RNA-dependent RNA polymerase (RdRp) that is active in RNA viruses produces genomic copies. In addition, almost all RNA virus replication mechanisms lie in the cytoplasm rather than the nucleus of the host cell. For positive-strand RNA viruses, host ribosomes quickly translate the genomic RNA to produce viral protein products following entry and uncoating. Positive-sense RNA virus reverse genetics systems mainly concentrate on the delivery of either delivery of cDNA under the control of a viral transcription promoter such as T7 or CMV or transcribed genomic RNA into the cell cytoplasm because the virus rarely needs to package additional non-structural proteins in the virion (3-10). However, the introduction of the RdRp and other crucial proteins to start genomic replication is frequently necessitated by the employment of extra helper constructions



in negative-strand and double-strand RNA virus reverse genetic systems. Utilizing host polymerases like Pol I and Pol II to synthesize viral RNA and promote the generation of host mRNA is a more modern alternative strategy that has been used in influenza research (11-12). This method has improved the efficiency of recovering recombinant viruses while streamlining the logistics of plasmid transfection (13). The bacterial artificial chromosome (BAC) is another tool that has been used to create infectious RNA virus clones. BAC constructs are single-copy DNA plasmids based on the F-plasmid of bacteria that can insert large DNA segments and allow them to be transcribed under the direction of a transcriptional promoter like T7. These constructs are genetically stable in *E. coli*. Few RNA viruses alter host gene expression and result in oncogenesis because the majority of RNA virus replication techniques are separated from the host genome and replication apparatus. As a result, host cell transformation is often not taken into consideration by RNA reverse genetic methods. Then again due to decreased genomic stability and decreased fidelity and proofreading activity of the viral RdRp, the majority of RNA viruses have tiny genome sizes, with the majority fewer than 15 kb. Due to their higher versatility in manipulating and changing the virus genome, cDNA genetic clones are frequently used in reverse genetic techniques (14-20).

III. BIOLOGY OF POSITIVE STRAND RNA VIRUS & VACCINE DEVELOPMENT

The genomes of positive-strand RNA viruses are contagious as soon as they enter host cells. Viral RNA is translated into one or more polyproteins by host ribosomes upon entry, which is then processed by either host or viral proteases (21-24). Positive-strand RNA virus clones have been produced using a variety of reverse genetics platforms, but almost all of them aim to incorporate sense genomic RNA transcripts directly or cDNA for transcription by an integrated RdRp. Here, we discuss methods used to create picornavirus and flavivirus infectious clones, as well as how these methods have been and are still being used to create vaccines (25).

1.1. Picornaviruses

A group of non-enveloped, positive-strand RNA viruses known as picornaviruses infects a variety of human and animal hosts. Illnesses caused by human picornaviral pathogens range from the common cold to poliomyelitis. The RNA genome of the picornavirus is surrounded by an icosahedral capsid structure. As two of the most thoroughly investigated picornaviruses to date, poliovirus and rhinovirus serve as model systems for the investigation of RNA virus biology, pathology, and epidemiology. Since there are no non-human primate reservoirs known to exist in nature, significant efforts were made to create and execute a polio vaccine in the 1950s with the goal of eradicating the disease. Increased attempts were made to create and design vaccines for numerous other human infections as a result of the efficacy of the inactivated

(Salk) and live-attenuated (Sabin) vaccines (26). The goal of global immunization campaigns is to eradicate the illness. The development and use of poliovirus vaccines with great success allowed for the evaluation of novel ideas and ways to research and comprehend the biology of RNA viruses. Racaniello and Baltimore's 1981 introduction of a full-length poliovirus cDNA clone and subsequent recovery of infectious virus was a significant advancement (2). Modern reverse genetics techniques for recovering picornaviruses mostly use cDNA-based systems, which are more effective and attributable to developments in the knowledge of and creation of plasmids for gene delivery. Theiler's virus [27], Hepatitis A virus [26], Foot and Mouth Disease virus [28], Swine Vesicular Disease virus [29], and two Echoviruses [30,31] are just a few of the picornaviruses for which reverse genetics techniques have been developed.

Since they were discovered more than 50 years ago, rhinoviruses continue to be the principal cause of the common cold throughout the world. However, overcoming the challenging issue of creating an immunogenic platform that can confer protective immunity to the more than 100 rhinovirus serotypes that are currently prevalent in nature is the key to generating a broadly protective rhinovirus vaccine (32-33). Four different serotype-specific proteins (VP1, VP2, VP3, and VP4) that make the rhinovirus capsid are known to be immunogenic and may cause cross-reactive antibodies (32,34-36). However, creating a polyvalent vaccination with different serotype variants of the immunogenic capsid proteins is likely necessary in order to produce broadly-neutralizing rhinovirus protection (36). The broad immunogenic memory required for effective polyvalent rhinovirus vaccine formulations has not yet been achieved. However, it has been discovered that certain areas of VP0 (the precursor to VP2 and VP4) are conserved throughout A and B group rhinoviruses, and when combined with a TH1-promoting adjuvant, mice had a cross-serotype immunological response (34). The recent development of chimeric or new reverse genetics platforms, which includes a mouse model of infection, and the discovery of conserved epitopes may open up a fresh path for the effective presentation of epitopes from the diversity of rhinoviruses (37-39).

1.2. Flaviviruses

Positive-strand RNA viruses called flaviviruses are enveloped and small that infect a variety of hosts (40-52). Most flaviviruses are transmitted by arthropod vectors because their genomes are significantly smaller which is 10-12 kb in size. Asymptomatic to severe neurological conditions such as encephalitis, meningitis, and myelitis are all caused by flaviviruses (53). The first human viral disease to be identified was the Yellow Fever Virus (YFV), a lethal flavivirus that causes over 30,000 deaths annually (according to the WHO) (54-56). The significance of creating reverse genetic platforms and effective vaccinations has been highlighted by outbreaks and sicknesses linked to flaviviruses. Similar to picornaviruses,



almost all flavivirus reverse genetics platforms use a bacterial artificial chromosome (BAC) platform or in vitro transcription of full-length or ligated cDNA segments of the genome, which are then transfected or electroporated into suitable cells. Many flaviviruses, including YFV (57,58), Dengue Types 1-4 (59-66), JEV (67), Kunjin virus (68), Tick-borne encephalitis virus (TBEV) (69-71), Langat virus (72, 73), West Nile virus (WNV) (6,74), have been the subject of reverse genetics platforms. Recombinant flavivirus technology advancements have been crucial to efforts to develop novel vaccines (75). Developing inactivated or live-attenuated strains for vaccines against flaviviruses was one of the early vaccine initiatives. Using a serially passaged virus that was originally isolated from an African patient, Max Theiler created the safe YFV live-attenuated vaccine 17D in 1937 (76). The current vaccine composition is virtually unchanged from that which was initially created more than 70 years ago, and it offers protective protection for more than 30 years (77-79). JEV was discovered and researched for the first time in the 1930s, and the first inactivated vaccine made from mouse brain was created in Japan in 1954 (53,80).

Worldwide, the dengue virus is endemic to tropical and subtropical regions. There are currently five different dengue virus serotypes known, one of which was discovered in 2013 (53,81). When a secondary infection occurs with a different serotype after an initial infection with one serotype, the illness becomes more severe (82). Therefore, any dengue virus vaccine must either offer protection against all serotypes to the fullest extent possible to prevent setting the stage for an increase in disease from a heterotypic infection, or it must get rid of the immunogenic parts of the virus that lead to an increase in disease severity following heterotypic infections.

IV. BIOLOGY OF NEGATIVE STRAND RNA VIRUS & VACCINE DEVELOPMENT

Genomes and antigenomes of negative-strand RNA viruses are unable to function as mRNA. They must form ribonucleoprotein complexes and be encapsidated with nucleocapsid in order to be a substrate of RdRp (RNPs) (83-85). The antigenomes of negative-strand RNA viruses are generally co-transfected with one or more plasmids containing the nucleocapsid and replicase machinery. They are delivered either as a linearized cDNA or as a plasmid under a T7 promoter. Negative-strand RNA virus reverse genetics systems have developed more slowly than positive-strand viral systems as a result of these biological prerequisites for starting an infection (86). Initially, helper viruses were used to recover viruses in order to create infectious clones because they could provide the viral genes and proteins required for replication. Isolating the desired mutants was challenging with these methods, though. In 1994, the rabies virus was successfully recovered completely from cDNA for the first time (87,88). Despite the biological constraints and difficulties associated with working with negative-strand RNA viruses, a number of successful negative-strand RNA virus vaccines have been

developed, and many more are currently undergoing clinical trials at various stages.

1.3. Paramyxoviruses

Numerous illnesses affecting humans and animals are brought on by paramyxovirus which is enveloped, single-stranded RNA viruses with a negative sense. There are known human paramyxoviruses that cause the common cold, measles, mumps, pneumonia, and other illnesses. The genome of a paramyxovirus, which is normally between 15 and 19 kb long, is only carried in a single copy. Paramyxoviruses must insert their replication apparatus, including the RdRp, into the virion during assembly, just like other negative-strand RNA viruses do. Very similar methods are used in the reverse genetic systems of paramyxoviruses. First, a promoter like T7 RNA polymerase is used to clone the full-length genome or antigenome as cDNA, together with helper plasmids that express nucleocapsid and polymerase proteins. Co-transfection of the plasmids into permissive cell lines. However, the majority of contemporary reverse genetics techniques use a T7 cell line or transfect a T7 plasmid. Earlier reverse genetics systems used a co-infection approach with a vaccinia virus-producing T7. Respiratory syncytial virus (RSV) was recently the subject of the first BAC-based reverse genetics system for a negative-strand RNA virus (21). For various paramyxoviruses, including the measles virus (89), mumps virus (90), Hendra virus (91), Nipah virus (92), and RSV (21,93), reverse genetics techniques have been developed. The success of developing paramyxovirus vaccines has varied despite the availability of reverse genetics technologies. In the 1950s, the first paramyxovirus vaccine was developed. A measles virus cultivation system was developed by John Enders, who also cultured the Edmonston strain of an attenuated measles virus (named after the child from which it was isolated). Despite being originally under-attenuated, the Edmonston strain was later modified and helped to successfully license a measles vaccine in 1963 (94). Building on the success of the live-attenuated measles vaccine, Maurice Hilleman was able to cultivate and modify a strain of the mumps he named Jeryl Lynn after his daughter, from whom it was isolated (95). Hilleman later contributed to the creation of the Wistar RA 27/3 strain of the MMR vaccine, a live-attenuated combination vaccination that was originally approved for use in 1971 (96). New efforts to create vaccinations against other deadly RNA viruses have been made in response to the early success of the measles and mumps vaccines. The creation of a vaccine for the respiratory syncytial virus continues to be one of the biggest challenges in the design of viral vaccines (RSV). The first chimpanzee with upper respiratory disease and the presence of respiratory syncytial virus (RSV) was discovered in 1955 (97). Since its discovery, RSV has been acknowledged as the virus that kills most infants globally [98,99]. Over 100,000 hospitalizations a year are solely attributable to RSV upper and lower respiratory infections in the US (98-100)]. Despite a significant clinical



burden, there are currently no authorized RSV vaccines, and the only existing therapies are expensive and only provide passive immunity by giving prophylactic antibodies (101–103). Young newborns are the population most vulnerable to RSV infection (98). The optimal RSV vaccine must therefore be immunogenic, genetically stable, and suitable for use in young children.

1.4. Orthomyxoviruses

Orthomyxoviruses are enveloped, negative-sense RNA viruses with genomes that are 12 to 15 kb in size and made up of several linear segments. Similar to paramyxoviruses, numerous orthomyxoviruses, most notably avian influenza and swine influenza, cause respiratory infections in humans and animals. The co-infection of a cell allows for the reassortment of the virus genomes, which is a crucial aspect of orthomyxovirus evolution. Influenza viruses require a functionally active viral ribonucleoprotein complex (RNP), which is made up of the viral genomic RNA, nucleoprotein (NP), and viral RdRp, in order to be transcriptionally active (comprised of PB1, PB2, and PA proteins) (104–112). For the influenza A virus (IAV), the first reverse genetics techniques were created, but these methods used helper viruses that had to be rebuffed in order to retrieve recombinants. Around the turn of the millennium, the first helper virus-free systems were developed (113,114). These initial systems needed co-transfection of eight plasmids expressing the eight viral RNA segments along with four or more plasmids under the control of a Pol II promoter. Depending on the number of viral genomic segments and how the helper protein constructs were organized, different numbers of plasmids had to be co-transfected for recovery, ranging from 10 (which expressed two helper proteins on each of two plasmids using the Pol II promoter and an IRES) to as many as 17 (113–118). However, employing a bidirectional expression system, recent developments in reverse genetics platforms have decreased the total number of plasmids required to 8 or less (11, 12). In this system, viral RNA synthesis is driven by a human Pol I promoter in conjunction with either a murine Pol I terminator or a CMV Pol II promoter, while viral mRNA synthesis is driven by the CMV Pol II promoter (11). However, despite this increased IAV production, only a small number of mammalian cell lines, such as the Vero or Madin-Darby canine kidney (MDCK) cell lines, can produce recombinant viruses for vaccines (119,120). The effectiveness of both uni- and bidirectional methods, however, has been hampered by the poor transfectability of certain cell lines and discrepancies in Pol I and Pol II compatibility. In several of these cell lines, the incorporation of species-specific polymerase promoters has boosted the effectiveness of recovery (119,120). The combination of up to 8 Pol I driven IAV genes on one plasmid and up to 3 Pol II drive genes on another plasmid has recently been developed; this method increases the likelihood that a single cell will receive all necessary plasmids during recovery (121). Influenza vaccination tactics have been transformed by the development of practical reverse genetics systems for

analyzing and determining the structure and function of influenza proteins. The present trivalent vaccination has been around for a while, although it used to contain three distinct strains (2 A strains and 1 B strain), which were chosen based on WHO guidelines prior to the upcoming flu season. However, the virus used to produce these vaccines were either inactivated or live-attenuated (via cold adaptation). Tetravalent or quadrivalent vaccines, which may be generated in animal cell cultures rather than chicken eggs, or virus-like particles (VLPs) in cultures of *S. frugiperda* insect (Sf9) cells are examples of next-generation immunization techniques (122–129). A seasonal influenza vaccine made of pure HA proteins and created utilizing a baculovirus-expression technology was given FDA approval in 2013 (130). These recombinant vaccines were effectively utilized to immunize people between the ages of 18 and 49, and they represent a significant advancement in influenza vaccine design and implementation because they require less time to produce than the traditional egg-based method (131).

V. REVERSE GENETIC APPROACHES FOR RNA VIRUS VACCINE DEVELOPMENT AND SYNTHETIC BIOLOGY

1.5. Challenges and Limitations

The development of novel vaccines and treatments, as well as the enhancement of the use and efficacy of those already on the market, represent a significant frontier of synthetic biology. The present gap in time between vaccine design and manufacture is a significant obstacle to the effective use of vaccines. Seasonal vaccinations against influenza viruses are necessary, but there is little time for development and deployment when the correct formulations are chosen. Reverse genetics technology has made recent strides that could shorten recuperation and production times from months to weeks. For instance, Dormitzer et. al. has shown that it is possible to create a recombinant influenza virus in just five days using the existing reverse genetics technique (132). Rapid techniques to recovering and adapting viruses will be crucial in the public health response as new emerging viruses continue to emerge and pandemics continue to occur. To improve the effectiveness and timing of delivery during epidemic and pandemic outbreaks, common chimeric reverse genetic platforms for quick cloning and production of surface antigens from emerging diseases and pathogens are being developed. Genetic stability is a significant barrier to the creation of successful live-attenuated vaccines. The lower fidelity of the RdRp causes RNA viruses to often have significant mutation rates. However, recent research has demonstrated that bias in codon usage can be used to change virus translation and, as a result, reproduction. Codon-deoptimization, also known as substituting non-preferred codons based on host cell codon use bias into the poliovirus genome, has been demonstrated by a number of research groups to reduce plaque areas and virus yields (133,134). The amount of viral protein can be adjusted without affecting function by altering codons rather than amino



acids. More importantly, the quantity of codon modifications made to the coding sequence severely restricts the possibility of reversion. This strategy represents a promising new direction for the creation of genetically stable, attenuated vaccination platforms.

VI. CONCLUSION

Despite the fact that many human infections have reverse genetics systems, not all of them have led to effective vaccine platforms. New reverse genetic techniques have been developed as a result of significant advances in biotechnology. These approaches could be used to create next-generation vaccinations. Optimizing antigens for display in vaccinations has become a widespread method known as structural vaccinology or structure-based antigen design (135). RSV continues to be a significant barrier to lowering viral childhood morbidity, as was previously indicated. Much effort has been put into analyzing the antigenic regions of the RSV fusion (F) protein and improving F expression constructs for greater immunogenicity since the discovery and use of palivizumab, the only licensed prophylactic inhibitory approach to RSV infection at the moment (105). The generation and identification of powerful neutralizing antibodies with higher neutralizing potencies than palivizumab have been made possible by current structural vaccinology research to assess the pre-fusion and post-fusion antigenic forms of RSV F protein (13). Predictive structural modeling and structure-based antigen design are now realistic choices for new efforts to create vaccines, for which there are now no vaccinations. This is due to the availability of new structures and sequences. The area of virology has been influenced by biotechnology developments just as much as any other branch of research. The science of vaccine creation has undergone a revolution thanks to the ability to create reverse genetics platforms for the analysis and manipulation of RNA viral genomes. Here, we have discussed the reverse genetics of RNA viruses as well as historical and present efforts to create vaccines to protect against several human RNA virus infections. The construction of next-generation vaccines against pathogens for which we already have vaccination techniques as well as those for which we do not will continue to be fuelled by new developments in reverse genetics technologies, adjuvants, non-human models of infection, and surveillance. Current shifts in human demographics and healthcare accessibility are likely to alter cost-benefit calculations and prompt the allocation of fresh funds for research and vaccine development. High efficacy, safety, and stability are the distinguishing characteristics of an effective viral vaccine, despite the constant innovation of vaccine design and use, and will always be a matter of concern.

Conflict of interest

The authors declared no conflicts of interest.

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