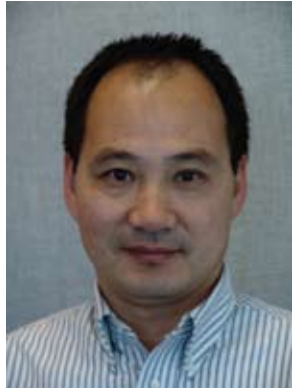


Reverse Genetics for Live Attenuated Virus Vaccine Development

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Abstract

Live attenuated RNA viruses make highly efficient vaccines and offer the promise of providing more effective immunity against respiratory viral diseases than subunit or split inactivated vaccines. However, the developments of such attenuated vaccines present unique challenges because RNA viruses undergo frequent antigenic change and some mutants may become highly infectious in infant and immunocompromised persons. The successful use of live attenuated viral vaccines depends not only on the proper choice and delivery of the attenuated strains, but also on maintaining the genetic stability and the sufficient potency required for an immune response during manufacturing, storage, and administration. The ideal vaccine candidate should use a thoroughly accredited master strain in which the gene elements or segment(s) contributed to the attenuation phenotype have been well characterized and determined and allow the ready detection of revertants if these occur. This review will primarily cover the past progresses and recent advances in live-attenuated RNA virus vaccine development with the focus on using recent reverse genetics technology for live-attenuated influenza virus vaccine.

Introduction

Live attenuated RNA virus vaccines have been a great success in preventing and curbing the spread of human viral diseases. A number of these vaccines are in routine use and have made dramatic historic impact on public health. Currently available live RNA virus vaccines include measles, mumps, rubella, polio, yellow fever, rotavirus and influenza. These live-attenuated vaccine strains retain the ability to replicate and elicit immune response in vaccinated individuals, but do not cause clinical disease. The first live attenuated virus vaccine was developed for smallpox, an acute DNA virus disease caused by infection with the variola virus that belongs to the genus orthopoxvirus of Poxviridae. Since 1798, live attenuated cowpox and/or vaccinia virus has been used to immunize against smallpox. After successful vaccination campaigns throughout the 19th and 20th centuries, smallpox, as the only naturally occurring human infectious disease, was completely eradicated in 1979⁽¹⁾. The first live-attenuated RNA virus vaccine was rabies vaccine developed in 1885, and the attenuated mutant strain was developed by chemical treatment of the wild-type virus, followed by selection of the mutant with reduced virulence⁽²⁾. Since 1962, the development and world-wide use of live-attenuated polio virus vaccine has brought polio close to eradication⁽²⁾.

The subunit vaccines based on individual recombinant proteins may be less effective in eliciting protective immune responses due to possible incorrect protein folding and/or antigen presentation to the immune system. In contrast, attenuated vaccines elicit immune response in a more effective and authentic way because they mimic natural infections of their parental wild-type viruses. In addition, the live-attenuated virus vaccine preserve almost all the essential epitopes, and are readily recognized by the immune system, and the immune system can present antigens in a more authentic conformation. In comparison to the inactivated or

subunit viral vaccines, the live-attenuated vaccines possess a number of advantages: (i) live-attenuated virus vaccines induce both the innate and cell-mediated immunity; (ii) the live attenuated virus vaccines elicit dominant type 1 T helper (Th-1) cell response and/or cytotoxic lymphocyte (CTL) response; (iii) the live attenuated virus vaccine induce systemic IgG antibody response as well as secretory IgA antibody response to viruses that replicate in mucosal tissues; (iv) a single dosage inoculation of live-attenuated virus vaccine induces a broad long-lived immune response. However, there are some obstacles to successfully developing live-attenuated vaccines. The major obstacle is the safety concern and the challenge to obtain a balance between the satisfactory attenuation without compromising immunogenicity. The other concern is the stability of the attenuated phenotype after several passages in the nature host. In addition, an attenuated virus can still replicate in the vaccinated individuals, therefore, the virus has the potential to revert to virulent phenotypes. Moreover, some of live vaccines can be transmitted from the vaccinated person to nonimmunized individuals, which can be a major safety concerns for immunocompromised individuals. These safety concerns are particular important for certain human RNA viruses such as human parainfluenza virus (PIV), respiratory syncytial virus (RSV) and HIV, since these RNA viruses, whose RNA-dependent RNA polymerases do not have a proofreading function and a high mutation rate could occur during virus replication. Therefore, the ideal live virus vaccine candidate should select and use

the strains with the stable mutations, with preference to those which have multiple mutations associated with the attenuated phenotype.

Classical methods for generation of live-attenuated virus vaccine

The classical technologies refer the ones that do not involve the use of recombinant DNA and genetic engineering technology for development of live-attenuated virus vaccines. The technical strategies used for licensed live-attenuated virus vaccines in the United States are outlined in Table 1. Historical methods for developing live-attenuated virus vaccines involved strategies such as extensive passage in cell culture at

TABLE 1: *Live-attenuated Virus Vaccines Licensed in the United States for Human Use*

Live-attenuated Virus Vaccine Type	Year Licensed	Strategies for generation of attenuated vaccine strain(s)
Smallpox	1798	Select and use of a related animal virus
Rabies	1885	Use of chemical mutagenesis to generate attenuated mutant
Yellow fever	1935	Extensive passage in mice and chicken embryos
Poliovirus	1962	Extensive passage in cell culture at increasingly suboptimal virus conditions followed by selection cell-adapted mutants that were highly immunogenic but attenuated.
Measles	1963	
Mumps	1967	
Rubella	1969	Passage cell culture at suboptimal low temperature to select strains with cold adaptation and attenuation
Adenovirus	1971	Extensive passage in cell culture followed by a selection cell-adapted mutants that were highly immunogenic but attenuated
Varicella	1995	
Influenza	2003	Use of reassortant processes to select 6:2 reassortant vaccine strain with six internal gene segments from cold-adapted attenuated donor virus while the HA and NA gene segments from circulating wild type virus
Rotavirus	1998 and 2006	Use of bovine-human and/or rhesus-human reassortant viruses with the vp7 gene from human strain and other gene segments from related animal rotavirus.

increasingly suboptimal temperatures or chemical mutagenesis, followed by a selection/characterization process to identify these mutants that were highly immunogenic but with reduced virulence. The first successful live virus vaccine was developed in 1798, using a related animal poxvirus as the vaccine strain for smallpox. The vaccine used a cowpox (vaccinia) virus for human vaccination against smallpox caused by variola virus. In the 1950s, cell culture technology was established and successfully used for propagating virus. Soon after that, it was found that repeated passages in cell culture can reduce the pathogenicity of wild-type viruses and the cell culture adapted mutants that were selected as attenuated vaccine strains⁽²⁾. These cell adapted strain(s) replicate efficiently in vitro but with a reduced virulence or pathogenicity in their natural host. This approach resulted in the successful development of numerous attenuated RNA virus vaccines licensed in the US (Table 1), including an oral poliovirus vaccine and injected vaccines such as measles, mumps, and rubella. Cell culture became an important technology for generation of reassortant viruses with segmented genomes, such as influenza virus (segmented negative-sense ssRNA virus) and rotavirus (segmented dsRNA virus). The reassortant virus is generated by coinfection of cells with two viruses and followed by selecting the reassortant whose genome contains genes from two parental viruses. The recent licensed attenuated influenza and rotavirus vaccines are the examples developed using gene segments reassorted technology. The reassortant strains used for production of rotavirus vaccines are the bovine-human and/or rhesus-human reassortants in which the vp7 gene derived from a human strain and other gene segments from related animal rotavirus (bovine or rhesus). The reassortant strains for influenza vaccine are produced by coinfection of cells or eggs with a cold-adapted attenuated master donor strain with a circulating wild type influenza virus. The licensed live influenza vaccine is an intranasal deliver vaccine containing the mixture of three virus strains: two types of A viruses (one H1N1 and one H3N2 subtype) and one Type B strain. Each attenuated strain is generated by reassortant between a wild-type virus that provide hemagglutinin (HA) and neuraminidase (NA) and a cold-adapted master donor virus, such as influenza A/Ann Arbor/6/60⁽³⁾ that supply the other six segments [PB2, PB1, PA, NP, M, NS]. The 6:2 reassortant with six internal gene segments from the attenuated donor virus and the other two gene segments [HA and NA] from a circulated wildtype virus of each of the three types was used for vaccine production. The six internal segments conferred the phenotypes for temperature-sensitivity (ts), cold-adaptation (ca) and attenuated (att) for virulence⁽⁴⁾.

Reverse genetics system for generation of infectious viruses

Historical methods for developing live-attenuated vaccines, such as extensive passage in cell culture at increasingly suboptimal temperatures or chemical mutagenesis to attenuate virus pathogens, can be

very labor intensive and may take years to establish a thoroughly accredited vaccine strain, in which the gene

elements or segment(s) contributed to the attenuation phenotype are well defined and characterized. In some cases, the traditional attenuating process is not well controlled, may not provide the stable attenuation phenotypes and is not always successful due to the fact that most of the mutants are generated through a point mutation in the virus genome. Consider the case of human RSV⁽⁴⁾ and PIV⁽⁵⁾. Historical methods generated a series of live vaccine candidates that were attenuated in an animal model but retained a certain level of residual virulence that is not acceptable for clinical evaluation. With the advancement of molecular biology and genetic engineering technologies, along with the increased understanding at the molecular levels of immune response, new approaches on live-attenuated virus vaccine development have emerged. One approach for molecular attenuation of RNA viruses is reverse genetics, a process of generating a recombinant virus from a cloned complementary DNA (cDNA) copy of a viral genome. Reverse genetics is a powerful molecular tool to attenuate virus and understand the molecular determinants related to virus attenuation, tissue tropism, and virulence factor(s). In recent years, reverse genetics has markedly accelerated the development of virus vaccines due to the feasibility that the viral genomes can be modified at will through a cDNA intermediate. More stable attenuated mutants can be generated by rational designs such as point mutations, deleted genes, changed virus gene expression order, and substituted gene or attenuating mutations from related viruses. In addition, the system provides reliable methods to identify and develop new types of attenuating mutations, including deletion of nonessential genes for virus replication and construct marker vaccine viruses containing the specific sequence tag or restriction enzyme site(s) that distinguish the vaccinated groups from the infected group. In addition to attenuated virus vaccine development, the reverse genetics system is also useful as biotechnological tools to produce recombinant viruses that can be designed with specific properties such as a virus deliver vectors for gene and cancer therapeutics.

In 1976, Goff and Berg first successfully produced a recombinant SV40 virus by transfecting cells with plasmids encoding SV40 and lambda phage DNA segments⁽⁶⁾. Racaniello and Baltimore in 1981 were first to rescue

poliovirus, a plus-strand RNA virus, using cloned cDNA that contained the entire poliovirus RNA genome⁽⁷⁾. After that, a number of positive-sense RNA viruses were generated using synthetic RNA transcript produced by T7 or T3 RNA polymerase on a cloned cDNA template (for review see⁽⁸⁾). For viruses with a positive-sense viral RNA (+vRNA), +vRNA is infectious and can serve as mRNA and initiate virus replication using cell RNA dependent RNA polymerase (RdRp). Therefore, simply transfecting DNA plasmids or +vRNA transcribed from plasmids containing their virus genomes into susceptible cells resulted in the recovery of infectious viruses⁽⁹⁻¹⁴⁾. In contrast, the genome of negative-strand RNA is not infectious and the negative-sense viral RNA (-vRNA) itself cannot initiate virus replication. Only -vRNA molecules encapsidated with viral polymerase and nucleoprotein complex are able to initiate a viral replication and transcription cycle. In 1990, Enami et al⁽¹⁵⁾ developed the first systems to rescue influenza virus, a segmented negative stranded RNA virus, and generated an influenza virus containing a specific mutation in the NA gene of the eight viral gene segments. Schenell et al in 1994⁽¹⁶⁾ rescued rabies virus entirely from a full-length cDNA clone of the viral genome under the control of the T7 RNA polymerase promoter. The components of the viral polymerase complex including virus nucleic protein (NP) were provided from protein expression plasmids that were controlled by the T7 RNA polymerase promoter. Transfection of cells that expressed the T7 RNA polymerase with the full-length cDNA plasmid of rabies virus antigenome along with plasmids for expression of viral polymerase complex was sufficient to produce infectious rabies virus⁽¹⁷⁾. Soon after that, a number of investigators generated non-segmented negative-sense RNA viruses from cloned cDNA using synthetic RNA transcripts, including vesicular stomatitis virus^(18,19), measles virus⁽²⁰⁾, respiratory syncytial virus⁽²¹⁾, sendai virus^(22,23), rinderpest virus⁽²⁴⁾, human parainfluenza virus^(25,26), simian virus⁽²⁷⁾, newcastle disease virus⁽²⁸⁾, and human severe acute respiratory syndrome corona virus⁽²⁹⁾. In 1990, Roner et al⁽³⁰⁾ developed an infectious system for a segmented dsRNA reovirus by transfecting cells with a combination of ssRNA, dsRNA, in vitro translated reovirus products, and complemented with a helper virus of different serotype. The resulting virus was discriminated from the helper virus by a plaque assay. In 1996, Mundt and Vakharia⁽³¹⁾ generated infectious bursal disease virus, a 2-segmented double-strand RNA virus, entirely from synthetic transcripts derived from cloned cDNAs. Komoto et al in 2006⁽³²⁾ rescued infectious rotavirus, a 10-12 segmented dsRNA virus, by providing the full-length VP4 viral RNA transcripts inside the cell by a vaccinia virus driven T7 RNA polymerase system, in conjunction with providing a helper virus of another human strain of rotavirus. However, in this system, the use of helper virus was necessary. In 1999, reverse genetics system for

generation of influenza virus, an 8-segmented negative-sense ssRNA virus, entirely from cloned cDNA without helper virus, was fully established^(33,34). The reverse genetics system for human influenza virus has greatly facilitated the comprehensive studies of influenza virus proteins and RNA elements in replication and pathogenesis, and design of a new generation of live vaccines.

Reverse genetics for live-attenuate influenza virus vaccine

Influenza infection remains the most common cause of respiratory infectious disease in humans and animals worldwide. Epidemic influenza occurs annually and results in increased morbidity, mortality and economic loss each epidemic year. Vaccination is the primary means to prevent and control the disease. However, influenza viruses undergo constant antigenic changes that require the annual reformulation of trivalent influenza vaccines. The influenza virus genome consists of 8 negative-sense ssRNA gene segments, which allows for producing traditional reassortment between two viruses. The selected vaccine manufacturing strains are the selected 6:2 reassortants with that six internal gene segments from cold-adapted attenuated donor virus such as influenza A/Ann Arbor/6/60 and the other two gene segments [HA and NA] from a circulated wildtype virus of each of the three types.

The negative-sense of viral RNAs (vRNA) of influenza viruses is not infectious. Only vRNA molecules encapsidated with the four viral polymerase complex proteins (PB1, PB2, PA, and NP) are able to initiate a viral replication and transcription/translation cycle. After the ribonucleoprotein complexes (RNPs) penetrate the cell nucleus, the associated proteins begin to transcribe the native sense vRNAs into mRNAs and complemented positive-sense cRNAs. These cRNAs serve as templates for the synthesis of vRNAs in the nucleus of infected cells. In 1990, Enami and Palese⁽¹⁵⁾ designed a first "reverse genetics" approach for generation of recombinant influenza virus, in which the vRNA templates are prepared by in vitro transcription of cloned cDNA plasmids and mixed with purified viral polymerase complex (RNPs). Transfection of the RNPs complex into cells along with infection with an influenza virus, results in the generation of viruses possessing a gene derived from cloned cDNA⁽¹⁵⁾. However, in this approach, the presence of helper virus is undesired since a selection system is required to eliminate helper virus. In 1999, a reverse genetics system using an 8-plasmid or 12-plasmid strategy was developed and influenza virus can be generated entirely from cloned cDNA without helper virus^(35,36). In the 12-plasmid system, 8 plasmids contain cloned cDNAs with each encoding

one of the 8-segment of influenza A virus under the control of an RNA polymerase I (pol I) promoter and terminator. Transfection of the eight plasmids, together with the four plasmids expressed viral RNA polymerase and nucleic protein (PA, PB1, PB2 and NP) into 293T or Madin–Darby canine kidney cells (MDCK) cells, resulted in the production of infectious influenza virus⁽³⁵⁾. In the 8-plasmid system, in which each plasmid encoding one of the influenza virus gene segment is inserted between an RNA polymerase I (pol I) promoter and terminator sequences, thereby resulting in the expression of vRNA, which are in turn flanked by an RNA polymerase II (pol II) promoter and a polyadenylation signal, thereby resulting in the expression of viral mRNA. Transfection of these 8-plasmid alone into the 293T, COS7 and/or MDCK cells, resulted in the generation of infectious influenza A viruses (H1N1 and H3N2)⁽³⁴⁻³⁶⁾ as well as influenza B virus⁽³⁷⁾. Although the 293T, COS7 and MDCK cells were used for the rescue of influenza viruses, but these cell lines imposes specific safety concerns for human vaccine production due to the potential tumorigenicity concern of these transformed cell lines⁽³⁸⁾. However, African Green Monkey Kidney (Vero) cell line is certified by the World Health Organization (WHO) for the production of human vaccines⁽³⁹⁾. The plasmid-based reverse genetics was used for the generation of high-yield influenza A/PuertoRico/8/34 (PR8 strain) virus in Vero cells⁽⁴⁰⁾. This reassortant virus is produced by transfecting into cells the expression plasmids that contain the PB1, PB2, PA, NP and M genes from the PR8 strain, the NS gene (NS1 and NS2) from the A/England/1/53 influenza strain, and the HA and NA genes from an influenza virus of interest⁽⁴⁰⁾. The PR8 virus has been used routinely for production of inactivated influenza vaccine for the past 30 years and the strain was well adapted for growing in the embryonated eggs with a high yield of virus titer without killing the chicken embryos. In addition to the conventional influenza strains, the plasmid based reverse genetics was also used to generate a recombinant influenza virus with the modified HA of highly pathogenic strains such as H1N1⁽⁴¹⁾, H5N1^(42,43) and H9N2⁽⁴⁴⁾ reassortant influenza virus, in which the HA has been modified so that the polybasic cleavage peptide in HA protein to a single basic amino acids. The rescued viruses with the modified HA protein replicate significantly slower with reduced virulence in the animal model^(45,46).

Traditional production of attenuated influenza vaccine strain relies on generation of 6:2 reassortant viruses, which are derived from coinfection of cell or chicken eggs with cold-adapted master donor virus that provide for the 6 internal gene segments and a circulated wild-type donor virus that for HA and NA segments. The reassortant process is time-consuming and cumbersome and sometimes can take about 3-4 months to produce a qualified manufacturing work virus seed. In addition,

the clinical isolate(s) and the reassortant process may contain unknown contaminants, especially low amount of other human respiratory viral pathogens that have the potential to grow in the chicken embryonated eggs, therefore adversely affect the quality of the vaccine strain. The plasmid based reverse genetic can overcome these safety concerns, because the system and process are well controlled and the molecular cloning process for the rescue system can eliminate the potential contaminants if present in the clinical isolates. In addition, the reverse genetic system can shorten the time needed for generation of the 6:2 reassortant vaccine simply because the rescue system eliminates the time needed for the reassortant process and 6:2 reassortant screening process. Therefore, the plasmid based reverse genetics make it possible for routine and rapid generation of strains for either inactivated or live attenuated influenza vaccine production.

Conclusion

Reverse genetics offers new approaches for the development of novel live-attenuated virus candidates, based on the generation of genetically engineered viruses from cDNA clones. More stable attenuated gene mutation or deletion can be designed and incorporated these mutations into the recombinant viruses produced by the reverse genetics. In addition, the technology also provide an important tool for the development of pandemic influenza vaccines such as highly pathogenic avian H5N1 influenza A virus, because the reverse genetics provides reliable technology to remove virulence determinants found in highly pathogenic avian strains, and generate a new H5N1 and A/Ann Arbor/6/60 reassortant and/or H5N1/PR8 reassortant with these new modifications in the HA or NA proteins⁽⁴¹⁻⁴⁶⁾. Highly pathogenic H5N1 virus of field isolates kills the chicken quickly and cannot produce high titers of virus stock in embryonated eggs and/or cell culture system. In addition, the field isolates of highly pathogenic H5N1 virus have to be handled in BL3 or BL4 facilities, which make it very challenges or impracticable to be use in conventional vaccine production. These limitations have prompted considerable efforts directed towards the development of cell culture systems for vaccine production. The reverse genetics has greatly simplified the procedures to develop mutant virus strains that propagate well in cell cultures while maintain desirable characteristics such as high growth rate and genetic stable attenuated phenotype. Therefore, the future of influenza prevention and control for these highly pathogenic influenza viruses depend on this powerful technology for the generation of safe and more effective live attenuate influenza vaccines.

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