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MINI-REVIEW

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Influenza vaccines: recent advances in production technologies

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Abstract In spite of ongoing annual vaccination programs, the seasonal influenza epidemics remain a major cause of high morbidity and mortality. The currently used "inactivated" vaccines provide very short-term and highly specific humoral immunity due to the frequent antigenic variations in the influenza virion. These intra-muscularly administered vaccines also fail to induce protective mucosal immunity at the portal of viral entry and destruction of the virally infected cells by induction of cytotoxic T lymphocytes. Therefore, it is necessary to develop immunologically superior vaccines. This article highlights some of the recent developments in investigational influenza vaccines. The most notable recent developments of interest include the use of immunopotentiators, development of DNA vaccines, use of reverse genetics, and the feasibility of mammalian cell-based production processes. Presently, due to their safety and efficacy, the cold-adapted "live attenuated" vaccines are seen as viable alternatives to the "inactivated vaccines". The DNA vaccines are gaining importance due to the induction of broad-spectrum immunity. In addition, recent advances in recombinant technologies have shown the possibility of constructing pre-made libraries of vaccine strains, so that adequately preparations can be made for epidemics and pandemics.

Introduction

The epidemics caused by influenza viruses A and B affect up to 500 million people annually. The highest rate of influenza attack is reported among children. Although a self-limiting disease, primary viral and secondary bacterial pneumonia caused by pneumococcus, staphylococcus or non-group *H. influenzae* accounts for >90% of influenza-related deaths among the elderly and immune-compromised persons. This mortality rate is expected to increase further due to enhanced life expectancy. In order to reduce the disease severity, the viral inhibitors of neuraminidase (NA) (zanamivir, oseltamivir) and M2 (amantadine, rimantadine) are found to be effective but cannot be prescribed as a first choice for prophylaxis. Hence, vaccination remains the most effective approach for mass immunization with >250 million doses of "inactivated" vaccines being brought to the market annually. However, in spite of considerable benefits and proven safety, these vaccines remain under-utilized due to the very short-term immunity received. As a result, over 40,000 deaths and 100,000 hospitalizations occur annually in the US due to influenza viruses (Kemble and Greenberg 2003).

The present situation can be improved by the development of superior vaccines and changes in vaccination strategies. For better vaccine design, knowledge of the structure and function of the virions, the mechanisms of "antigenic variations", and the possibility of short-term evolution in other viral genes are providing much-needed stimulus. This review summarizes some of the recent advances in influenza vaccine production technologies.

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Structure of influenza virion and the antigenic variations

The general structure of a functionally infective influenza virus (virion A, B, and C) belonging to the family *Orthomyxoviridae* is illustrated in Fig. 1. The pleomorphic (80–120 nm) virion is made up of a lipid bilayer envelope derived from the host cell, proteins, and negatively stranded RNA genome of eight segments. The highly antigenic membrane glycoprotein hemagglutinin (HA) facilitates virion entry into the human epithelial cells by attachment to sialic acid receptors containing α-2,6 galactose linkages. NA is an enzyme that prevents aggregation of the virions within the host cell and facilitates cell-to-cell spread by cleavage of glycosidic linkages to sialic acid. The virions A and B can

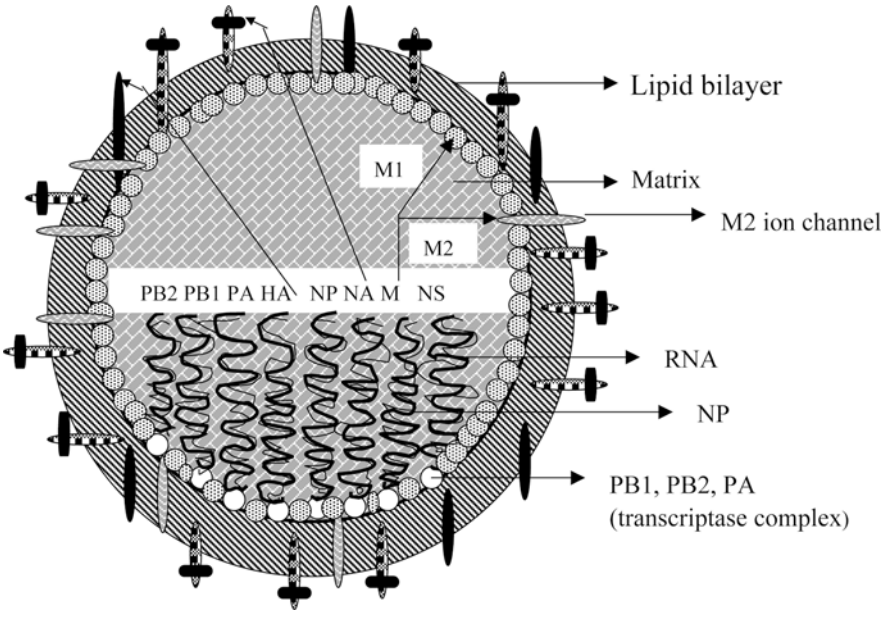

Fig. 1 Structure of the influenza virion. *NP* RNA segment-coating nucleoprotein; *PB1, PB2, PA* polymerases

be differentiated only by the inability for cross-reactivity of the antibodies with the corresponding antigenic proteins of the two viral subtypes.

The M1 protein located within the envelope interacts with the cell genome and nuclear export factor, and facilitates virion assembly. The M2 protein forms an ion channel and serves as a link between the interior and the outer environment. It also provides a low pH necessary for HA synthesis and virus uncoating. The other viral components such as non-structural protein (NS1) antagonize interferon suppression in the host cell (Hilleman 2002). The RNA segment-coating nucleoprotein (NP), and polymerases PB1, PB2, and PA form the transcription complex essential for replication. The epidemiological behaviour of influenza virions depends upon two types of antigenic variation in HA and NA molecules known as "drift" and "shift" as discussed below.

The frequent point mutations due to lack of "proof-reading" enzymes in the influenza virions result in amino acid substitutions in the HA and NA proteins. These substitutions occur more commonly in the five antigenic regions (A–E) on the globular domain of HA1 (Caton et al. 1982), which are crucial for binding with the virus-neutralizing antibodies. As a result of these antigenic variations, the mutant virion can escape recognition by the host antibodies. This phenomenon is known as "drift". The rate of drift varies among the genes and the virus types. This is a major challenge facing vaccine manufacturers since vaccines have to be reformulated every year to ensure that the HA and the NA molecules exactly match those found in the circulating strains. Otherwise, the protection rate of the vaccine decreases.

The fragmented genome of the influenza virus is more vulnerable to genetic variations in comparison to monogenic viruses. For example, in a doubly infected cell, rare gene exchange of single or multiple RNA molecules between a human viral strain and an avian or equine viral strain can result in a new genotype. This phenomenon is called "antigenic shift". The epidemiological studies in humans indicate that only A viruses are amenable to "shift" and cause pandemics after 10–40 years of quiescence. Until now, 15 HA and nine NA subtypes of A viruses have been reported. Among these, the historically important pandemics were caused by H1N1 (Spanish flu, 1918), H2N2 (Asian flu, 1957), and H3N2 (Hong Kong flu, 1968). Currently, two subtypes of A (H1N1 and H3N2) and one subtype of B are circulating in human beings with sporadic appearances of H5N1 (Hong Kong, 1997) and H9N2. More details on the virology, pathogenesis, and epidemiology of human influenza can be found elsewhere (Hilleman 2002; Nicholson et al. 2003).

Recent advances in influenza vaccine designing

A brief summary of the characteristics of the various types of investigational influenza vaccines is given in Table 1. The important vaccines in significant developmental stages include the "inactivated" vaccines, vaccines with immunopotentiators, virosomes, live attenuated vaccines, and DNA vaccines. Presently, the highly specific antigenicity of the HA and NA glycoproteins in the vaccine is customarily matched with the randomly mutating molecular structure of the "drift" strain as per the biannual recommendations provided by the WHO Influenza Surveillance Program. The details on the annual production cycle for influenza vaccines are reported by Gerdil (2003).

Inactivated influenza vaccines

The currently used bacterial endotoxin-free trivalent influenza vaccines (TIV) are formulated with 15 μg HA from each A/H1N1, A/H3N2, and virus B. The conventional production process of these vaccines is illustrated in Fig. 2. In brief, the seed strain is prepared by simultaneously in-

Table 1 Types of investigational influenza vaccines. *CMI* Cell-mediated immunity, *GMT* geometric mean titer, *ca* cold adapted

Characteristics	Disadvantages
Inactivated influenza vaccines	
Induction of serum HA-inhibition antibodies	Parenteral delivery
Efficacy: 70–90% (healthy adult), 30–70% (elderly)	Low levels of CMI
No interference by type A vaccine strains with type B	Effective only against antigenically matched strains
Contains thimerosol as preservative	Adverse reactions in children due to egg proteins
Vaccines with immunopotentiators	
TIV (FluAD®): 15 μg of HA and MF59 (5% squalene, 0.5% Tween 80, 0.5% sorbitan trioleate)	Reactogenic causing local pain, temperature, erythema, induration, and mylagia
Increase (1.5-fold) in average GMT in the serum antibody, 100% sero-conversion	Safety with synthetic immuno-modulants or cytokines may restrict use in near future
Licensed in 12 western European countries	Complex production protocols
Immune-stimulating complexes (ISCOMS): Quil A, QS-21, Iscoprep 703, onjisaponins, GM-CSF with TIV, INFLUSOME-VAC [combination of cytokines IL-2 or GM-CSF with immunogens contained in lipid vesicles]	
Stronge B-cell, T-cell, Th-cell, and CTL responses in non-human primates	
Virosomes	
Increased $CD8^+$ CTL-mediated immunity	Assessment of reactogenicity (adverse reactions) profile is need
FluINsure: first proteosome-based nonlive, intranasal subunit vaccine patented in USA	Cases of Bell"s palsy with NasalFlu® containing heat labile toxin from *Escherichia coli*
Live attenuated vaccines; ca vaccine	
FluMist™: derived from the ca master donor A/Ann Arbor/6/60, B/Ann Arbor/1/66 and A/Wuhan/359/95 strains licensed in 2003 in the USA as intranasal vaccine	Lower serum antibody response
Induce local IgA, secretory IgA, cytokines, and priming of CTL responses	Increased antigen concentration or a combination of "live" and "inactivated vaccines needed
Increase in the antigenic load due to replication in the host	Lower serological response to B vaccine strain due to interference by the type A vaccine strains
Exposure to the endogenous processing mechanisms	Interference of H3N2 vaccine reassortant with H1N1 reassortant
Reduce viral shedding and transmission	Increased dose of type B strain in the vaccine formulation or administration of multiple doses needed
DNA vaccines	
Improved utility of the vaccines due to the absence of protein component and lack of immune response against the plasmid itself	Technology is in the developmental stage
	Gene delivery mechanisms not identified
	Safety and efficacy trials in humans needed

fecting the allantoic sac of the chicken embryo with a laboratory-adapted high-growth phenotype of H1N1 (A/PR/8/34) and the epidemic strain. The resulting high-growth reassortants (new genotype) are tested to confirm the absence of genes encoding PR8 or PR8-like surface glycoproteins. This selected seed strain containing HA and NA components of the epidemic strain is mass propagated in fresh eggs to obtain sufficient quantities of vaccine virus. Seed strains for producing influenza B vaccines are field isolates because no high-growth master B strain has yet

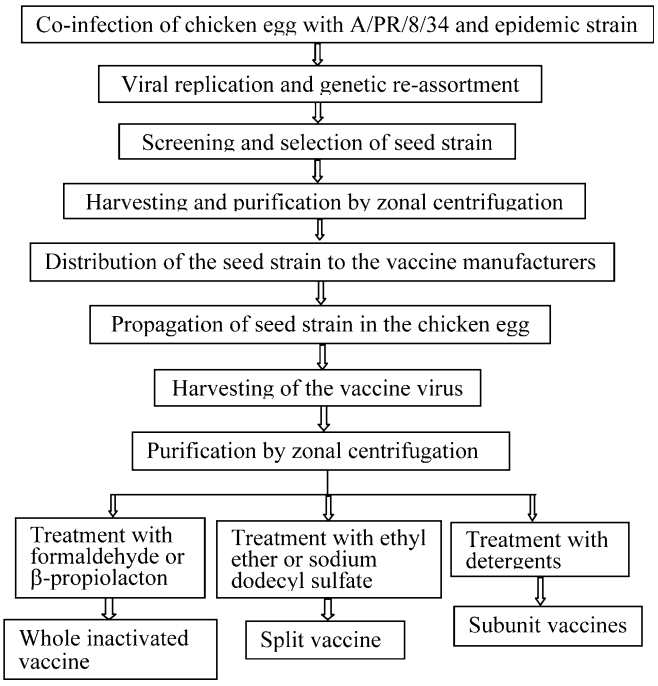

Fig. 2 Production of "inactivated" influenza vaccines in chicken egg

been found. The various inactivated vaccines are then prepared by chemical treatments of the purified viruses. Details on the protective efficacy of influenza vaccination can be found in Hannoun et al. (2004).

Vaccines with immunopotentiators

The immunopotentiators (Table 1) are known to increase the immunogenicity of TIV, thereby increasing the available doses without a compensatory increase in egg production. For example, an aluminum adjuvanted whole virus vaccine with 1.9–7.5 μg HA was immunogenically similar to a "whole virus" vaccine with 15 μg HA without alum. More details on the mode of action of these adjuvants (from the Latin verb adjuvare—to help), efficacy, and safety can be found elsewhere (Morein et al. 1984; Babai et al. 2001; Nagai et al. 2001; Podda 2001; Rimmelzwaan et al. 2001; Ramanathan et al. 2002).

Virosomes

The reconstituted virus-like particles (VLP; diameter 150 nm) contained in the lipid bilayer of phosphatidylcholine and phosphatidylethanolamine possess the viral-cell binding and membrane fusion activities but lack of the viral genetic material. A virosome vaccine, Inflexal-V, is used in Switzerland and Italy. More details on the action and efficacy of these vaccines can be found in Plante et al. (2001) and Herzog et al. (2002).

Live attenuated vaccines

The easily re-assortable nature of the influenza viral genome is being exploited to create stable multiple attenuating lesions on all six non-surface antigen genes in an extensively tested laboratory phenotype. Three types of variants; temperature sensitive (ts), host range (hr), and cold-adapted (ca) "live attenuated" vaccines have been extensively studied (Murphy et al. 1972; Clements et al. 1986). However, due to doubtful genetic stability and possibility of reversion to the virulent phenotype, interest in the ts and hr vaccines has subsided (Wareing and Tannock 2001). In the case of ca vaccines, attenuation of the master strains was carried out by serial passage in either primary chicken kidney cells or embryonated chicken eggs at successive, low temperatures ranging from 33°C to 25°C (Maassab and DeBorde 1985). This attenuated master strain which is unable to cause significant illness in humans but is able to donate the genes other than those of the HA and NA is then co-infected with the contemporary epidemic strain to produce live attenuated vaccine following the traditional egg-based process (Jin et al. 2003). The produced reassortants carry HA and NA similar to the epidemic strain but the required degree of avirulence due to stable mutations in all three polymerase genes, viz. PA, PB1, and PB2. The ca vaccines have been proved to be safe and effective in millions of people (Kendal 1997). Details on these vaccine are provided by Pfleiderer et al. (2002) and Belshe (2004).

In recent years, production of replication defective "recombinant live attenuated" viruses by deletion of a major portion of NS1 and M2 has also been reported (Watanabe et al. 2002a). Similarly, the VLP are produced by leaving out one of the eight gene segments essential for the replication when transfecting into the cell line. The cells are then "trans-complemented" by transient or constitutive expression of the missing viral protein (Rimmelzwaan and Osterhaus 2001). Although, these technologies are in the very early stages of developmental research, the VLP incapable of replication and spread to other cells due to NS2 deletion are expected to be good vaccine candidates (Watanabe et al. 2002b).

DNA vaccines

The possibility of vaccination by direct intra-muscular injection of DNA expression cassettes was first demonstrated by Wolff et al. (1990). To construct a DNA vaccine, a simple *Escherichia coli*-derived non-infectious and non-replicative plasmid that encodes only the proteins of interest can be used. Ljungberg et al. (2000) demonstrated the rapid and flexible construction of DNA plasmid vectors that can meet the needs of antigenic drift. Through homologous recombination, they successfully cloned the variable antigenic determinant domains of the HA gene into a vector handle derived from a H3N2 strain followed by the production of plasmid DNA vaccines in *E. coli* XL1 blue.

Hoffmann et al. (2002) reported an alternative to the classic method by creating virus seed strains through a generation of 6+2 reassortants using an eight-plasmid system. They used six genes from a high-yield H1N1 virus (A/PR/8/34) and HA and NA genes of the circulating strains. The method does not require cumbersome screening and selection procedures unlike the conventional technique that produces 2^8 types of progeny viruses. The de novo generation of 6+2 viruses by this PR-8 plasmid method eliminates the need for multiple passage in eggs, thereby reducing the time required for vaccine production.

An another technique called "reverse genetics" is being employed to specially design "recombinant" A and B viruses using plasmid DNAs (Neumann et al. 1994, 1999; Palese et al. 1997). In this technique, for the transcription of vRNA and mRNA from the plasmids, the ribonucleoprotein polymerase complex (NP, PB1, PB2, PA) is provided by cotransfecting plasmids from which mRNAs for these proteins are transcribed from a pol II promoter, with the plasmids from which vRNA is transcribed. Hoffmann et al. (2000) used bi-directional plasmids from which vRNA and mRNA can be transcribed from pol I and pol II promoters, respectively. The prevention of contamination with extraneous viruses is the major advantage of reverse genetics because only relevant genes are cloned into plasmids. In addition, a simple selection of a desired gene segment can rapidly produce even well-defined assortants with a "high growth" or ca phenotype (Rimmelzwaan and Osterhaus 2001).

Additionally, Li et al. (1999) proposed the use of reverse genetics to safely produce a vaccine strain against the deadly A/Hong Kong/97 virus (chicken H5N1 virus), where conventional techniques cannot be employed. They modified the HA gene by deleting a series of basic amino acids associated with virulence. Subsequently, by reverse genetics, the modified HA and NA genes were rescued from the wild-type H5N1 virus and inserted into ca A/Ann Arbor/6/60 virus. These experiments indicated the potential of this technique for the development of live and inactivated vaccines for both pandemic and interpandemic use.

Recently, Horimoto et al. (2004) used reverse genetics to eliminate the interference among the vaccine viruses A and B that affect the efficacy of live attenuated vaccines by restricting their replication. They created a chimeric virus (A/B) possessing chimeric (A/B) HA, and full-length B type NA in the background of a type A vaccine virus. This study provided a novel method for creating live attenuated vaccine from a single master strain.

The efficacy of the plasmid DNA vaccines expressing HA, NP, M2, and NP/M1 alone or in combination with one another or together with DNA encoding various cytokines has also been demonstrated in several animal models (Robinson et al. 1993; Slepushkin et al. 1995; Donnelly et al. 1997; Neirynck et al. 1999; Okuda et al. 2001; Ulmer 2002). During vaccination with DNA, the foreign gene is endogenously expressed, and the proteins subsequently processed, mimicking the viral processing during actual infection. The DNA vaccines are shown to elicit broad-based humoral (IgG2a, IgGI, serum HI antibodies) and cellular immunity ($CD4^+$, $CD8^+$ T cells, CTL, anti-HA IgG, and IgA secreting cells) against both the homologous and heterosubtypic challenges of the influenza viruses (Justewicz et al. 1995). In addition, alterations in the vector, dose of the DNA, inclusion of CpG-ODN motifs, fusion with specific Th cell, CTL epitopes, and appropriate vaccine delivery mechanisms are expected to further improve the efficacy of these vaccines (Bowersock and Martin 1999; Joseph et al. 2002).

Production of influenza vaccines in mammalian cells

In recent years, several continuous cell lines such as the Madin Darby canine kidney (MDCK), African green monkey kidney Vero cells (Kistner et al. 1998; Youil et al. 2004), human cell line Per.C6 (Pau et al. 2001), and St. Jude porcine lung epithelial cell line (Seo et al. 2001) have been tested for the production of influenza vaccines. Comparative analysis has also been made for large-scale vaccine production in these cell lines with the conventional egg-based process (Tree et al. 2001; Youil et al. 2004) as summarized in Table 2. Due to the ability of the MDCK cells to yield high quantities of influenza virus, large-scale culture systems have been made available for culturing these adherent cells such as fixed bed, fluidized bed, and microcarrier-based reactors. A simplified flow diagram

Table 2 Comparison of influenza vaccine production in chicken egg and mammalian cell. *MDCK* Madin Darby canine kidney

Advantages	Disadvantages
Production in chicken egg	
High yield of the viral strains	Requirement of extensive advanced planning
Lack of replication of adventitious agents	Man power intensive
Good antigenicity of the vaccine	Requires 1–2 eggs/dose of vaccine produced
Inability to cause tumor	Difficulty in handling and control
Less adverse reactions	Antigenic changes upon egg passages
	Lethality of H5N1 to embryo
	Requirements for high level bio security
Production in mammalian cell lines	
Easier supply of substrate	Low viral yield
Ease in process controllability and scalability	Propagation of adventitious agents due to multiple passages
Reduced risks of microbial contaminations, elimination of Thimerosol	Changes in the pH requirement for replication due to changes in the HA2 portion
Similar antigenicity	Requirement for immobilization, trypsin, and fetal calf serum
Preservation of receptor specificity	Increased local reactogenicity with MDCK-based vaccine

depicting the production of influenza vaccines in these mammalian cell lines is presented in Fig. 3.

Tree et al. (2001) assessed porous and solid microcarriers for large-scale culture of influenza virus in the MDCK cells. The solid microcarriers were found to give higher titers of A/PR8/34 virus (1.3×10^9 PFU/ml) compared to the porous one (4.0×10^8 PFU/ml). However, embryonated egg was reported to yield the highest titers (3.9×10^9 PFU/ml).

In another study, Youil et al. (2004) reported comparative propagation ability of type A and type B cold-adapted Russian strains and wild type influenza viruses in MDCK and Vero cells. The results indicted a more rapid viral growth of both type A and B influenza viruses in MDCK compared with Vero cells. However, for the type A viruses, the ratio of genome copies to infectious unit was more favourable for Vero cells than for MDCK cells, indicating a substantial difference between the two cell lines with respect to viral growth kinetics and in the nature of the viral output. The feasibility of the industrial production of vaccines derived from MDCK and Vero cell lines has been demonstrated at 400 l and 1,200 l, respectively. The latter cell line yielded 200,000 dose equivalents for one strain. A European national license has been granted to two vaccines derived from these cell lines (Kemble and Greenberg 2003). However, the future wide-scale application of these mammalian cell lines will depend on meeting the needs of high viral yield, appropriate permissiveness, and ability to support replication of all A and B viruses to high titers in short time. In addition, a complete knowledge of exposure to animal-derived products, history of its derivation, and passaging will be essential to fulfill the regulatory and safety standards.

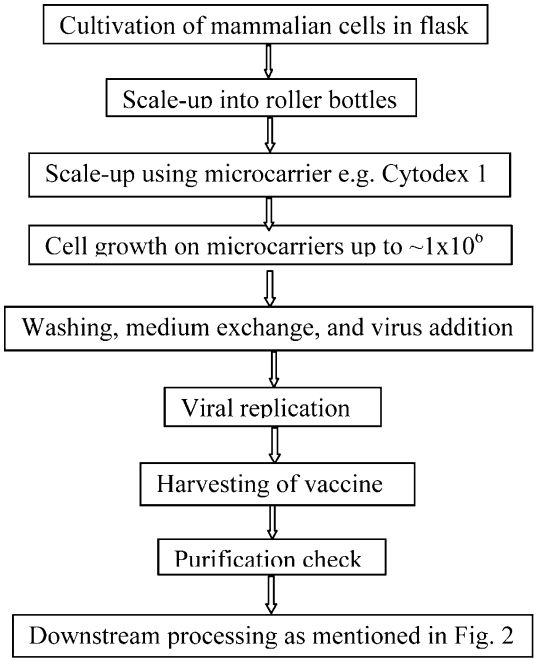

Fig. 3 Production process of influenza vaccine in mammalian cells

Summary

Presently, the two extremely variable viral membrane glycoproteins, HA and NA, are used for vaccination due to their ability to induce virus-neutralizing antibodies. Unfortunately, these antibodies, developed due to natural infection or vaccination fail to recognize the influenza virions during subsequent encounters. Therefore, to reduce the social and economic burden of influenza, rapidly producible vaccines with broad-spectrum immunogenicity and acceptable safety levels are being developed and tested. The recombinant techniques enable the construction of premade libraries of all possible reassortant antigenic subtypes that have high probability for transmission to mammals. Similarly, the high growth reassortants can be produced by reverse genetics. Significant achievements have been demonstrated in the optimization of immunogenicity, development of novel antigen delivery mechanisms, and production in mammalian cell culture systems. Presently, the genetically well-defined and stable live attenuated ca vaccines are expected to provide viable alternatives to the inactivated vaccines. The DNA vaccines may be good candidates for vaccination if safety and efficacy in humans can be proved. In addition, an increase in vaccination coverage, paying careful attention to public health policies, and regional organization of resources can improve public health and human productivity with respect to influenza.

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