Current Advances in Developments of New Influenza Vaccines

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Abstract: We have been now experiencing the first pandemic in the 21st century by a 2009 novel influenza A (H1N1) virus infection. The use of effective vaccination is the most reliable prophylactic measures against influenza virus infection. Hemagglutinin (HA) of surface viral glycoproteins plays a principal role as immunogenecity induced by natural infection or vaccination in our bodies. A split-product vaccine is prepared from inactivated influenza virus particles of epidemic strains and used worldwide. However, the administration of inactivated vaccine is not always effective. The antigenicity of HA proteins is continuously changed according to mutations in its gene for escaping from host immune systems. Therefore, vaccination against epidemic strains with mutations is needed to repeat annually. Contrary to epidemic strains, it is difficult to propagate pandemic strains of influenza viruses owing to the virulence. Consequently, a sufficient quantity of antigen cannot be obtained. Thus, currently licensed influenza vaccine has a lot of inevitable problems. New developments for influenza vaccination, such as live cold-adapted vaccine, reverse genetics vaccine, DNA vaccine, universal vaccine and co-administration with adjuvant, have been tested in order to solve the problems. It is noted that the development of vaccine preparation using genetic engineering progressed rapidly. This article, therefore, reviews recent knowledge regarding (1) influenza virus HA, (2) currently licensed influenza vaccines, and (3) new devices for developing influenza vaccines.

Keywords: Influenza virus, hemagglutinin, live cold-adapted vaccine, reverse genetics vaccine, universal vaccine, adjuvant.

1. INTRODUCTION

Influenza is a highly contagious acute illness of the respiratory tract caused by influenza virus infection. Influenza is self-limited but can cause complications that lead to hospitalization and death. Influenza virus hemagglutinin (HA) that is one of the surface proteins embedded into the envelope exhibits the principal immunogenicity in our bodies. That is, antibodies against HA can prevent reinfection with influenza viruses that have infected once. However, the viruses with selected point mutations within two surface glycoproteins, such as HA and neuraminidase (NA), are emerged in the body during the replication cycles by the host immunological pressure, resulting in new epidemic strains every 1 to 2 years. Although the epidemics of influenza virus infection stay in local areas, it has been estimated that 500,000 people died from severe complications associated with influenza virus infection worldwide every year. In contrast to epidemics, pandemics are rare events that occur every 10 to 50 years. The reassortment of viral segmented genes among human, swine and avian viruses appears to arise pandemic virus strains. During the 20th century, three influenza pandemics have occurred. The death toll has been estimated as 1 million to 50 million during the past pandemics. As adjusted for today's world population, it has been calculated that death toll would be ranging from 1.7 million to 180 million when a new pandemic of influenza virus infection occurred.

We have been now experiencing the first pandemic in the 21st century by a 2009 novel influenza A (H1N1) virus infection. Human infection with the novel virus has been initially confirmed in Mexico since March 2009 and spread out to 9 countries within one and half of months [1]. Hereby, World Health Organization (WHO) has considered the influenza to be a pandemic threat and raised the influenza pandemic alert level to phase-5 on the 29th of April 2009. Phylogenetic analysis has revealed that the novel influenza A (H1N1) virus was genetically related to recent swine influenza viruses, while the genetic profile regarding HA, NA and matrix protein (M) genes was not detected among those of viruses infecting previously to either swine or human populations [2]. Hemagglutination inhibition tests using antisera obtained from ferrets have demonstrated that the novel influenza A (H1N1) virus strains isolated from different areas, such as North America, Europe and Oceania, were antigenically homogeneous but distinct from currently circulating seasonal influenza A (H1N1) viruses. However, their antigenicities were the most closely related to those of the past seasonal influenza virus strain of A/California/7/2009 (H1N1) and similar to those of strains of triple-reassortant A (H1N1) swine influenza virus strains (e.g. A/Illinois/09/2007) in North American lineage that have circulated in pigs over the last 10 years in the USA and occasionally infected into humans during the same period. Therefore, the 2009 pandemic influenza in humans is named as "swine influenza" or H1N1 influenza A. As of the 31st January 2010,

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1876-5181/10

worldwide more than 209 countries have reported laboratory confirmed cases of pandemic influenza H1N1 2009, including at least 15,174 deaths.

Furthermore, we have still faced to the threat of infection with highly pathogenic avian influenza viruses. In 1997, human infection with a highly pathogenic avian influenza A (H5N1) virus has been reported for the first time in Hong Kong. Eighteen cases with H5N1 virus infection have been confirmed, six of them have died from acute respiratory distress syndrome or multiple organ failure, followed by serious outbreak of chicken infection with H5N1 virus in poultry farms. The strains of H5N1 viruses isolated from human have been apparently avian origin but not had swine type viral genome segments. These facts indicate that the avian viruses can directly infect to humans, providing a sign of an incipient pandemic of the highly pathogenic avian influenza A (H5N1) virus infection in humans.

Individual species, such as human, bird, pig, horse and sea animals, can be infected with respective species-specific influenza viruses, the infection with which usually stay in the respective species. However, these viruses rarely cause infection in irrelevant species by acquiring transmissibility *via* two principal mechanisms, such as reassortment of viral genome segments and adaptive mutations in viral genes. An intermediate host of influenza viruses having an ability of infection to human is considered swine, while avian virus directly infect to human as shown in Fig. (**1**).

Influenza viruses infected to human are divided into three types, A, B and C, on the basis of serological properties of the major internal proteins, such as nucleoprotein (NP) and M1. Influenza virus type A is further classified into subtypes according to the serotypes of the surface glycoproteins, such as HA and NA. HA facilitates virus attachment to cell surface receptors and virus invasion into cells with the membrane fusion activity. It is well known that antibodies to neutralize virus infection consistently present in anti-HA antibodies. Anti-NA antibodies do not neutralize virus infection, however, they inhibit spread of virus infection through the suppressive effect on virus release from host cells. Anti-M2 antibodies also exhibit suppressive effect on virus proliferation. Intracellular domain of M2 protein presents antigenicity, while antibodies against the region do not neutralize virus infection because they cannot pass the envelop of viruses.

A split-product vaccine is prepared from inactivated influenza virus particles of epidemic strains and widely used worldwide. However, inactivated vaccines have a lot of inevitable problems. New devices for influenza vaccination, such as live attenuated vaccine, reverse genetics vaccine, DNA vaccine, universal vaccine and co-administration with adjuvant, have been developed in order to solve the problems. Accordingly, to understand the availabilities and limits of current inactivated vaccines in the prevention of influenza virus infection, knowledge of structures, functions and antigenicities of HA is important. This article, therefore, reviews recent knowledge regarding (1) influenza virus HA, (2) currently licensed influenza vaccines, and (3) new devices for developing influenza vaccines.

2. HEMAGGLUTININ PLAYING CRITICAL ROLE IN INFECTIVITY

2.1. Characteristic Structures of HA

Influenza virus HA is a glycoprotein on virus surface as spikes composed of homotrimer. The schematic structure of HA of A/Hong Kong/68 (H3N2) is shown in Fig. (**2**). The structure of HA can be divided into conservative and variable regions. The signal peptide, trans-membrane and fusion peptide domains, the cleavage site and disulfide bound site are located within the conservative regions. Contrary, the variable region contains the receptor-binding domain, the domain surrounding the cleavage site, the binding sites for oligosaccharide chain, and the antigenic domains that are recognized by antibodies. The variations in HA protein lead the alteration of immunogenicity against influenza virus infection, resulting in the emergence of epidemic strains.

The basic structures including an amino acid sequence of various HA subtypes are well established and compared

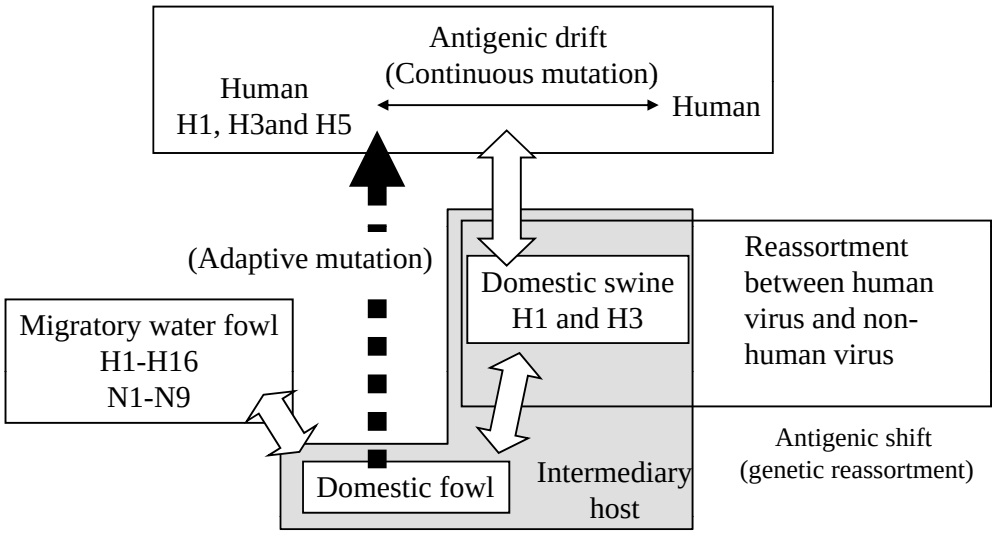

Fig. (1). Scheme of antigen drift and shift of influenza virus.

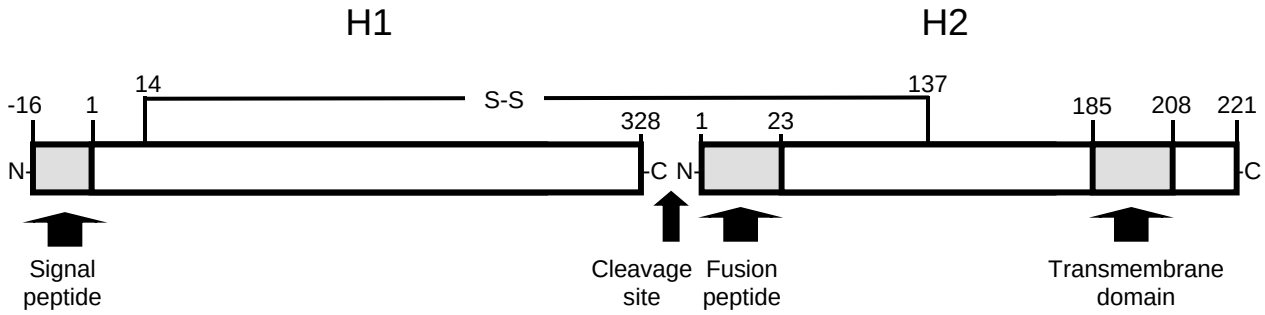

Fig. (2). Structures of HA1 and HA2 subunits of influenza A/Hong Kong/68 virus (H3N2).

among them [3]. HA is synthesized as a single polypeptide precursor, HA0. For an instance, HA0 of A/Hong Kong/68 (H3N2) is composed of 550 amino acid residues. The HA0 is located into the endoplasmic reticulum through the signal peptide that is consisted of 16 amino acid residues placed at *N*-terminal end, in which they form homotrimer associating with molecular chaperons. Thereafter, the HA0 is modified (e.g. glycosylation) and translocated to the cell surface through the Golgi apparatus [4]. HA0 polypeptides of all subtypes of influenza A virus are cleaved into two subunits, HA1 and HA2, by trypsin-like proteases present in respiratory and intestinal tracts or by ubiquitous subtilisin-like proteases present in the Golgi apparatus [5]. The cleavage of HA0 precursor into HA1 and HA2 subunits is carried out on the cell surface or on whole virus particles after the release from cells. The limited distribution of trypsin-like protease is responsible for the limited tissue tropism of influenza viruses.

Analysis of amino acid sequences has revealed that the cleavage domains are slightly but significantly different among high pathogenic H5N1 subtypes and low pathogenic H5N2 and H1N1 subtypes [3, 5, 6]. The cleavage sequence of high pathogenic H5N1 subtype is composed of "RRKKR/G" consisting with polybasic amino acids with positive charge (amino acid positions ranging from 342 to 347; a slash mark means cleavage point by protease), whereas those of H5N2 and H1N1 are composed of "QRETR/G and SIQSR/G", respectively, with a small number of basic amino acid (amino acid positions ranging from 338 to 343 of H5 and from 325 to 330 of H1). The HA0 polypeptides for the H1, H2 and H3 subtypes of previous epidemic strains may be cleaved by the serine protease, tryptase Clara derived from Clara cells in the bronchiolar epithelium [7, 8]. The trypsin-like enzyme specifically recognize the amino acid sequence with a little or low content of arginine. For some of the HAs of the H5 and H9 subtypes, the HA1 and HA2 subunits are separated by polybasic amino acid sequences with high content of arginine, which is cleaved with subtilisin-like enzyme, furin present in the Golgi apparatus. As shown in Table **1**, amino acid sequences at the cleavage sites are conserved in each group of HA subtypes, while there is a difference in amino acid sequences between high and low pathogenic viruses with H5 subtypes. It is predicted that the difference in amino acid sequences reflects the protease susceptibility. The polybasic amino acid sequence of HA with H5 subtype seems to be related to a broad tissue tropism, resulting in the high pathogenicity [11].

HA0 polypeptide is cleaved into HA1 and HA2 subunits by proteases [12]. HA1 subunit is linked to HA2 subunit with a single disulfide bond [13]. HA1 forms a globular head at the tip of the HA spike and contains biologically significant domains, such as five antigenic moieties and a receptor-binding site. The exposure of hydrophobic domain at the *N*-terminus of HA2 subunit embedded in the viral envelope is prerequisite for fusion with the inner layer of the endosome membrane, resulting in the formation of a fusion pore.

2.2. Subtype Classification of HA

The influenza virus genome consists of several single-stranded negative-sense RNA segments. Each influenza A and B virus contains eight RNA segments, while influenza C virus contains seven RNA segments. HA and NA of influenza A virus are encoded by RNA segments 4 and 6, respectively. Influenza A viruses are classified into subtypes according to the antigenic properties of HA and NA proteins. In avian viruses, 16 subtypes of HA and 9 subtypes of NA have been currently identified [14]. In the combination of 16 HA subtypes and 9 NA subtypes, it is presumed that 144 subtypes of influenza A virus are presented. Although many subtypes are not recognized in influenza B virus, influenza B virus is classified into only two types, such as Victoria and Yamagata.

Influenza A viruses have been isolated from humans and various animals, such as domestic animals (i.e. swine and equine), sea mammals (i.e. seals and whales), wild waterfowls (ducks), and poultry [15]. Of the 16 HA subtypes (H1-H16), H3 and H7 subtype viruses infect equine. H1 and H3 subtype viruses infect swine. H2, H4, H5, and H9 subtype viruses rarely infect swine. H1 and H3 subtype viruses infect human. In addition, avian viruses, such as H2 (A/Japan/170/63; H2N2), H5 (A/Hong Kong/97; H5N1), H7 (A/Seal/Mass/1/80 and A/Fowl Plague/Dutch27; H7N7), and H9 (A/Hong Kong/99; H9N2), rarely infect human.

Liu *et al.* have analyzed the phylogenetic diversity and the distribution among HA genes selected from approximately 23,000 influenza A virus strains registered in the database of GenBank. They generated two panorama phylogenetic trees of influenza A viruses covering all the 16 HA subtypes and 9 NA subtypes [16, 17]. As shown in Table **2**, lineages and sublineages were classified according to genetic distances, topology of the phylogenetic trees and distributions of the viruses in hosts, regions and time.

Table 1. Comparison of Amino Acid Sequences of the Cleavage Site of HA0 by Trypsin- Like Protease

Subtypes	Strains	Sequence							References
H1	A/PuertoRoco/8/34	P	S	I	Q	S	R	G	[3]
H1	A/South Calrolina/1/18	P	S	I	Q	S	R	G	[3]
H1	A/sweine/Iowa/15/30	P	S	I	Q	S	R	G	[3]
H2	A/Singapore/1/57	P	Q	I	E	S	R	G	[3]
H3	A/Duck/Ukraina/63	P	E	K	Q	T	R	G	[9]
H3	A/Aichi/68	P	E	K	Q	T	R	G	[9]
H3	A/Duck/Hokkaido/5/77	P	E	K	Q	T	R	G	[9]
H3	A/Aichi/2/68	P	E	K	Q	T	R	G	[3]
H3	A/Aichi/2/68	P	E	K	Q	T	R	G	[10]
H5*	A/Hong Kong/157/97	R	R	R	K	K	R	G	[3]
H5	A/Duck/Singapor/97	P	Q	R	E	T	R	G	[10]
H5*	A/Hong Kong/486/97	R	R	R	K	K	R	G	[10]
H5N1*	QINGHAI	R	R	R	K	K	R	G	[6]
H5N2	USDA	P	Q	P	E	T	R	G	[6]
H9	A/Hong Kong/1073/99	P	A	R	S	S	R	G	[3]
H9	A/Sweine/9/97	P	A	R	S	S	R	G	[10]

* Highly pathogenic strains.

Table 2. Host Distribution of 14,328 Influenza A Viruses with HA Sequences

Host / Subtype	Birds	Humans	Pigs	Horses	Others
H1	71	1,994	311	0	1
H2	77	84	2	0	0
H3	215	6,868	179	154	9
H4	129	0	2	0	1
H5	2,120	240	11	0	27
H6	374	0	0	0	0
H7	446	6	0	12	2
H8	12	0	0	0	0
H9	759	7	34	0	0
H10	58	0	0	0	0
H11	65	0	0	0	0
H12	24	0	0	0	0
H13	14	0	0	0	1
H14	3	0	0	0	0
H15	6	0	0	0	0
H16	10	0	0	0	0

2.3. Antigenicity of HA

2.3.1. Antigenic Drifts and Shifts

The HA of surface protein is the major target for host immune responses as it plays a critical role in the attachment to and penetration into host cells. Influenza A virus possesses a unique capability of altering antigenicity in order to evade from neutralization with antibodies. Viruses bearing HA genes with point mutations that lead unresponsiveness against antibodies are selectively proliferated under host immunological pressures. An accumulation of such mutations associates with “antigenic drift”, resulting in the pro-

duction of potential new epidemic strains potentially [18, 19]. Pandemic strains of influenza A viruses are resulting from "antigenic shift" which reflects a major change in the HA and possibly NA. Antigenic shift occurs as a result of genetic reassortment among genome pools of avian, swine and human viruses in swine as a mixing vessel [20, 21]. Since there is little or no background immunity in the population to the new viruses with antigen shifts, human infection with the new viruses spreads rapidly and extensively.

2.3.2. Studies of Antigenic Structures

(a) Antigenicities Against Panels of Monoclonal Antibodies

H1 Subtype

Caton *et al*. have demonstrated the antigenic structure of HA of influenza A/PR/8/34 (H1N1) virus strain by using a large number of monoclonal antibodies. Five immunodominant antigenic sites (designated Sa, Sb, Ca1, Ca2 and Cb) presented on the globular domain. The changes of amino acid residues at these sites were detected in mutant strains, the antigenicity of which were different each other among H1 subtypes [22]. Shen *et al*. have analyzed the antigenic properties of HA among hundreds of H1 subtypes of viruses isolated between 1918-2008, suggesting the evolutionary trends of antigenic properties by host selection through immune response, host-driven antigenic drift [23].

H2 and H3 Subtypes

Antigenic structure and properties among 6 HA molecules of H2 subtype have been analyzed using 19 monoclonal antibodies. Distinctive 6 antigenic sites (designated I-A to I-D and II-A and II-B) have been identified. The study suggested that the structures of 5 antigenic sites were similar to those of the H3 subtype HA, while a highly conserved antigenic site in the stem domain of HA was significantly different from that of H3 subtype HA [24].

H5 Subtype

The antigenic sites on HA molecules of 16 mutant strains of H5N2 virus (A/Mallard/Pennsylvania/10218/84) have been analyzed using a panel containing 5 monoclonal antibodies. In the study, 5 epitope sites were identified and defined as group-1 to group-5, the positions of amino acids on which were as follows: Group 1 at positions of 156 and 193; group 2 at a position of 62; group 3 at a position of 46; group 4 within a 3-amino-acid gap in the alignment between 119 and 120 of positions; group 5 at a position of 145; ungrouped at a position of 186. Three of five epitopes closely corresponded with those of H3 subtype HA. The group 1 was either in antigenic site B or between sites A and B. The groups 5 and 2 were located within sites A and E, respectively. However, the other two epitopes on H5 subtype HA did not correspond with those of H3 subtype [25]. Based on the results from the phylogenic tree of influenza A viruses, Liu has demonstrated that H5 and H2 subtype HA molecules were placed on exceedingly mutually near position in the tree, however, the antigenicities of them were different each other significantly.

H9 Subtype

Okamoto *et al*. have analyzed the antigenic structure of H9 subtype HA of 21 strains. Based on the reactivity patterns using eight monoclonal antibodies, 5 non-overlapping antigenic sites and seven distinct epitopes designated as I-VII at least were identified [26]. The reactivity patterns with the panel of monoclonal antibodies suggested that strains used in the study can be antigenically divided into 7 distinct groups, and that the structures of epitopes were changed by point mutations in viral genome.

(b) Nucleotide Sequence-Based Study for Evolution

Huang *et al*. have developed a new method for identifying antigenic critical amino acid positions, rules and co-mutated positions for antigenic variants, using 45 HA sequences (genetic data) and 181 hemagglutination inhibition data (antigenic data). They have considered amino acid positions with both highly antigenic discriminating score and highly genetic diversity as antigenic critical positions. Their results have demonstrated that such positions were highly correlated to site for an antigenic drift, and that most of these antigenic critical positions were located on five epitopes or on the surface based on the HA structure. The method may be useful for studying influenza virus evolution and vaccine development [27].

These results highlight the urgency to understand the interplay between antigenic drift and receptor binding in HA evolution, and provide molecular signatures for monitoring future antigen drifts in epidemic and pandemic influenza viruses.

3. CURRENTLY LICENSED INFLUENZA VACCINES

Prophylaxis and therapeutics of influenza virus infection are practiced by an immunization with vaccines and chemotherapy with antiviral drugs, such as M2 inhibitors and NA inhibitors, respectively. The use of antiviral drugs is also a temporal strategy for prophylaxis in limited cases because the use of antiviral drugs induces the emergence of drug resistant viruses frequently.

Vaccination is an acquisition of protective immunity in advance by administration with viral antigenic glycoproteins, such as HA and NA. Therefore, the vaccination is widely considered to be the first line of defense for protecting populations in advance against influenza virus infection. Since anti-HA antibody in the serum has the most consistent relation to the immunity against influenza virus, it is considered that the HA as transmembrane protein exhibits the principal immunogenicity in our bodies [28]. Moreover, other transmembrane proteins, such as NA and M2, are also considered candidates for immunization. Currently available and under developed vaccines are listed in Fig. (**3**).

The effectiveness of vaccine administration is determined by the antigenicities of viruses used for vaccine preparation. Based on a continuous worldwide surveillance for circulating influenza viruses, WHO recommends potential virus strains for vaccine preparation for a coming season [29].

3.1. Split Vaccines

Three types of influenza vaccine using inactivated whole virion, split-products and purified surface antigens are currently available worldwide for parenteral administration. Since the administration of inactivated whole virion vaccine

- Licensed Vaccines (Cell-derived or Egg-derived)
  - Inactivated Vaccines
    - Whole particles
    - Split ingredients
    - Purified surface antigens
  - Live-cold attenuated reassortment Vaccines
- Vaccines in the development
  - Development of vaccine strains
    - Reverse genetics
  - Development of adjuvants
  - New type of Vaccines
    - DNA vaccines
    - Recombinant Vaccines
      - Virus vectors
      - Bacteria vectors
    - Universal vaccines

Fig. (3). Licensed and under developing vaccines.

is associated with frequent systemic adverse effects, it is unsuitable for young children and elderly, and unlicensed in many countries except for some countries. The split-product vaccine is widely used worldwide. It is prepared from highly purified influenza viruses that were disrupted by treatment with either detergent or ether, and then inactivated with formaldehyde. The surface antigen vaccine contains predominantly purified HA and NA. The cost for preparation is relatively high. These licensed vaccines usually contain 15 μg of each HA protein derived from two strains of influenza A viruses with different subtypes (e.g. H1N1 and N3N2) and one strain of influenza B virus.

3.1.1. Preparation

The split-product vaccine is produced using vaccine strains that are reassortant viruses. The prevalent preparation processes of inactivated vaccines are shown in Fig. (**4**) [30]. A laboratory-adapted influenza strain, A/ PR/8/34 (H1N1), and the epidemic strain are co-inoculated in the allantoic sac in fertile chicken eggs for preparing seed strains in order to obtain reassortant viruses with high growth ability. For selection of seed strains, the reassortant viruses are tested to confirm the absence of genes encoding PR8 or PR8-like surface glycoproteins using specific antibodies. The seed strain containing HA and NA of the epidemic strain is selected for a vaccine strain and propagated in large quantities in fertile eggs. Seed strains of influenza B virus are isolated from fields. The inactivated viruses are prepared from seed strains by chemical treatments. This procedure is recommended by WHO.

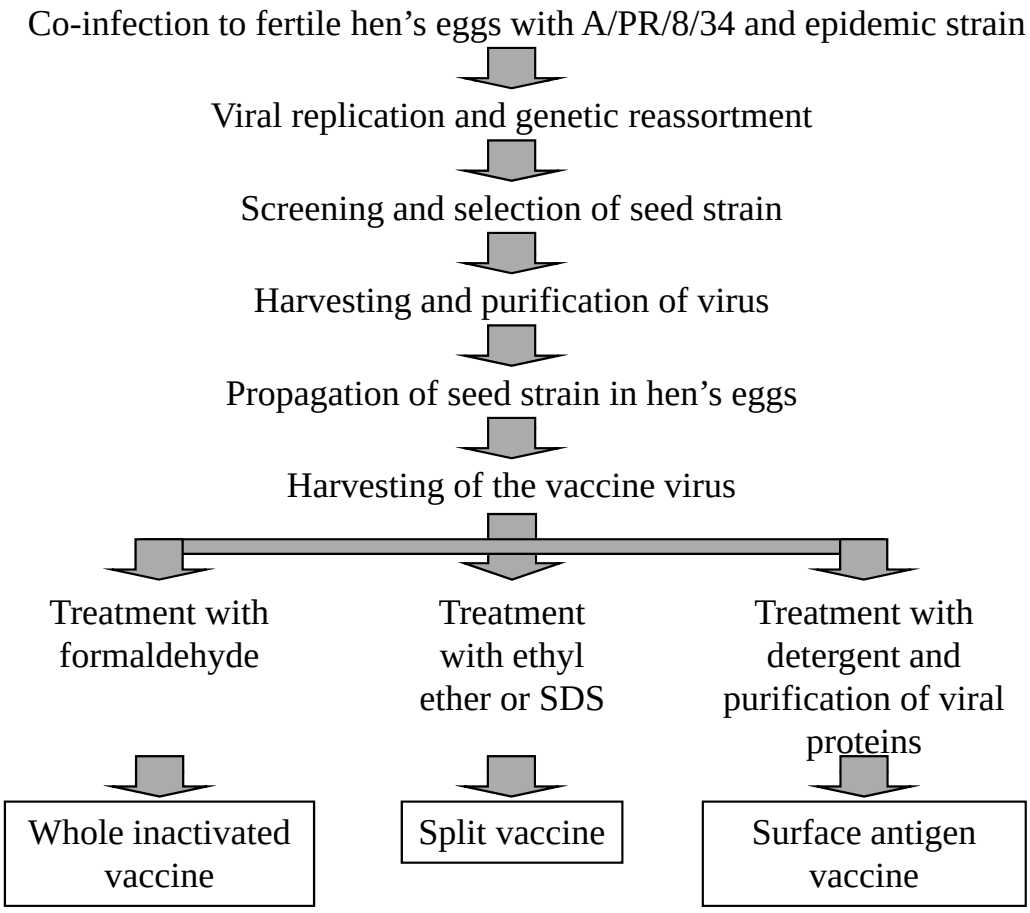

Fig. (4). Scheme of production of inactivated influenza vaccines in hen's eggs.

In recent years, certain cell lines, such as MDCK, Vero and Per.C6 cells, have been used for vaccine production instead of eggs [31, 32]. The use of cell lines for vaccine production has advantages over the conventional method using eggs, such as high yield of viruses, less adverse reaction, good antigenicity, easier supply, and reduced risk of contamination with microbes [33]. The innovation using cell lines may contribute for the supply of safe vaccine products in a large-scale in a future.

3.1.2. Immune Response

Although the respiratory tract mucosa is the site for influenza virus infection, the tissue plays a role in the primary barriers against the viral infection. The primary defense mechanisms involves the innate immune system constructing from mucus, IgA antibody, macrophages, dendritic cells, natural killer cells and interferons (IFNs) α and β. In the respiratory tract, viruses are neutralized with secretory IgA antibody that is synthesized by IgA-producing cells and transported to the epithelial cell surface. IgA antibody, therefore, plays an important role in the protection at the early step of the infection rather than IgG in the humoral immunity [34]. When viruses defeated the innate defense barriers, the following adaptive immune system should eliminate viruses. $CD8^+$ cytotoxic T lymphocytes (CTL) should destruct the virus-infected cells. The parenteral administration of currently licensed vaccines can induce systemic IgG antibody but neither mucosa IgA antibody or heterosubtypic CTL (Table **3**). Accordingly, the immunization with split-product vaccine can induce the higher protective effect against infection with vaccine strains but not against infection with the other epidemic or pandemic strains.

It is generally believed that inactivated vaccines including split-product, whole virion and purified surface antigens do not induce influenza virus-specific CTL because they do not produce virus-derived endogenous antigens in cells that can stimulate epitope-specific CTL *via* an MHC class I-restricted antigen processing pathway subunit (direct presentation). However, it has been recently reported that vaccination with inactivated whole virus can induce cross-protective CTL presumably by cross presentation [35].

3.1.3. Safety

The split-product vaccine induces a good protective effect in healthy older children and adults, although the protective effect is lower in young children and the elderly [29] (Table **4**). After vaccination with split-products and surface antigens, the local erythema and tenderness are documented, while the incidence of adverse reactions including systemic symptoms, fever and allergic reactions, such as urticaria and anaphylaxis, is similar to that of placebo [36, 37]. Recent randomized and controlled trials in a large cohort study have confirmed the safety of these vaccines in patients with asthma [38]. Although the current influenza vaccines contain a small quantity of egg protein, it has been confirmed that even sufferers having the allergic reactions probably resulted from hypersensitivity to residual egg protein can safely receive these vaccines [39, 40]. The worldwide investigations including above reports suggest that these vaccines are well tolerated and extremely safe [41].

As described in the above, inactivated vaccines provide us a benefit through the induction of highly specific humoral immunity. However, they have some inevitable problems. The effective period after vaccination is relatively short as 3-4 years because variant viruses with selected mutations appear frequently. The vaccination fails to induce protective mucosal and cellular immunities. Infants showed a significantly poor antibody rise and lymphocyte response as com-

Table 3. Characteristics of Current and Developing Vaccines

Vaccine	Immunresponse (production of Ig type)		
	IgA	IgG	CTL
Current licensed vaccines			
whole, split-product, and subunit vaccines (parentanal)	-	++	-
Cold-adapted live virus vaccine (intranasal)	+++	++	+++
Developing or new vaccines (not licenced)			
Inactivated vaccines			
Ajdubant-combined vaccines (parentanal)	-	+++	+
Ajdubant-combined vaccines (intranasal)	+++	++	+
whole, split-product, and subunit vaccines (intranasal)	++	+	-
DNA vaccines			
prentanal type	-	+	+
intranasal type	++	+	+
Live virus vaccines (intranasal)			
Virus-vectored vaccines	++	+	++
Recombinant virus vaccines	+	+	+

Table 4. Responses of Hemagglutinin-Inhibition Antibody in Serum Induced by the Inactivated Influenza Vaccines

Vaccine Virus Strain	Age	Rise (%)	Titer of Abs		≧40 (%)	
			Pre Vac	Pro Vac	Pre Vac	Pro Vac
A/Soloman/ Island/03/06 (H1N1)	Adults	52	92	279	38	100
	Elderly	92	14	129	8	100
	Children	96	6	457	79	63
A/Wisconsin/67/05 (H3N2)	Adults	50	95	457	79	100
	Elderly	54	66	267	58	100
	Children	86	8	48	17	69
B/Malasia/2506/04	Adults	54	27	220	46	100
	Elderly	42	30	82	54	88
	Children	72	8	65	14	72

pared with young children [42]. Consequently, the development of immunologically superior vaccines has been required.

3.2. Live Cold-Adapted Vaccines

The live cold-adapted (CA) vaccines have been developed as one of immunologically superior vaccines and licensed in Russia and the USA. The CA and attenuated virus strains replicate well at 25-33 ºC, while the replication at 37 ºC is reduced. The live CA vaccine should be administrated intranasally except for the high-risk groups, such as infants, immunodeficient patients and pregnant women.

3.2.1. Preparation

CA type, temperature sensitive type and host range type have been proposed for live attenuated vaccines in the earlier studies [43, 44]. Due to the uncertain genetic stability and a possible reversion to virulent phenotypes, live CA vaccines are of interested and eventually selected for live-attenuated vaccines [45].

The live CA vaccine is prepared from reassortant viruses that contain genes for HA and NA of the wild-type virus strains and the six non-surface genes from one of CA master virus strains. Either influenza A/Ann Arbor/6/60 (H2N2) or B/Ann Aber/1/66 virus is used for the CA master strain. The CA master viruses are prepared by serial passage under low temperature conditions at 25 ºC and 33 ºC, which are attenuated and unable to replicate at human body temperature [46]. After co-infection of cultured cells with the CA master virus strain and the wild-type virus strain, reassortant viruses are selected using plaque purification techniques. The reassortant viruses are proliferated in the presence of antiserum to HA and NA of the CA master virus strain. The replicated reassortant viruses carry HA and NA of the wild-type virus strain and exhibit the avirulence accompanied by stable mutations derived from the CA master virus strain.

3.2.2. Immune Response

The subcutaneous or intramuscular administration of inactivated vaccine can induce humoral immunity but neither local immunity in the nasal cavity and pharynx where are infected with viruses firstly or cellular immunity. However, the intranasal administration of live CA vaccine can mimic natural infection and produce a larger quantity of antigen than inactivated vaccines, providing an advantage of broader immunological responses. The intranasal administration of live CA vaccines induces the cross-protective immunological responses including secretory IgA and humoral IgG antibodies and CTL responses to both HA and NA. It is notable that the immune responses induced by live CA vaccine are greater than its parental vaccines [47]. The live CA vaccine can reduce the amount and frequency of viral shedding in comparison with the inactivated vaccines [48].

After the intranasal administration of live CA vaccine to children, the efficacy of vaccination in preventing culture-confirmed influenza was estimated as 87% after the first administration and 96% after the second administration. Furthermore, live CA vaccine has been efficacious against infection with a variant virus strain, which was not contained in the vaccine strains [47]. In animal models, it has been demonstrated that the live CA vaccine was safe and immunogenic against infection with H9N2 and H5N1 avian influenza viruses [49-51].

3.2.3. Safety

The live CA vaccines are suitable for use in children and elderly. CA reassortant viruses have been established to be genotypically stable and suitable for administration to children with asthma. The live CA vaccines are well tolerated in children with minor upper respiratory symptoms including rhinorrhoea or sore throat [52]. Excess systemic complaints including myalgia, headache and lethargy occur not generally in healthy adult subjects [53]. Furthermore, these are well tolerated by elderly in nursing homes and patients with asthma, chronic obstructive airways disease or cardiac conditions [54]. Good efficacy in clinical trials including young children has been confirmed in Asia and Europe. Recently, another CA master virus strains of A (H1N1) and A (H3N2) subtypes have bee developed by growth of wild type of influenza virus in Vero cells at 25 ºC, which were also safe and

well tolerated and exhibits immunogenicity after intranasal immunization in young adult volunteers [55].

Current report has demonstrated that live CA vaccines were prepared from reassortant viruses possessed genes encoding a modified HA and a wild-type N1 subtype NA from influenza A (H5N1) viruses isolated in Hong Kong and Vietnam in 1997, 2003 and 2004, and the remaining genes derived from CA master strain, influenza A/Ann Arbor/6/60 (H2N2). In mice, the H5N1 CA vaccines exhibited immunogenicity and completely protected from the lethal challenge with wild-type H5N1 viruses and different subtype viruses isolated in Asia between 1997 and 2005 [51].

CA vaccine and inactivated vaccine do not differ by systemic reactions after administration, however, it is not ruled out that there can be unfavorable reactions in vaccination of persons with allergy to the chicken-embryo proteins as well as in cases of persistence/reversion of CA strain observed in vaccination of persons with primary impairments of the immune system [56]. Therefore, CA vaccine still has disadvantages.

4. NEW DEVICES FOR DEVELOPING INFLUENZA VACCINES

4.1. Necessity for Developing New Type Vaccines

In Hong Kong in 1997, a highly pathogenic avian influenza A (H5N1) virus has been apparently transmitted directly from chickens to human with no intermediate mammalian hosts. The catastrophe with influenza pandemic (e.g. with H5 strains) will undoubtedly occur as issued a warning by WHO. WHO has estimated that there globally are about 1.2 billion people at high risk for severe influenza outcomes: 385 million elderly over 65 years of age, 140 million infants, and several hundred million children and adults with underlying chronic health problem. At the present time, the world's total vaccine production capacity is limited to about 900 million doses, which are not sufficient for the global high-risk population. Therefore, it is obvious that a timely manufacturing, distribution and delivery of pandemic influenza vaccines cannot be carried out.

Current dosage of vaccines (i.e. 15 μg HA) induces immune responses in most persons. Since it has been established that the increase in vaccine dosage (antigen dosage) enhances the serum anti-HA antibody response [57], a lager amount of dosage will induce a higher immune response. However, the infection of eggs with high pathogenic influenza virus strains results in the rapid death of embryos. Therefore, adequate viral titers for the preparation of vaccines cannot be reached. It has been attempted to settle this problem by using low pathogenic strains having the same HA subtypes instead of high pathogenic strains. Consequently, new devices, such as reverse genenetics technology, are needed for developing vaccines for highly pathogenic viruses.

Current vaccines can immunize against highly specific virus-membrane constituents, HA and NA, although these vaccines are not effective against antigenic shift or drift variants of the original viruses, and also take a lot of time for preparation. Since CTL response is one of the most important reactions to eliminate virus particles from the body, it is difficult to induce the CTL response against variable viral proteins, such as HA and NA, with currently available vaccines. Accordingly, new devices for vaccination have been desired urgently, which can induce a protective cellular immune response to conserved viral proteins. As such new devices, DNA vaccines and universal vaccines have been improved.

4.2. Reverse Genetics Vaccines

When human infection with the highly pathogenic H5N1 virus has occurred in Hong Kong in 1997, an antigenically related but nonpathogenic H5N3 virus strain has been isolated. The nonpathogenic H5N3 virus strain itself was grown well, however, the growth of attenuated H5N3 reassortant viruses with human PR8 strain was slow. Reassortant virus carrying properties, such as higher growth ability in eggs, low pathogenicity and antigenicity of the pandemic virus, will be suitable for large-scale production of an adequate vaccine. Therefore, alternative strategies for the production of a safe pandemic vaccine strain have been required. It is now possible to prepare a vaccine against any potential pandemic influenza virus strain, such as the avian H5N1 strain, using genetic technologies [58, 59].

As shown in Fig. (**5**), the reverse genetics was conducted using six plasmids carrying genes of PR8 and two plasmids carrying modified HA genes with deletion of basic amino acid sequences and NA gene of H5N1 virus. These plasmids were co-transfected into Vero cells together with four plasmids encoding acidic polymerase (PA), basic polymerase (PB) 1, PB2 and NP genes, which were prerequisite for the replication of viral genes, of PR8 virus. The generated viruses possess a similar antigenicity to high pathogenic virus strains. They grow and adapt to cell culture similar to low pathogenic master strains [60, 61]. The high pathogenicity of avian strains is associating with polybasic amino acids at the HA cleavage site that allowed cleavage by ubiquitous furin-like protease as described previously. Using simple genetic engineering techniques, the extra basic amino acids at the cleavage site can be excised from a cloned copy of the avian HA gene [62].

Improved reverse genetics have reduced the number of plasmids required for the generation of influenza A virus particles as compared with that of previously utilized methods and allowed the generation of reassortant viruses [61]. The cDNA for each of the eight influenza virus RNA segments was inserted between the RNA polymerase I (pol I) promoter and terminator sequences. This entire pol I transcription unit was flanked by an RNA polymerase II promoter and a polyadenylation site. The orientation of the two transcription units allowed the synthesis of negative-sense viral RNA and positive-sense mRNA from one viral cDNA template. In other words, functions of both transcription of viral gene and expression of viral proteins have been engineered into one plasmid, making it possible to require only eight plasmids. This approach has provided advantages in the generation of reverse genetics viral strains (reassortants) carrying sufficient viral titers for vaccine production on demands, and in the elimination of contamination with any potential pathogenic agents, such as other respiratory virus present in the original specimen and unknown components from cells used to isolate the virus.

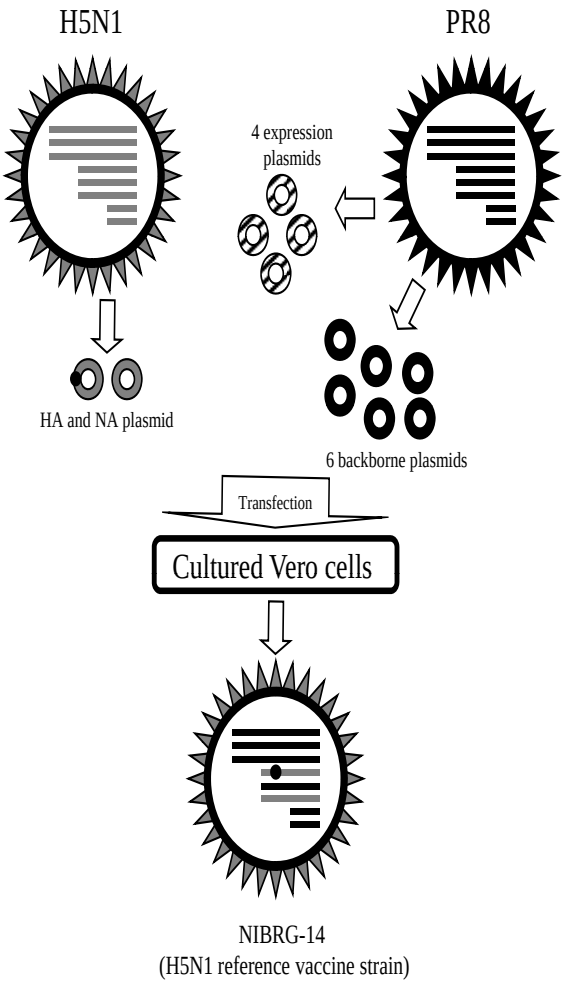

Fig. (5). NIBRG-14 virus is a reassortant containing six RNA segments from PR8 (black lines), and two from the wild type H5N1 strain (gray lines). The HA segment is modified to delete the basic amino acid sequence domains (dot spot) conferring the high pathogenicity.

4.3. DNA Vaccines

Wolff *et al*. have demonstrated for the first time a possibility of vaccination by direct intramuscular injection of DNA expression vectors containing genes for chloramphenicol acetyltransferase, luciferase and β-galactosidase in mice [63]. To generate a viral antigen for presentation to the immune system, plasmid DNA encoding NP of influenza A virus was injected into the muscle of mice. This resulted in the generation of NP-specific CTL and protection from a subsequent challenge with influenza A virus, as measured by decreased viral lung titers, inhibition of mass loss, and increased survival [64]. Experiments in mice have shown that intramuscular and intranasal administrations of plasmid containing HA sequences induced IgG and IgA against HA protein [65].

Many studies have demonstrated that HA-based DNA vaccines induced the protective immunity against influenza virus infection. Delivering DNA vaccines using "gene-gun" inoculation techniques has shown to induce the protection against influenza virus infection [66] and the long-life maintenance of protective B cell response and immunity [67, 68]. Gold particle-mediated epidermal deliveries, improvement of plasmid vectors, and antigen planning have been examined in order to enhance the efficacy of DNA vaccines [69-71]. Ljungberg *et al*. have successfully cloned the variable antigenic determinant domains of the HA gene derived from a strain of H3N2 subtype and inserted it into a vector, followed by the production of plasmid DNA vaccines in *E. coli* XL1 [72]. Furthermore, it has been shown that both humoral and cellular responses were induced by a very small amount of plasmid encoding HA gene [73].

Following vaccine application and uptake by host cells, the HA gene was transcribed into RNA and exported to the cytoplasm for protein synthesis. The HA protein synthesized in cells is processed *via* MHC class I proteins, which stimulates CTL through $CD8^+$ T-cell stimulation and interleukin (IL)-2 and IFN-γ, or is presented by MHC class II molecules for stimulation of humoral immunity through $CD4^+$ T-cell and ILs-4, -5, -6, -10, and -13. In this regard, the mechanism of immune induction of both neutralizing antibodies and CTL is similar to that of live-virus vaccines [70, 74-79].

Many cytokines have been examined as adjuvant because an addition of certain cytokine enhanced the immune response induced by vaccination and subsequent protection from virus infection. IL-6 molecule has been shown to enhance two immunologic responses including the induction of IgA and the stimulation of CTL. Furthermore, it has been reported that co-administration of DNA encoding IL-6 and HA genes conferred effective immune response against a lethal challenge of influenza virus, and that the long-term protection was maintained [80]. These results suggest that IL-6 molecule is useful adjuvant for vaccination against influenza virus infection.

DNA vaccines also have excellent safety profiles and have shown no toxicity unlike other vaccines. These unique features of DNA vaccine make it to be attractive. However, some problems have been remained. In some cases, no DNA vaccines have been licensed and manufactured for use because of the high cost for manufacturing and of the requirement of multiple vaccinations to achieve protective immunity. There are also some concerns with a possibility of inducing anti-double-stranded DNA antibodies and activating the Toll-like receptors. Currently, there are several DNA vaccines that have been developed for veterinary and human purposes worldwide, while none have as yet proceeded beyond phase 2 trials. Much effort is needed to improve their performance in the near future to fulfill their promise.

4.4. Universal Vaccines

It is predicted that millions people die from a new influenza pandemic before vaccination matching to the pandemic strain because it takes more than 6 months for manufacturing those vaccines. To stand up to the potential threat of influenza pandemic, it is looking forward to preparing a vaccine that can protect against any influenza viruses universally, so-called "universal vaccines".

Transmembrane M2 protein encoded viral genes is scarcely present on the virus but is abundantly expressed on virus-infected cells. The external domain, M2e, composed of 23 amino acids is highly conservative because the sequence has hardly changed since the first isolation of influenza virus strain in 1933, despite numerous epidemics and several pandemics [81, 82]. As M2e-specific antibodies have been shown to reduce the severity of infection in animals, M2e is being studied for its capability of providing protection against a broad range of influenza A virus strains. However, the antigenicity of M2e domain in humans is weak [82, 83].

The synthetic M2e peptide with both incomplete Freund's and aluminum adjuvant induced M2e-specific IgG antibodies and also provoked M2e-specific T cell response in mice. The immunization with M2e peptide protected mice from a lethal challenge with influenza virus [84]. Without adjuvant, immunization with fusion protein of M2e and Toll-like receptor ligand developed M2e-specific antibody responses that were quantitatively superior to those observed with M2e peptide delivered in aluminum [85]. Intraperitoneal or intranasal administration of M2 protein fused with hepatitis B virus core protein protected mice from a lethal challenge with various types of influenza viruses [86]. Using a synthetic multiple antigenic peptide (MAP) vaccine system, M2e-MAP induced higher levels of anti-M2e antibody against defined subtypes of viruses [87]. Recently, phase I clinical studies with M2e vaccines have been completed. Further development in humans pursue the protective efficacy of universal vaccines against influenza A viruses [88].

The fusion peptide region of HA0 is highly conserved among all HA subtypes of influenza A viruses, a part of which forms a loop on the virus-infected host cells [89]. Vaccination with a peptide spanning the HA1/HA2 joining region decreased symptoms and mortality after influenza virus infection in mice. The immunity against the HA1/HA2 joining region was more robust than M2e-specific immunity [90, 91]. These findings suggest that the HA1/HA2 joining region is a candidate for universal vaccines against influenza virus infection.

4.5. Co-administration with Adjuvants

The co-administration of immuno-stimulating complexes with vaccine has accelerated both antibody and T-cell responses [92]. Subunit influenza vaccine with adjuvant MF59, an emulsion of squalene in water for parenteral use, is licensed in some European countries. MF59 significantly increased immune responses to interpandemic influenza A and influenza B antigens, particularly in elderly with chronic diseases [93, 94]. Virosomes can be constructed from virus surface proteins embedded in phospholipids bilayers. Vaccination with the virosomes induced higher concentrations of protective antibody and higher rates of immunization than that with inactivated vaccines without adjuvant [95]. A complex of heat-labile *E. coli* toxin, lecithin as vesicles and inactivated trivalent influenza virus preparations is considered another adjuvant candidate for intranasal administration [96]. Ichinohe *et al*. have demonstrated that poly (I:C) was a new and effective intranasal adjuvant for influenza virus vaccines [97]. As previously described in the subsection of DNA vaccines, IL-6 molecule appears to be useful as an adjuvant for vaccination against influenza virus.

5. CONCLUSION

The pandemic influenza (H1N1) 2009 has been spreading to many countries and recognized as a most devastating infectious disease of this century accompanied with many deaths. Mutations in the virus genes may potentially lead more dangerous outbreaks. Monitoring antigen drift and shift in influenza A viruses is important not only to predict changes of the virulence but also to prepare influenza vaccines matching to circulating strains in a timely fashion. However, currently licensed influenza vaccines have many inevitable problems to be solved. New devices for influenza vaccination have been required and developed in order to solve the problems. As described in this article, the live CA vaccine, the reverse genetics vaccines, the DNA vaccines, the universal vaccines and the co-administration with adjuvant are promising for the new devices. We will be sure to control epidemics and pandemics of influenza virus infection using new technologies for vaccination in a near future.

REFERENCES

[1] Fraser C, Donnelly CA, Cauchemez S, *et al*. Pandemic potential of a strain of influenza A (H1N1): early findings. Science 2009; 324: 1557-61.

[2] Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, *et al*. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 2009; 360: 2605-15.

[3] Stevens J, Corper AL, Basler CF, *et al*. Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. Science 2004; 303: 1866-70.

[4] Copeland CS, Zimmer KP, Wagner KR, Healey GA, Mellman I, Helenius A. Folding, trimerization, and transport are sequential events in the biogenesis of influenza virus hemagglutinin. Cell 1988; 53: 197-209.

[5] Guo XL, Li L, Wei DQ, Zhu YS, Chou KC. Cleavage mechanism of the H5N1 hemagglutinin by trypsin and furin. Amino Acids 2008; 35: 375-82.

[6] Rumschlag-Booms E, Guo Y, Wang J, Caffrey M, Rong L. Comparative analysis between a low pathogenic and a high pathogenic influenza H5 hemagglutinin in cell entry. Virol J 2009; 6: 76.

[7] Kido H, Yokogoshi Y, Sakai K, *et al*. Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. J Biol Chem 1992; 267: 13573-9.

[8] Tashiro M, Yokogoshi Y, Tobita K, Seto JT, Rott R, Kido H. Tryptase Clara, an activating protease for Sendai virus in rat lungs, is involved in pneumopathogenicity. J Virol 1992; 66: 7211-6.

[9] Ha Y, Stevens DJ, Skehel JJ, Wiley DC. X-ray structure of the hemagglutinin of a potential H3 avian progenitor of the 1968 Hong Kong pandemic influenza virus. Virology 2003; 309: 209-18.

[10] Ha Y, Stevens DJ, Skehel JJ, Wiley DC. H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. EMBO J 2002; 21: 865-75.

[11] Perdue ML, Suarez DL. Structural features of the avian influenza virus hemagglutinin that influence virulence. Vet Microbiol 2000; 74: 77-86.

[12] Garten W, Klenk HD. Characterization of the carboxypeptidase involved in the proteolytic cleavage of the influenza haemagglutinin. J Gen Virol 1983; 64 : 2127-37.

[13] Segal MS, Bye JM, Sambrook JF, Gething MJ. Disulfide bond formation during the folding of influenza virus hemagglutinin. J Cell Biol 1992; 118: 227-44.

[14] Fouchier RA, Munster V, Wallensten A, *et al*. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005; 79: 2814-22.

[15] Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev 1992; 56: 152-79.

[16] Liu S, Ji K, Chen J, *et al*. Panorama phylogenetic diversity and distribution of Type A influenza virus. PLoS One 2009; 4: e5022.

[17] Chen JM, Sun YX, Chen JW, *et al*. Panorama phylogenetic diversity and distribution of type A influenza viruses based on their six internal gene sequences. Virol J 2009; 6: 137.

[18] Meiklejohn G, Eickhoff TC, Graves P, I J. Antigenic drift and efficacy of influenza virus vaccines. J Infect Dis 1978; 138: 618-24.

[19] Both GW, Sleigh MJ, Cox NJ, Kendal AP. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J Virol 1983; 48: 52-60.

[20] Smith GJ, Bahl J, Vijaykrishna D, *et al*. Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci USA 2009; 106: 11709-12.

[21] Zell R, Motzke S, Krumbholz A, Wutzler P, Herwig V, Dürrwald R. Novel reassortant of swine influenza H1N2 virus in Germany. J Gen Virol 2008; 89: 271-6.

[22] Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 1982; 31: 417-27.

[23] Shen J, Ma J, Wang Q. Evolutionary trends of A (H1N1) influenza virus hemagglutinin since 1918. PLoS One 2009; 4: e7789.

[24] Tsuchiya E, Sugawara K, Hongo S, *et al*. Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus. J Gen Virol 2001; 82: 2475-84.

[25] Philpott M, Hioe C, Sheerar M, Hinshaw VS. Hemagglutinin mutations related to attenuation and altered cell tropism of a virulent avian influenza A virus. J Virol 1990; 64: 2941-7.

[26] Okamatsu M, Sakoda Y, Kishida N, Isoda N, Kida H. Antigenic structure of the hemagglutinin of H9N2 influenza viruses. Arch Virol 2008; 153: 2189-95.

[27] Huang JW, King CC, Yang JM. Co-evolution positions and rules for antigenic variants of human influenza A/H3N2 viruses. BMC Bioinformatics 2009; 10(Suppl 1): S41.

[28] Ennis FA, Yi-Hua Q, Schild GC. Antibody and cytotoxic T lymphocyte responses of humans to live and inactivated influenza vaccines. J Gen Virol 1982; 58: 273-81.

[29] Couch RB. Seasonal inactivated influenza virus vaccines. Vaccine 2008; 26(Suppl 4): D5-9.

[30] Bardiya N, Bae JH. Influenza vaccines: recent advances in production technologies. Appl Microbiol Biotechnol 2005; 67: 299-305.

[31] Youil R, Su Q, Toner TJ, *et al*. Comparative study of influenza virus replication in Vero and MDCK cell lines. J Virol Methods 2004; 120: 23-31.

[32] Pau MG, Ophorst C, Koldijk MH, Schouten G, Mehtali M, Uytdehaag F. The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines. Vaccine 2001; 19: 2716-21.

[33] Tree JA, Richardson C, Fooks AR, Clegg JC, Looby D. Comparison of large-scale mammalian cell culture systems with egg culture for the production of influenza virus A vaccine strains. Vaccine 2001; 19: 3444-50.

[34] Tamura S, Tanimoto T, Kurata T. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. Jpn J Infect Dis 2005; 58: 195-207.

[35] Furuya Y, Chan J, Regner M, *et al*. Cytotoxic T cells are the predominant players providing cross-protective immunity induced by g-irradiated influenza A viruses. J Virol 2010; 84: 4212-21.

[36] Margolis KL, Nichol KL, Poland GA, Pluhar RE. Frequency of adverse reactions to influenza vaccine in the elderly. A randomized, placebo-controlled trial. JAMA 1990; 264: 1139-41.

[37] Govaert TM, Dinant GJ, Aretz K, Masurel N, Sprenger MJ, Knottnerus JA. Adverse reactions to influenza vaccine in elderly people: randomised double blind placebo controlled trial. Br Med J 1993; 307: 988-90.

[38] The American Lung Association Asthma Clinical Research Centers. The safety of inactivated influenza vaccine in adults and children with asthma. N Engl J Med 2001; 345: 1529-36.

[39] Bierman CW, Shapiro GG, Pierson WE, Taylor JW, Foy HM, Fox JP. Safety of influenza vaccination in allergic children. J Infect Dis 1977; 136: S652-5.

[40] James JM, Zeiger RS, Lester MR, *et al*. Safe administration of influenza vaccine to patients with egg allergy. J Pediatr 1998; 133: 624-8.

[41] Turner D, Wailoo A, Nicholson K, Cooper N, Sutton A, Abrams K. Systematic review and economic decision modelling for the prevention and treatment of influenza A and B. Health Technol Assess 2003; 7: iii-iv 1-170. xi-xiii.

[42] Kumagai T, Nagai K, Okui T, *et al*. Poor immune responses to influenza vaccination in infants. Vaccine 2004; 22: 3404-10.

[43] Snyder MH, Clements ML, Betts RF, *et al*. Evaluation of live avian-human reassortant influenza A H3N2 and H1N1 virus vaccines in seronegative adult volunteers. J Clin Microbiol 1986; 23: 852-7.

[44] Clements ML, Snyder MH, Buckler-White AJ, Tierney EL, London WT, Murphy BR. Evaluation of avian-human reassortant influenza A/Washington/897/80 x A/Pintail/119/79 virus in monkeys and adult volunteers. J Clin Microbiol 1986; 24: 47-51.

[45] Wareing MD, Tannock GA. Live attenuated vaccines against influenza; an historical review. Vaccine 2001; 19: 3320-30.

[46] Maassab HF, DeBorde DC. Development and characterization of cold-adapted viruses for use as live virus vaccines. Vaccine 1985; 3: 355-69.

[47] Belshe RB, Gruber WC, Mendelman PM, *et al*. Efficacy of vaccination with live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine against a variant (A/Sydney) not contained in the vaccine. J Pediatr 2000; 136: 168-75.

[48] Chen H, Matsuoka Y, Swayne D, *et al*. Generation and characterization of a cold-adapted influenza A H9N2 reassortant as a live pandemic influenza virus vaccine candidate. Vaccine 2003; 21: 4430-6.

[49] Goings SA, Kulle TJ, Bascom R, *et al*. Effect of nitrogen dioxide exposure on susceptibility to influenza A virus infection in healthy adults. Am Rev Respir Dis 1989; 139: 1075-81.

[50] i S, Liu C, Klimov A, *et al*. Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. J Infect Dis 1999; 179: 1132-8.

[51] Suguitan AL Jr, McAuliffe J, Mills KL, *et al*. Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. PLoS Med 2006; 3: e360.

[52] Belshe RB, Mendelman PM, Treanor J, *et al*. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. N Engl J Med 1998; 338: 1405-12.

[53] Edwards KM, Dupont WD, Westrich MK, Plummer WD Jr, Palmer PS, Wright PF. A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. J Infect Dis 1994; 169: 68-76.

[54] Gorse GJ, Belshe RB, Munn NJ. Superiority of live attenuated compared with inactivated influenza A virus vaccines in older, chronically ill adults. Chest 1991; 100: 977-84.

[55] Romanova J, Katinger D, Ferko B, *et al*. Live cold-adapted influenza A vaccine produced in Vero cell line. Virus Res 2004; 103: 187-93.

[56] Gendon IuZ. Advantages and disadvantages of inactivated and live influenza vaccine. Vopr Virusol 2004; 49: 4-12.

[57] Mostow SR, Schoenbaum SC, Dowdle WR, Coleman MT, Kaye HS, Hierholzer JC. Studies on inactivated influenza vaccines. II. Effect of increasing dosage on antibody response and adverse reactions in man. Am J Epidemiol 1970; 92: 248-56.

[58] Subbarao K, Chen H, Swayne D, *et al*. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. Virology 2003; 305: 192-200.

[59] Wood JM, Robertson JS. From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. Nat Rev Microbiol 2004; 2: 842-7.

[60] tech J, Garn H, Wegmann M, Wagner R, Klenk HD. A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin. Nat Med 2005; 11: 683-9.

[61] Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA 2000; 97: 6108-13.

[62] Ozaki H, Govorkova EA, Li C, Xiong X, Webster RG, Webby RJ. Generation of high-yielding influenza A viruses in African green monkey kidney (Vero) cells by reverse genetics. J Virol 2004; 78: 1851-7.

[63] Wolff JA, Malone RW, Williams P, *et al*. Direct gene transfer into mouse muscle *in vivo*. Science 1990; 247: 1465-8.

[64] Ulmer JB, Donnelly JJ, Parker SE, *et al*. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993; 259: 1745-9.

[65] Soboll G, Nelson KM, Leuthner ES, *et al*. Mucosal co-administration of cholera toxin and influenza virus hemagglutinin-DNA in ponies generates a local IgA response. Vaccine 2003; 21: 3081-92.

[66] Kodihalli S, Goto H, Kobasa DL, Krauss S, Kawaoka Y, Webster RG. DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. J Virol 1999; 73: 2094-8.

[67] Justewicz DM, Webster RG. Long-term maintenance of B cell immunity to influenza virus hemagglutinin in mice following DNA-based immunization. Virology 1996; 224: 10-7.

[68] Kodihalli S, Haynes JR, Robinson HL, Webster RG. Cross-protection among lethal H5N2 influenza viruses induced by DNA vaccine to the hemagglutinin. J Virol 1997; 71: 3391-6.

[69] Sharpe M, Lynch D, Topham S, Major D, Wood J, Loudon P. Protection of mice from H5N1 influenza challenge by prophylactic DNA vaccination using particle mediated epidermal delivery. Vaccine 2007; 25: 6392-8.

[70] Jiang Y, Yu K, Zhang H, *et al.* Enhanced protective efficacy of H5 subtype avian influenza DNA vaccine with codon optimized HA gene in a pCAGGS plasmid vector. Antiviral Res 2007; 75: 234-41.

[71] Wang S, Taaffe J, Parker C, Solórzano A, Cao H, García-Sastre A, Lu S. Hemagglutinin (HA) proteins from H1 and H3 serotypes of influenza A viruses require different antigen designs for the induction of optimal protective antibody responses as studied by codon-optimized HA DNA vaccines. J Virol 2006; 80: 11628-37.

[72] Ljungberg K, Wahren B, Almqvist J, Hinkula J, Linde A, Winberg G. Effective construction of DNA vaccines against variable influenza genes by homologous recombination. Virology 2000; 268: 244-50.

[73] Forde GM. Rapid-response vaccines - does DNA offer a solution? Nat Biotechnol 2005; 23: 1059-62.

[74] Fynan EF, Robinson HL, Webster RG. Use of DNA encoding influenza hemagglutinin as an avian influenza vaccine. DNA Cell Biol 1993; 12: 785-9.

[75] Kodihalli S, Haynes JR, Robinson HL, Webster RG. Cross-protection among lethal H5N2 influenza viruses induced by DNA vaccine to the hemagglutinin. J Virol 1997; 71: 3391-6.

[76] Le Gall-Reculé G, Cherbonnel M, Pelotte N, Blanchard P, Morin Y, Jestin V. Importance of a prime-boost DNA/protein vaccination to protect chickens against low-pathogenic H7 avian influenza infection. Avian Dis 2007; 51: 490-4.

[77] Robinson HL, Hunt LA, Webster RG. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine 1993; 11: 957-60.

[78] Suarez DL, Schultz-Cherry S. The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Dis 2000; 44: 861-8.

[79] Seder RA, Gurunathan S. DNA vaccines--designer vaccines for the 21st century. N Engl J Med. 1999; 341: 277-8.

[80] Lee SW, Youn JW, Seong BL, Sung YC. IL-6 induces long-term protective immunity against a lethal challenge of influenza virus. Vaccine 1999; 17: 490-6.

[81] Pinto LH, Lamb RA. The M2 proton channels of influenza A and B viruses. J Biol Chem 2006; 281: 8997-9000.

[82] Fiers W, De Filette M, Birkett A, Neirynck S, Min Jou W. A "universal" human influenza A vaccine. Virus Res 2004; 103: 173-6.

[83] Feng J, Zhang M, Mozdzanowska K, *et al.* Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. Virol J 2006; 3: 102.

[84] Wu F, Huang JH, Yuan XY, Huang WS, Chen YH. Characterization of immunity induced by M2e of influenza virus. Vaccine 2007; 25: 8868-73.

[85] Huleatt JW, Nakaar V, Desai P, *et al*. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 2008; 26: 201-14.

[86] Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med 1999; 5: 1157-63.

[87] Mozdzanowska K, Zharikova D, Cudic M, Otvos L, Gerhard W. Roles of adjuvant and route of vaccination in antibody response and protection engendered by a synthetic matrix protein 2-based influenza A virus vaccine in the mouse. Virol J 2007; 4: 118.

[88] Chun S, Li C, Van Domselaar G, *et al.* Universal antibodies and their applications to the quantitative determination of virtually all subtypes of the influenza A viral hemagglutinins. Vaccine 2008; 26: 6068-76.

[89] Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell 1998; 95: 409-17.

[90] Horváth A, Tóth GK, Gogolák P, *et al.* A hemagglutinin-based multipeptide construct elicits enhanced protective immune response in mice against influenza A virus infection. Immunol Lett 1998; 60: 127-36.

[91] Bianchi E, Liang X, Ingallinella P, *et al*. Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. J Virol 2005; 79: 7380-8.

[92] Rimmelzwaan GF, Nieuwkoop N, Brandenburg A, *et al*. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines. Vaccine 2000; 19: 1180-7.

[93] Banzhoff A, Pellegrini M, Del Giudice G, Fragapane E, Groth N, Podda A. MF59-adjuvanted vaccines for seasonal and pandemic influenza prophylaxis. Influenza Other Respi Viruses 2008; 2: 243-9.

[94] O'Hagan DT, Wack A, Podda A. MF59 is a safe and potent vaccine adjuvant for flu vaccines in humans: what did we learn during its development? Clin Pharmacol Ther 2007; 82: 740-4.

[95] Sambhara S, Kurichh A, Miranda R, *et al*. Heterosubtypic immunity against human influenza A viruses, including recently emerged avian H5 and H9 viruses, induced by FLU-ISCOM vaccine in mice requires both cytotoxic T-lymphocyte and macrophage function. Cell Immunol 2001; 211: 143-53.

[96] Tamura SI, Kurata T. A proposal for safety standards for human use of cholera toxin (or Escherichia coli heat-labile enterotoxin) derivatives as an adjuvant of nasal inactivated influenza vaccine. Jpn J Infect Dis 2000; 53: 98-106.

[97] Ichinohe T, Watanabe I, Ito S, *et al.* Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol 2005; 79: 2910-9.

Received: January 10, 2010 Revised: May 05, 2010 Accepted: May 09, 2010