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MODERN MARKET OF INFLUENZA VACCINE PRODUCTION

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ABSTRACT

Influenza virus is a negative stranded RNA virus that causes seasonal flu infections and is the reason for several epidemics that have occurred in the previous century. The high mutagenicity of the virus is mediated by error-prone RNA polymerase, which incorporates mutations into the viral genome during every replication cycle. Mutations lead to the emergence of new virus clades within subgroups, which leads to the periodic reevaluation of seasonal and pandemic vaccine contents. Vaccines usually prevent severe symptoms of flu infection only if a patient is vaccinated and infected with the same influenza subtype.

Hence, there is the constant possibility of the emergence of new influenza pandemic virus. This could be prevented by either increasing the scope of vaccine efficiency or by developing methods of rapid vaccine production against any emerging subtype or clade.

This article reviews vaccines against influenza virus subtypes, and modern and prospective alternative ways to increase vaccine range, breadth, and efficiency in both healthy adults and people in risk groups.

Key words: vaccine, influenza, epidemics, hemagglutinin, neuraminidase, T-cells

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INTRODUCTION

Influenza viruses cause flu in mammals and aquatic birds. The most popular hosts for the virus are humans, pigs, horses, chicken, ducks, pigeons and salmons. Three types of influenza are distinguished, according to the hosts, varieties in molecular structure and genetic sequences. Influenza A is the most common: it had caused pandemic outbreaks in the past and has a wide host variety, including humans, swine and chicken. Influenza type B is pathogenic only for humans, while type C affects both swine and humans [1,2].

As the influenza type A has caused all outbreaks of flu pandemics during previous century, the vaccines are mostly developed against this flu type. Vivid examples are H3N2v, H5N1, H5N6, H6N1, H7N3, H7N9 and H10N8 from animal reservoir, which have caused morbidity and mortality, so the development of vaccines against this pre-pandemic potential threat is significant. Influenza type B lacks an animal reservoir that might be a proposal of its inability to cause pandemics [1].

Molecular structure of influenza virus

Influenza virus is asingle stranded RNA virus. The genome consists of six-eight separate negative-sense RNA strands; only the virion containing all RNA strains is able to infect its host. Genome segments encode one protein or two with similar functions [1].

The genome encodes eleven proteins of influenza virus, nine of which are assembled into new virions. Two nonstructural (NS1 and NS2 or NEP) proteins facilitate virion assembly, others included are RNA polymerase proteins: basic 1 (PB1), basic 2 (PB2) and acidic (PA), external glycoproteins hemagglutinin and neuraminidase (HA and NA, respectively), matrix protein 1 (M1), surface protein (M2), and nucleoprotein (NP) [2].

RNA polymerase of influenza type A is error prone, and it incorporates mutations into nucleotide sequence almost per replication cycle [3]. However, some protein regions contain more conserved sequences, because they are significant for the virus functionality and are supported by evolutionary natural selection forces. The HA head domain contains more variable sites in nucleotide sequence and it is more immunodominant, while HA stem is more conserved [4]. The general structure of the most immunodominant molecule – hemagglutinin is on Figure 1.

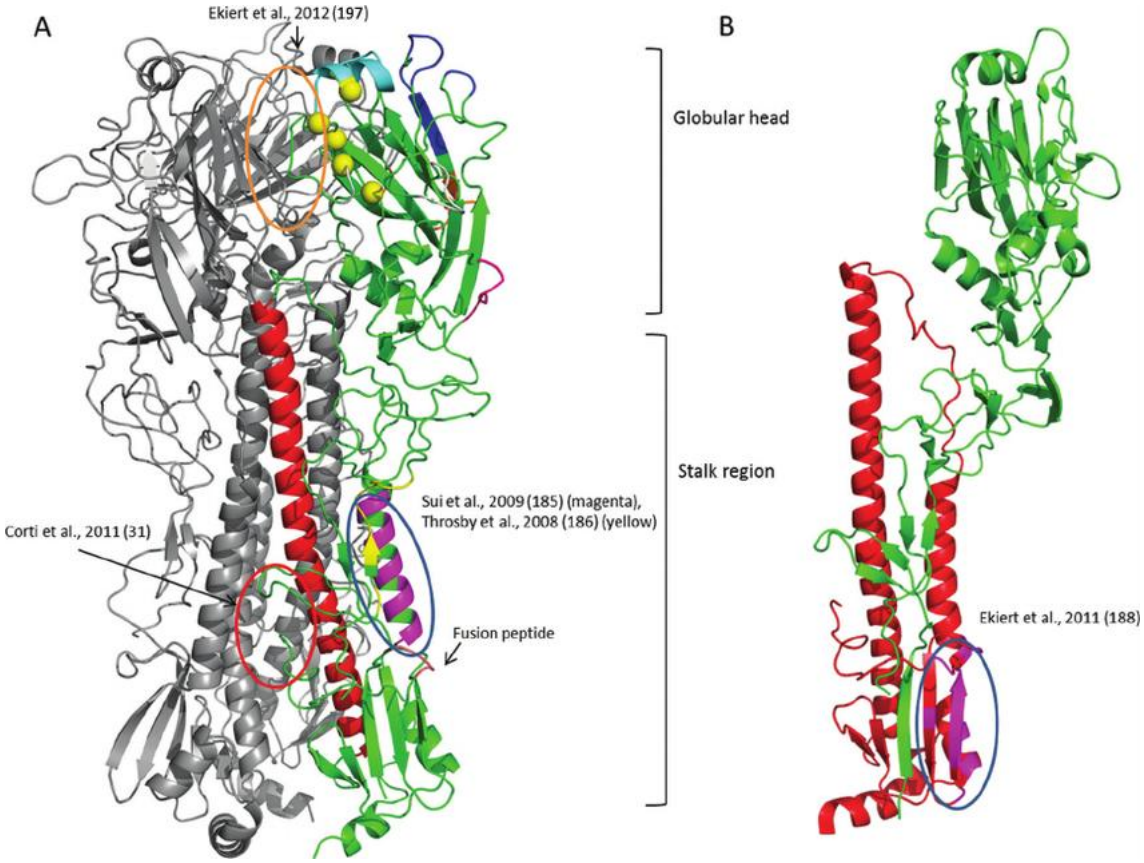

Fig. 1. Schematic representation of hemagglutinin molecular structure [4].

The immunodominance mediates antibody production mostly against HA headand other viral proteins are left untouched by immune system of a host. If the mutation has evolved within HA head, the immune system would need time to synthesize new antibodies against them. However, if the antibodies were also produced against HA stem or NP protein, the influenza infection could be less severe, as immune system would be triggered and activate its humoral and cellular responses.

New antigenic variants of A/H3N2 viruses appear every 3–5 years, whereas new antigenic variants of A/H1N1 and influenza B viruses appear less frequently (2–5 years for A/H3N2 viruses compared with 3–8 years for A/H1N1 and influenza B viruses) [5].

Viral entrance into the host and activation of immune responses

Upon initial exposure to the virus, the mucosal barrier creates a layer of mucus rich in sialic acid, which acts as a decoy by binding to the viral HA protein and thus traps a substantial portion of viruses, effectively reducing the infectious dose [6].

Mucosal responses are largely mediated by immunoglobulin A (IgA) antibodies in the upper respiratory tract, which are able to cross the epithelial barrier from the blood to the upper respiratory tract lumen in order to bind to and neutralize influenza viruses, thus preventing the infection of host cell [7].

Upon viral entrance into the cell, Toll-like receptor signaling and interferon-mediated responses are triggered [1]. This process results in the activation of an antiviral state that limits the permeability of neighboring cells to virus particles. The efficiency of innate immune selection is dependent on infectious dose.

RNA viruses such as bunyaviruses, paramyxoviruses and rhadboviruses replicate in the cytoplasm, but influenza virus, to the contrary, replicates in the nucleus [1]. In that site the virus steals a 5-capped end of host cell mRNAs and thus increases the synthesis of its own proteins, enlarging new virions concentration.

The virus replicates in upper respiratory tract of a human, because its HA molecule binds to α2-6 sialylated receptors mostly expressed there. The transmission of the influenza from human to human is promoted by respiratory droplets, direct contact and fomites.

The immune system of a human is ready to fight the flu by its own resources. However, the risk group is children, people with chronic disease, pregnant women and the older over 60 years old. To prevent and reduce flu infections risks, the vaccines are used to teach immune system recognize the pathogen and activate B-lymphocytes production [6].

T-cell response in humans is targeted against several types of internal influenza proteins: HA, NA, M1 M2, and NP, but in different levels. M1 and NP proteins are more conserved than HA and N. So, M1 and NP induce cytotoxic T lymphocyte (CTL) responses, which are cross reactive within different subtype clade. This feature is not able to prevent infection at all, but it can greatly reduce the severity, duration and lethality of the infection [7].

Molecular structure of the most common vaccines

There are three main types of vaccines available in actual market: the parenteral inactivated influenza vaccine, the intranasal live attenuated influenza vaccine, and recombinant HA vaccines. [6]

The inactivated influenza vaccine (IIV) contains viral subunits or a split virion or recombinant HA based vaccines that are administered intramuscularly. The vaccines are standardized according to hemagglutinin level, commonly 15 μg HA per strain. However, for people in risk group vaccines containing 60 μg HA per strain have recently been licensed [6-8].

There are two main types of IIV: tertiary and quaternary split virions. Tetriary IIV consists of two influenza A strains and one influenza B strain, which are currently circulating in the surroundings. The most popular trivalent influenza vaccine covers influenza A H3N2, H1N1 and one influenza B strain.

Quaternary IIV contains two influenza A and two influenza B strains, thus providing wider protection. Two types of administration are used in USA: 15mg of each purified HA protein intra-muscularly or 9mg of each purified HA protein administered

intradermally. The type of administration is related to the infection severity and patient's age and condition [6].

Five main companies currently offer trivalent or quadrivalent seasonal influenza vaccines: GlaxoSmithKline, Green Cross Corp., Hualan Bio, Novartis, and Sanofi Pasteur. Seasonal trivalent LAIV are mostly offered by Serum Institute of India. Split virions and engineered vaccines require WHO qualification [9].

The IIVs are used for all age groups starting from infants older than 6 months. They induce a strain-specific serum IgG antibody response and are effective against current circulating strain type [10].

The second popular vaccine product is the live attenuated influenza vaccine (LAIV). It was firstly used in Russia, and now is shipped worldwide.

Attenuated vaccines are produced by random mutagenesis followed by several rounds of selection in special, usually non-physiological conditions. This process requires time and usually produces few prospective vaccine candidates. In addition, these vaccines should be reformulated annually due to the frequent incorporation of new mutations and antigenic drift and low cross-protection against other strains [11].

Recent findings also suggest that in USA and China the quadrivalent LAIVs used over 2015-2018 years have not been protective as stated during the development [12].

This vaccine also contains a mixture of the same four influenza strains as the quaternary inactivated influenza vaccines, but is administered intranasally as a spray. The LAIV contains live viruses with temperature-sensitive and attenuating mutations [13].

LAIV are produced by reverse genetic tool by fusing HA and NA genes from circulating viruses with an attenuated, temperature-sensitive, cold adapted virus backbone. The structure of backbone prevents replication at temperatures above 33 °C, which is a general temperature of upper respiratory tract. That implies that the virus cannot replicate in lower respiratory tract and thus causes much milder type of infection.

LIAV production was licensed in Russia in 1980 year, and then USA made an independent research to generate and license its own LIAV in 2003 [11].

Vaccination with LAIV results in the production of strain-specific serum IgG as well as mucosal IgA and T cell responses [13]. LAIV is also effective against some antigenically drifted strains of influenza.

The advantage of LAIV over IIV is the stimulation of both humoral and cell-mediated immune responses [12]. It includes IgG, IgA, and antigen-specific cytokine-secreting T-cells activation. IgG neutralizes HA and NA antibodies, while IgA acts locally.

LAIV and IIV both require chicken embryonated eggs for vaccine production. This is a significant disadvantage, because the generation of a new vaccine requires time, and in the case of epidemics there could be a problem to satisfy egg need. However, the third licensed vaccine type is recombinant HA vaccine, which contains HA proteins split from different subtypes. The example is FluBlok, and HA proteins are expressed in insect cells by baculovirus vectors [12].

Recombinant HA vaccines could be alternatively produced by the binding of HA peptides to the Ii-Key moiety of the class 2 Major Histocompatibility Complex. This vaccine was firstly produced by Antigen Express. Other common examples are FluBlock manufactured by Protein Sciences Corporation and Flucelvax by Novartis (fig.2).

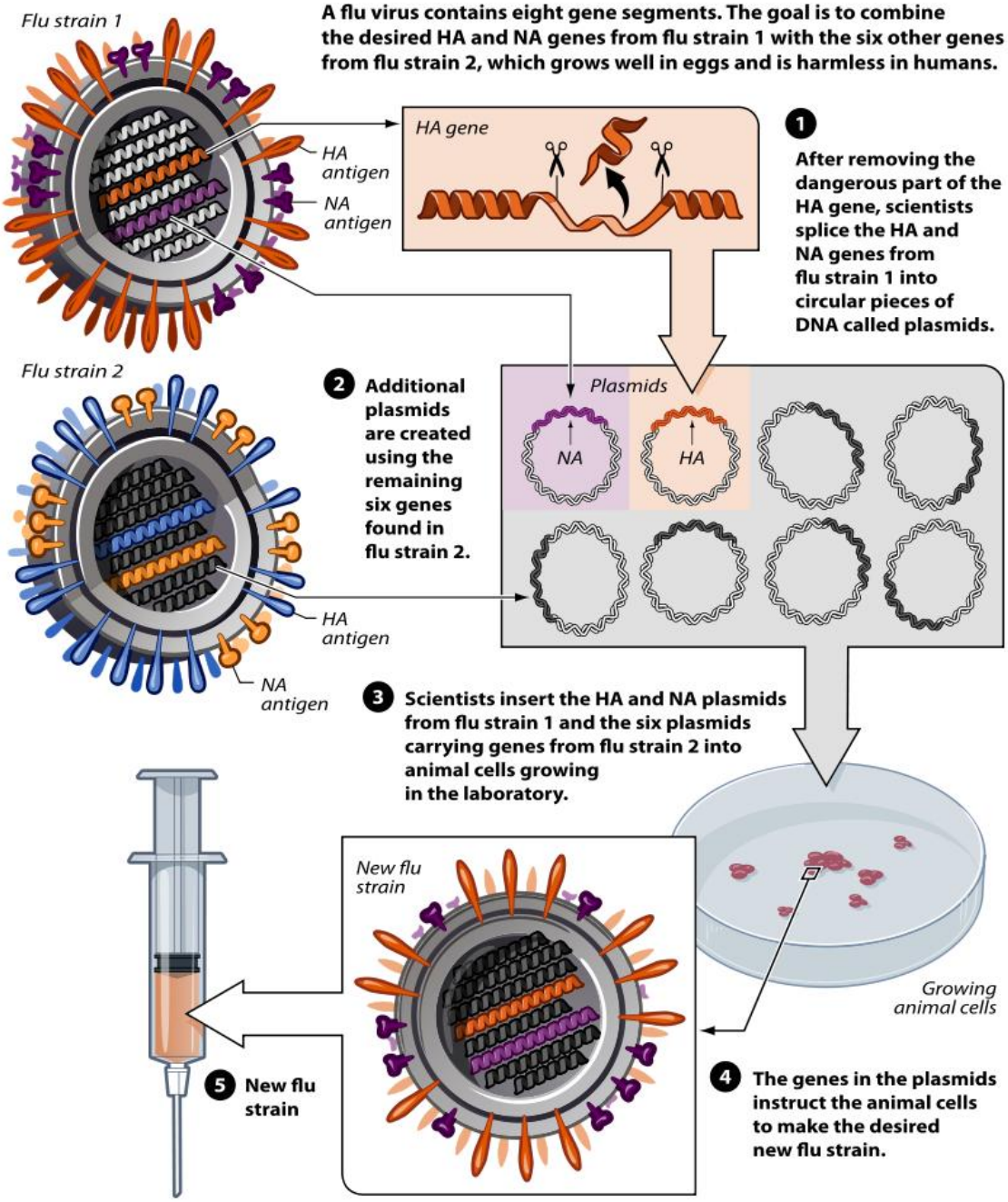

Fig. 2. The development of influenza vaccine by the use of recombinant genetic technique [14] .

The fusion constructs could be also generated with other influenza proteins or immune-stimulant molecules [9].

Ways to improve the scope of vaccines

Vaccines are working against a particular strain, sometimes against two or three influenza clades or subtypes. Vaccine does not prevent a receiver from an influenza attack if the strain contains any changes within the protein content.

The research worldwide mostly aims to enhance the scope of vaccine efficiency by increasing protection at least within influenza clades, and then intra- and inter-groups. Theoretically, this might be done by inducing antibodies against other influenza molecules, by reducing immunodominance of HA head and by upgrading vaccine manufacture.

One of the perspective approaches to widen the breadth of vaccine efficiency is to induce antibodies formation directed at the conserved region of HA stem.

HA consists of a stem and a globular head. The head contains the receptor binding site and several well-defined antigenic sites, which accumulate mutations per replication cycle during the influenza adaptation under immune pressure [1]. So, the majority of antibodies generated during the infection target HA head, because it excites strong immune response. On the contrary, HA stem is much more conservative. Antibodies targeting HA stem are cross-reactive and potentially could be induced during different clade infection.

HA proteins are phylogenetically classified into two groups. The first group includes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18, while group 2 comprises H3, H4, H7, H10, H14, and H15 [1].

HA stem antibodies could potentially recognize subtypes within the same group, and a few demonstrate binding even across both groups. There are several assumptions how stem antibodiesfunction. They inhibit fusion of the viral membrane through steric hindrance, or maturation of the virus if the antibody binds to the uncleaved HA protein, or clear infected cells through antibody-dependent cell-mediated cytotoxicity (ADCC). [15].

Another strategy to trigger a broader and more stable immune response against seasonal influenza is based on heterologous prime–boost experiment. This type of regimen has already been tested in mice, in ferrets and in nonhuman primates; however it requires investigation of humans before its introduction into the global market. A DNA vaccine with a hemagglutinin from a seasonal influenza virus is administered first (prime), followed by the administration of typical trivalent IIV (boost).

Mice that received the prime–boost regimen obtained much milder infection mediated by broader immunity.

To increase vaccine production, the time consumed for the preparation could be reduced by the usage of special cell lines or cellular substrates. In addition, cellular substrates are able to reduce the need of embryonated chicken eggs needed for vaccine formulation and facilitate process scaling. Widely used cell lines are Madin–Darbey canine kidney cells, Vero cells (African green monkey) and Per.C6 cells (human), which were established for influenza virus vaccine production [5, 14, 11]

Other common expression systems for influenza proteins are baculoviruses, insects, *Nicotiana* species due to *Agrobacterium* species, and *Escherichia coli*.

Vaccines production in plants

Some companies already produce or develop vaccines against influenza by using plants expression system such as *Nicotiana* species. Influenza antigens are produced in agroinfiltrated *N. benthamiana* plants as an HA antigenic domains fused with a carrier protein.

The protein expression in plants is much easier in comparison with microorganisms or insects, because the care of plants is unpretentious. *Nicotiana* species require good aeration, temperature around 37˚C, sufficient humidity and moderate watering.

The production of recombinant influenza antigens in plants is performed through the expression of the HA ectodomain bound to a KDEL peptide. This is done to increase accumulation through retention in the endoplasmic reticulum together with a poly-histidine purification tag. Examples are HA ectodomains from a human seasonal influenza strain (A/Wyoming/03/03 (H3N2) and highly pathogenic avian strains A/Indonesia/5/05, A/Bar-headed Goose/Qinghai/1A/05 and A/Anhui/1/05 [16].

Proteins produced through the plant expression might lack a sufficient glycosylation profile, thus decreasing the level of HA or NA recognition by host

antibodies. This also affects the rate of antibodies production. To overcome this problem, plants might be genetically altered to increase glycosylation or chemically induced

In addition, the proteins should be investigated of lipid content and residual impurities presence. If present, antibodies production and functioning should be investigated, because altered proteins might induce a different immunogenic profile. Methods of purification could be also applied to reduce risks concerning vaccine production and efficiency [17].

One of the influenza vaccines produced through plants expression is based on influenza virus-like particles (VLP), noninfectious virion.

Medicago based in Quebec, Canada, has developed a plant-based VLP manufacturing platform, which allows the large-scale production of influenza VLPs. The platform introduces complex-type *N*-glycans having core α(1,3)-fucose, core β(1,2)-xylose epitopes and Lewis^a extensions. However, clinical trials have shown that neither hypersensitivity nor IgG or IgE activation directed towards those glycan structures took place. Plant made influenza vaccines are pure VPL preparations: they do not contain contaminants from extraction enzymes nor Agrobacterium specific molecules. However, they do contain glucosylceramides from plant lipids [17].

In addition, plants cannot synthesize sialic acid residues, so all the glycoproteins obtained do not contain sialylated substrates for cell surface attachment. So, those virus-like particles are perspective for pandemic vaccine, because only HA coding sequence is required to initiate vaccine expression, which greatly reduces time and process of its manufacturing. HA usage in VLPs upon expression in *N. benthamiana* has now been demonstrated for several HAs from type A influenza, including H2, H3, H6 and H9, and from type B influenza (HAB) [18].

Alternative approaches for novel vaccine types

Nowadays, HA is the most prominent molecule used to generate antibodies against influenza. However, high mutation rate in HA head requires constant reevaluation of seasonal vaccines, which takes time and resources. In case of pandemics, accessible amount of vaccine could rapidly finish as it had happened in 2009. The production of new HA vaccines takes time, efforts and resources, so it is more perspective to base vaccines on other molecules such as M or NP, or construct vaccines alternatively [6]

Recently, the M protein of influenza started to be a potential target in vaccine engineering. Several studies deeply investigated the efficacy of vaccines targeting ion channel protein.

Wang et al used a transposon mutagenesis to generate a set of M gene mutants. [12] The M gene codes for M1 and M2 ion channel proteins to generate a metabolite exchange with the environment. The goal was to identify a mutant strain, which would successfully trigger an immune response in mice and would cause only mild symptoms of infection.

The mice were infected with obtained mutant strains following viral inoculation. According to the research results, three main clusters of mutant strains were identified bearing different replication profiles. The first cluster contained infection causing viruses due to their rapid replication cycle. The second one consisted of viruses with slow replication profile, which could be explained by strong immune response or decreased viral fitness. And the third group, the most suitable one, was represented by rapidly replicating viruses during first 6 infection days followed by immune suppression. From the later one a perspective strain W-7791 was selected to be used in vaccines. Mice were vaccinated by a vaccine containing W-7791 and even the first administration of W-7791 protected animals from lethality caused by influenza

heterologous strain. W-7791 elicited a strong immune response, including T-cell activation and antibody synthesis [19].

M2 vaccines could also become a prospective vaccine target. It activates synthesis of antibodies and increases antibody dependent cell cytotoxicity. This type of vaccine generates broader immune response, because M2 contains more conservative regions in comparison with HA and NA. Thus it could trigger immune response even during infection by another influenza clade [19].

M2e vaccine constructs are tetrameric and multimeric M2e, VLP-displayed M2e and flagellin-fused M2e. All of them were tested in mice and primates and showed suitable efficiency against a panel of divergent influenza viruses. M2e-based vaccines protection is probably mediated by ADCC.

However, M1 and M2 vaccines are still in need to be reevaluated per year due to antigenic shift and incorporated mutations, which in turn leads to the emergence of novel viruses.

COBRA

COBRA HA is a good potential pool for influenza vaccines generation. COBRA is a consensus sequence, which represent the most common amino acids on each HA position. The sequence was produced through HA protein analysis isolated from vast influenza samples.

Conserved HA regions induce the production of cross-reactive antibodies, which results in less pathology once influenza infection occurs. H5N1 COBRA vaccine was shown to be an effective prophylaxis against multiple H5N1 clades in monkeys [20].

Viral vectors

Viral vectors are non-pathogenic genetic tool, which allows direct introduction of target proteins into the cell. By the use of viral vectors, high and stable amount of antigens against influenza could be produced within the host cell [21]. Viral vectors are able to function as adjuvants, increasing the immune response in the host.

An example of this vaccine type is a modified vaccinia virus Ankara (MVA), which expresses a fusion protein of influenza NP and matrix 1 protein (M1). NP and M1 contained within the vaccine induce T cell response which is not followed by antibodies neutralization [21].

Adjuvants

Another important approach is to use viral vectors together with adjuvants. Vaccine targets synthesis of antibodies against neuraminidase and broadens immune response by activating innate immune signaling. The advantages of using this type of vaccine are increased immunogenicity and small dosage.

One of the most formulated adjuvants is MF59 and AS03 and it was shown that they are able to enhance vaccines effectiveness [11, 22].

MF59 vaccine was licensed by WHO and marketed in many European countries under the name Fluad manufactured by Novartis, Switzerland.

Iron particles

A vaccine based on influenza HA trimers bound to the ferritin particles was shown to induce stronger and broader immune response [11].

CONCLUSION

Influenza viruses present a constant threat to the health of all humans. The periodic outburst of influenza pandemics or epidemics requires annual revision of

vaccines available on the global market. Vaccines accessible often show low efficacy of protection when the seasonal flu does not match viral particles contained within the vaccine.

So in order to achieve wider protection towards influenza several methods are studies and already under use including adjuvants, plants expression, different molecular targets instead of common HA and NA. All the clinical trials and research are mostly aimed to construct a universal vaccine, which would give a wide HA intra and inter-group protection.

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