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REVIEW

Trends in development of the influenza vaccine with broader cross-protection

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Summary. – Influenza A viruses cause in humans acute respiratory infections, which spread yearly in the form of epidemics or pandemics. A high variability and broad host specificity of influenza A viruses are the main reasons of repeated influenza infections. Therefore, no effective prevention against influenza is available today. The main problem of insufficient protection efficacy is that virus-neutralizing antibodies induced by current vaccines are closely strain-specific and the vaccines need to be updated each year. Therefore, various novel approaches to vaccine preparation have been developed with the aim to widen the spectrum of their efficacy. These approaches comprise using new adjuvants as components of the inactivated vaccines, new techniques of live attenuated vaccine preparation (reverse genetics), and new vaccine design focused on the conserved antigens of influenza A viruses inducing protective immunity not only against the influenza viruses antigenically similar (homologous) to vaccine strains, but also against heterologous viruses, even of different subtypes. In this review examples of new approaches to the induction of intersubtype immunity against influenza and their utilization in vaccine preparation are described.

Keywords: influenza A virus; immune response; adjuvants; conserved antigens; live vaccine; vaccine efficacy

Contents:

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Abbreviations: APC(s) = antigen presenting cell(s); ca = cold adapted; CTB = cholera toxin B; CTL = cytotoxic T lymphocyte; HA = haemagglutinin; HA1 gp = HA glycopolypeptide 1; HA2 gp = HA glycopolypeptide 2; IL = interleukin; IFN = interferon; LTB = termolabile toxin B from *E. coli*; MAb = monoclonal antibody; M1 = matrix protein 1; M2 = ion channel protein; M2-e = extracellular domain of M2 protein; MHC = major histocompatibility complex; NA = neuraminidase; NEP = nuclear export protein; NK cells = natural killer cells; NP = nucleoprotein; NS = non-structural protein; RNP = ribonucleoprotein; Th cells = T helper cells; TLR3 = toll-like receptor 3; TNFα = tumor necrosis factor α

1. Introduction

The influenza A viruses are human pathogens causing acute respiratory infection sometimes with fatal impact. Worldwide, about half a million people are dying each year in the consequence of influenza infection (Wright *et al*., 2007).

Though these viruses have been intensively studied for many years, there is no universal prevention against influenza. The reason is the unpredictable variability of influenza A viruses as well as their ability to infect a broad spectrum of hosts. Besides humans, influenza A viruses infect also other mammals and birds.

Influenza A viruses are divided into subtypes according to the antigenic properties of two surface antigens – hemagglutinin (HA) and neuraminidase (NA). Up to now, 16 HA and 9 NA subtypes have been recognized. Since all of them were found in waterfowl, it is believed that aquatic birds serve as a natural reservoir of influenza A viruses (Fouchier *et al.*, 2005). However, only three HA subtypes (H1-H3) and two NA subtypes (N1, N2) of influenza virus circulate in human population. Avian influenza viruses are not commonly transmitted to the humans, but since 1997 several cases of the human infection with influenza virus of avian origin with severe symptoms and even fatal impact have been described (Gillim-Ross and Subbarao, 2006; Sandrock and Kelly, 2007). Recently, a growing number of human infections with a new influenza A virus of swine origin of H1 subtype have been reported (Shinde *et al.*, 2009; Garten *et al.*, 2009; Smith *et al.*, 2009; Itoh *et al.*, 2009; Wood, 2009). The threat of a pandemic caused by a new influenza virus of HA subtype, which previously did not infect humans and against which there is no immunity in human population (Michaelis *et al.*, 2009), led to the development of a new vaccine preparation strategy.

2. Structure and biological properties of influenza A viruses

Influenza virus is a 80–120 nm large particle of spherical shape composed of the viral "core" (Fig. 1) consisting of nucleocapsid and matrix protein M1, which is surrounded by a lipid bilayer (Ruigrok, 1998). Viral genome consists of 8 ssRNA segments of negative polarity. Together 13,000–14,000 nucleotides of genomic RNA comprise the genetic information for 12 viral proteins, of which 9 are structural components of the virus. Five proteins form the viral core: nucleoprotein (NP), polymerase proteins PA, PB1, PB2, and nuclear export protein (NEP), previously considered as non-structural protein NS2 (Richardson and Akkina, 1991; Steinhauer and Skehel, 2002; Palese and Shaw, 2007). M1 protein mediates interaction between core proteins and transmembrane proteins in the lipid bilayer. The surface glycoproteins (HA and NA) and non-glycosylated protein (M2) with ion channel activity are embedded in the viral membrane. M1 protein is the most abundant virion protein that participates in the export of ribonucleoprotein (RNP) from nucleus and in the inhibition of viral mRNA transcription (Martin and Helenius, 1991). Genome of influenza

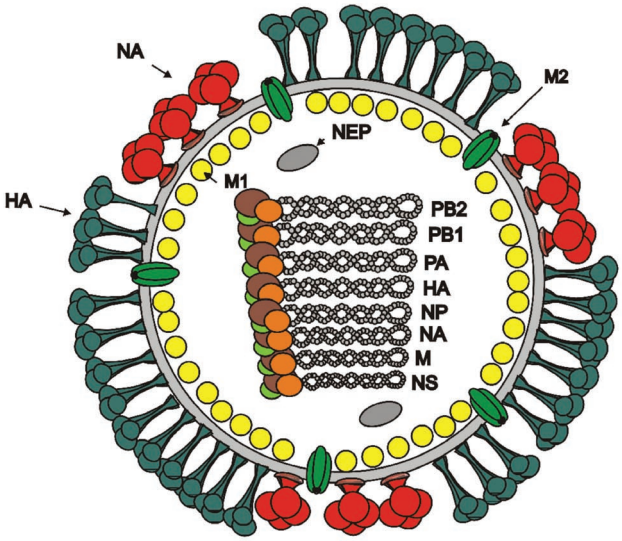

Fig. 1

Schematic structure of influenza A virus

HA – hemagglutinin; NA – neuraminidase; M1 – matrix protein; M2 – protein with ion channel activity; NP – nucleoprotein; PA, PB1, PB2 – RNA polymerase complex proteins; NEP –nuclear export protein.

A virus encodes also 3 non-structural proteins: NS1, PB1-F2 and recently described protein N40. NS1 blocks synthesis of IFNα that binds to the dsRNA and inhibits processing of host pre-mRNAs (Talon *et al.*, 2000). PB1-F2 protein interacts with the inner mitochondrial membrane and affects apoptosis of the infected cells (Chen *et al.*, 2001; Zamarin *et al.*, 2005). The function of N40 protein has not been elucidated until now (Wise *et al.*, 2009). Each segment of RNA genome is associated with NP and forms circular helical structure of RNP. Each segment of RNP contains RNA polymerase complex composed of PB1, PB2 and PA proteins. Genomic RNA(-) serves as a template for transcription into mRNA(+) and replication via complementary cRNA(+) intermediate. Transcription and replication of viral RNA occurs in the cell nucleus (Mikulášová *et al.*, 2000; Elton *et al.*, 2006; Palese and Shaw, 2007).

Due to the low fidelity of viral RNA-polymerase, influenza virus genome undergoes frequent changes that are of special significance in the case of segments encoding HA and NA. The impact of these changes (substitution or deletion) depends on their extent. The accumulation of point mutations in genes encoding HA or NA together with the selection pressure of the immune system leads to the amino acid changes in viral surface antigens resulting in small antigenic changes known as "*antigenic drift*". The segmented genome enables also another genetic change, reassortment of the RNA segments. It occurs after simultaneous infection of the host by two or more influenza A viruses, what produces the chance

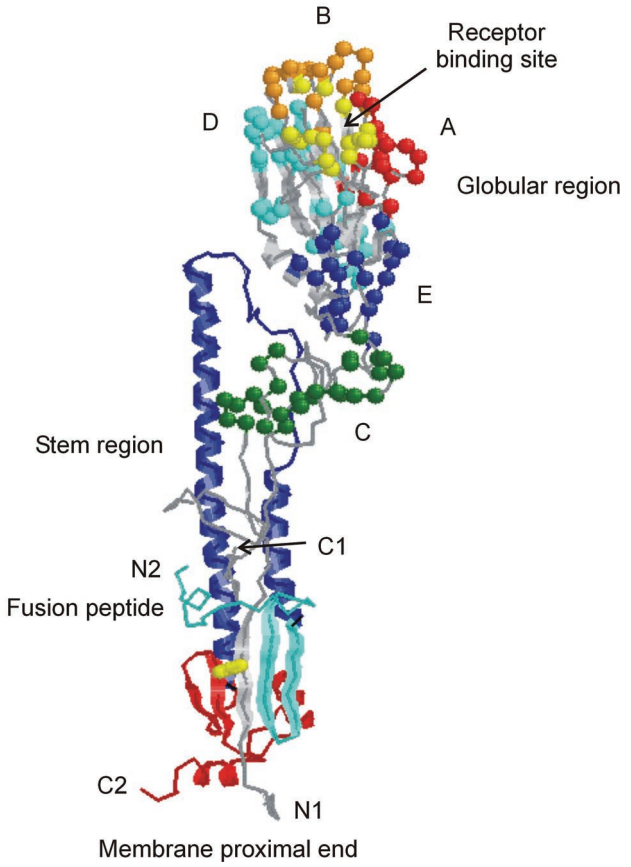

Fig. 2

Scheme of HA monomer of H3 subtype at neutral pH

The antigenic sites A, B, C, D, and E were localized according to the Influenza Sequence Database (Macken *et al.*, 2001). N-termini (N1, N2) and C-termini (C1, C2) of HA1 gp and HA2 gp, respectively.

of RNA segments exchange between different strains. In that case a novel reassortant virus contains genes from two or three different influenza viruses (Shinde *et al.*, 2009). In this way a new virus can arise presenting such HA or NA subtype that has not been circulating in human population previously. This antigenic change is known as "*antigenic shift*" (Steinhauer and Skehel, 2002; Palese and Shaw, 2007).

Frequent antigenic changes allow the influenza virus to escape virus-neutralizing antibodies induced by a previous infection with older epidemic strains or vaccination. This is the reason of repeated flu epidemics and pandemics in human population (Lewis, 2006).

2.1 Structure and function of influenza A hemagglutinin

Replication of influenza virus is initiated by the attachment of virus to the host cell and its subsequent endocytosis into the cell. HA is the main surface antigen of influenza virus and is responsible for the virus attachment to target cell and for the fusion of viral and cell membranes in endosomes (White *et al.*, 1997). It is synthesized as a precursor molecule HA0, which is trimerized, glycosylated and acylated. HA0 is postranslationally cleaved into two glycopolypeptides, HA1 gp and HA2 gp. Mature HA is a transmembrane glycoprotein formed by three identical monomers. Each monomer is composed of HA1 gp and HA2 gp joined by a disulphide bond. HA1 gp creates a globular head of the monomer and HA2 gp forms mainly a filamentous stem-like structure (Fig. 2). HA trimer is anchored into the viral membrane via C-terminus of HA2 gp that is acylated.

Influenza A viruses bind to the terminal sialic acid of the epithelial cell surface glycoproteins or glycolipids (Wiley and Skehel, 1987) attached to galactose by α 2,3 (recognized by avian viruses) or by α 2,6 (recognized by human viruses) bond. The highly conserved receptor-binding site localized on HA1 gp, is surrounded by the amino acids creating antigenic sites variable among different strains (Weis *et al.*, 1988; Skehel and Wiley, 2000). These antigenic sites are recognized by neutralizing antibodies that block the binding of the virus to the receptor-binding site and consequently, the initiation of infection is prevented. In case of amino acid changes in these sites, neutralizing antibodies bind less effectively or not at all to the receptor binding site and consequently lose their virus-neutralizing ability, i.e. fail to prevent infection (Wiley *et al.*, 1981).

The light chain, HA2 gp, mediates the fusion of viral and endosomal membranes. The cleavage of HA0 into HA1 and HA2 gp is essential for the infectivity of influenza A, since it releases the N-terminus of HA2 gp – the fusion peptide. The first 11 amino acids of the fusion peptide are highly conserved within various subtypes of influenza A virus. At neutral pH, end of the fusion peptide is inserted into the inter-space of HA trimer. At low pH that triggers the fusion process, exposed N-terminus of the fusion peptide is inserted into the target membrane, being anchored in both pre-fused membranes simultaneously, e.g. in the viral membrane by the C-terminus of HA2 and in endosomal membrane by its N-terminus (Skehel and Wiley 2000). It was observed that the conformational change of HA as well as the fusion process is pH- and temperature-dependent (Skehel *et al.*, 1982), e.g. the higher is the temperature, the closer to pH 7 is the value needed for activation of the fusion potential (Daniels *et al.*, 1985).

3. Innate and adaptive immunity against influenza virus

Immune response elicited during the influenza virus infection is a complex process involving the mechanisms of innate and adaptive immunity (Ada and Jones, 1986). At first, viruses are recognized and eliminated by a non-specific innate immune mechanisms, represented by macrophages,

dendritic cells, natural killer (NK) cells, cytokines as interferon (IFN) type I (α, β), tumor necrosis factor α (TNF-α), interleukin 12 (IL-12), chemokines and complement (Seo and Webster, 2002; Wright *et al.*, 2007). Viruses that were able to escape the primary immune mechanisms are further recognized and eliminated by the mechanisms of adaptive immunity. The effect of mechanism of the adaptive immunity can be influenced by components of the influenza virus via toll-like receptors (TLR) present on the surface of macrophages and dendritic cells of the respiratory tract. Influenza virus is recognized by at least two TLR, namely TLR3 and TLR7. TLR3 activates a cascade leading to IFN stimulation that is mediated by ds RNA. TLR7 induces production of IFN-α and inflammatory cytokines, namely IL-6 that is mediated by ssRNA (Wright *et al.*, 2007).

The adaptive immunity is a result of cooperation between specific cell and humoral immune response of the host acting against the infection. The antiviral protection is mediated by secretory IgA antibodies, serum IgG antibodies and CD8+ cytotoxic T lymphocytes (CTL) (Wiley *et al.*, 2001). Secretory IgA antibodies are transferred to the epithelium of respiratory tract by a trans-epithelial transport (Brandtzaeg *et al.*,1994). IgG antibodies distributed in the alveolar epitel are transferred from serum to the mucous membranes of the respiratory tract by transudation (Palladino *et al.*, 1995; Gerhard, 2001). CD8 receptors present on CTL recognize conserved epitopes of internal virus proteins NP or M1 complexed with MHC-I (major histocompatibility complex) molecule (Taylor and Askonas, 1986; Yewdell *et al.*, 1985; Grebe *et al.*, 2008).

The surface glycoproteins HA and NA are the main antigens recognized by CD4+ T helper cells (Th) in the complex peptide-MHC-II molecule present on the surface of antigen presenting cell (APC) (Johansson *et al.*, 1989; Grebe *et al.*, 2008). The result of Th cell stimulation is activation of B cells, their maturation and production of antibodies specific to HA and NA. Anti-HA antibodies neutralize the viral infectivity by prevention of virus attachment to the cell surface. In contrast, anti-NA antibodies prevent release of budding viral particles from the surface of infected cells (Nash, 1998). Antibodies induced by M2 protein that contains conserved epitopes within viruses of various subtypes are cross-protective. However, their level elicited during the natural infection is low and not sufficient for a significant protection (Tamura *et al.*, 2005). On the other hand, *in vivo* protection mediated by antibodies against the conserved internal proteins NP or M1 has not been demonstrated, though these proteins induce a significant antibody response during infection.

3.1 Influenza A virus hemagglutinin as an inductor of antibody response

Specific antibody response plays the most important role in the protection against influenza infection. Antibodies against HA, NA, NP, and M1 proteins are produced during infection, but only HA induces virus-neutralizing antibodies that are able to prevent the infection (Gerhard, 2001; Wright *et al.*, 2007).

HA is the most immunogenic antigen of influenza virus and its both glycopolypeptides HA1 gp and HA2 gp are good immunogens able to induce specific antibody response during the natural infection (Styk *et al.*, 1979; Wiley *et al.*, 1981; Gerhard, 2001; Kostolanský *et al.*, 2002; Fislová *et al.*, 2005). The glycopolypeptides have a complex antigenic structure with defined antigenic sites. Five antigenic sites were defined on HA1 gp (Fig. 2) (Skehel and Wiley, 2000) and four on HA2 gp (Varečková *et al.*, 2003a) (Fig. 3). While HA1-specific antibodies are mostly virus-neutralizing, HA2-specific antibodies do not neutralize virus (Skehel and Wiley, 2000; Becht *et al.*, 1984; Russ *et al.*, 1987), but reduce the virus replication (Varečková *et al.*, 2003b; Stropkovská *et al.*, 2009).

The mechanism of virus neutralization by HA1-specific antibodies consists of blocking of virus attachment to the cell surface and consequent prevention of infection. However, not all HA1-specific antibodies are virus-neutralizing. The more distantly from the receptor-binding site they bind, the less effective is their inhibition of virus attachment to the cell (Skehel and Wiley, 2000; Gerhard, 2001). HA1 gp is located in the exposed position on viral particle and is easily accessible to the antibodies posing a selection pressure. Therefore, mutant viruses able to escape the action of neutralizing antibodies often emerge. Furthermore, the intracellular inhibition of virus-cell fusion with antibodies bound away from the receptor-binding site is an alternative mechanism of virus neutralization or reduction of virus replication (Vanlandschoot *et al.*, 1998; Edwards and Dimmock, 2000, 2001; Gerhard, 2001). These antiviral antibodies bind to the virus before its attachment to the cell and afterward are endocytosed together with the virus. It is supposed that these two mechanisms can be employed simultaneously. Okuno *et al.* (1993) described the inhibition of virus-cell fusion by monoclonal antibody (MAb) specific to the conformational epitope involving HA1 gp and HA2 gp that led to an impaired virus replication. After binding of MAb to the HA trimer, the conformational change of HA required for the fusion activation was prevented. Thus, the fusion was blocked and RNP could not be released to the cytoplasm and start the replication of virus.

Mechanism of biological effect of HA2-specific antibodies is different. They do not neutralize the virus (Becht *et al.*, 1984; Russ *et al.*, 1987), but are able to inhibit the fusion activity of HA (Varečková *et al.*, 2003a). HA2-specific antibodies reduce virus-mediated hemolysis, virus-liposome fusion, and fusion of cells expressing HA. It should be emphasized that HA2-specific antibodies reduce *in vitro* replication of influenza viruses of various HA subtypes (Varečková *et al.*,

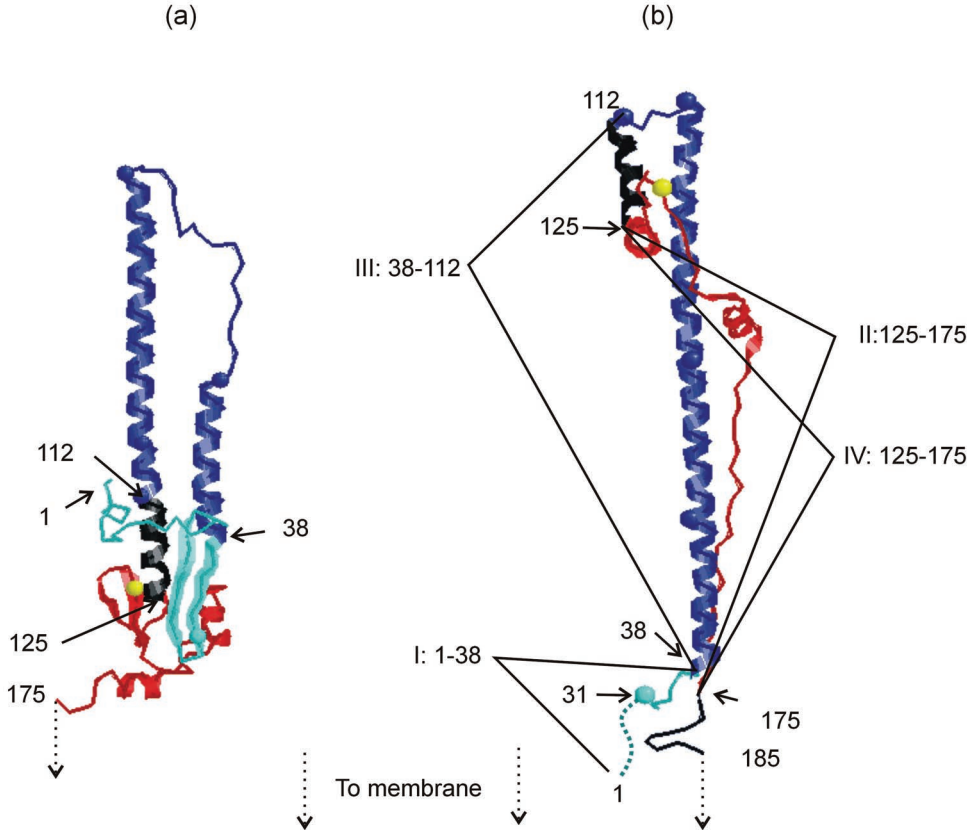

Fig. 3

Scheme of HA2 gp of H3 subtype in the native (a) and post-fusogenic (b) conformation

Native conformation is depicted with 1–175 amino acids and post-fusogenic conformation with 31–185 amino acids (Chen *et al.*, 1995). Antigenic sites are marked as I, II, III, and IV (Varečková *et al.*, 2003a).

2003b; Stropkovská *et al.*, 2009) and protect mice against the lethal infection with influenza virus (Gocník *et al.*, 2007). Biological activity and broad cross-reactivity of the antibodies described above indicate the significance not only of HA1- but also of HA2-specific antibodies in heterosubtypic immunity (Becht, 1984; Russ *et al.*, 1987; Sanchez-Fauquier *et al.*, 1987; Tkáčová *et al.*, 1997; Varečková *et al.*, 2002, 2008). Their role was later confirmed by several authors describing antibodies specific to HA2 gp or to the stem of HA trimer that reduced *in vitro* or *in vivo* virus replication and at the same time they were protective against different strains of influenza virus (Gocník *et al.*, 2007, 2008; Lim *et al.*, 2008; Throsby *et al.*, 2008; Eckiert *et al.*, 2009; Prabhu *et al.*, 2009; Sui *et al.*, 2009).

4. The prevention of influenza infection

Vaccination represents the most effective protection of humans against influenza infection. General attribute of presently used vaccines against influenza is their ability to induce virus-neutralizing antibodies recognizing epitopes surrounding the receptor binding site present on the globular part of HA. Influenza vaccines are relatively well-tolerated and still new vaccines with higher immunogenicity are developed. Presently, two types of vaccines are used: inactivated and live (cold adapted – ca) vaccines. They differ in the stimulation of the immune system and also in the mode of their administration. While inactivated vaccines are usually administered intramuscularly, live attenuated vaccines are applied intranasally. Live vaccines stimulate the complex immune response. The temperature of the cell environment in the upper respiratory tract is relatively low (cca 25°C) and therefore, the replication of the live vaccine virus is limited and does not produce any clinical symptoms in the host. In contrast to live vaccines, inactivated vaccines stimulate preferentially B-cell and CD4 T-cell immune response and induce only minimal CTL response. After vaccination the acquired protection is restricted to the virus variants that are antigenically similar to the vaccine strains. However, the

efficacy of live vaccines is broader, but not universal. A study of the induction of heterosubtypic immunity and the evaluation of CD8, CTL and CD4 T-cell response of inactivated vaccines has not yet been completed.

4.1 Inactivated vaccines

4.1.1 Currently used inactivated vaccine

Inactivated vaccines are most widely used in prevention of influenza in humans. Two types of inactivated influenza vaccines are currently used, e.g. vaccine containing the whole virions or subunit vaccine containing components of the viruses. Both types of vaccines are trivalent, what means they are composed of two currently circulating influenza A viruses or their subunits (HA and NA) of H1N1 and H3N2 subtypes as well as of influenza B virus (Palese and Garcia-Sastre, 2002; Tamura *et al.*, 2005; Katz *et al.*, 2006). The whole-virion vaccine contains viral particles inactivated by formaldehyde or by organic solvents (ether), which remove lipids from the virus particle resulting in split virions (Palese, 2006). The subunit vaccines contains highly purified surface glycoproteins HA and NA. However, the whole-virion vaccine is more immunogenic than the subunit vaccine. Immunogenicity of the whole-virion vaccine is enhanced due to the adjuvant effect of ssRNA present in the vaccine (Diebold *et al.*, 2004). On the other hand, subunit vaccines are considered as relatively high protective, though they are less immunogenic. Inactivated vaccines induce preferentially the production of IgG antibodies, which are protective against actual homologous epidemic strains. Nevertheless, they are less effective in protection against the infection caused by drifted epidemic strains and they do not protect against the infection caused by heterologous viruses of other subtypes. Statistical analysis confirmed about 70–80% efficacy in prevention against the influenza infection caused by seasonal epidemic strains. The main advantage of inactivated vaccines is their safety, because they cannot revert to the original pathogenic strain. Therefore, new approaches to the inactivated vaccine preparation are developed.

New inactivated vaccines. To enhance the relatively low immunogenicity of presently used inactivated vaccines, various adjuvants are included to the vaccine preparations. There are several types of parenteral vaccines combined with the new adjuvants (Tamura *et al.*, 2005, Katz *et al.*, 2006). In virosomal subunit vaccine, HA and NA are incorporated into the biodegradable unilamelar liposomes containing phosphatidylcholin. Due to the lipid content and repetitive arrangements of HA molecules on their surface, the vaccine effectively induce the antibody response (De Bruijn *et al.*, 2007). Immunization with "virus like particles", the self-assembled non-infectious particles 80–120 nm in diameter carrying HA of various subtypes (H3, H7 or H5), successfully protects against the infection with heterologous viruses within a HA subtype (Pushko *et al.*, 2005; Bright *et al.* 2007, 2008; Kang *et al.*, 2009).

Another example of the vaccine with enhanced immunogenicity is an emulsified subunit vaccine containing new adjuvant MF59. Emulsion of MF59 (with 0.05% Tween 80, 0.5% sorbilin trioleat and 4.3% squalen) enhances the antibody response due to the induction of Th2-type immune response (De Bruijn *et al.*, 2007). Aluminium hydroxide or aluminium phosphate belongs to the very effective alternate adjuvant substances suitable for the use in whole-virus inactivated vaccines (Squarcione *et al.*, 2003).

Nasal vaccines containing whole or split virion without adjuvants. It was shown that intranasal application of inactivated whole or split virus vaccine in the form of aerosol effectively induces local HA-specific IgA antibodies that prevent development of influenza infection at the site of virus entry. It leads to the enhanced production of both local IgA as well as hemagglutination-inhibiting antibodies in the serum. Vaccine containing formaldehyde-inactivated virus has broader spectrum of protection in comparison to the vaccine containing ether-inactivated virus due to the presence of ssRNA in the viral particles, which simulate the adjuvant via TLR7 receptor (Lund *et al.*, 2004).

Nasal vaccines containing whole or split virion with adjuvants. Intranasal immunization with whole inactivated vaccines confers broader spectrum of protection against drifted viruses in humans than subunit vaccines (Takada *et al.*, 2003). Immunogenicity of intranasal vaccines containing ether-split virus can be enhanced by the subunits of cholera toxin B (CTB) (0.1% of toxin) or subunits of termolabile toxin B from *Escherichia coli* (LTB) (0.5% of toxin). This type of vaccine confers a cross-protection in the upper respiratory tract against various variants of influenza A virus within one subtype or against the variants of influenza B virus (Tamura *et al.*, 1994, 1988; Mbawuike *et al.*, 1993). The enhancement of mucosal immune response against influenza viral antigens with LTB or CTB is mediated by innate immune mechanism that reduces the replication of virus nonspecifically in the upper respiratory tract. Reduction of virus replication correlated with the activation of APCs (macrophages, dendritic cells). This mechanism can be utilized in the search for the substances stimulating APCs and ligands of some TLRs with the aim to find a new more effective adjuvant. However, the application of nasal vaccines with adjuvants like CTB or LTB may not be quite safe, since after their application have occurred several cases of face paralysis (Durrer *et al.*, 2003).

Epidermal vaccine. Application of the epidermal vaccine is an alternative route of stimulation of the protective immunity**.** The vaccine can be also applied intramuscularly, but the epidermal application results in a much higher titer of antibodies, which are elicited by the better recognition

of antigens by Langerhans and dendritic cells. This type of vaccine induces preferentially serum IgG antibodies while the level of mucosal IgA antibodies is lower (Chen *et al.*, 2000, Tamura *et al.*, 2005).

4.1.2 New design of inactivated vaccine preparation

Components of improved vaccines. Inactivated vaccines effectively induce antibodies preventing virus infection, but their disadvantage is the narrow strain specificity. The reason is a high variability of antigenic sites recognized by virus-neutralizing antibodies. Therefore, these vaccines need to be updated yearly. Though implementation of various new adjuvants into vaccines improved their cross-protectivity, the final effect is far from satisfactory. Recently, a new approach to broaden the vaccine effectiveness was described. It is based on the selection of conserved antigens (or epitopes) of influenza virus that can induce protective immune response. Such antigens are M2 protein, NA, and conserved parts of HA.

M2 protein. Membrane protein M2 of influenza A virus has the function of ion channel. The attention of researchers is focused on M2 extracellular domain (M2-e) consisting of 24 amino acids. The comparison of known human influenza M2-e amino acid sequences with the 1933 influenza A viral isolate showed that almost all sequences are conserved, and in addition, M2-e does not undergo antigenic shift or drift. Amino acid substitutions were identified in two positions gly16glu and asp21gly (Neirynck *et al.*, 1999; Fiers *et al.*, 2001). It is supposed, however, that these changes do not influence the immunogenicity of the vaccine based on M2-e. This domain of M2 protein is now considered to be the most promising candidate for a new vaccine conferring more effective cross-protection against the new circulating influenza A viruses of different subtypes (Fiers *et al.*, 2001; Fan *et al.*, 2004; De Filette *et al.*, 2006, 2008; Ernst *et al.*, 2006; Wu *et al.*, 2008).

Neuraminidase. NA belongs to the important antigens of influenza A virus able to induce immune response that contribute to the protection against influenza infection. NA added to the conventional vaccines improves the prophylaxis against influenza and confers better cross-protection (Couch *et al.*, 1974; Johansson *et al.*, 1989, 1999; Kilbourne *et al.*, 2004; Tamura 2005).

Conserved epitopes of hemagglutinin. HA is the most variable antigen of influenza A virus. Nevertheless, it contains several regions conserved not only within one, but also among various subtypes (Nobusawa *et al.*, 1991). These conserved regions are located in the interface or stem of HA trimer and conclusively, they are not readily accessible. However, they can be disclosed after removing HA1 globular part of the HA trimer (Graves, 1983). The antigenic determinants in conserved parts of HA molecule induce cross-protective antibodies against influenza infection with viruses of various subtypes. Mutant molecules of HA with the globular part deleted could be therefore a suitable component of the new vaccine ensuring inter-subtype protective efficacy. Recent *in vivo* studies showed that some HA2 epitopes induce antibodies protecting mice against the lethal influenza infection. The immunization of mice with recombinant vaccinia virus expressing HA2 gp results in the protection against lethal influenza infection (Gocník *et al.*, 2008). Very promising results were obtained with intravenous administration of MAbs recognizing the most conserved part of HA, the fusion peptide (N-terminus of HA2 gp), which protected mice against the lethal infection (Gocník *et al.*, 2007; Prabhu *et al.*, 2009). In addition, MAbs recognizing other epitopes on HA2 gp or on the stem of HA trimer reduced or prevented replication of virus *in vitro* or *in vivo* (Lipatov *et al.,* 1997; Smirnov *et al.,* 2000; Gocník *et al.*, 2007; Throsby *et al.*, 2008; Lim *et al.*, 2008; Sui *et al.*, 2009; Eckiert *et al.*, 2009). Therefore, it can be supposed that the enrichment of a vaccine with HA2 gp could contribute to the widening of cross-protectivity of vaccine and bring us closer to the construction of universal influenza vaccine.

The latest studies showed that some epitopes on HA1 gp localized close to the receptor site can induce the cross-protective antibodies that neutralize virus (Chen *et al.*, 2009; Yoshida *et al.*, 2009; Wang and Palese, 2009).

4.2 Live vaccines

Currently used inactivated vaccines confer the effective protection against the infection with a homologous virus by inducing serum HA-specific IgG antibodies. However, these vaccines are not effective enough against the infection with heterologous viruses (Couch and Kasel, 1983; Tamura *et al.*, 2005).

On the other hand, the application of live viral vaccines inducing IgA, IgG antibodies and CTL can trigger some health problems, most often the inflammation of the nose epithelium, sore throat, or elevated body temperature. Therefore, there is still a room to improve and to develop a safe live vaccine. To avoid undesired complications associated with the application of current live vaccines, various approaches to attenuation of vaccine strains have been developed.

Live attenuated vaccines. Cold-adapted attenuated virus vaccines (*ca*-virus vaccines) were prepared and licenced in Russia and USA. *Ca*-virus vaccine consists of reassortant viruses, which contain two genes from the wild type virus encoding surface proteins HA and NA and remaining 6 genes from a cold-adapted virus (*ca*-master). Cold-adapted viruses are prepared by co-infection of host cells with the wild type virus and *ca*-master strain. Serial passages under the temperature decreased to 25°C cause genetic changes resulting in the different growth properties of cold-adapted viruses in

comparison to the parental wild type virus (Tamura *et al.*, 2005). The mechanism of this attenuation, however, has not yet been fully explained.

Ca-adapted viral vaccine is applied as a nasal spray simulating the natural infection. It confers the local cross-protection by inducing the serum IgA, IgG antibodies and CTLs (Murphy and Clements, 1989). In some cases, c*a*-vaccines trigger respiratory problems. In spite of this, they are considered as safe and genetically stabile. The application of the live vaccine is permitted in the age group of 5–49 year in the USA. The high risk groups involving elderly, newborns, pregnant women as well as immune-deficient patients are excluded from vaccination with the nasal spray (Palese 2006; Tamura, 2005).

Recombinant live virus vaccines. Advanced molecular biology techniques such as reverse genetics bring new possibilities for the improvement of vaccines. The reverse genetics allows construction of a new generation of influenza viruses from cloned plasmid DNA (Fodor *et al.*, 1999; Neumann *et al.*, 1999; Hoffmann *et al.*, 2002). The reverse genetics system composed of 8 plasmids is utilized in the preparation of attenuated viruses. Six of these plasmids encode genes for internal proteins of the donor (attenuated) virus and two plasmids encode genes for HA and NA derived from the circulating epidemic strains. Such reconstructed virus displays a limited replication in the epithelial cells of upper respiratory tract. In addition, reverse genetics enables a modification of the virulency motifs in HA and NA genes, as the deletion of multibasic cleavage site from HA gene of pathogenic avian strain virus (Luke and Subbarao, 2006). Incorporation of multiple attenuating mutations into the vaccine strain can enhance the stability of the changed phenotype and decrease a probability of reassortment of the vaccine strain with a circulating strain resulting in a virulent variant. Another alternative approach to attenuate influenza virus is the preparation of a virus with genetically modified NS1 protein exhibiting a reduced ability to act as an antagonist of IFN type I. The modified virus allows the initiation of IFN production, what limits viral replication in the upper respiratory tract (Garcia-Sastre *et al.*, 1998; Neumann and Kawaoka, 2002; Ferko *et al.*, 2004, 2006; Tamura, 2005; Palese, 2006; Romanova *et al.*, 2009). Preparation of the viral particles with defective gene encoding NEP (previously named NS2) seems to be very promising, since the replication of defective virus in the respiratory tract is limited to only one replication cycle (Watanabe *et al.*, 2002a). Similar approach was used to prepare attenuated mutant virus expressing M2 protein lacking transmembrane region critical for the ion channel activity (Palese and Garcia-Sastre, 2002; Watanabe *et al.*, 2002b).

Virus-vector vaccines. Another type of live influenza vaccine represents the vaccine consisting of replication-defective human adenovirus vector encoding HA (Wesley *et al.*, 2004; Van Kampen *et al.*, 2005). It was shown that this vaccine is able to induce the humoral immune response specific to HA1 gp and HA2 gp, but also cellular immune response effective in protection against the homologous, as well as antigenically different H5 viral strains. This vaccine is based on cell-cultivated virus and therefore, this approach could be utilized even for influenza strains growing poorly in chick embryos (Hoelscher *et al.*, 2006).

4.3 DNA vaccines

The possibility of DNA vaccine application is considered as the alternative of prophylaxis against the influenza epidemics in the upcoming years. DNA vaccines are non-infectious, non-replicating plasmids derived from *E. coli* that contain transcription machinery encoding only proteins of interest and consequently inducing immune response only to the protein of interest. Intramuscular immunization of mice with DNA encoding NP derived from the influenza virus of H1 subtype led to the induction of NP-specific CTL and to heterosubtypic cross-protection against infection with the influenza A virus of H3 subtype (Ulmer *et al.*, 1993, 2002). However, the application of plasmid DNA expressing NA, HA or HA-C3d(3) (HA fused with 3 copies of complement C3d) was more effective (Mitchell *et al.*, 2003). Good protection was also achieved in mice and ferrets immunized by DNA vaccine composed of H5 HA, NP and M2 plasmids (Lalor *et al.*, 2008). Usually, small amount of DNA induce a long-lasting immune response and therefore, DNA vaccines have a potential to be used for the prevention. However, limited data are available concerning their imunogenicity in humans.

5. Conclusions

Vaccine strategies using various adjuvants and alternate systems for the antigen delivery to immune system of the host contribute to the improvement of inactivated vaccines that are considered as the safest. The development of new technologies for the vaccine preparation, particularly reverse genetics techniques, can significantly contribute to the improvement of live attenuated vaccines and shorten a time needed for the preparation of both, inactivated or live vaccines. It enables to ensure a better safety of the products and widen the protection against different subtypes of HA including newly emerging ones. Reverse genetics method was applied also for the preparation of new vaccines against avian viruses (Gillim-Ross and Subbarao, 2006). Furthermore, this method supports the rapid development of a vaccine against newly emerging viruses such as "swine flu 2009" H1N1 virus that currently gives rise to the pandemics in humans. The advantage of live vaccines

reside in their ability to stimulate not only mucosal IgA and serum IgG antibodies, but also CTL that are important for the inter-subype cross-protection. The protective potential of conserved antigens of influenza A virus can be utilized for the preparation of "multi-epitope" based vaccine, what could subsequently lead to the development of the "universal" vaccine effective against influenza A viruses of any HA and NA subtype (Ben-Yedidia and Arnon, 2005; Arnon, 2006; Gerhard *et al.*, 2006; Grebe *et al.*, 2008).

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