

**Inactivated influenza vaccine for intranasal immunization.**

**Author**

Zichria Zakay-Rones PhD

**Address:**

Department of Biochemistry  
and Molecular Biology and  
Chanock Center of Virology,  
IMRIC, the Hebrew  
University-Hadassah Medical  
School, POB 12272,  
Jerusalem, 91120, Israel. Tel:  
972 -2- 6758556 Fax: 972-2-  
6784010  
[zichriar@ekmd.huji.ac.il](mailto:zichriar@ekmd.huji.ac.il)

**Abstract**

Influenza is a major winter contagious respiratory disease that takes a high toll on the population due to wide range morbidity, complications, hospitalization and mortality. At present, vaccination is the most effective means for controlling influenza infection. Licensed vaccines include several forms of inactivated vaccines (trivalent, quadrivalent, whole virus, split and subunit) delivered by injection with or without adjuvants, and live attenuated vaccines for intranasal administration (LAIV). Type of vaccine and mode of administration dictate the induced immune response and efficiency of vaccination. It has been suggested that intranasal administration induces better protection, as it neutralizes the virus at its entry site, eliciting production of secretory IgA (SIgA) antibodies and a serum barrier. In contrast, following injection, SIgA antibodies are produced only in negligible amounts. Another type of vaccine under clinical investigation is inactivated virus for intranasal application. This type of vaccine induces local (SIgA) and serum responses similar to the LAIV vaccines. Moreover, inactivated vaccines may be suitable for high-risk groups, which are constantly expanding, for whom live vaccines (approved only for healthy 2-49 years old) are inappropriate. The main obstacles toward successful vaccination are the annual changes in the virus (drift and occasional shift), appearance of new pandemic strains not included in the vaccine and transmission of pathogenic avian strains to humans. Efforts to develop a universal vaccine, which will protect against all A strains, might be a solution. Intranasal application of a universal vaccine to mice protected vaccinated animals from lethal infection by heterologous strains and furthermore reduced the transmission of virus from vaccinated to non-vaccinated mice. It may be concluded that intranasal inactivated virus formulas, either whole (without adjuvant), split (with adjuvant) or universal, may be the future vaccine for influenza.

**Keywords:** influenza vaccines, inactivated vaccine, universal vaccines, intranasal administration

## **Inactivated influenza vaccine for intranasal immunization.**

### **1. Introduction**

Influenza is a major winter contagious respiratory disease that every season affects millions of people, substantially increasing hospitalization (1) and thus leading to a high burden on health services (2-4). Ten-20% of the population is infected with A and B influenza viruses and the disease is responsible for a worldwide annual mortality of up to 500,000 individuals. These numbers are even higher when epidemic outbreaks occur (30-50% of the population is infected), often leading to severe illness, complications and excess mortality (5-9). The entrance site of influenza virus to the host is the respiratory tract where replication takes place and disease symptoms are manifested. All influenza strains A (human, avian and other animals), B and C (which cause only sporadic cases), have to overcome the primary local defense obstacles (such as mucin layer, cilia, proteases and different cell population: macrophages, dendritic cells, NK cells, cytokines and the interferon system). The virus replicates in the ciliated column epithelial cells, and is transferred to other victims by droplet infection. World wide spread of the virus is nowadays faster due to globalization and modern transportation. The balance between the elicited innate and adaptive immunity and previous exposure of the host on the one hand, and virus type, transmissibility and virulence on the other, dictate the outcome and severity of infection.

### **2.0 Prevention and Control**

The most effective means for controlling infection and thereby reducing morbidity, complications and mortality, is vaccination (7, 10-12). Vaccination is regulated and approved by the Centers for Disease Control that selects and updates (together with the WHO) the strains for every season, according to predicted strains that would be in circulation during the following year (13, 14). The vaccines are trivalent, with recently

quadrivalent vaccines available, thus reducing possible mismatching (which occurs in about 15% of cases) between circulating strains and those included in the vaccine, a fact that reduces vaccine effectiveness (15-17) (Table 1).

### **2.1 Target groups for vaccination**

According to the CDC recommendations (13) all persons aged 50 years and older, with and without chronic health conditions, are considered at risk. This group also includes residents of long-term care facilities and nursing homes, a population that is constantly expanding due to increased longevity. Adults and children with chronic health conditions (diabetes, obesity, heart conditions and immune- deficiency patients following transplantation, irradiation or chemotherapy) are also targets for vaccinations. Vaccination is also highly recommended for children aged 6–59 months. It is advised for pregnant women, who are more vulnerable to infection, to get the vaccine in order to protect themselves and to provide passive protection to the newborn, as there is no vaccine available until the age of 6 months. Vaccination of health-care personnel, who provide direct patient care, as well as contacts of risk groups is also of great benefit to the community. The optimal goal is vaccination of the entire population, assuming a sufficient supply of vaccine doses is available.

### **2.2 Inactive vaccines for parenteral administration**

Inactivated “killed” influenza vaccines that cannot cause disease have been in use since the 1940s with many improvements introduced throughout the years, primarily in production technologies and in the addition of adjuvants. Several experimental vaccines are under development and at different phases of clinical trials (e.g. DNA vaccines, production of antigens by cloning in different vehicles) (11). After whole inactivated virus vaccines

### **Inactivated influenza vaccine for intranasal immunization.**

(WIV), not approved for children, were developed, split and subunit vaccines were introduced. These vaccines inflict less local and systemic side effects, but are not as immunogenic. To improve their efficacy, the addition of adjuvants and other methods to increase immunogenicity, such as liposomes, virosomes and virus like particles (VLP), with different supplements, such as IL-2 and TLR ligands, were attempted (18). The split and subunit inactivated vaccines are approved for the entire population from the age of 6 months, and are highly recommended for high-risk groups (see 2.1). The vaccine is produced for parenteral immunization (intramuscular, subcutaneous or intradermal). Vaccines are prepared in embryonated eggs and the infected allantoic fluids are clarified and purified. The history of inactivated influenza, from the discovery of the influenza virus onwards, was summarized by Couch (19). In the last few years, inactivated vaccines prepared from virus grown in cell culture (VERO and MDCK) are also available (20-25). The MDCK cell line is useful due to its high sensitivity to virus growth. Vero cells, which are already approved for the production of other human vaccines, are suitable for productive replication of most, but not all, influenza A strains. A major advantage of the cell-cultivated virus is its greater similarity and higher homology to the human virus found in clinical isolates, thus avoiding the selection of egg variants and producing a more effective vaccine. Another advantage of cell cultures is that individuals allergic to eggs can also be vaccinated. Both live vaccines, delivered by nasal spray, and inactivated vaccines delivered by intranasal or intramuscular injection, are produced in eggs. In addition, a large number of vaccines are currently manufactured in cell culture. Recently, a baculovirus-expressed influenza vaccine (Flublok), a trivalent recombinant hemagglutinin (HA) vaccine, was licensed by the FDA (2013) for the prevention of seasonal

influenza, for adults 18–49 years of age. In 2016, the vaccine was licensed for all adults older than 18 years. Current influenza vaccines are aimed at inducing neutralizing antibodies specific for HA and to neuraminidase (NA) (these antibodies are not neutralizing; they limit release, load, and spread of the virus as well as disease manifestations)(11). Due to antigenic variability and immune evasion of circulating strains, annual vaccination, based on current strains, is necessary. Regarding the vaccine dose, usually 15µg HA /strain is delivered. Recently, a vaccine designated for the elderly, which is used at a high dose (60µg/strain), was produced (Fluzone). This vaccine evoked higher serum anti HA and anti NA (N1 and N2) antibody titers in the elderly (26-28).

The protection rate of the inactivated vaccines, based on anti HA neutralizing antibodies, ranges from 50 to 90%, while efficacy is only 40 to 70%. Immunogenicity may be as low as 20-30%, particularly among the immuno-compromised and the elderly that have a diminished immune response to influenza vaccination compared to young healthy adults (29, 30). Even when vaccine efficiency is reduced to 23%, vaccination still prevents some illness and serious influenza-related complications, including thousands of hospitalizations and death cases (31). In children with high-risk conditions, vaccine efficiency is 51% compared to 61% in non-high-risk children (32).

### **2.3 The rationale behind intranasal vaccination**

The rationale behind the development of an alternative for parenteral vaccination was multifactorial. The inactivated vaccines designated for systemic vaccination were of low efficiency, especially in the high-risk and in the aged populations. The immune response and protection evoked by parenteral

## **Inactivated influenza vaccine for intranasal immunization.**

vaccination was restricted to the specific anti hemagglutinin antibodies, and there was none or only negligible mucosal response. Therefore, a defense barrier in the respiratory tract was absent. Since the entrance site of the virus is through the upper respiratory tract, serum antibodies could not neutralize the virus efficiently at the entrance site, and thus viral replication and infection were not prevented. It was hypothesized that intranasal application of influenza antigens may be able to mount mucosal immunity that would neutralize the virus at the entry site, improve protection and possibly prevent infection. Thus, local vaccination may overcome the limitations of routinely used inactivated influenza vaccines and confer potent immunity against viruses with new pandemic potential. The intranasal vaccine may also lead to an increased enrollment to vaccination programs by avoiding “fear of the needle”.

### **2.4 Live Attenuated Influenza Vaccine (LAIV)**

The need for more effective influenza vaccines resulted in the development of cold-adapted, live-attenuated vaccines, created using internal protein-coding gene segments from the cold-adapted temperature-sensitive master donor virus A/Ann Arbor/6/1960 and HA/NA gene segments from circulating viruses (Flu mist®, Medimmune Vaccines Inc. in the USA and Fluenz Tetra MedImmune UK Limited (Speke, Liverpool, UK) in Europe). The Food and Drug Administration (FDA) approved its administration in 2003 only for healthy 2-49 year old individuals for intranasal delivery. The vaccine now contains four strains against influenza A/H1N1, A/H3N2 and two representatives of influenza B: B-Victoria, and B-Yamagata lineages (33). In contrast to the inactive vaccine, activity of the live vaccine is dependent on the replication of the virus in the upper respiratory tract and may cause mild signs of influenza infection. The

attenuated vaccine has the potential to induce a secretory and systemic immune response that more closely resembles the immune response detected after natural infection (34). The vaccine is prepared either in embryonated eggs or in cell cultures and includes the same selected strains as the inactivated vaccine. This vaccine was shown to be safe, stable and hardly transmitted to siblings (35). Following treatment with this vaccine, a long lasting broader humoral immune response, also against drifted viruses, was detected, and a cellular response was evoked (36). Efficacy of the attenuated vaccine is higher than that of the inactive vaccine administered by injection and is appropriate for vaccination against pandemic and zoonotic strains (10, 11, 13, 36, 37).

### **2.5 Inactivated vaccines for intranasal administration**

**Past development:** The same approach that led to the development of live vaccines aiming toward local immunization was behind the attempt to develop inactivated vaccines for intranasal immunization. In addition to protection from the virus at the entry site through the establishment of local immunity, this type of vaccine may allow individuals from high risk groups (which are constantly expanding due to life longevity and more immune-deficient patients) for whom the live vaccine is inappropriate, to be safely included in vaccination programs. Intranasal immunization with inactivated influenza vaccines was investigated as early as a few decades ago, and whole and split virus vaccines were evaluated with or without the addition of different types of adjuvants. Waldman (38) has already shown in 1969 that in man, aerosol immunization with an inactivated influenza vaccine stimulates higher levels of secreted antibodies in the respiratory tract than subcutaneous immunization. They described the presence of a secretory immunological system that

### **Inactivated influenza vaccine for intranasal immunization.**

may- act as a "first line of defense" in protecting mucous surfaces against invasion by pathogens. In 1997, Kuno-Sakai (39) suggested that inactive viruses for intranasal vaccination are preferable, since they are usually given to people with some degree of immunity due to past infection or immunization, and therefore they may have a booster-like effect. Potter and Jennings (40) discuss the background, advantages and disadvantages of the development of inactivated influenza vaccines for intranasal administration as compared to parenteral vaccination and intranasal administration of live viruses.

A trivalent inactivated whole influenza virus vaccine, consisting of 20µg HA of each strain, was applied intranasally to groups of volunteers of different age groups: two doses to 61 elderly people in the community and one dose to 21 residents in a nursing home (with a group that was injected intramuscularly and served as control) (41-43). The vaccine was also given to 28 children (12-14 years old, a single dose)(44). Volunteers from the community (182 vaccinees), 12-60 years old, received one dose (45) and 102 young adult nurses and medical students, 21-28 years old, received one or two doses (46). The vaccine was effective in all age groups, with a significant production of local secretory IgA (SIgA) anti-influenza antibodies and serum HI antibodies (IgG) against the three strains in the vaccine. Thus, producing a double barrier against virus infection and complications, the intranasal vaccine was significantly more effective than the intramuscular vaccine in inducing a mucosal SIgA response. Morbidity was also prevented, and vaccination was associated with a significant reduction in respiratory illness among vaccinated healthy older children and adults in the community compared to the placebo group (46, 47).

Although neuraminidase (NA), the second major viral surface glycoprotein, was not

quantified in the vaccine, and its concentration might have been low, antibodies against N1 and N2 in nasal washings were evident, but were absent or negligible following intramuscular immunization (48). Although these antibodies do not neutralize the virus, they reduce viral load and severity of disease. Similar results were obtained in mice following intranasal immunization with formalin-inactivated intact virus, but not ether-split vaccines. A broad spectrum of heterosubtypic protective immunity, possibly mediated by the mucosal immune response, was demonstrated in mice. These were most likely secretory IgA antibodies to viral proteins (49). An inactivated virosomal-subunit influenza vaccine licensed in Switzerland (Nasalflu, Berna Biotech, October 2000) was the first licensed intranasal influenza vaccine for humans in the world and was available for the 2000–2001 influenza season; it contained *Escherichia coli* heat-labile toxin as a mucosal adjuvant (50). In spite of the satisfactory response, this vaccine is no longer in clinical use, due to a suggested strong association between the inactivated intranasal influenza vaccine and Bell's palsy (51).

#### **2.6 Revisiting intranasal inactivated vaccines**

The use of intranasal inactivated vaccines was neglected when the LAIV was licensed, in 2002/3. However the restriction of the LAIV use to 2-49 years only, and the contraindication for its use in infants, asthmatic patients, those with respiratory infections, the elderly and the frail, immunodeficient individuals, their house hold and contacts (52), led to the exclusion of the sector in the population which has most to gain from vaccination against influenza. In addition, the failure of the vaccine in the last seasons (3% efficacy only) and withdrawal of the vaccine, at least temporarily (53), justified and encouraged the renewed interest in the

## **Inactivated influenza vaccine for intranasal immunization.**

inactivated vaccine for intranasal application. This approach is being evaluated so far only in animal models and in preclinical trials in humans. There is not yet a licensed vaccine.

Following is a summary of research carried out in animal models and in humans using inactivated whole virus or subunit vaccines with or without adjuvants, applied intranasally.

### **2.6.1 Animal models**

Intranasal immunization of mice with inactivated influenza virus A/PR8 (H1N1) provided complete protection against the homologous virus and a drift virus within the same subtype, A/WSN (H1N1), but not against the heterosubtypic virus A/Philippines (H3N2). However, co-administration of inactivated virus with cholera toxin as an adjuvant conferred complete heterosubtypic protection (54). Another group started evaluating inactivated vaccines applied intranasally as early as 1990, with promising results in mice and human adult volunteers (55). Intranasal delivery of an enterotoxin B-combined vaccine enhanced the production of serum hemagglutination inhibition antibody as well as SIgA antibodies in the respiratory tract compared to the nasal vaccine alone (56, 57).

Research focused on the cholera toxin B subunit (CTB) as adjuvant (56, 57). Immunization provided cross-protection against variants within a subtype of the A virus (or variants within the B virus), and induced highly cross-reactive SIgA antibodies to viral HA, and weak cross-reactive IgG antibodies in the respiratory tract (56, 57). However, since CTB was prohibited, and mucosal adjuvant was necessary as the inactivated virus is not a good immunogen, particularly in naïve individuals, search for new mucosal adjuvants was carried out. The result was Ampligen®, poly(I):poly(C(12)U,

a Toll-like receptor (TLR)3 agonist (58). For its evaluation, Ampligen was used with a seasonal trivalent inactivated intranasal vaccine. Cross-reactivity of mucosal IgA and serum IgG with the H5N1 virus, a reduced H5N1 virus titer in nasal-wash samples and increased survival after challenge with the H5N1 virus were recorded in vaccinated mice. Subcutaneous inoculation did not induce a cross-reactive IgA response and did not afford protection against H5N1 viral infection (59). Similar results were obtained in vaccinated cynomolgus macaques (60). Another adjuvant, Endocine™, was evaluated in ferrets. Intranasal administration of inactivated pandemic H1N1/California/2009 split antigen or whole virus antigen mixed with the adjuvant induced high levels of serum hemagglutination inhibition and virus neutralization antibody titers. The antibodies were also cross-reactive with distant swine viruses of the same subtype. Hemagglutination inhibition and virus neutralizing antibody titers as well as protection from challenge with a homologous virus were found after a single nasal immunization. Immunized ferrets were fully protected from virus replication in the lungs and were largely protected against body weight loss, virus replication in the upper respiratory tract and pathological changes in the respiratory tract (61). Adding a TLR2 agonist to an existing seasonal detergent-split influenza vaccine with a TLR2 agonist-based lipopeptide adjuvant (R4Pam2Cys) improved influenza vaccine efficacy by inducing immediate short-term non-specific antiviral protection. The vaccine also provided antigen-specific responses that elicited homologous and heterologous immunity in mice (62).

The addition of carboxy-vinyl polymer (CVP) increased the subunit vaccine-specific IgA antibody responses of the intranasal vaccinated cynomolgus macaques. The

## **Inactivated influenza vaccine for intranasal immunization.**

vaccine was retained significantly longer in the nasal cavity of both mice and nonhuman primates (63). Whole virus, inactivated by  $\gamma$ -ray radiation, was also reported to induce local response in mice (64). It was shown that intranasal immunization of mice with a formalin-inactivated whole-virion vaccine from MDCK cell-cultures protected them against challenge with lethal influenza viruses of homologous and heterologous subtypes. This is another step towards establishing the use of this type of vaccine (65).

### **2.6.2 Humans**

In 2006, trivalent influenza virus vaccine preparations of highly purified hemagglutinin and neuraminidase were used in a randomized, controlled, dose-ranging phase I study. As it is claimed that inactivated influenza virus antigens are not reliably immunogenic when delivered to the mucosa, the potent mucosal adjuvant LTK63 (a nontoxic derivative of the native holotoxin of *E. coli*, (51)) was added. As anticipated, the largest increase in circulating antibodies was detected in response to intramuscular vaccination; the largest mucosal immunoglobulin A (IgA) response was found in response to mucosal vaccination (66). Aina et al (67) intranasally vaccinated 50 healthy adults aged 22 to 69 years, with some immunological memory for seasonal influenza viruses, twice at 3 week intervals with an inactivated whole virus vaccine (45 $\mu$ g HA per dose), without the addition of mucosal adjuvant. All parameters associated with an effective outcome of vaccination in the criteria defined by the European Medicines Agency were met. Serum and nasal hemagglutination inhibition and neutralizing antibody responses consisted of HA-specific IgG and IgA antibody responses, with IgG and IgA antibodies being dominant in serum and nasal responses, respectively. It may be

concluded that the inactivated whole virus vaccine appears to be a promising vaccine. However, a split vaccine may need adjuvant to improve immunogenicity, which is not required by the whole virus vaccine. The higher immunogenicity of the whole virus vaccine may be explained by the adjuvant action of single-stranded viral RNAs that activate toll-like receptors. Viral RNA is present in the inactivated virus particles, but is absent in split-product vaccine formulations (67).

An intranasal human inactivated whole virus vaccine without adjuvant induced both innate and adaptive immune responses and cross protection against variants within a virus subtype, mainly through a humoral immune response. The cross-protection elicited by the whole virus vaccine and the split vaccine with mucosal adjuvants in intranasal immunized mice was very similar (67-70).

Based on the results in animals and the finding that Ampligen<sup>®</sup> proved to be safe in a Phase III human trial with Flumist, it may be interesting to try the adjuvant with an inactivated vaccine, to further develop this mode of vaccination (71).

### **2.7 Reasons for the need for annual vaccination**

The main problem of all the described vaccines is the need for annual vaccination. Although the vaccines can prevent symptoms of disease, complications and mortality, efficiency is dependent on the successful matching of the vaccine strains with the circulating strains. The sudden appearance of epidemic strains, such as A/pdm California/2009 H1N1 (8), and the transmission of avian strains to humans (A/H5N1, with about 60% mortality, H7N9, H10N8 and others) may stand in the way of successful vaccination (72, 73).

## Inactivated influenza vaccine for intranasal immunization.

The main obstacle is continuous change in the virus. The HA and NA surface glycoproteins change due to two different processes. a) Antigenic drift, a gradual accumulation of point mutations (partially due to immunological pressure) that results in the appearance of new antigenic epitopes. Drift-mutated HA and NA sequences that either change the protein sequence or shield it by glycosylation are selected, resulting in a continual antigenic drift of the circulating viruses. This change occurs in A as well as in B influenza genera. Once in 2-5 years a new strain appears, not recognized by previous antibodies. A large enough naïve population allows the burst of an outbreak or epidemic. b) Antigenic shift, a major antigenic change in A strains only, occurring every 10-50 years. Antigenic shift is dependent on the large reservoir of A strains in nature, mostly in fowls but also in other species, and lately also found in bats (74). The influenza viruses (Orthomyxoviridae) are RNA viruses consisting of eight segments, coding to 11-13 proteins. When a cell or a host is infected with two different strains, re-assortment can take place, resulting in the formation of a strain with a new HA and /or NA subtype introduced into the pool of human strains. The mixing vessel is considered to be in most cases the pig, since it possesses the specific receptors for both human and avian strains. Re-assortment can also take place in humans, seals and some poultry species. The reemergence of a previous strain or adaptation of an avian strain can also lead to a pandemic.

### 2.8 Universal vaccines

The fact that all the current influenza inactivated and live vaccines are effective only against a narrow range of virus strains and that vaccine-induced protection against influenza may decline within one season (75), make it necessary to develop universal vaccines. Such vaccines should aim toward long term and broader anti-influenza

responses, in order to provide protection against multiple strains and thus eliminating the need for annual vaccine production and vaccination.

The basis for studying the potential of a universal vaccine is the accumulated evidence that long-term immunity does exist. Preexisting antibodies against A/pdm California H1N1 2009 were detected in a high percentage of the population. The prevalence of these antibodies was dependent on age: older people had a high titer, while 44 years old and younger were sera negative (76). Gostic et al (77) found that birth year determined the level of protective immunity to avian strains (IAV) from two different phylogenetic clades (H5N1 and H7N9) and IAV strains circulating during an individual's childhood confer long-term protection against novel HA subtypes from the same phylogenetic group. Based on these findings, she proposed that immunity was dependent on antibodies targeted to the HA stem (see below). Monoclonal antibodies isolated from infected mice or humans recognized distinct conserved epitopes in the stem region of the A/ HAs (78). Some antibodies reacted with almost all influenza A group strains, including H3N2 and H7N7. Based on this data, the 18 diverse HAs were grouped into two broad clades of viruses. Each group seems to share a sequence conserved in the stem, which could serve as a target for broadly neutralizing antibodies. These findings led the way for the establishment of vaccines inducing a broad immune response based on invariant regions or common proteins (11). The basic approaches and challenges of creating such a vaccine are discussed by Egorov (79). Several such potential candidates (also listed in Table 2) are:

1. Hemagglutinin (80, 81), the major surface glycoprotein anchored in the virus membrane, is a trimer composed of two chains, HA1 and HA2, linked by disulfide bonds. In A strains, each chain consists of a



**Inactivated influenza vaccine for intranasal immunization.**

globular part with the receptor-binding site and five antigenic sites. Antibodies to the globular part are responsible for neutralizing the virus by inhibiting adsorption of the virus to the host cell receptor. The HA1 subunit is characterized by high variability (drift), resulting in considerable diversity in the amino acid sequences of the HA protein responsible for the large number of strains. In contrast, the stem of the HA, which is primarily contributed by the HA2 domain and is located in close proximity to the viral membrane, is conserved among a large variety of strains. The main function of the HA2 subunit is to ensure fusion of the viral and the endosomal membranes by means of a fusion peptide. Unmasking these invariable regions by the removal of the head of the HA produced "headless hemagglutinin". Vaccination of mice with headless HA conferred protection against a lethal influenza virus. However, as there are two unrelated HA clusters, two preparations are needed in order to cover all A strains (78). Corti et al (82) selected an antibody after infection and vaccination with the live attenuated vaccine, which reacted against all the 16 HAs known at the time. This opened the way to design a new immunogen. In B strains, the conserved region is located around the cleavage site on HA0, and can elicit a protective immune response against a lethal challenge with viruses belonging to either one of the representative, non-antigenically cross-reactive, influenza B virus 2 lineages (83). The headless hemagglutinin epitopes were recognized by neutralizing antibodies that induced broad protective/heterosubtypic immunity in animal models by binding to HAs within the phylogenetic group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18) or group 2 (H3, H4, H7, H10, H14, H15) including zoonotic strains that are transmitted to humans (H5N1) (84). One monoclonal antibody even recognized

influenza B HAs (85). These antibodies, directed against the stem, do not act in the classic way of neutralization by blocking viral entry, but rather provide effective protection via antibody dependent cellular cytotoxicity (ADCC) (86). It appears that elicitation of non-neutralizing antibodies by an HA stem-only can provide broad protection against severe disease and should be considered in strategies to develop universal influenza vaccines (87).

2. The ectodomain of the M2 protein of influenza A viruses is an encoded integral membrane protein. The highly conserved, 24-amino-acid extracellular domain of M2 is similar in all the A strains, and unlike HA and NA (NA is the second most abundant surface glycoprotein), is not subjected to immune selection and is therefore stable. Although its immunogenicity is weak and M2-e specific antibodies do not neutralize the virus, they contribute to antibody dependent NK cell activity (88-90). By binding to M2 on virus-infected cells, they can prevent viral replication in mice lungs, illness and death from lethal H5N1, H1 N1 and other heterologous strains. In B strains, only B/M2 is present but it is not equivalent to A/M2 as candidate for a vaccine (91).

3. Nucleoprotein (NP). An additional candidate for a universal vaccine is NP, a stable and uniform protein. Epitopes of conserved internal influenza virus proteins can induce heterosubtypic immunity to different influenza virus strains of the same type (A or B). NP is an internal structural component, well conserved, exposed on infected cells during virus replication. For this reason, its role in protection and in accelerating virus clearance in mice and humans is not by neutralization, but could be related to the generation of cross-reactive CD8+ cytotoxic T cells (CTLs) that have the potential to destroy infected cells (86, 92-94). It is likely that a future vaccine will include,

## **Inactivated influenza vaccine for intranasal immunization.**

in addition to combinations of HA and NA, several other viral components. For example, NP, M1 (which also activate CD8<sup>+</sup> cytotoxic lymphocytes) and M2-e. Such a T cell-stimulating vaccine, with the addition of the non-structural NS1 protein, will be capable of inducing a broad and long-term immune response and might be a promising candidate for incorporation into a broad-spectrum influenza vaccine (95, 96).

Several experimental vaccines based on invariant regions of the virus are now being clinically evaluated for priming or as booster for various vaccine formulas.

### **2.9 Universal vaccine for intranasal administration**

As it seems that intranasal delivery is the preferable mode of immunization, the question was raised whether this route could deliver the universal vaccine. It was demonstrated by Tamura in 1996 (97) that mice immunized intranasally with adjuvant-combined NP showed accelerated virus clearance from the nasal site, morbidity was diminished and survival rate increased (98). In animal models, cross-protection by vaccines based on conserved antigens does not completely prevent infection, but greatly reduces morbidity, mortality, virus replication and, as a result, viral shedding and spread. Such immunity is especially effective and long lasting with mucosal administration. Cross-protective immunity in humans is controversial, but is suggested by some epidemiological findings. Universal vaccines, protective against all influenza A viruses, might substantially reduce severity of infection and limit spread of disease during outbreaks. Price and colleagues (99) demonstrated that a candidate universal influenza vaccine protects vaccinated animals from lethal infection and reduces the transmission of virus from vaccinated to non-vaccinated mice. Their vaccine, consisting of NP and M2, induced immunity against

proteins conserved among all known influenza A virus strains and subtypes. Thus, the vaccine could be used early in a pandemic before conventional strain-matched vaccines are available and could potentially reduce the spread of infection in the community.

Vaccination of mice with a mixture of virus-like particle individually displaying H1, H3, H5, or H7 HAs, showed significant protection following challenge with influenza viruses expressing 1918 H1, 1957 H2, avian H5, H6, H7, H10, and H11 hemagglutinin subtypes (100).

### **3.0 Understanding the immune response (Table 3)**

#### **3.1 Different compartments of the immune system are activated, depending on the type of immunization**

The immune response elicited by the current vaccines is dependent on the route of administration (parenterally or mucosal) (101). The type of antigen (killed or attenuated live), the formulation of the vaccine (supplementation with adjuvant) and the vaccinee's profile (age, health condition and previous exposure to influenza antigens), all affect the immune response. Parenteral immunization with inactivated vaccines induces mostly strain-specific immunity: humoral IgM and IgG antibody response to HA and NA in the lower respiratory tract that may protect against pneumonia. However, none or only negligible mucosal immunity develops at the entry site of the virus. LAIV can boost mucosal heterosubtypic immunity and serum antibodies as well as virus-specific CTLs by mimicking natural infection. This is in contrast to inactivated antigens that do not induce strong CTL responses. Mucosal delivery strategies, particularly when using LAIV that can replicate, but also following intranasal application of an inactivated vaccine, may mount a mucosal barrier that inhibits infection.

## **Inactivated influenza vaccine for intranasal immunization.**

### **3.1.1 SIgA response**

One of the main differences between parenteral vaccination and local administration (LAIV or inactivated vaccine) is the elicitation of SIgA antibodies (mucosal response), the major immunoglobulin on mucosal surfaces.

SIgA displays different molecular forms depending on its production in plasma cells or as monomeric IgA in serum and tissues (102). The secretory IgA antibodies are dimers that exhibit a broad spectrum of activity; they react with homologous HA and cross-react with heterogeneous viral A strains and avian strains. The secretory form is more effective than the monomer serum IgA and IgG. The findings of Suzuki et al (103) that trimeric, tetrameric, and larger polymeric structures displayed increased neutralizing potency against influenza A viruses compared with dimeric SIgA, may explain these differences. These results suggest that polymerization of IgA enhances its antiviral effect, but does not increase the number of influenza virus strains neutralized by the IgA (104).

### **3.2 Immune response following infection**

Following natural infection in humans and in animal models, innate immunity, the first response generated, is known to play an important role in inhibition and viral replication and spread of influenza A virus (69). Type I alpha/beta interferons (IFN- $\alpha/\beta$ ), and recently type III IFNs (IFN- $\lambda$ ) were identified as the major response to intranasal infection with this virus (105). The next stage of the immune response is the activation of adaptive immunity: long lasting cellular and humoral responses in the serum and on mucosal surfaces (69). Importantly, anti HA stem antibodies, the basis for a universal vaccine, are induced following infection, but only low titers are produced following

vaccination (106). ADCC-antibody titers increased following experimental influenza virus infection in adults and after split vaccine administration in both children and adults, but not after LAIV. Preexisting ADCC antibodies also conferred protection (86).

Understanding the immune response that develops during infection and the role of the various adjuvants in the correct TH1/TH2 balance, may enable the development of a more efficient vaccine.

### **4.0 Summary and Conclusions**

Currently available vaccines, although the major means to control and prevent influenza infection, are far from being optimal. Protection by inactivated vaccines is strain-specific and balances the level of anti HA (neutralizing) and anti NA (non-neutralizing) antibodies. LAIV protection is dependent mostly on mucosal immune response and, although it exhibits a broader activity against heterologous A strains, is still not sufficient. Inactivated vaccines delivered nasally seem to be better than current inactivated injectable vaccines due to mode of administration (avoiding fear of the needle) and their higher efficacy. In addition, intranasal vaccines are appropriate for those target groups that LAIV is not approved for, and furthermore the immune response is similar to the response to LAIV. Universal vaccines might be the solution for the constantly changing virus. Introducing a new generation of vaccines calls for new criteria for evaluation, as titer of hemagglutination inhibition antibodies, which correlates with protection following vaccination by injection, is not always relevant. This is the case, for example, for intranasal application or following the usage of non-hem agglutinating moieties in universal vaccines. The requirements for licensing these new vaccines and how they might be used in the future is discussed (96).

**Inactivated influenza vaccine for intranasal immunization.**

Taken together, it seems that intranasal delivery is the preferable mode of immunization. Applying a universal vaccine by this route may further augment its potential and may elicit a broad-spectrum immune response. Including the entire population in vaccination programs may be future practice.

A crucial question is whether the flu will stay with us forever, or whether there is a possibility of eradicating it. In this regard, influenza B and C should be differentiated from influenza A. B and C usually infect only man (the reservoirs in seals for B and in pigs for C play only a negligible role in epidemiology and spread of the disease to man). The C type is responsible for only

sporadic cases and is not, therefore, included in the annual vaccine. The variation of the B type is only by drift (minor antigenic changes due to acquisition of point mutations) and only two lineages are recorded. While both influenza B and C may be considered suitable for eradication, or at least elimination, influenza A, a zoonotic disease, with its constant genetic turbulence, entirely contradicts the requirements for eradication. Since the available vaccines presently protect only against a specific strain/s and not against new ones, universal vaccines administered by injection or intranasally may be the only way to control and prevent infection, particularly of the avian strains.

## Inactivated influenza vaccine for intranasal immunization.

### References

1. Kyeyagalire R, Tempia S, Cohen AL, Smith AD, McAnerney JM, Dermaux-Msimang V, et al. Hospitalizations associated with influenza and respiratory syncytial virus among patients attending a network of private hospitals in South Africa, 2007-2012. *BMC infectious diseases*. 2014;14:694.
2. Nair H, Brooks WA, Katz M, Roca A, Berkley JA, Madhi SA, et al. Global burden of respiratory infections due to seasonal influenza in young children: a systematic review and meta-analysis. *Lancet*. 2011;378(9807):1917-30.
3. Lafond KE, Nair H, Rasooly MH, Valente F, Booy R, Rahman M, et al. Global Role and Burden of Influenza in Pediatric Respiratory Hospitalizations, 1982-2012: A Systematic Analysis. *PLoS medicine*. 2016;13(3):e1001977.
4. Matias G, Taylor R, Haguinet F, Schuck-Paim C, Lustig R, Shinde V. Estimates of mortality attributable to influenza and RSV in the United States during 1997-2009 by influenza type or subtype, age, cause of death, and risk status. *Influenza and other respiratory viruses*. 2014;8(5):507-15.
5. Nicholson KG, Wood JM, Zambon M. Influenza. *Lancet*. 2003;362(9397):1733-45.
6. Lemaitre M, Carrat F, Rey G, Miller M, Simonsen L, Viboud C. Mortality burden of the 2009 A/H1N1 influenza pandemic in France: comparison to seasonal influenza and the A/H3N2 pandemic. *PloS one*. 2012;7(9):e45051.
7. Hibbert CL, Piedra PA, McLaurin KK, Vesikari T, Mauskopf J, Mahadevia PJ. Cost-effectiveness of live-attenuated influenza vaccine, trivalent in preventing influenza in young children attending day-care centres. *Vaccine*. 2007;25(47):8010-20.
8. Writing Committee of the WHO CoCAoPI, Bautista E, Chotpitayasonondh T, Gao Z, Harper SA, Shaw M, et al. Clinical aspects of pandemic 2009 influenza A (H1N1) virus infection. *The New England journal of medicine*. 2010;362(18):1708-19.
9. Estimates of deaths associated with seasonal influenza - United States, 1976-2007. *MMWR Morbidity and mortality weekly report*. 59:1057-6.
10. Luce BR, Nichol KL, Belshe RB, Frick KD, Li SX, Boscoe A, et al. Cost-effectiveness of live attenuated influenza vaccine versus inactivated influenza vaccine among children aged 24-59 months in the United States. *Vaccine*. 2008;26(23):2841-8.
11. Zakay-Rones Z. Human influenza vaccines and assessment of immunogenicity. *Expert review of vaccines*. 2010;9(12):1423-39.
12. Oliveira Jde F, Boing AF, Waldman EA, Antunes JL. Ecological study on mortality from influenza and pneumonia before and after influenza vaccination in the Northeast and South of Brazil. *Cadernos de saude publica*. 2013;29(12):2535-45.
13. Grohskopf LA, Sokolow LZ, Broder KR, Olsen SJ, Karron RA, Jernigan DB, et al. Prevention and Control of Seasonal Influenza with Vaccines. *MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports*. 2016;65(5):1-54.
14. Stohr K, Bucher D, Colgate T, Wood J. Influenza virus surveillance, vaccine strain selection, and manufacture. *Methods in molecular biology*. 2012;865:147-62.
15. Greenberg DP, Robertson CA, Noss MJ, Blatter MM, Biedenbender R, Decker MD. Safety and immunogenicity of a quadrivalent inactivated influenza vaccine compared to licensed trivalent inactivated influenza vaccines in adults. *Vaccine*. 2013;31(5):770-6.
16. Ambrose CS, Levin MJ. The rationale for quadrivalent influenza vaccines. *Human*

**Inactivated influenza vaccine for intranasal immunization.**

- vaccines & immunotherapeutics. 2012;8(1):81-8.
17. Antunes JL, Waldman EA, Borrell C, Paiva TM. Effectiveness of influenza vaccination and its impact on health inequalities. *International journal of epidemiology*. 2007;36(6):1319-26.
  18. Ben-Yehuda A, Joseph A, Zeira E, Even-Chen S, Louria-Hayon I, Babai I, et al. Immunogenicity and safety of a novel liposomal influenza subunit vaccine (INFLUSOME-VAC) in young adults. *Journal of medical virology*. 2003;69(4):560-7.
  19. Couch RB. Seasonal inactivated influenza virus vaccines. *Vaccine*. 2008;26 Suppl 4:D5-9.
  20. Montomoli E, Khadang B, Piccirella S, Trombetta C, Mennitto E, Manini I, et al. Cell culture-derived influenza vaccines from Vero cells: a new horizon for vaccine production. *Expert review of vaccines*. 2012;11(5):587-94.
  21. Barrett PN, Berezuk G, Fritsch S, Aichinger G, Hart MK, El-Amin W, et al. Efficacy, safety, and immunogenicity of a Vero-cell-culture-derived trivalent influenza vaccine: a multicentre, double-blind, randomised, placebo-controlled trial. *Lancet*. 2011;377(9767):751-9.
  22. Barrett PN, Portsmouth D, Ehrlich HJ. Vero cell culture-derived pandemic influenza vaccines: preclinical and clinical development. *Expert review of vaccines*. 2013;12(4):395-413.
  23. Ehrlich HJ, Muller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, et al. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. *The New England journal of medicine*. 2008;358(24):2573-84.
  24. Ehrlich HJ, Singer J, Berezuk G, Fritsch S, Aichinger G, Hart MK, et al. A cell culture-derived influenza vaccine provides consistent protection against infection and reduces the duration and severity of disease in infected individuals. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2012;54(7):946-54.
  25. Shin D, Park KJ, Lee H, Cho EY, Kim MS, Hwang MH, et al. Comparison of immunogenicity of cell-and egg-passaged viruses for manufacturing MDCK cell culture-based influenza vaccines. *Virus research*. 2015;204:40-6.
  26. DiazGranados CA, Dunning AJ, Robertson CA, Talbot HK, Landolfi V, Greenberg DP. Effect of Previous-Year Vaccination on the Efficacy, Immunogenicity, and Safety of High-Dose Inactivated Influenza Vaccine in Older Adults. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2016;62(9):1092-9.
  27. Robertson CA, DiazGranados CA, Decker MD, Chit A, Mercer M, Greenberg DP. Fluzone(R) High-Dose Influenza Vaccine. *Expert review of vaccines*. 2016;15(12):1495-505.
  28. Cate TR, Rayford Y, Nino D, Winokur P, Brady R, Belshe R, et al. A high dosage influenza vaccine induced significantly more neuraminidase antibody than standard vaccine among elderly subjects. *Vaccine*. 2010;28(9):2076-9.
  29. Keren G, Segev S, Morag A, Zakay-Rones Z, Barzilai A, Rubinstein E. Failure of influenza vaccination in the aged. *Journal of medical virology*. 1988;25(1):85-9.
  30. Turner DA, Wailoo AJ, Cooper NJ, Sutton AJ, Abrams KR, Nicholson KG. The cost-effectiveness of influenza vaccination of healthy adults 50-64 years of age. *Vaccine*. 2006;24(7):1035-43.
  31. Flannery B, Clippard J, Zimmerman RK, Nowalk MP, Jackson ML, Jackson LA, et al. Early estimates of seasonal influenza vaccine effectiveness - United States, January 2015. *MMWR Morbidity and mortality weekly report*. 2015;64(1):10-5.

**Inactivated influenza vaccine for intranasal immunization.**

32. Flannery B, Reynolds SB, Blanton L, Santibanez TA, O'Halloran A, Lu PJ, et al. Influenza Vaccine Effectiveness Against Pediatric Deaths: 2010-2014. *Pediatrics*. 2017;139(5).
33. Recommendations of the ACIP: prevention and control of seasonal influenza with vaccines  
In: CDC M, editor. 2009. p. 1-52
34. Gasparini R, Amicizia D, Lai PL, Panatto D. Live attenuated influenza vaccine--a review. *Journal of preventive medicine and hygiene*. 2011;52(3):95-101.
35. Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, et al. Live attenuated versus inactivated influenza vaccine in infants and young children. *The New England journal of medicine*. 2007;356(7):685-96.
36. He XS, Holmes TH, Zhang C, Mahmood K, Kemble GW, Lewis DB, et al. Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. *Journal of virology*. 2006;80(23):11756-66.
37. Wang L, Liu SY, Chen HW, Xu J, Chapon M, Zhang T, et al. Generation of a Live Attenuated Influenza Vaccine that Elicits Broad Protection in Mice and Ferrets. *Cell host & microbe*. 2017;21(3):334-43.
38. Waldman RH, Mann JJ, Small PA, Jr. Immunization against influenza. Prevention of illness in man by aerosolized inactivated vaccine. *Jama*. 1969;207(3):520-4.
39. Kuno-Sakai H. [Intranasal application of inactivated influenza virus vaccines]. *Nihon rinsho Japanese journal of clinical medicine*. 1997;55(10):2738-45.
40. Potter CW, Jennings R. Intranasal immunization with inactivated influenza vaccine. *Pharmaceutical science & technology today*. 1999;2(10):402-8.
41. Muszkat M, Friedman G, Schein MH, Naveh P, Greenbaum E, Schlesinger M, et al. Local SIgA response following administration of a novel intranasal inactivated influenza virus vaccine in community residing elderly. *Vaccine*. 2000;18(16):1696-9.
42. Muszkat M, Yehuda AB, Schein MH, Friedlander Y, Naveh P, Greenbaum E, et al. Local and systemic immune response in community-dwelling elderly after intranasal or intramuscular immunization with inactivated influenza vaccine. *Journal of medical virology*. 2000;61(1):100-6.
43. Muszkat M, Greenbaum E, Ben-Yehuda A, Oster M, Yeu'l E, Heimann S, et al. Local and systemic immune response in nursing-home elderly following intranasal or intramuscular immunization with inactivated influenza vaccine. *Vaccine*. 2003;21(11-12):1180-6.
44. Greenbaum E, Furst A, Kiderman A, Stewart B, Levy R, Schlesinger M, et al. Serum and mucosal immunologic responses in children following the administration of a new inactivated intranasal anti-influenza vaccine. *Journal of medical virology*. 2001;65(1):178-84.
45. Greenbaum E, Furst A, Kiderman A, Stewart B, Levy R, Schlesinger M, et al. Mucosal [SIgA] and serum [IgG] immunologic responses in the community after a single intra-nasal immunization with a new inactivated trivalent influenza vaccine. *Vaccine*. 2002;20(7-8):1232-9.
46. Greenbaum E, Engelhard D, Levy R, Schlesinger M, Morag A, Zakay-Rones Z. Mucosal (SIgA) and serum (IgG) immunologic responses in young adults following intranasal administration of one or two doses of inactivated, trivalent anti-influenza vaccine. *Vaccine*. 2004;22(20):2566-77.
47. Kiderman A, Furst A, Stewart B, Greenbaum E, Morag A, Zakay-Rones Z. A double-blind trial of a new inactivated, trivalent, intra-nasal anti-influenza vaccine in general practice: relationship between immunogenicity and respiratory morbidity over the winter of 1997-98. *Journal of clinical virology : the official publication of the Pan*

**Inactivated influenza vaccine for intranasal immunization.**

- American Society for Clinical Virology. 2001;20(3):155-61.
48. Muhamed G, Greenbaum E, Zakay-Rones Z. Neuraminidase antibody response to inactivated influenza virus vaccine following intranasal and intramuscular vaccination. *The Israel Medical Association journal : IMAJ*. 2006;8(3):155-8.
49. Takada A, Matsushita S, Ninomiya A, Kawaoka Y, Kida H. Intranasal immunization with formalin-inactivated virus vaccine induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice. *Vaccine*. 2003;21(23):3212-8.
50. Gluck R, Mischler R, Durrer P, Furer E, Lang AB, Herzog C, et al. Safety and immunogenicity of intranasally administered inactivated trivalent virosome-formulated influenza vaccine containing *Escherichia coli* heat-labile toxin as a mucosal adjuvant. *The Journal of infectious diseases*. 2000;181(3):1129-32.
51. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *The New England journal of medicine*. 2004;350(9):896-903.
52. Fiore AE, et al. Prevention and control of seasonal influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep*: 2009.
53. Ambrose CS, Bright H, Mallory R. Letter to the editor: Potential causes of the decreased effectiveness of the influenza A(H1N1)pdm09 strain in live attenuated influenza vaccines. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin*. 2016;21(45).
54. Quan FS, Compans RW, Nguyen HH, Kang SM. Induction of heterosubtypic immunity to influenza virus by intranasal immunization. *Journal of virology*. 2008;82(3):1350-9.
55. Hashigucci K, Ogawa H, Ishidate T, Yamashita R, Kamiya H, Watanabe K, et al. Antibody responses in volunteers induced by nasal influenza vaccine combined with *Escherichia coli* heat-labile enterotoxin B subunit containing a trace amount of the holotoxin. *Vaccine*. 1996;14(2):113-9.
56. Tamura S. Studies on the usefulness of intranasal inactivated influenza vaccines. *Vaccine*. 2010;28(38):6393-7.
57. Tamura SI, Samegai Y, Kurata H, Kikuta K, Nagamine T, Aizawa C, et al. Enhancement of protective antibody responses by cholera toxin B subunit inoculated intranasally with influenza vaccine. *Vaccine*. 1989;7(3):257-62.
58. Hasegawa H, Ichinohe T, Aina A, Tamura S, Kurata T. Development of mucosal adjuvants for intranasal vaccine for H5N1 influenza viruses. *Therapeutics and clinical risk management*. 2009;5(1):125-32.
59. Ichinohe T, Tamura S, Kawaguchi A, Ninomiya A, Imai M, Itamura S, et al. Cross-protection against H5N1 influenza virus infection is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine. *The Journal of infectious diseases*. 2007;196(9):1313-20.
60. Ichinohe T, Aina A, Ami Y, Nagata N, Iwata N, Kawaguchi A, et al. Intranasal administration of adjuvant-combined vaccine protects monkeys from challenge with the highly pathogenic influenza A H5N1 virus. *Journal of medical virology*. 2010;82(10):1754-61.
61. Maltais AK, Stittelaar KJ, Veldhuis Kroeze EJ, van Amerongen G, Dijkshoorn ML, Krestin GP, et al. Intranasally administered Endocine formulated 2009 pandemic influenza H1N1 vaccine induces broad specific antibody responses and confers protection in ferrets. *Vaccine*. 2014;32(26):3307-15.
62. Chua BY, Wong CY, Mifsud EJ, Edenborough KM, Sekiya T, Tan AC, et al. Inactivated Influenza Vaccine That Provides



**Inactivated influenza vaccine for intranasal immunization.**

- Rapid, Innate-Immune-System-Mediated Protection and Subsequent Long-Term Adaptive Immunity. *mBio*. 2015;6(6):e01024-15.
63. Saito S, Ainai A, Suzuki T, Harada N, Ami Y, Yuki Y, et al. The effect of mucoadhesive excipient on the nasal retention time of and the antibody responses induced by an intranasal influenza vaccine. *Vaccine*. 2016;34(9):1201-7.
64. Furuya Y. Return of inactivated whole-virus vaccine for superior efficacy. *Immunology and cell biology*. 2012;90(6):571-8.
65. Haredy AM, Takenaka N, Yamada H, Sakoda Y, Okamatsu M, Yamamoto N, et al. An MDCK cell culture-derived formalin-inactivated influenza virus whole-virion vaccine from an influenza virus library confers cross-protective immunity by intranasal administration in mice. *Clinical and vaccine immunology : CVI*. 2013;20(7):998-1007.
66. Stephenson I, Zambon MC, Rudin A, Colegate A, Podda A, Bugarini R, et al. Phase I evaluation of intranasal trivalent inactivated influenza vaccine with nontoxicogenic *Escherichia coli* enterotoxin and novel biovector as mucosal adjuvants, using adult volunteers. *Journal of virology*. 2006;80(10):4962-70.
67. Ainai A, Tamura S, Suzuki T, van Riet E, Ito R, Odagiri T, et al. Intranasal vaccination with an inactivated whole influenza virus vaccine induces strong antibody responses in serum and nasal mucus of healthy adults. *Human vaccines & immunotherapeutics*. 2013;9(9):1962-70.
68. Ainai A, Tamura S, Suzuki T, Ito R, Asanuma H, Tanimoto T, et al. Characterization of neutralizing antibodies in adults after intranasal vaccination with an inactivated influenza vaccine. *Journal of medical virology*. 2012;84(2):336-44.
69. Tamura S, Ainai A, Suzuki T, Kurata T, Hasegawa H. Intranasal Inactivated Influenza Vaccines: a Reasonable Approach to Improve the Efficacy of Influenza Vaccine? *Japanese journal of infectious diseases*. 2016;69(3):165-79.
70. Okamoto S, Matsuoka S, Takenaka N, Haredy AM, Tanimoto T, Gomi Y, et al. Intranasal immunization with a formalin-inactivated human influenza A virus whole-virion vaccine alone and intranasal immunization with a split-virion vaccine with mucosal adjuvants show similar levels of cross-protection. *Clinical and vaccine immunology : CVI*. 2012;19(7):979-90.
71. Overton ET, Goepfert PA, Cunningham P, Carter WA, Horvath J, Young D, et al. Intranasal seasonal influenza vaccine and a TLR-3 agonist, rintatolimod, induced cross-reactive IgA antibody formation against avian H5N1 and H7N9 influenza HA in humans. *Vaccine*. 2014;32(42):5490-5.
72. Li Q, Zhou L, Zhou M, Chen Z, Li F, Wu H, et al. Epidemiology of human infections with avian influenza A(H7N9) virus in China. *The New England journal of medicine*. 2014;370(6):520-32.
73. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, et al. Human infection with a novel avian-origin influenza A (H7N9) virus. *The New England journal of medicine*. 2013;368(20):1888-97.
74. Tong S, Li Y, Rivaille P, Conrardy C, Castillo DA, Chen LM, et al. A distinct lineage of influenza A virus from bats. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109(11):4269-74.
75. Belongia EA, Sundaram ME, McClure DL, Meece JK, Ferdinands J, VanWormer JJ. Waning vaccine protection against influenza A (H3N2) illness in children and older adults during a single season. *Vaccine*. 2015;33(1):246-51.
76. Liu X, Liu Y, Zhang Y, Chen Z, Tang Z, Xu Q, et al. Pre-existing immunity with high neutralizing activity to 2009 pandemic

**Inactivated influenza vaccine for intranasal immunization.**

- H1N1 influenza virus in Shanghai population. *PloS one*. 2013;8(3):e58810.
77. Gostic KM, Ambrose M, Worobey M, Lloyd-Smith JO. Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. *Science*. 2016;354(6313):722-6.
78. Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, et al. A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science*. 2011;333(6044):843-50.
79. Egorov AY. The challenges of creating a universal influenza vaccine. *MIR Journal*. 2016;3(1).
80. Nabel GJ, Fauci AS. Induction of unnatural immunity: prospects for a broadly protective universal influenza vaccine. *Nature medicine*. 2010;16(12):1389-91.
81. Steel J, Lowen AC, Wang TT, Yondola M, Gao Q, Haye K, et al. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *mBio*. 2010;1(1).
82. Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, et al. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science*. 2011;333(6044):850-6.
83. Bianchi E, Liang X, Ingallinella P, Finotto M, Chastain MA, Fan J, et al. Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *Journal of virology*. 2005;79(12):7380-8.
84. Krammer F. Novel universal influenza virus vaccine approaches. *Current opinion in virology*. 2016;17:95-103.
85. Dreyfus C, Laursen NS, Kwaks T, Zuijdgheest D, Khayat R, Ekiert DC, et al. Highly conserved protective epitopes on influenza B viruses. *Science*. 2012;337(6100):1343-8.
86. Vandervan HA, Ana-Sosa-Batiz F, Jegaskanda S, Rockman S, Laurie K, Barr I, et al. What Lies Beneath: Antibody Dependent Natural Killer Cell Activation by Antibodies to Internal Influenza Virus Proteins. *EBioMedicine*. 2016;8:277-90.
87. Yassine HM, Boyington JC, McTamney PM, Wei CJ, Kanekiyo M, Kong WP, et al. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nature medicine*. 2015;21(9):1065-70.
88. El Bakkouri K, Descamps F, De Filette M, Smet A, Festjens E, Birkett A, et al. Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. *Journal of immunology*. 2011;186(2):1022-31.
89. Schotsaert M, De Filette M, Fiers W, Saelens X. Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments. *Expert review of vaccines*. 2009;8(4):499-508.
90. De Filette M, Min Jou W, Birkett A, Lyons K, Schultz B, Tonkyro A, et al. Universal influenza A vaccine: optimization of M2-based constructs. *Virology*. 2005;337(1):149-61.
91. Hatta M, Kawaoka Y. The NB protein of influenza B virus is not necessary for virus replication in vitro. *Journal of virology*. 2003;77(10):6050-4.
92. Wraith DC, Vessey AE, Askonas BA. Purified influenza virus nucleoprotein protects mice from lethal infection. *The Journal of general virology*. 1987;68 ( Pt 2):433-40.
93. Epstein SL, Kong WP, Mispion JA, Lo CY, Tumpey TM, Xu L, et al. Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. *Vaccine*. 2005;23(46-47):5404-10.
94. Gschoesser C, Almanzar G, Hainz U, Ortin J, Schonitzer D, Schild H, et al. CD4+ and CD8+ mediated cellular immune response to recombinant influenza nucleoprotein. *Vaccine*. 2002;20(31-32):3731-8.

**Inactivated influenza vaccine for intranasal immunization.**

95. Zhirnov OP, Isaeva EI, Konakova TE, Thoidis G, Piskareva LM, Akopova, II, et al. Protection against mouse and avian influenza A strains via vaccination with a combination of conserved proteins NP, M1 and NS1. *Influenza and other respiratory viruses*. 2007;1(2):71-9.
96. Gilbert SC. Advances in the development of universal influenza vaccines. *Influenza and other respiratory viruses*. 2013;7(5):750-8.
97. Tamura S, Miyata K, Matsuo K, Asanuma H, Takahashi H, Nakajima K, et al. Acceleration of influenza virus clearance by Th1 cells in the nasal site of mice immunized intranasally with adjuvant-combined recombinant nucleoprotein. *Journal of immunology*. 1996;156(10):3892-900.
98. Epstein SL, Price GE. Cross-protective immunity to influenza A viruses. *Expert review of vaccines*. 2010;9(11):1325-41.
99. Price GE, Lo CY, Mispion JA, Epstein SL. Mucosal immunization with a candidate universal influenza vaccine reduces virus transmission in a mouse model. *Journal of virology*. 2014;88(11):6019-30.
100. Schwartzman LM, Cathcart AL, Pujanauski LM, Qi L, Kash JC, Taubenberger JK. An Intranasal Virus-Like Particle Vaccine Broadly Protects Mice from Multiple Subtypes of Influenza A Virus. *mBio*. 2015;6(4):e01044.
101. Belyakov IM, Ahlers JD. What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? *Journal of immunology*. 2009;183(11):6883-92.
102. Corthesy B. Multi-faceted functions of secretory IgA at mucosal surfaces. *Frontiers in immunology*. 2013;4:185.
103. Suzuki T, Kawaguchi A, Aina A, Tamura S, Ito R, Multihartina P, et al. Relationship of the quaternary structure of human secretory IgA to neutralization of influenza virus. *Proceedings of the National Academy of Sciences of the United States of America*. 2015;112(25):7809-14.
104. Tanimoto T, Haredy AM, Takenaka N, Tamura S, Okuno Y, Mori Y, et al. Comparison of the cross-reactive anti-influenza neutralizing activity of polymeric and monomeric IgA monoclonal antibodies. *Viral immunology*. 2012;25(5):433-9.
105. Jewell NA, Cline T, Mertz SE, Smirnov SV, Flano E, Schindler C, et al. Lambda interferon is the predominant interferon induced by influenza A virus infection in vivo. *Journal of virology*. 2010;84(21):11515-22.
106. Krammer F, Pica N, Hai R, Tan GS, Palese P. Hemagglutinin Stalk-Reactive Antibodies Are Boosted following Sequential Infection with Seasonal and Pandemic H1N1 Influenza Virus in Mice. *Journal of virology*. 2012;86(19):10302-7.

**Inactivated influenza vaccine for intranasal immunization.**

**Table 1:** Available influenza vaccines

| <b>Vaccine</b>  | <b>Production system</b>                      | <b>Target group</b>                                | <b>Status</b>  |
|---|---|--|--|
| <p><b>a: Trivalent/ quadrivalent inactivated (formalin, BPL) by injection.</b></p> <p>Recently produced by all leading companies</p>              | <p>Eggs</p> <p>Cell Cultures (VERO,MDCK)</p>  | Different age groups                               | Licensed world wide                                  |
| 1. Whole Inactivated Virus  | <p>Eggs</p> <p>Cell Cultures (VERO,MDCK)</p>  | Not for children                                   | Licensed world wide                                  |
| 2. Split  | <p>Eggs</p> <p>Cell Cultures (VERO,MDCK)</p>  | From 6 months                                      | Licensed world wide                                  |
| <p>3. Subunit</p> <p>4. Fluzone high dose (60ug, Sanofi Pasteur)</p>  | <p>Eggs</p> <p>Cell Cultures (VERO,MDCK)</p>  | <p>From 6 months</p> <p>≥65</p>                    | <p>Licensed world wide</p> <p>FDA 2010</p>           |
| <p><b>b: Monovalent: A/H5N1,A/H1N1(2009) produced by all leading companies</b></p>  | <p>Eggs</p> <p>Cell Cultures (VERO,MDCK)</p>  | From 6 months                                      | Licensed world wide                                  |
| <p>1. LAIV (FluMist®) Trivalent, quadrivalent for intranasal administration</p> <p>2. Monovalent (A/H1N1 /2009) for intranasal administration</p> | <p>Eggs</p> <p>Cell Cultures (VERO, MDCK)</p> | <p>For healthy (non-pregnant)</p> <p>2-49 yrs.</p> | World wide   |
| 3. Flublok Recombinant influenza vaccine RIV3 trivalent and quadrivalent  | Insect cells Baculovirus technology           |  | <p>Approved 2013, 18-49 yrs.</p> <p>2016, ≥18yrs</p> |

**Inactivated influenza vaccine for intranasal immunization.**

**Table 2:** Viral Polypeptides and Components Relevant for Universal Vaccine

| Function   | Polypeptide/s  | RNA Segment |
|--|--|-------------|
| Transcriptase binds to cap structure on host cell MRNAS  | PB2  | 1           |
| Transcriptase elongation and endonuclease  | PB1 (F-2 pro-apoptotic)  | 2           |
| Transcriptase protease activity  | PA<br>PA-x(in A strains) inhibits INF expression   | 3           |
| Hemagglutinin, surface glycoprotein<br>Binds to receptor, fusion activity  | <b>HA</b><br><b>Conserved</b> stem part , two lineages found each sharing the same component | 4           |
| Nucleoprotein RNA binding; part of transcriptase complex ,nuclear/cytoplasmic transport of RNA   | <b>NP</b><br><b>Conserved</b> , common to all A or B strains                                 | 5           |
| Neuraminadase; surface glycoprotein, virus penetration and release neuraminidase activity  | <b>NA</b>  | 6           |
| Matrix protein major component of virion, plays a vital role in assembly by recruiting viral components to the site of assembly, has an essential role in budding process and viral particles(morphogenesis) | <b>M1</b><br><b>Conserved</b> , shared by all members of group A or B                        | 7           |
| Integral membrane protein-ion channel inserted into viral envelope and project from virus surface as tetramers(97aa ,19 in lipid 54 cytoplasmic  | <b>M2</b><br><i>M2-e 24aa extra-membranal exposed</i> <b>Conserved</b> (in A strains only)   | 7           |
| Nonstructural nucleus, effects on cellular NA transport ,splicing ,translation <b>antagonist to interferon</b>   | NS1  | 8           |
| Non- structural nuclear export protein   | NEP  |             |

**Inactivated influenza vaccine for intranasal immunization.**

**Table 3:** Response to infection and immunization

| <b>Response</b>  | <b>Infection</b>                            | <b>Parenteral Vaccination with Inactivated Vaccine</b>     | <b>Intranasal Vaccination with LAIV Vaccine</b> | <b>Intranasal Vaccination with Inactivated Vaccine</b> |
|--|---|--|---|--|
| <b>Innate Immunity</b>   |   |  |   |  |
| Cytokines<br>Interleukins<br>Interferon gamma<br>Interferon Type I<br>Type III | ++  | +  | ++  | ++   |
| <b>Adaptive Immunity</b>   |   |  |   |  |
| IgM, IgG (neutralizing antibodies)   | +++   | +++  | ++  | ++   |
| HAI  | +++   | +++  | +   | ++   |
| Anti NA  | +++   | ++   | +   | ++   |
| Mucosal SIgA   | +++   | +/-  | +++   | +++  |
| Cross protection   | ++  | +/_-   | ++  | ++   |
| ADCC   | +++   | +++  | -   | ++   |
| CD8 CTLs T cells   | ++  | +/-  | ++  | ++   |
| CD4 TH cells   | ++  | +/-  | +++   | ++   |
| Memory cells   | ++  | +  | ++  | +  |
| <b>Spectrum of Response</b>  | Broader to heterologous strains             | Narrow specific to homologous strains                      | Broader to heterologous strains                 | Broader to heterologous strains                        |
| <b>Defense Mounted</b>   | Mucosal entrance site of virus and in serum | Serum, lower respiratory tract. Negligible mucosal defense | Mucosal entrance site of virus and in serum     | Mucosal entrance site of virus and in serum            |

+ is to give an estimate of an existing response. Addition of adjuvant, as well as other factors, may shift results