H5N1 influenza vaccine quality is affected by hemagglutinin conformational stability

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ABSTRACT

Since 1997, highly pathogenic H5N1 avian influenza viruses have circulated in wild and domestic birds and sporadically have infected humans. Conventional inactivated vaccines made from these viruses were shown to have decreased HA content and immunogenicity compared to seasonal preparations. We assumed that the high pH threshold (5.6–6.0) known for the HA conformational change (pH of fusion or activation) of avian highly pathogenic influenza viruses was the reason of the low stability of native HA conformation and affected the vaccine quality. The S8Lys→Ile mutation introduced into the HA2 subunit of the HA of A/chicken/Kurgan/5/05 (H5N1) virus decreased the pH threshold of the HA activation. The mutant virus demonstrated increased HA stability toward acidic pH and elevated temperature, decreased binding efficiency to the monoclonal antibody IIF4 that recognizes the HA low pH form, and increased HA resistance to trypsin digestion. Virus with modified HA was less susceptible to freezing stress and showed an increased content of immunocompetent HA in inactivated vaccine preparation compared to the analogous virus with original HA. Therefore, we have shown a way to increase the quality of inactivated vaccines made from highly pathogenic avian influenza viruses.

INTRODUCTION

Since 1997, highly pathogenic avian influenza (HPAI) H5N1 viruses have continued to circulate and sporadically infect humans, and that poses a pandemic threat. Vaccination is recognized as the best strategy to prevent virus spreading. Conventional inactivated vaccines made from these viruses were shown to have decreased content of immunocompetent hemagglutinin (HA) and immunogenicity compared to seasonal preparations. The current international guidelines for an inactivated seasonal vaccine require one dose containing 15 μg of each of three/four HA antigens (two A-strains: H1N1 and H3N2 and one/two B-strains: Victoria and Yamagata lineages). The requirements of EU Committee for Proprietary Medicinal Products (CPMP) for effectiveness of inactivated interpandemic vaccine include the following specifications: (1) number of fourfold increases in anti-HA antibody titers is greater than 40%; (2) increase in geometric mean antibody titer exceeds 2.5; (3) number of individuals achieving anti-HA titer in hemagglutination inhibition (HAI) test >1:40 is greater than 70%. For the inactivated split H5N1 vaccine, the two-dose regimen, with 7.5 to 30 μg of HA, met the CHMP criteria. Additionally the percentage of seropositive subjects was not high enough even when the highest dose supplemented with aluminum hydroxide adjuvant was applied [1]. For subvirion H5N1 vaccine, two doses of 45–90 μg was necessary to get an antibody response in more than half of the subjects according to either HAI or microneutralization assays (MNA) [2]. Slightly better performance was shown for a whole virion vaccine, although the antibodies were revealed more often by MNA than by standard HAI test and remained at low levels [3]. The reason for such a low immunogenicity of H5N1 vaccines remains unknown so far.

Our previous studies have demonstrated that certain host range mutations in the HA of human influenza viruses lead to the enhancement of the pH threshold of HA activation which in turn contributed to the decreased virus stability and impaired HA antigen content of inactivated vaccine preparations [4]. Based on these data, we assumed that the higher pH threshold of HA conformational change known for the HPAI viruses (5.6–6.0) versus that for human viruses (5.0–5.4) [5, 6] might result in the low stability of the native HA conformation and decreased quality of the corresponding inactivated vaccines. The goal of this study was to investigate the effect of HA conformational stability on the HA antigen content and quality of whole virion inactivated influenza vaccine preparation made from H5N1 avian influenza virus.

MATERIALS AND METHODS

Cells and viruses

A Vero (WHO-certified) cell line was obtained from the European Collection of Cell Cultures and was adapted
and further cultivated at 37°C and 5% CO₂ in a serum-free Opti-pro medium (Invitrogen) containing 4 mM L-glutamine (Invitrogen). Madin-Darby canine kidney (MDCK; ATCC CCL-34) cells were cultivated at 37°C and 5% CO₂ in DMEM medium (Invitrogen) comprising 2% Fetal Bovine Serum (FBS, Invitrogen) and 2 mM L-glutamine.

Differentially cultured cells of human tracheo-bronchial epithelial cells (HTBE cultures) were prepared as described before [7, 8]. In brief, commercial primary human cells (Lonza) were expanded on plastic, and stored in aliquots. These first passage cells were propagated on membrane supports (12 mm Transwell-Clear, Corning Inc.) at an air–liquid interface (ALI) in serum-free, hormone- and growth factor-supplemented growth medium (GM) [7]. Fully differentiated 5- to 7-week-old cultures were used for the experiments.

All H5N1 recombinant viruses used in the present study were obtained by reverse genetics on Vero cells. The generation of reassortant viruses was performed by cotransfecting Vero cells with the respective plasmids, as described previously [9].

The HA and NA sequences of A/chicken/Kurgan/5/05 HPAI virus were obtained by direct sequencing. The GenBank accession numbers for HA and NA are DQ449632 and DQ449639. The sequences of HA and NA genes for viruses A/Vietnam/1203/04 (VN1203), A/Hong Kong/156/97 (HK156), A/Indonesia/5/05 (IND05), A/Anhui/1/05 (ANH01), A/turkey/Turkey/1/05 (tkTK01) were taken from the GenBank. All of the sequences were cloned into the pHW2006 plasmid, a synthetically produced analogue of pHW2000 [10]. The HA polybasic cleavage site was replaced by the more stable sequence TETR/GLF described for low pathogenic avian influenza viruses [11]. The 58Lys→Ile mutation in the HA2 subunit of A/chicken/Kurgan/5/05 HA was introduced by mutagenesis kit (Stratagene, Germany). Reassortants KG05 and KG05-58 inherited HA and NA genes from A/chicken/Kurgan/5/05 virus and genes coding six internal proteins from the A/PR/8/34 (H1N1) (PR8) strain and differed by a single mutation K58I in the HA2.

Additionally, a number of reassortants were generated containing HA and NA genes from VN1203, HK156, IND05, ANH01, tkTK01 viruses in combination with other genes from the IVR-116 vaccine strain. Virus IVR-116 is a reassortant containing HA and NA genes from the virus A/New Caledonia/20/99 (H1N1), PB1 gene from A/Texas/1/77 (H3N2) and all other genes from PR8.

Epidemic viruses A/Brisbane/59/07 (H1N1) (BR59) and A/Brisbane/10/07 (BR10) were obtained from NIBSC (National Institute of Biological Standards and Controls, GB). The primary isolate A/Vienna/25/07 (VI25) antigenically characterized as A/Wisconsin/67/05-like (H3N2) was provided by the Institute of Virology, Vienna, Austria. Human primary isolate A/St. Petersburg/14/10 (H1N1pdm) (SP14) and H5N3 low pathogenic virus A/duck/Singapore/5/97 (dkSG03) was provided by Research Institute of Influenza, Saint Petersburg, Russia.

Virus propagation and titration

Egg-derived viruses were propagated in the allantoic cavity of 9–11-day old embryonated specific pathogen free (SPF) chicken eggs (CEF). Allantoic fluid was collected 48 h post infection. Vero or MDCK cells were inoculated with viruses diluted in OptiPRO SFM medium and incubated at 37°C for 30 min. Cells were cultivated in OptiPRO SFM supplemented with 4 mM L-glutamine, 250 ng/ml Amphotericin B (Bristol-Myers Squibb, Austria) and 5 μg/ml trypsin (Sigma–Aldrich, Austria) at 37°C with 5% CO₂ for 72 h. Infectious virus titers were determined on CE, Vero or MDCK cells and expressed in 50% Embryo Infective Dose (EID₅₀/ml), calculated according to Reed and Muench method [12].

Hemolysis assay

Titers of investigated viruses were adjusted to 128 HAU. Diluted viruses (50 μl) were mixed with 150 μl of 1% chicken red blood cells (cRBC) and incubated on ice for 1 h to allow virus to bind. Mixtures were centrifuged at 400 g and supernatants removed. After the addition of 250 μl of 0.15 M sodium citrate buffered saline to the solid precipitate at various pH values (from 5.1 to 7.0) mixtures were incubated at 37°C for 1 h and finally centrifuged at 400 g. Then, 200 μl of supernatants were transferred to 96-well flat-bottom plates and the amount of hemoglobin released by virus-cell fusion induced hemolysis was determined by measuring the optical density (OD) at 415 nm. The presented results are the mean values of three replicates at the indicated pH plus/minus standard deviation (SD).

Thermal stability

Viruses were incubated in a thermo block at 58°C for different time intervals from 0 to 60 min. In parallel, control samples were incubated on ice for 60 min. Subsequently, the HA titer was measured with a 0.5% suspension of cRBC.

Characterization of virus HA conformation by binding with IIF4 antibodies

The assay protocol was adapted from Vareckova et al. [13]. Briefly, maxisorp 96-well plates (Nunc, Denmark) were coated with 40 HAU/well of virus diluted in PBS. After overnight incubation at 4°C, the wells were treated with 300 μl of MES-based buffers, adjusted to pH in the range from 4.6 to 7.5 and incubated at 37°C for 15 min. After buffer removal, the wells were washed with PBS and blocked with 0.5% BLOTTO and 0.5% Tween 20 in PBS. The presence of HA in low-pH conformation was assessed by 1 h incubation of plates with 100 ng/well of IIF4 monoclonal antibodies (Dr. E. Vareckova, Slovak Academy of Sciences) at room temperature, followed by detection with HRP labeled goat anti-mouse IgG(γ) (KPL, USA). The HRP enzyme activity was assayed with the Peroxidase Substrate TMB (KPL, USA). The enzyme reaction was stopped by the addition of 1 N H₂SO₄ following by the OD measurement at 450 nm. The reported results...
are the mean values of 4 replicates at the indicated pH plus/minus SD.

**Infectivity of viruses at different pH**

Vero cell monolayers were inoculated with viruses diluted to the multiplicity of infection (MOI) of 2 in MES infection buffer containing 250 ng/ml Amphotericin B, adjusted to pH 5.2, 5.4, 5.6, 5.8, 6.0, or 7.5, for 30 min at 37°C. After that, the inoculum was removed and the cells were incubated in OptiPRO SFM containing 4 mM L-glutamine in the presence of 5% CO₂ at 37°C for 5 h. Then, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, Austria), permeabilized with 1% Triton X-100 (Merck, Germany), and incubated overnight with 1% BSA (Sigma-Aldrich, Austria) in PBS. After that, the cells were stained with mouse anti-influenza virus A NP antibodies (Millipore, USA) followed by treatment with goat anti-mouse Alexa Fluor 488 antibodies (Invitrogen, Austria). The infected cells were detected by fluorescence microscopy.

HTBE cultures were inoculated with virus suspensions in MES buffers (pH 5.6, 5.8, 7.5) at MOI-1 for 1 h at 35°C. After removal of the inoculum, the cultures were washed 3 times with PBS and incubated at air-liquid interface for an additional 8 h. Then, the cultures were fixed and immunostained for viral nucleic acid as described above using peroxidase-labeled secondary antibodies and DAB peroxidase substrate.

MDCK cells were inoculated with viruses (MOI 2) in MES buffer at different pH as described above. After 5 h of incubation, the cells were collected from the solid support surface by 0.2% solution of trypsin-EDTA ("Cellogro") and fixed by Fix/Perm buffer (BD Cytofix/Cytoperm Plus Kit). After that, the cells were treated with 1% solution of fetal calf serum in PBS overnight, permeabilized and stained with FITC labeled anti-influenza NP antibodies ("Imagen", UK). The concentration of the infected cells was measured by flow cytometry using EXPO 32 software (Immunotech Inc., a Beckman Coulter company).

**Preparation of H5N1 inactivated vaccine samples**

Viruses were grown in the embryonated chicken eggs or cell cultures (Vero, MDCK) as described above. Harvested virus suspensions were mixed with formalin (final concentration 0.015%) and incubated at 32°C for 24.5 h for complete virus inactivation. Viral proteins were separated in a polyacrylamide gel by SDS-PAGE. Qubit Protein Assay (Invitrogen) was used to estimate the amount of protein in each preparation.

**Determination of HA content in vaccine preparations**

The influenza HA antigen content in the vaccine preparations was determined by single radial immuno-diffusion (SRID) assay according to Wood et al. [14].

**Treatment with trypsin**

The purified concentrated virus preparations were diluted to a concentration of 15 μg/ml protein and treated with 100 μg/ml trypsin (TPCK-treated, Sigma) in PBS at pH 7.4 and 37°C for 2 h. Then, each sample was mixed with a loading buffer, heated for 5 min at 95°C and the viral proteins were separated in a polyacrylamide gel by SDS-PAGE. Qubit Protein Assay (Invitrogen) was used to estimate the amount of protein in each preparation.

**RESULTS**

**Avian H5N1 influenza viruses are less stable at acidic pH than human viruses**

To find out whether the H5N1 viruses are less stable at acidic pH than human seasonal strains, we compared their infectivity at acidic pH in differentiated cultures of human tracheo-bronchial epithelial cells (HTBE). Primary human isolates V125 (H3N2), SP14 (H1N1pdm) and the reassortant VN1203 (H5N1) virus were adjusted to a similar MOI and mixed with acidic (pH 5.6 and 5.8) or neutral (pH 7.5) buffer, followed by inoculation into the cultures. In 1 h, the buffer was changed to the neutral medium and viruses were further incubated for 8 h at 37°C. The effectiveness of the infection was determined by visualization with anti-influenza nucleoprotein (NP) antibodies. As shown in Fig. 1, both human viruses V125 and SP14, infected HTBE cultures at pH 5.6 and 5.8 while virus VN1203 infected human cells at pH 7.5 and pH 5.8, but was not infectious at pH 5.6.

In order to check more H5N1 viruses, the reassortants with HA and NA genes from the avian viruses of different clades, such as HK156, (clade 1), IND05 (clade 2.1), ANH01 (clade 2.2), tkTK01 (clade 2.2), and KG05 (clade 2.2), were generated. The infectivity of obtained viruses at different pH was compared in the analogous experiment in MDCK cells at pH range 5.2-7.5 followed by the determination of the number of infected cells by flow cytometry (Fig. 2A). The virus was considered stable if it had infected more than 50% of the cells at the indicated pH.

We found that human strains BR59 (H1N1) and BR10 (H3N2) were the only ones infecting cells at pH ≥ 5.4 (62% and 68% respectively). Viruses dkSG03 (H5N3) and HK156 (H5N1) infected MDCK cells at pH ≥ 5.6 (74% and 76% at pH 5.6, respectively), and the rest of H5N1 strains were infectious only at pH ≥5.8. Based on the obtained results the stability chart was constructed (Fig. 2B).

**Stability of KG05 and mutant KG05-58 at low pH and elevated temperature**

It was shown that the substitution 58Lys→Ile introduced into the HA2 subunit of H5N1 avian virus decreases the pH threshold of the HA conformational change and increases virus stability toward acidic pH and elevated temperature [15-18]. We introduced this change in the HA of KG05 (Fig. 3). As a result, the constructed reassortant
Fig. 1. Infectivity of viruses at different pH in HTBE cultures.
Wells with differentiated cultures of human tracheobronchial epithelial cells were infected with SP14 (H1N1pdm), VI25 (H5N2) seasonal human viruses and with H5N1 virus (VN1203) at a different pH. The infected cells were visualized by immunostaining for viral NP.

Fig. 2. Infectivity of viruses at different pH in MDCK cells.
(A) Viruses were mixed with MES buffer at indicated pH and used for infection of MDCK cells at MOI 2. After incubating for 5 h, influenza NP was visualized by immunostaining followed by flow cytometry. The indicated numbers show the percentage of infected cells. (B) The diagram of stability of studied influenza viruses toward low pH.
KG05-58 differed from the original virus KG05 by a single 58Lys→Ile mutation in HA protein. Both viruses KG05 and KG05-58 demonstrated high growth in CE with the maximal HA titer of 512 HAU/50 μl and infectious activity of 9.0 logEID₅₀/ml (Fig. 4 A, B).

To investigate the effect of the introduced mutation on the pH threshold of the HA activation, viruses were compared in a hemolysis assay. As shown in Fig. 5A, virus KG05 triggered fusion at pH 5.6–5.7, while the same fusion rate for the mutant virus KG05-58 was observed at the pH 5.3–5.4.

Next, we compared the HA binding activity of KG05 and KG05-58 viruses to the IIF4 monoclonal antibody known to recognize the HA low pH conformation. The results show that IIF4 bound efficiently to the KG05 virus at pH 5.6 while the equivalent binding of IIF4 to KG05-58 virus was observed at pH 5.3 (Fig. 5B).

Then we investigated whether the mutant virus was more stable at an elevated temperature. Viruses were incubated at 58°C for various time intervals (from 0 to 60 min) and analyzed for the residual HA activity. The data presented in Fig. 5C, show that the complete HA inactivation for the virus KG05 was observed in 20 min, while for the mutant KG05-58 in 40 min.

Investigation of the virus infectivity under acidic conditions in Vero cells also revealed that the mutant KG05-58 gained the resistance to acidic pH compared to the original virus and infected Vero cells at pH ≥ 5.4, whereas the KG05 virus was infectious at pH ≥ 5.8 (Fig. 5D).

The obtained data demonstrate that introduction of 58Lys→Ile mutation leads to enhancement of HA protein stability of KG05 influenza virus toward an acidic pH and elevated temperature.

**Characterization of mutant and original viruses by electron microscopy**

The structure and morphology of the purified inactivated KG05 and KG05-58 viral particles was compared by means of electron microscopy. In addition to native virus preparations, we compared samples after one cycle of freezing/thawing. As it is shown in Fig. 6, the HA spikes of both viruses appeared as rods with a well-defined length. However, after one cycle of freezing/thawing a number of spikes of the KG05 virus disappeared and some of the remaining spikes were damaged. In contrast to that, the consistency of spikes and the virion morphology of KG05-58 did not change.

Thus, in the course of the freezing/thawing process, the KG05 virus was significantly destroyed, whereas the mutant virus KG05-58 was unaffected by this process.

**Susceptibility of original and mutant viruses to trypsin digestion**

The HA protein in its native state is resistant to the proteolysis by trypsin and other proteases, but becomes susceptible to it in low pH conformation [20]. In order to reveal the HA structural rearrangements after one cycle of freezing/thawing the HA integrity was assessed by SDS-PAGE before and after trypsin treatment. Virus preparations KG05 and KG05-58 were adjusted to the same protein concentration and treated with trypsin. The results show that, without trypsin digestion, no difference in the pattern of HA specific bands between viruses was observed (Fig. 7). Treatment with trypsin resulted in the disappearance of HA band for KG05 while the HA of mutant virus KG05-58 appeared to be resistant to trypsin digestion. This observation confirms that virus KG05 after one cycle of freezing/thawing...

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**Fig. 3.** Side view of the HA molecule three-dimensional structure. Residues responsible for protein stability are highlighted. The figure was generated with the Chimera v. 1.5.3 [19] structure 2IBX (Protein Data Bank).
H5N1 HA Stability and Vaccine Quality

Fig. 4. The kinetic of KG05 and KG05-58 growth in CE
(A) The infectious titer of viruses grown in CE. (B) The HA titer of viruses grown in CE at different time points. The results are presented as the mean of three independent experiments.

Fig. 5. The stability toward low pH and elevated temperature of KG05 and KG05-58 viruses
(A) Hemolysis assay of KG05 and KG05-58 made with chicken erythrocytes. (B) Binding affinity of the monoclonal antibody IIF4 to the HA of KG05 and KG05-58. (C) Thermal stability of KG05 and KG05-58 at elevated temperatures. (D) Infectivity of indicated viruses under acidic conditions in Vero cells at pH range 5.2-7.5.

contains more HA in the low pH conformation sensitive to trypsin compared to the mutant virus KG05-58.

Measurement of HA content in vaccine preparations
The most common test to estimate the inactivated influenza vaccine potency is the SRID. This assay measures the amount of the immunocompetent HA reacting with antibodies present in the standard antiserum. To find out whether the substrate used for the virus propagation has any impact on the vaccine potency, we propagated KG05 and KG05-58 in CE, Vero and MDCK cell lines and determined the relative HA content of each inactivated virus preparation (Table 1). All virus-containing fluids were inactivated, purified, and concentrated by the same procedure and then diluted to the protein content of 100 μg/ml. The results show that the relative amount of immunocompetent HA in the KG05 grown in MDCK was 1.8 times lower than that of the mutant KG05-58 (Fig. 8C).

For the virus grown in the Vero cell line, the relative HA content for the mutant KG05-58 was 1.14 times higher than that of the KG05 (Fig. 8B). Inactivated viruses produced in CE did not show any substantial difference in HA content (Fig. 8A). It is interesting to note that the total protein amount in preparations derived from the modified virus KG05-58 grown on MDCK and Vero was 1.9 and 1.5 times higher, respectively, than that of the original KG05 virus (Table 1).

DISCUSSION
In the current study, we investigated the effect of the HA conformational stability of HPAI H5N1 viruses on the properties of inactivated vaccine preparations. We found that H5N1 viruses of different clades are more susceptible to acidic inactivation than the human BR59 (H1N1) and BN10 (H3N2) seasonal strains.
We showed that the H5N1 virus stability could be improved by the introduction of a single amino acid substitution 58Lys→Ile in the HA2 subunit of HA molecule. The amino acid at position 58 is located in the HA2 coiled-coil domain and presumably stabilizes the HA trimer structure providing additional intermolecular hydrophobic interactions with the neighboring monomers (Fig. 5) [21]. Consequently, the HA of mutant virus KG05-58 underwent fusion with erythrocytes at pH close to that of the human viruses (pH 5.0–5.4) [22]. The mutant virus also became more resistant to elevated temperature and trypsin digestion than the original virus. Finally, the introduced mutation contributed to the resistance of HA spikes to freezing/thawing stress induced by pH shift because of the changes of the local salt concentration of PBS buffer during freezing [23].

As a result, the purified virus preparation of mutant virus contained more immunocompetent HA as measured by SRID assay compared to the preparation made from the original virus. The observed difference depended on the substrate used for virus propagation. The major increase was detected for the virus preparations produced on MDCK cells. The corresponding effect for the Vero-grown strains was less pronounced. No difference was revealed for viruses grown in CE. Since the total protein content for the mutant and original viruses was equal, it is reasonable to explain the decreased amount of immunocompetent HA in the original virus preparation by the HA “damage” in the course of purification and concentration steps. The absence of difference for the egg-grown viruses could be explained by the higher pH (8.0–8.3) of the allantoic fluid in comparison to that of the cell media (pH 7.4) and the possible stabilizing action of the allantoic proteins [24].

Harvey et al. [25] showed that H5N1 vaccine preparations contain up to 22% less immunocompetent HA than vaccines prepared from the human influenza viruses. The authors concluded that this effect is partially due to the relatively lower HA protein content in the H5N1 virus particles. According to our results, the low content of immunocompetent HA of H5N1 vaccines is rather attributed to the HA conformational instability of H5N1 viruses.

The natural structure of HA represents the metastable state, which is triggered by acidic pH to the thermodynamically more stable irreversible conformation by overcoming the kinetic barrier. As a result the HA antigenic determinants located on the surface of the HA1 globular part and responsible for the development of the neutralizing antibodies, are being masked, while the HA2 epitopes, hidden in the natural HA structure, become exposed. The HA2 epitopes are known to induce antibodies, which do not react in HAI
Table 1. The HA titers and total protein contents of KG05 and KG05-58 purified inactivated virus preparations grown in different substrates.

| Substrate | Virus   | Viral Suspension | Purified inactivated virus preparations |
|-----------|---------|------------------|-----------------------------------------|
|           |         | Volume, ml       | HA Titer | Volume, ml | HA Titer | Total Protein, mg/ml |
| CE        | KG05    | 100              | 128      | 0.5        | 12800    | 1.78                 |
|           | KG05-58 | 100              | 128      | 0.5        | 12800    | 1.92                 |
| Vero      | KG05    | 100              | 16       | 0.4        | 400      | 0.40                 |
|           | KG05-58 | 100              | 16       | 0.4        | 800      | 0.61                 |
| MDCK      | KG05    | 100              | 16-32    | 0.25       | 320      | 0.30                 |
|           | KG05-58 | 100              | 16       | 0.25       | 640      | 0.59                 |

Fig. 8. The HA content in the KG05 and KG05-58 purified virus preparations measured by SRID assay.
Relative HA content in purified inactivated virus preparations grown in different substrates was measured as precipitation area (mm²): (A) CE, (B) Vero cells, (C) MDCK. Viruses were adjusted to the same protein content.
test. Thus, the low HA conformational stability of H5N1 viruses might be related to the decreased amount of antibodies reacting in the HA1 test revealed for H5N1 vaccines[26, 27]. Our assumption is confirmed by Khurana et al. [28], who reported the enhanced production of the antibodies to the HA2 antigenic determinants in patients vaccinated with the H5N1 vaccine. The authors showed that the antibodies in the sera of individuals had a low binding affinity to the properly folded HA1 subunit (peptide 28-319), but showed high reactivity with the HA2 epitopes. The amount of neutralizing antibodies directed to the native HA increased when the vaccine was administered together with adjuvant MF59. It could possibly be explained by the stabilizing action of the oil adjuvant MF59.

The HA conformational change could be triggered not only by pH shift but also by elevated temperature and denaturation agents [29]. Therefore, the H5N1 viruses are highly susceptible to different destabilizing factors during the vaccine production, such as centrifugation, filtration, chromatography, chemical disruption, and storage.

It should be mentioned that the low HA conformational stability of H5N1 viruses increases the tendency of virions to form aggregates by interaction of the hydrophobic HA2 domens [30]. The presence of aggregates in vaccine preparations is extremely undesirable because they are known to induce additional side effects in humans after the vaccination [31].

Earlier, we have shown that the efficiency of intranasal vaccination with the live virus also depends on the HA conformational stability [15, 32]. The correct native HA composition is critical for the effective virus attachment and penetration in the epithelial cells of the upper respiratory tract and assures the higher immune response. These results are in full agreement with the data published by Imai et al. [33], who discovered that the increased pH of HA activation of the avian highly pathogenic virus strains is one of the factors for their low airborne transmission in ferrets.

In summary, we showed that the decreased content of the immunocompetent HA in the HPAI vaccine preparations is determined by the high HA activation pH leading to the low HA conformational stability of these viruses. This could also explain the low immunogenicity of the H5N1 vaccines, but the latter assumption requires further investigation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no commercial or financial conflict of interest.

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