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REVIEW

Influenza vaccines manufacturing in continuous cell lines: problems and solutions

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ABSTRACT

In order to decrease the morbidity and mortality caused by seasonal influenza outbreaks, several hundred million vaccine doses are produced worldwide each year. The predominant substrate for the production of the influenza vaccine today is fertilized hen's eggs. The substitution of the technology based on living organisms by the cell culture-based process offers many advantages, including easier scalability and reduced dependence on the availability of eggs. The African green monkey kidney and Madin Darby canine kidney cell lines support the efficient growth of influenza viruses of different subtypes and, therefore, are considered to be the two most promising alternative substrates for the production of the human influenza vaccine.

However, the pH of endosomes in both of these cell lines is higher than the pH essential for triggering a conformational change of the hemagglutinin (HA) of human influenza viruses, which enables the viral-cellular membrane fusion. This mismatch gives rise to mutations in the HA that lead to an increase of the optimum pH of HA conformational change. As of a result of these mismatches, the HA, and consequently the whole virus, has reduced stability to low pH and elevated temperatures. The production of a vaccine from less stable virus will lead to an elevated HA content in the low pH conformation that can affect the safety, potency, infectivity, and protective efficacy of the final inactivated and live attenuated influenza vaccines.

The main limitations of the cell line-based influenza vaccine technology and the possibilities to preserve the viral stability over the course of influenza vaccine production are discussed in the review.

INTRODUCTION

According to data from the World Health Organization (WHO), seasonal influenza epidemics cause 250,000 to 500,000 deaths worldwide every year. Vaccination remains the primary measure to prevent the spread of this virus and to reduce the morbidity and mortality caused by influenza disease.

The influenza virus belongs to the family *Orthomyxoviridae* that comprises five genera: influenza A, B, C, *Thogotovirus*, and *Isavirus*. Influenza A and B viruses are the only types that infect humans. The host antibodies are generated to the influenza virus surface glycoproteins: hemagglutinin (HA or H) and neuraminidase (NA or N). These proteins are responsible for the virus attachment to the target cells and subsequent release of new virus particles. To date, 18 variants of HAs and 11 variants of NAs are known for the influenza A virus. The combination of different HAs and NAs in virus defines the influenza A virus subtype. Only the viruses of three subtypes, *i.e.* A(H1N1), A(H2N2), and A(H3N2), have been circulating in the human population that have caused outbreaks and pandemics. There are no known subtypes for the influenza B virus strains, although two antigenically different lineages have been identified, B/Victoria and B/Yamagata.

The WHO performs permanent surveillance and control of new influenza viruses based on the data provided by the National Influenza Centers. The National Centers isolate the viruses from infected humans and characterize them in terms of their antigenic properties and the HA and NA primary structure. The isolation is performed in fertilized hen's eggs or in Madin Darby canine kidney (MDCK) cell line. Two times per year, the WHO selects the most dominating strains and recommends them for the vaccine composition for Northern and Southern hemisphere, respectively (http://www.who.int/influenza/en/). The vaccine composition includes two strains of influenza A (H3N2 and H1N1) and one strain of B virus for the production of trivalent preparation. The high growth seed viruses of influenza A are generated by the genetic reassortment of the H1N1 and H3N2 viruses provided by the WHO Collaborative Centers with egg-adapted virus A/Puerto Rico/8/34 (H1N1) (PR8). Influenza B strains are used without any reassortment. All seed viruses are then distributed to the manufacturers. It is important to notice that only egg-isolated virus strains are allowed for use in the vaccine production because of the established safety parameters for this substrate.

Reports about the sporadic infections of humans with low pathogenic (H9N2, H7N2, H7N3) and highly pathogenic (H5N1, H7N3, H7N7, and H7N9) avian influenza viruses (LPAIVs and HPAIVs, respectively) are constantly being published [1-4]. The wild aquatic birds are the main source of LPAIVs. These viruses replicate predominantly in the intestinal tract of birds and induce asymptomatic infection [5]. Sporadically, LPAIVs infect domestic animals and poultry, and can infect humans working on the animal and poultry farms causing the symptomatic disease. The disease induced by HPAIVs is more severe. These viruses infect wild birds as well as poultry and induce infection with a mortality rate close to 100%. The reassortment of any avian virus with a human strain can result in the emergence of the virus with new antigenic proteins. Such a virus would have pandemic potential since the human population lacks immunity to the avian HA and NA proteins and, consequently, a pre-pandemic vaccine should be produced in this case.

The vast majority (approx. 500 million doses) of all the existing vaccines are produced in hen's eggs – an old production system developed in the 1950s [6]. The production of the influenza vaccine in eggs is a cumbersome and time-consuming process. The duration of the whole process is about 6 months. The eggs for vaccine manufacture must be free of any pathogens (specific pathogen free, SPF) and should be ordered in advance to insure the continuous process. The estimated current capacity of all vaccine producing companies is 1.42 billion doses per year, which is not enough to supply the world's population in the case of a pandemic threat [6]. Substitution of the egg-based vaccine production technology by the process based on continuous cell lines offers many advantages including easier scalability and reduced dependence on eggs availability. Several cell lines, such as Vero, MDCK, human embryonic retinal (PER.C6), and human embryonic kidney (HEK293) cells, are considered as candidates for vaccine production. The Vero and MDCK are the two most developed to date cell lines recommended by the WHO as safe substrates for the production of human influenza vaccine. Vero cell line is widely used for the production of Inactivated Polio Vaccine (IPV), rotavirus (RotaTeq and Rotarix) and smallpox (ACAM2000) vaccines. Baxter International Inc. developed the technology for the production of influenza vaccine in Vero cells grown on Cytodex microcarriers. Trivalent Vero-derived influenza vaccine against seasonal influenza, Preflucel, was licensed in European Union (EU) in 2010. The pandemic vaccine Celvapan© against H1N1pdm09 virus and pre-pandemic vaccine Vepacel against H5N1 virus were also licensed in EU [7]. However, in 2011, 300,000 doses of Preflucel vaccine were withdrawn from the market because of a high percentage of side effects noticed after the vaccination. Sanofi Pasteur had undertaken an attempt to develop the production of the inactivated influenza vaccine in PER.C6 cells, but this project was discontinued. First influenza MDCK-derived vaccine Influvac was licensed in the Netherlands by Solvay Biologicals as a split virus preparation produced in cells cultivated in Cytodex microcarriers [8]. Nevertheless, after the acquisition of the Solvay Biologicals by Abbott, the production of Influvac vaccine was discontinued. The attempts undertaken by MedImmune and Nobilon to develop the production of the two live attenuated influenza (LAI) vaccines in MDCK cells were also stopped. The only one MDCK-derived vaccine approved in EU [9] and the US today is Optaflu/Flucelvax (Novartis) – the trivalent subunit vaccine against seasonal influenza. In addition, Protein Sciences [10] licensed in the US the first recombinant trivalent influenza vaccine FluBlok produced in insect *expres*SF cells using a baculovirus expression system. The main limitations of the cell line-based technology for influenza vaccine production are discussed in this review.

Differences between human and avian influenza viruses

The binding affinity of virus HA protein to the cells of different species is one of the parameters that defines the species barrier for influenza viruses. The influenza viruses initiate the infection via attachment to the ciliated epithelial cells in the upper respiratory tract (URT). The HAs of human influenza viruses recognize and bind to oligosaccharide chains terminated with the sialic acid-α2,6-galactose (SAα2,6Gal), which are prevalent on the human upper respiratory tract epithelial cells [11]. The avian influenza viruses bind to the SAα2,3Gal-terminated glycans that dominate on the surface of avian epithelial tissues [12]. Cells expressing the SAα2,3Gal-saccharides are known to be present in human bronchial epithelium and dominate in human lungs. Therefore, the avian influenza viruses can induce the disease in humans if the virus reaches lungs of the exposed person, for example, upon the close contact with infected bird.

One of the innate mechanisms of defense in mammals is the acidification of the mucosal surfaces of the nasal passageways in response to irritation or inflammation. The degree of acidification of these surfaces in mammals varies depending on the distribution and activity of the submucosal glands. The pH of the human passageway has a range from 5.2 to 8.0 [13-18]. Therefore, the efficient infection of human URT cells requires a certain level of virus stability to acidic pH. The acidic pH triggers the HA irreversible conformational change to the low pH form. This HA structural change happens in the endosome upon acidification and enables the fusion of the endosomal and viral membranes in order to release the viral genome to the cell cytoplasm. The pH threshold necessary for the HA conformational change is called the pH of fusion. The HAs of human and LPAIVs are known to change the conformation at the pH range from 5.1 to 5.4 [19, 20]. The HAs of HPAIVs are characterized by an elevated pH of fusion (from 5.6 to 6.0) [20] and, therefore, these viruses are much less stable to the acidic pH than LPAIVs and seasonal influenza viruses [21].

The essential prerequisite for influenza virus replication is the HA cleavage to HA1 and HA2 subunits by cell proteases that is necessary for release the fusion peptide. The HA of the human and LPAI viruses contains

a single arginine (-R-) cleavage site that is cleaved by the trypsin-like serine proteases present in the human airways [22]. The HAs of HPAIVs contain the polybasic cleavage site (e.g. R-S-S-R or R-S-R-R) that is cleaved by intracellular furin-like serine proteases expressed in different tissues [23]. This feature enables virus spreading to different organs and the induction of systemic infection. Therefore, the HPAIVs are highly virulent for humans and cause influenza disease with a mortality rate exceeding 50%. All of these differences between human and avian influenza viruses have to be taken into account when developing the vaccine production technology.

Virus adaptation passages and their consequences

As it was already mentioned, the primary influenza viruses are isolated in the fertilized hen's eggs. The cells of the amniotic and chorioallantoic membranes of the egg are the main target cells for influenza virus replication. These cells express both types of sialylated glycans, but predominantly with SAα2,3Gal linkage. The shortage of the human-like receptors in eggs is the first barrier for the effective replication of some human influenza isolates. Thus, the insufficient growth of virus A/Fujian/411/2002 (H3N2) induced the delayed vaccine production in the season of 2003-2004 [24]. For the same reason in 2008, it was necessary to substitute the poorly growing virus A/Brisbane/10/2007 (H3N2) by the A/Uruguay/716/2007 (H3N2) virus. In order to overcome this limitation, the primary isolates are usually subjected to several consecutive passages in eggs. The other approach is to perform the reassortment process with PR8 virus in the primary chicken embryo kidney cells instead of eggs. Both ways are associated with the emergence of the adaptation mutations in the HA protein that can change the receptor-binding and antigenic properties, or change the pH of HA fusion [25-27].

Earlier, we investigated the emergence of HA mutations upon the isolation of human influenza viruses from clinical material in eggs, as well as in the MDCK and Vero cell lines [28]. We found that the isolation of influenza A viruses in eggs was always accompanied by HA mutations. The amino acid substitutions found for the H1N1 viruses (225Asp→Gly, 226Gln→Arg) or for the H3N2 viruses (194Leu→Ile, 220Arg→Ser) were located near the HA receptor-binding site (RBS), and were associated with its specificity [29]. One (220Arg→Ser) or two (133Asn→Asp, 218Gly→Glu) substitutions located near the HA RBS were found in H3N2 viruses isolated in the MDCK cells as opposed to the Vero cells. These mutations were associated with the glycosylation pattern of the MDCK cells [28]. The sequence of the HA1 subunit of H1N1 viruses isolated in both Vero and MDCK cells was identical to that of the human virus present in swab. However, mutations in the HA2 fusion peptide were often identified for H1N1 viruses (unpublished data).

The Vero and MDCK continuous cell lines are known to have an equal amount of cells containing the saccharides with both types of sialic acid linkages (SAα2,3Gal as well as SAα2,6Gal) on the surface [30]. Most of the human influenza primary isolates easily grow in MDCK cells, while their growth in Vero cells is less efficient. The authors of several publications have shown [25, 31] that the adaptation of influenza viruses to the growth in MDCK and Vero cell lines induces mutations that increase the pH of fusion of the influenza virus HA. According to the experimental data, the endosomal pH in MDCK cells is around 6.4 and reaches even higher values in Vero cells [31, 32]. The endosomal pH in the chicken embryo fibroblasts (CEF) is around 6.4, however, there is no data available on the endosomal pH of the cells in the chorioallantoic or amniotic membranes [32]. All the above mentioned data lead to the conclusion that the relatively high endosomal pH might explain the selection of mutants with an increased pH of HA fusion post adaptation to the growth in MDCK and Vero cell lines.

Thus, none of the studied substrates are ideal for the cultivation of human influenza viruses. In order to assess the consequences of virus adaptation to various substrates, we performed 5 consecutive passages of H1N1, H3N2, and influenza B viruses in eggs as well as in the MDCK and Vero cell lines and compared the properties of obtained viruses [33]. The egg-derived seed viruses recommended for the vaccine production were selected as the starting material. Previously, we demonstrated [34] that HA mutations that increase the pH of HA fusion lead to decreased stability of the virus to the low pH and elevated temperatures. These vaccine candidates showed the decreased immunogenicity upon the intranasal immunization of ferrets. Therefore, the pH of HA fusion and virus stability were the main parameters assessed in this study.

According to the obtained data, no mutations were revealed after 5 passages of influenza B strain in any of these substrates. Five passages of A/Brisbane/59/2007 (H1N1) and A/Brisbane/10/2007 (H3N2) viruses in eggs also did not induce any changes in HA. On the other hand, 5 passages of A/Brisbane/59/2007 (H1N1) in Vero cells, as opposed to the MDCK cells, led to mutations that increased the pH of HA fusion by 0.4 units. This change was associated with HA1 mutation 190Asn→Val and HA2 stalk mutation 50Asn→Lys. The resultant virus became more sensitive to the acidic pH and elevated temperatures. The passages of A/Brisbane/10/2007 (H3N2) virus in both MDCK and Vero cells induced the enhancement of the pH of HA fusion by 0.1 unit (HA2 mutation 160Asp→Glu) that was associated with decreased stability to acidic pH and elevated temperatures. It is necessary to mention that the pH of HA fusion of initial egg-derived virus A/Brisbane/10/2007 was 6.0. This value is 0.4 units higher than that known for the human influenza viruses and particularly for the antigenically similar human isolate A/Nizhniy Novgorod/ 668/08. One can assume that the enhancement of pH of fusion of A/Brisbane/10/2007 virus HA happened upon the adaptation passages in eggs. Additional passages of this virus in both cell lines led to a further increase of the pH of HA fusion and to a decrease in its stability. For all of the obtained mutants, the increased pH threshold of HA conformational change was associated with reduced virus stability to acidic pH and elevated temperatures.

Furthermore, we showed that the concentrated purified preparation made from the less stable virus mutant contained about 50% less HA than the analogous preparation from the more stable virus according to the single radial immunodiffusion (SRID) assay. This assay measures the amount of HA molecules present in the vaccine in the native conformation [35].

The obtained data show that the passages of human influenza viruses in eggs as well as in the MDCK and Vero cell lines induce mutations that increase the pH of HA fusion, which in turn impair the virus stability toward low pH and elevated temperatures. The mutations related to this change were identified in various HA regions, including the receptor-binding domain [36], interface between the HA1 and HA2 subunits [37-39], the coiled-coil regions of the HA2 subunit [37, 40, 41], and the fusion peptide pocket [40, 42]. However, it seems that passages in eggs impair the virus stability less dramatically than the analogous passages in both cell lines. The effect of mutations decreasing virus stability on the quality of influenza vaccines is discussed below.

Effect of the influenza virus stability on the quality of inactivated vaccines

The inactivated influenza vaccines are available as a whole virion, split or subunit preparations that include 3 components (15 μg of each) and are designed for intramuscular or subcutaneous administration. The efficiency of the inactivated influenza vaccine is determined by the amount of antibodies induced to the native HA trimeric structure, which is measured by the hemagglutination inhibition assay (HAI).

The HA of influenza virus with increased pH of fusion is converted to the low pH form much easier. This conformational change is irreversible and happens not only at acidic pH, but also at elevated temperatures or upon exposure to the denaturing reagents [43]. Depending on the vaccine type, the downstream production process might include a number of steps, such as virus inactivation, concentration by ultracentrifugation, purification by chromatography, precipitation, sucrose-gradient centrifugation, chemical disruption, and sterile filtration. All of these steps can trigger the conversion of less stable HA to the low pH form. The HA in the low pH form expresses the hydrophobic domains, which are prone to form aggregates. The presence of viral aggregates in the vaccine can induce the severe adverse effects. Thus, the emergence of the new oculo-respiratory syndrome was detected in 2000-2001 season in adults after immunization with the vaccine that contained a high amount of aggregated unsplit virus particles [44, 45]. Viruses that contain the structurally modified HAs are known to become more sensitive to the cleavage with proteolytic enzymes [46]. In addition, the low pH form of HA expresses the epitopes that are hidden in the native HA conformation. These epitopes induce cross-reactive antibodies with less neutralizing potential [47, 48].

Similar problems were observed in the course of production of the pre-pandemic vaccines from the H5N1 HPAIVs. The inactivated vaccines produced from these viruses by conventional methods were found to have decreased HA content and immunogenicity compared to the vaccines against seasonal influenza viruses produced by the same methods [49, 50]. The antibodies induced post immunization poorly reacted in the HAI test [51]. In order to reach the protective level of antibodies post immunization with the H5N1 vaccine it was necessary to increase the vaccine dose up to 45-90 μg or to add the adjuvant. At the same time, in some studies the better heterosubtypic cross-protection was observed after immunization with H5N1 vaccines [52].

We hypothesized that the high pH of HA fusion known for the HA of HPAIVs is the main factor affecting the properties of vaccines produced from these viruses. In order to prove that, we introduced the mutation 58Lys→Ile, which is known to decrease the pH of HA fusion, in the HA2 subunit of A/Kurgan/05/2005 (H5N1) virus [53]. Two similar 6/2 reassortants, one containing the original HA and the other with the mutant HA, in combination with NA gene from A/Kurgan/05/2005 and all the other genes from the PR8 virus were constructed. The reassortant with the mutant HA was characterized by a decreased pH of HA fusion by 0.3 units [54] and increased stability toward an acidic pH and elevated temperatures compared to the reassortant with the original HA protein. Two inactivated whole virion vaccine preparations were produced using these reassortants and their properties were compared. We have shown that vaccine preparation from the virus with the mutant HA was more stable to freezing-thawing stress and more resistant to trypsin digestion. The HA content measured by the SRID test for this vaccine preparation was higher than that for the vaccine made from the virus with the original HA.

Therefore, the production of the seasonal and pre-pandemic inactivated influenza vaccines from viruses with reduced stability could lead to decreased vaccine quality.

Effect of influenza virus stability on the quality of live attenuated vaccines

Two LAI vaccines are approved for human use. The first vaccine is licensed in the US and Europe (FluMist®, MedImmune, USA) [55], while the second vaccine is licensed in Russia [56]. Both vaccines contain the cold adapted (CA) attenuated vaccine strains (6/2 reassortants) produced in eggs and are designed for intranasal application. It is known that the immunogenicity of a live vaccine correlates with the productive replication of the vaccine strains in the URT of humans [57].

The analysis of the clinical data shows that the immune response induced by both licensed vaccines is inconsistent. Thus, the pre-pandemic vaccine produced from the virus of H2N2 subtype (FluMist®) showed the restricted virus replication in the URT (24%) and immune response in only 24% of participants [58]. The vaccine strain used in this study was the master strain A/Ann Arbor/6/1960 (H2N2) that is used as a backbone for the seasonal vaccine strains. At the same time, the Russian pre-pandemic vaccine of H2N2 subtype, which contains the reassortant of the master stain

A/Leningrad/134/17/1957 CA with the A/California/1/1966 (H2N2) virus, showed a high replication rate in the URT of volunteers (78.6%) and a high number of responders (92.6%) [57]. The analysis of the origin of both vaccine strains revealed that the virus A/Ann Arbor/6/1960 CA was isolated and attenuated by serial passages in the primary chicken kidney cells and had decreased stability to the acidic pH compared to the original wild type strain [59]. The virus strain used in the Russian vaccine preparation was obtained by classical reassortment in eggs. The surface antigens of this vaccine strain were inherited from the epidemic egg-derived isolate A/California/1/1966 (H2N2) [60]. Based on our results discussed herein above, we can assume that the decreased stability of A/Ann Arbor/6/1960 CA virus is one of the reasons leading to its restricted immunogenicity.

Another reason for the low immunogenicity of the vaccine strains of the LAI vaccine could be the incompatibility of the HA and M genes originated from different strains. O'Donnell *et al*. [61] showed that 6/2 reassortants of A/Ann Arbor/6/1960 CA (H2N2) master strain with viruses of H1N1, H3N2, H2N2, H6N1, H7N3, H9N2, and H5N1 subtypes have an elevated pH of HA fusion and decreased stability to low pH and elevated temperatures compared to the corresponding epidemic viruses. Clinical trials performed with some of 6/2 reassortants showed their restricted replication in the respiratory tract of seronegative adult volunteers [58, 62, 63]. The authors of these papers have found that the decreased stability of the studied reassortants is connected with the nature of the M gene of A/Ann Arbor/6/1960 CA master strain, but the exact mechanism of this phenomenon was not discussed.

One of the proteins coded by M gene is the M2 that is known to have the ion channel activity. It was shown that the M2 protein plays a major role in the preservation of the HA native conformation, preventing the premature HA conformational change to the low pH form during the virus transport to the cell surface [64]. Earlier we demonstrated [65] that the M2 protein of the virus A/Singapore/1/1957 CA (H2N2) has lower ion channel activity than the M2 of A/Switzerland/5389/1995 (H1N1). The 6/2 and 5/3 reassortants that contain the HA and NA or HA, NA and M genes, respectively, from the A/Switzerland/5389/1995(H1N1) virus and all the other genes from the A/Singapore/1/1957 CA were constructed. The intranasal immunization of mice with these reassortants showed the clear difference in the amount of antibodies directed to the HA1 and HA2 subunits. Therefore, the 6/2 reassortant induced 1.7 times more antibodies to the HA2 subunit than to HA1. At the same time, the HA2/HA1 ratio of antibodies induced by 5/3 reassortant was almost similar to that induced by A/Switzerland/5389/1995 virus (1.1 and 1.0, respectively). These results indicate that the M2 protein of A/Singapore/1/1957 CA, which has low ion channel activity, did not prevent the premature conformational change of the A/Switzerland/5389/1995 HA protein to the low pH form during transport to the cell membrane. A similar mechanism can contribute to the premature conformational change of HA in 6/2 reassortants produced from the A/Ann Arbor/6/1960 CA (H2N2) strain and consequently cause their decreased stability.

The attempts to vaccinate humans with pre-pandemic H5 live influenza vaccines gave the contradictory results. The vaccine strains containing the surface antigens of HPAIVs A/Vietnam/1203/2004 or A/Hong Kong/213/2003 of H5N1 subtype produced using the A/Ann Arbor/6/1960 CA strain (MedImmune) failed to replicate in the human nasal mucosa and to induce a reasonable immune response [66]. At the same time, the vaccine strain containing the HA of the LPAIV A/Potsdam/1402-6/1986 (H5N2) and all other genes from the A/Leningrad/134/17/1957CA virus replicated efficiently in the URT of humans (up to 10 days) and caused the reasonable systemic and local immune response [67]. This contradiction could be explained by the different HA stability of the corresponding reassortants depending from which virus (HPAIVs or LPAIV) the HA gene was inherited.

In order to prove this hypothesis we introduced the mutation 58Lys→Ile, which is known to decrease the pH of HA fusion, in the HA2 of HPAIV A/Vietnam/1203/2004 (H5N1). Then two analogous 6/2 reassortant vaccine candidates – one with the mutant and the other with the original HA gene – were constructed based on PR8 virus. The attenuation of these viruses was provided by deletion of the NS1 mRNA open reading frame [21]. We have demonstrated that the virus with mutant HA was characterized by a decreased pH of HA fusion and enhanced stability toward low pH and elevated temperatures compared to the analogous virus with original HA sequence. Both viruses were used for the intranasal immunization of mice. The immunization with a more stable virus resulted in increased virus replication in the nasal mucosa, trachea, and lungs of mice as well as induced enhanced humoral and local immunity compared to the virus bearing the original HA.

Therefore, the HA stability of the LAI vaccine reassortants to the low pH and elevated temperatures is crucial for their effective replication in the URT of mammals that consequently could affect the vaccine efficacy.

CONCLUSION

The cultivation of the human influenza viruses in continuous cell lines such as MDCK and VERO often leads to the emergence of HA mutations that increase the pH of HA fusion and dramatically decrease the stability of HA protein, and consequently the whole virus, to different physical and chemical factors. These viruses are much more sensitive to the procedures of vaccine manufacturing. As a result, the vaccine virions contain a bigger portion of HA proteins in the low pH form, which can affect the safety, potency, infectivity, and protective efficacy of the final inactivated and live attenuated influenza vaccines.

We found a set of conditions that should be met in order to keep the optimal quality of vaccines produced in the cell lines. First, it is necessary to maintain the

HA sequence in the vaccine strain as close as possible to that of the original human virus isolate. In order to accomplish this, the HA and NA genes primary structure of the virus recommended for vaccine composition should be determined before any isolation passages. The construction of vaccine reassortants by reverse genetics methods allows for the cloning of the HA sequence identical to that of the virus present in the human swab, and for the rescue of the virus without any adaptation mutations. The next important goal is to prevent the emergence of HA mutations associated with increased pH of HA fusion during the vaccine production. We have demonstrated that the propagation of viruses at acidified conditions in Vero cell line helps to preserve the original HA primary structure and the virus stability [33]. Another possibility to prevent the emergence of adaptation mutations that increase the pH of HA fusion is to cultivate the virus in the presence of Amphotericin B promoting fusion of viral and cellular membranes [68]. Moreover, the new cell lines that support the effective replication of the human influenza viruses and have low endosomal pH are promising candidates for influenza vaccine production. Cultured mouse heart cells could be considered as an example, since they have more acidic endosomes (pH 5.5) than cultured mouse kidney cells (pH 6.0) [69].

In conclusion, all attempts to develop and produce new cell culture-based influenza vaccines, without taking into account HA mutations leading to an increase of the pH of HA conformational change, are doomed to failure. It is obviously that more research in both academia and industry is necessary to develop the new cell-based technologies for the influenza vaccine production.

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CONFLICT OF INTERESTS

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