

# Factors affecting the immunogenicity of the live attenuated influenza vaccine produced in continuous cell line

Andrea Wolkerstorfer<sup>1</sup>, Dietmar Katinger<sup>2</sup>, Julia Romanova<sup>1#</sup>

<sup>1</sup>Department of Biotechnology, University for Natural Resources and Life Sciences, Vienna, Austria

<sup>2</sup>Polymun Scientific Immunbiologische Forschung GmbH, Klosterneuburg, Austria

# Corresponding author: Julia Romanova, e-mail: jr.romanova@gmail.com

DOI: 10.18527/2500-2236-2016-3-1-13-24

**Keywords:** influenza, live vaccine, immunogenicity, reassortant, M2 protein, ion channel, Vero cell line

Received March 18, 2016

Accepted May 2, 2016

Published May 9, 2016

## ABSTRACT

The biological basis for the restricted immunogenicity of some live attenuated influenza vaccine strains generated on the backbone of the cold adapted (ca) A/Singapore/1/1957/ca (H2N2) influenza A virus master strain and produced in the Vero cells was investigated. According to our previous results the vaccine candidate made from A/Hong Kong/1035/1998 (H1N1) Vero-derived virus did not provoke a measurable antibody titers following the intranasal immunization of humans. We report here that the hemagglutinin (HA) of A/Hong Kong/1035/1998 virus contained the mutation 10Ile→Val in the HA2 subunit, that increased the pH threshold of HA conformational change (pH of activation) by 0.3 pH units and therefore might be responsible for the lack of immune response in humans. Similar effect was shown for the reassortant made from the Vero-derived A/Switzerland/5389/1995 (H1N1) (5389wt) virus which had the HA2 mutation 3Phe→Leu leading to the lack of immune response in mice. Another factor compromising the immunogenicity of a vaccine candidate is the incompatibility of epidemic virus HA with the M gene of the master strain. In mice the 6/2 A/Switzerland/5389/1995 reassortant induced antibodies that were directed predominantly to the HA2 subunit and were detectable by ELISA but not by a hemagglutination inhibition (HAI) test. In contrast, the 5/3 reassortant, bearing the HA, neuraminidase (NA), and M genes from the epidemic virus induced an equivalent amount of antibodies against the HA1 and HA2 subunits detected by HAI and ELISA. By comparing the sensitivity of the viruses to amantadine, we showed that the M2 ion channel of the master strain had lower activity than that of the A/Switzerland/5389/1995. These data suggest that M2 of the master strain was not sufficiently active to keep the pH of the *trans*-Golgi network high enough to prevent the conformational change of the acid sensitive HA to the low pH form.

Overall, the adaptation mutations in the HA of the vaccine candidate that increase the pH of HA activation as well as the incompatibility of HA and M genes must be taken into consideration when constructing the reassortant strains for the live attenuated vaccine.

## INTRODUCTION

Vaccination against influenza continues to be one of the most effective means to prevent the disease and to decrease the mortality rate during influenza epidemics. Inactivated and live attenuated vaccines are the two types of influenza vaccines available on the market. Currently, two live influenza vaccines are approved. First, an attenuated cold adapted (ca) vaccine was developed in Russia in the 1980s [1-5]. Later, a similar vaccine known by the trade name FluMist® was developed in the US using the same approach [6, 7]. The conventional vaccine strains for the live vaccine are virus reassortants, which combine two gene segments coding for surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) derived from actual epidemic viruses with the six internal protein gene segments of the attenuated master strain (6/2 combination). These reassortants are created by classical reassortment (Russian vaccine) or by reverse genetics (FluMist®). The master strains used

for both vaccines are H2N2 viruses attenuated by serial passages at a suboptimal temperature in embryonated hen's eggs or primary chicken kidney cells. Both vaccines are produced in embryonated eggs. This production system has several shortcomings including inflexibility, cumbersome processing, potential of allergic reactions to residual egg protein, and the inability of some strains to grow to high titers in eggs. The major drawback of the egg production system is the selection of receptor-binding mutants, which might have altered antigenic properties [8-10]. It is believed that the replacement of eggs by mammalian continuous cell lines could overcome the above-mentioned limitations.

Several cell lines have been approved for the production of an influenza vaccine. The African green monkey kidney (Vero) and the Madin Darby canine kidney (MDCK) cell lines are considered to be the most prominent substrates for vaccine production [11, 12].

The Vero cell line is very attractive because it was used for over 35 years for the production of polio and rabies virus vaccines for humans [13, 14]. Human influenza viruses grow to high yields in MDCK cells but do not reach high titers in Vero cells. It was found that Vero cells have a higher endosomal pH than MDCK cells [15]. The acidic environment of the endosome is necessary for the change of HA conformation to the low pH form in order to create the conditions for the subsequent fusion of the viral and endosomal membranes – the mandatory step for the delivery of the viral genome into the cytoplasm. Several adaptation passages are usually required to enable efficient influenza virus growth in Vero cells.

A new cold adapted master strain for the cell line-based influenza vaccine was generated by the adaptation of the virus A/Singapore/1/1957 (H2N2) to the growth on Vero cells at 25°C by serial passages. The resulting virus A/Singapore/1/1957/ca (Sing/ca) was shown to be attenuated for laboratory animals (mice and ferrets) [16]. Several vaccine candidates were obtained by the reassortment of Sing/ca with the epidemic viruses isolated directly in Vero cells. However, the preclinical experiments in mice and ferrets (unpublished data) as well as the clinical examination of the obtained strains revealed low immunogenicity for some of them. Thus, according to our previous data [16], the immunization of adult volunteers with the reassortant of wild type (wt) virus A/Switzerland/7729/1998 (H3N2) (7729wt) induced antibodies with the geometric mean titer (GMT) of 45.25 in the hemagglutination inhibition (HAI) test, while the use of the reassortant of A/Hong Kong/1035/1998 (H1N1) (1035wt) virus did not provoke a measurable antibody titer.

The goal of the current study was to investigate the biological basis for the restricted immunogenicity of some live attenuated influenza vaccine strains produced in the Vero cell line.

## MATERIALS AND METHODS

### Viruses and cells

Vero and MDCK cell lines were obtained from the American Type Culture Collection (ATCC). Vero cells were adapted to and further cultivated in DMEM/Ham's F12 medium (Biochrom F4815) with 4 mM L-glutamine and animal protein free supplement (proprietary formulation, Polymun Scientific GmbH, Austria). MDCK cells were cultivated in DMEM/Ham's F12 (1:1) medium supplemented with 2% of heat inactivated fetal calf serum (FCS) (Gibco).

Epidemic wild type viruses 5389wt (H1N1) and 7729wt (H3N2) were isolated from the human clinical sample kindly provided by Canton University Hospital (Geneva, Switzerland). Virus 1035wt (H1N1) was isolated from the human clinical material provided by Dr. Lee (Hong Kong). Influenza reassortants were obtained by classical reassortment in Vero cells and genotyped as described previously [16].

### Propagation and titration of viruses

Viruses were propagated in Vero cells in DMEM/Ham's F12 medium containing 1 µg/ml of trypsin (Sigma, T4549). A hemagglutination assay (HA assay) was performed with a 0.5% suspension of chicken red blood cells. The infectious titer of viruses was determined by a limiting dilution assay or by a plaque assay in Vero cells with agar overlay containing DMEM/Ham's F12 medium, 4 mM L-glutamine, 1 µg/ml of trypsin, 0.01% DEAE dextran (Pharmacia), 0.6% agar (Sigma) and expressed in TCID<sub>50</sub>/ml or plaque forming units (PFU), respectively.

### Mouse immunization

Four to six week old B6D2F1 hybrid mice (Charles River, Germany) were infected intranasally under ether anesthesia with 50 µl of virus containing cell culture supernatant. One or 3 days post infection, 4 mice from each group were sacrificed, the nasal turbinates and lungs were removed, homogenized and titrated for the presence of virus by a limiting dilution assay. The virus titer was expressed in log<sub>10</sub> TCID<sub>50</sub>/ml of 10% organ suspension. Mouse sera (4 animals per group) were collected three weeks after immunization. The antibody titers were measured by HAI assay and ELISA [17]. For the HAI assay, immune serum samples were treated with a receptor-destroying enzyme (Denka, Japan) to remove unspecific inhibitors, according to the supplier's recommendations.

### Virus purification

The supernatant of infected cells was clarified by centrifugation at 3,500 g for 15 min at 4°C. Virus particles were purified by centrifugation in an SW28 rotor (Beckman) through a 30% sucrose cushion in NTE buffer (0.1 M NaCl, 0.01 M Tris HCl, 0.001 M EDTA, pH 7.4) at 90,000 g for 150 min at 4°C. The pellet was resuspended in NTE buffer and loaded on the discontinuous gradient of 60 and 20% (w/v) sucrose in NTE buffer. The sucrose gradient was centrifuged in an SW41 rotor (Beckman) at 210,000 g for 16 h at 4°C. The virus-containing band was isolated, diluted in NTE buffer, pelleted by centrifugation in an SW41 rotor at 210,000 g for 30 min at 4°C and resuspended in phosphate buffered saline (PBS).

### Enzyme-linked immunosorbent assay (ELISA)

The ELISA protocol was performed as described previously [16]. For this purpose, sucrose purified 5389wt virus was adjusted to 20 HA units/50 µl in carbonate buffer (pH 9.6) and used for the coating of ELISA plates. Serial dilutions of pooled sera in PBS containing 1% bovine serum albumin (BSA) were added to the coated plates and incubated for 1 h at room temperature. After several washing steps, bound antibodies were detected with rabbit anti-mouse IgG conjugated with horseradish peroxidase (Sigma). Plates were stained with *o*-phenyldiamine as substrate, and absorbance at 450 nm was measured. The cutoff values were determined as the mean of the negative control samples value plus 3 standard deviations.

## Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and Western blot analysis

SDS PAGE of purified 5389wt virus preparation was carried out using Tris-Glycine 16% gels (Anamed). Western blotting was performed by the electrophoretic transfer of the proteins from the gel to a polyvinyl difluoride membrane (Millipore) for two hours at 400 mA. After overnight blocking in TPBS (PBS with 0.1% Tween 20) containing 3% of skim milk, the membrane was cut into separate single lane pieces that were incubated for one hour with a pool of polyclonal mouse antisera raised against different viruses that were diluted 1:100 in TPBS buffer containing 1% of skim milk. After washing with TPBS, the membrane was incubated for 1 h with alkaline phosphatase labeled anti-mouse antibody (Sigma) diluted 1:10,000 in TPBS containing 1% of skim milk. The blots were developed in staining buffer (100 mM Tris HCl; 100 mM NaCl; 5 mM MgCl<sub>2</sub>, pH 9.5) with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

## Determination of fusion pH by hemolysis

Sucrose purified viruses in PBS, adjusted to an HA titer of 1:128 (200 µl) were incubated in a 48-well plate on ice with 600 µl of 0.5% freshly washed human red blood cells (type 0, Rh negative) prepared in saline solution for 1 h to allow virus binding. The plate was then centrifuged at 800 g for 3 min and 700 µl of supernatant were removed. Another 700 µl of sodium citrate buffered saline (0.15 M) was added at various pH, covering the range 5.0-6.2, and incubated for 1 h at 37°C. After centrifugation at 800 g for 3 min, 300 µl of supernatant were transferred to a flat-bottom 96-well plate. The amount of hemoglobin in the supernatants was determined by measuring the absorbance at 405 nm. The reported results are the mean values ± standard deviations of 3 repeats at the indicated pH.

## Virus stability toward acidic pH

Influenza viruses were incubated in 0.15 M sodium citrate buffered saline with pH 5.2 at 37°C for different time intervals. At the appropriate time points, samples were placed on ice and neutralized with NaOH to pH 7.4. Subsequently, the TCID<sub>50</sub> titer was determined for all of the samples in Vero cells and expressed in log<sub>10</sub> TCID<sub>50</sub>/ml.

For the control sample, an equal amount of PBS was added instead of sodium citrate and the viruses were treated the same way.

## Focus reduction assay

MDCK cell cultures in 96-well plates were inoculated with 150 µl of serial twofold dilutions of amantadine (Sigma), ranging from 4 to 0.03 µg/ml in DMEM/Ham's F12 medium [18]. Then, 50 µl/well of virus with a multiplicity of infection (MOI) from 100 to 200 focus forming units were added to each well except the control well. After overnight incubation at 33°C without trypsin, cells were fixed with 4% paraformaldehyde for 1 h and permeabilized with a 0.2% solution of Triton X-100 in PBS for 20 min. All the steps were carried out at room temperature. Fixed cultures were immuno-stained for the expression of viral nucleoprotein by incubation for 1 h with monoclonal antibodies specific for the nucleoprotein of influenza A virus (kindly provided by Dr. Eva Varechkova at the Institute of Virology, Bratislava, Slovakia) followed by 1 h of incubation with peroxidase-labeled anti-mouse antibodies (Sigma) and a 30 min incubation with precipitate-forming peroxidase substrate (True Blue, KPL). Horse serum (10%) plus 0.005% Tween 20 in PBS was used for the preparation of working dilutions of immuno-reagents. Foci were counted visually and the results were expressed as the percentage of inhibition. The reported results are the mean values ± standard deviations of 3 replicate determinations at the indicated concentrations of amantadine.

## Gene amplification and sequencing

Viral RNA was extracted from the tissue culture supernatant of infected cells using Trizol reagent (Invitrogen). C-DNAs were synthesized using M-MuLV reverse transcriptase (MBI). The M gene and three overlapping parts of the HA gene were amplified by PCR using specific primers (sequence available upon request). The PCR products were purified using a Gel Band Purification Kit (Amersham Pharmacia Biotech) and were sequenced at SEQLAB GmbH (Germany). The sequences were analyzed using Lasergene 99 software. The H3 numbering system was used throughout the study for influenza A viruses. The obtained sequence data for HA and M genes were submitted to the GenBank (Table 1).

**Table 1.** Comparison of amino acid sequences of viruses used in this study.

Virus	Gene (protein)	Origin	Accession number in PubMed	Amino acid position	Residue
7729/wt	HA	Vero	AF382318.1	-	-
1035/wt	HA (HA2)	Vero	AF386776	10	Val
1035/wt	HA (HA2)	MDCK	AF386777	10	Ile
5389/wt	HA (HA2)	Vero	AF386773.1	3	Leu
5389/wt	HA (HA2)	MDCK	AF386774.1	3	Phe
Sing/ca	M (M2)	Vero	X08093.1	36, 43	Leu, Leu
5389/wt	M (M2)	Vero	DQ299489.1	36, 43	Met, Ile

## Statistical analysis

The obtained data were analyzed using GraphPad Prism version 6.00 for Windows. The significance of the differences between the groups was determined by a non-parametric Mann–Whitney test. P-values of  $\leq 0.05$  were considered significant.

## RESULTS

### HA sequences of epidemic influenza viruses isolated in Vero cells

In order to avoid any receptor binding mutations induced by the avian cells of embryonated eggs, we used epidemic viruses isolated in Vero cells directly from the human clinical material for the construction of vaccine candidates. According to our data, enhanced virus growth in Vero cells might be accompanied by the occurrence of mutations in the HA, which increase the pH threshold of HA conformational change (pH of fusion or activation) [19]. In our previous paper we showed that viruses with an increased pH of activation are characterized by decreased infectivity in the mouse respiratory tract and reduced immunogenicity in ferrets after intranasal immunization [20, 21].

The HA sequences of both reassortants used for human immunization were identical to the sequences of the corresponding Vero-derived wild type parent strains. Therefore the consecutive studies were performed using the wild type viruses. Initially we analyzed the HA of wild type viruses for the presence of mutations in comparison to the original human viruses. We found that the HA sequence of Vero-derived 7729wt virus fully coincided with the HA sequence of virus present in the clinical swab (Table 1). In contrast, the HA of Vero-derived 1035wt virus differed from that of the clinical sample by a single mutation 10Ile→Val located at the fusion peptide of HA2 subunit. It is known that mutations in the fusion peptide might increase the pH threshold of HA conformational change [22]. Therefore, as the next step in our study, we compared the pH of HA activation of the Vero-derived 1035wt virus with that of the MDCK-derived 1035wt virus, which did not have any substitutions, in a hemolysis test.

### The pH of the HA activation of Vero- and MDCK-derived 1035wt viruses

The pH of HA fusion was determined as the pH value at which the released hemoglobin was observed following the fusion of virus with human red blood cells. The results shown in Fig. 1A indicate that 50% of the fusion for the MDCK-derived 1035wt was observed at pH 5.5, while for the Vero-derived virus it was observed at pH 5.8. Therefore, mutation 10Ile→Val increases the threshold pH of HA activation of Vero-derived 1035wt virus by 0.3 pH units.

### The stability of 1035wt and 7729wt viruses to acidic pH

Next, we compared the stability of 1035wt and 7729wt viruses toward acidic pH (pH 5.2). As shown in Fig. 2, the

7729wt virus did not lose any infectious titer following incubation at pH 5.2 for 15 min, whereas the infectious activity of the 1035wt decreased by  $1.75 \log_{10}$  TCID<sub>50</sub>/ml. Therefore, virus 7729wt appeared to be more stable toward the acidic pH than virus 1035wt bearing the mutation 10Ile→Val. Based on our previous observations, it is possible to suggest that substitution 10Ile→Val in the HA of vaccine strain might be the reason for the absence of an immune response after the immunization of humans.

Besides mutations in HA, the reassortant gene constellation is known to affect virus properties [23–28]. Thus it was shown that the incompatibility of HA with M1 and M2 proteins coded by the M gene leads to the impaired virus yield and induces changes of the virion morphology [29–31]. Therefore, we investigated the possible influence of the M gene origin on the immunogenicity of reassortant viruses.

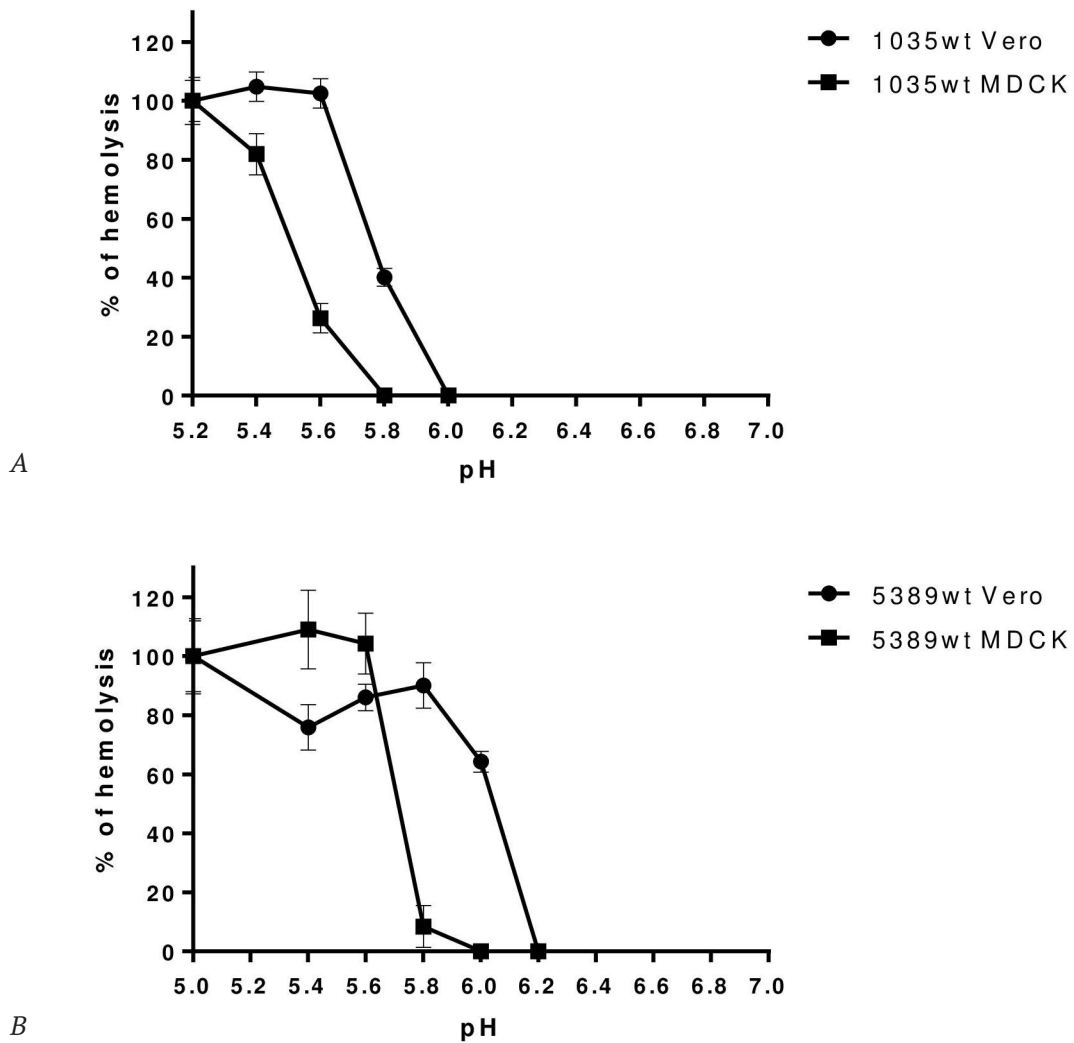
### Properties of 6/2 and 5/3 reassortants obtained from 5389wt and Sing/ca viruses

In order to investigate the influence of the M gene products on virus properties, the corresponding reassortants with two (HA and NA) or three (HA, NA and M) genes from the epidemic virus 5389wt (H1N1) and other genes from the Sing/ca master strain were generated. Virus 5389wt was isolated directly in Vero cells. The HA of Vero-derived virus 5389wt contained a single mutation 3Phe→Leu located at the HA fusion peptide when compared to the HA sequence of MDCK-derived virus or clinical isolate. Both reassortants, despite the M gene origin, displayed similar growth in Vero cells. The infectious titers of both viruses were comparable to the titer of 5389wt and were  $2.0 \log_{10}$  TCID<sub>50</sub>/ml lower than that of Sing/ca (Fig. 3). These results indicate that the origin of the M gene had no influence on the growth of reassortants in Vero cells.

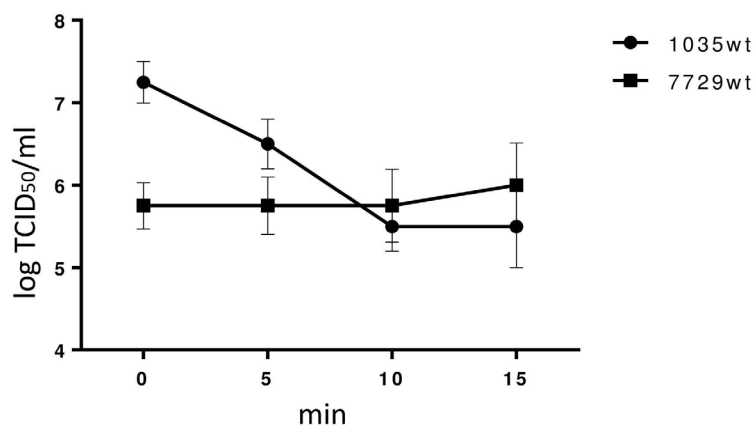
A comparison of the pH of HA fusion for the Vero-derived 5389wt with that of MDCK-derived virus was performed by the hemolysis assay. We demonstrated that the HA of MDCK-derived 5389wt virus showed 50% of hemolytic activity at pH 5.7 while the HA of Vero-derived virus at pH 6.0. (Fig. 1B). These data indicate that substitution 3Phe→Leu is also related to the increased pH threshold of the HA conformational change of Vero-derived 5389wt virus by 0.3 pH units.

### Growth of 6/2 and 5/3 reassortants in the mouse respiratory tract

Next, we compared the growth of Vero-derived 5389wt and both 6/2 and 5/3 reassortants in the upper and lower respiratory tract of mice after intranasal immunization. As shown earlier, the master strain Sing/ca is replicated in the upper respiratory tract of animals but not in the lungs [16]. The epidemic virus 5389wt demonstrated a high replication activity in the mouse nasal turbinates ( $6.0 \log_{10}$  TCID<sub>50</sub>/ml) as well as in the lungs ( $5.5 \log_{10}$  TCID<sub>50</sub>/ml). Reassortants 6/2 and 5/3 grew to titers not exceeding  $2.5 \log_{10}$  TCID<sub>50</sub>/ml in nasal tissues



**Fig. 1.** Fusogenic properties of Vero- and MDCK-derived viruses. (A) Fusogenic activity of 1035wt viruses. (B) Fusogenic activity of 5389wt viruses. The pH of HA fusion was determined as the value at which the released hemoglobin was observed following the fusion of the virus with human red blood cells. The amount of released hemoglobin was determined by measuring the absorbance at 405 nm. The reported results are the mean values  $\pm$  standard deviation of 3 repeats at each pH for each virus.



**Fig. 2.** The stability of 1035wt and 7729wt viruses toward acidic pH. Virus infectious titers were measured post incubation in 0.15 M sodium citrate buffered saline (pH 5.2) at 37°C from 5 to 15 min.

of mice and were not detectable in the lungs of animals (Table 2). These results indicate that the origin of the M gene products does not have any effect on the virus growth in the respiratory tract of mice.

### The immune response induced by 6/2 and 5/3 reassortants in mice

The immunogenicity of 6/2 and 5/3 reassortants was measured three weeks after a single intranasal immunization of mice by HAI and ELISA. The results showed that the highest immune response was induced by the 5389wt virus with GMT of 104 in HAI and GMT of 412 in ELISA (Table 2). Animals immunized with 6/2 reassortant revealed no antibodies in the HAI test (GMT<4), while the GMT of antibodies measured by ELISA was equal to 75. In contrast, the titers of the 5/3 reassortant-induced antibodies (GMT of 19.4 in HAI and 180 in ELISA) were significantly higher than those for the 6/2 reassortant. Thus, the 5/3 reassortant appeared to be more immunogenic than the 6/2 after single intranasal immunization of mice.

Next we determined the ratio of antibodies in mouse post infectious sera directed to the HA1 and HA2 subunits based on the density of HA bands in a Western blot (Fig. 4). The ratio of HA2/HA1 antibodies induced by 5389wt virus was used as a reference point equal to 1. It turned out that the 5/3 virus induced a comparable amount of antibodies binding to HA1 and to HA2 subunits with the HA2/HA1 ratio of 1.1. In contrast, the serum obtained after immunization with the 6/2 reassortant contained predominantly antibodies that recognized the HA2 subunit with the HA2/HA1 ratio of 1.7. These data indicate that upon replication in mouse cells the HA of 6/2 reassortant was transformed partially to the low pH form with the exposed epitopes of HA2 subunit.

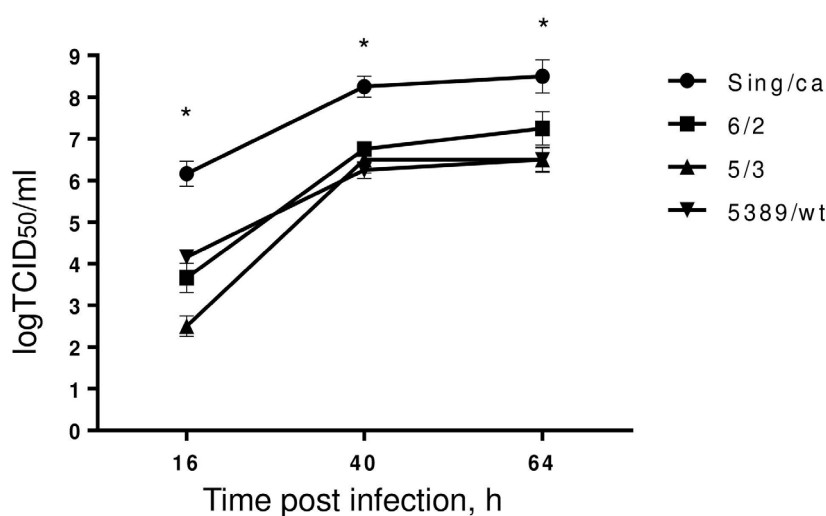
One of the functions of the M2 protein is to preserve the native HA conformation during the transport through the *trans*-Golgi network [29, 31, 32], i.e. to keep the pH above the threshold of HA conformational change. When the activity of the M2 ion channel is low, the HA can change the conformation to the low pH form. The HA at low pH conformation is known to express more epitopes of the HA2 subunit that are hidden in the native HA conformation. Therefore, we suggested that the ion channel activity of the Sing/ca M2 protein is not sufficient to keep the mutant HA of 5389wt virus in a correct native conformation in the *trans*-Golgi network. To check this hypothesis, we compared the ion channel activities of 6/2 and 5/3 reassortants.

### M2 ion channel activity of 6/2 and 5/3 reassortants

Amantadine is an antiviral drug that inhibits influenza virus replication by blocking the M2 ion channel activity. The sensitivity of viruses to amantadine correlates with the ion channel activity of M2 protein [33-35]. We compared the M2 ion channel activity of 6/2 and 5/3 reassortants by measuring their sensitivity to amantadine using the focus reduction assay.

The obtained results showed that, although both reassortants were sensitive to amantadine, the 6/2 reassortant appeared to be inhibited by amantadine to a greater extent than the reassortant 5/3 (Fig. 5). This result indicates that the M2 ion channel activity of Sing/ca virus is lower than that of 5389wt.

The comparison of M2 protein sequences of Sing/ca and 5389wt viruses revealed a difference in 7 amino acids. Two of them, 36Leu(Met) and 43Leu(Ile), are located in the transmembrane (TM) domain of the M2 protein responsible for the ion channel activity (Fig. 6) [36].



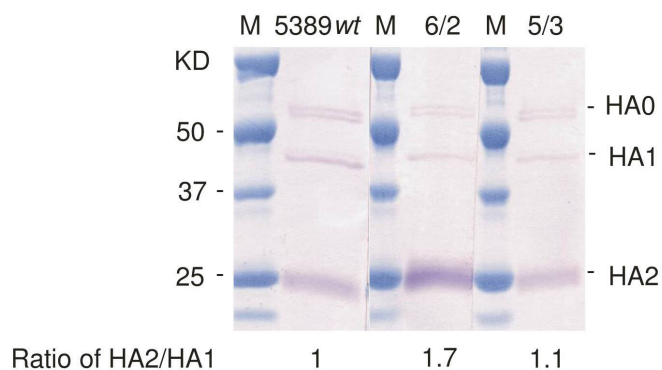
**Fig. 3.** Growth of Sing/ca, 5389wt, 6/2 and 5/3 viruses in Vero cells. Vero cells were infected at an MOI of 0.01. The virus titers were determined at indicated time points after infection. The presented results are the mean values of 3 titrations. The difference between the Sing/ca virus and others is significant at 16, 40, and 64 h post infection. \* indicates  $p < 0.02$  determined by a non-parametric Mann-Whitney test.

**Table 2.** Immune response and replication capacity of 5389wt, 6/2 and 5/3 viruses in mice.

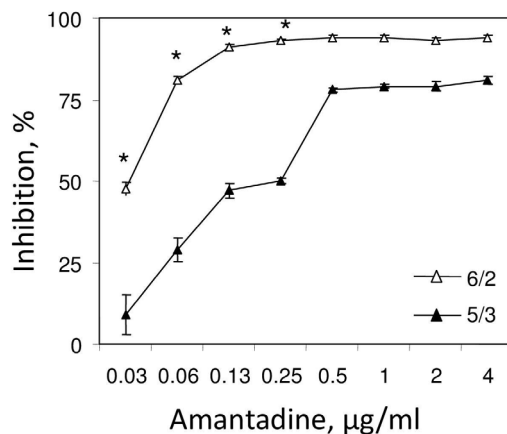
Virus	Reproduction in mouse organs, log <sub>10</sub> TCID <sub>50</sub> /ml <sup>a</sup>			Serum reciprocal antibody titers, GMT	
	Nasal turbinates/day		Lungs/day 3	HAI	ELISA, total IgG
	1	3			
5389wt	4.5±0.3	6.0±0.4	5.0 ± 0.2	104	412
6/2	2.5±0.2	2.5±0.2	<1.5	2	75
5/3	2.5±0.2	2.5±0.2	<1.5	19.4 <sup>b</sup>	180

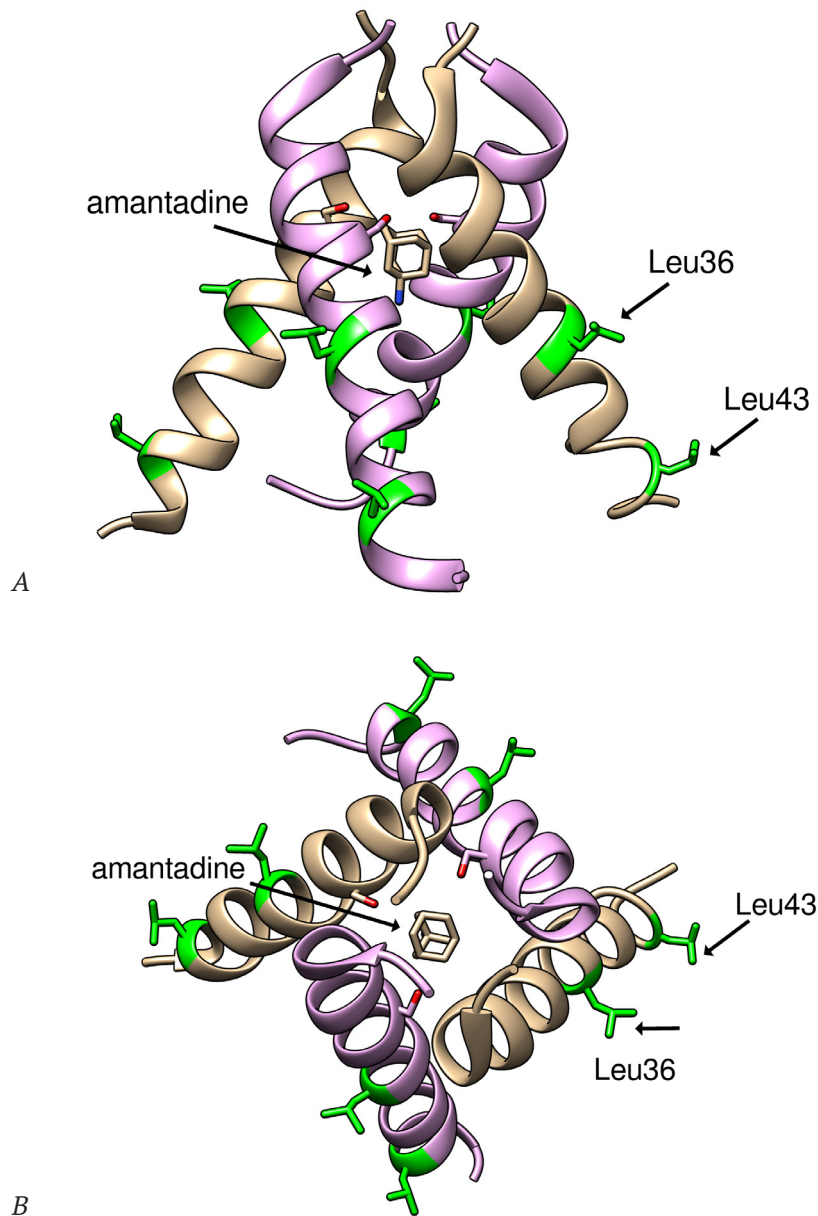
<sup>a</sup> Data are given as mean values for four mice.

<sup>b</sup> The difference between 6/2 and 5/3 reassortants is significant,  $p = 0.02$  determined by a non-parametric Mann-Whitney test.

**Fig. 4.** Distribution of antibodies in mouse post infection sera interacting with HA1 and HA2 subunits.

Purified influenza virus 5389wt was subjected to SDS PAGE and Western blotting as described in Materials and methods. After blotting, the membrane was incubated with 1:100 diluted pool of 4 mouse antisera obtained after immunization with the 5389wt, 6/2 or 5/3 virus. M - molecular size marker. The ratio of HA2/HA1 was measured by density analysis.

**Fig. 5.** Sensitivity of 6/2 and 5/3 reassortants to amantadine. Serial 2-fold dilutions of amantadine were added to MDCK cell cultures. Virus with an MOI ranging from 100 to 200 focus forming units was added to each well. Then cells were fixed, permeabilized and immunostained with monoclonal antibodies specific for the nucleoprotein of influenza virus. The reported results are mean values ± standard deviation of 5 repeats at each concentration of amantadine for each virus. The difference is statistically significant. \* indicates  $p < 0.01$  determined by a non-parametric Mann-Whitney test.



**Fig. 6.** Structure of the transmembrane domain of influenza virus M2 protein in complex with amantadine.(A) Side view. (B) Top view. Protein Data Bank model 3C9J was visualized using USCf Chimera v.1.10.2 [36]. Mutated amino acids are colored in green.

## DISCUSSION

For many years, the isolation of influenza viruses from patient clinical material was done in embryonated eggs. Due to the prevailing regulations, only egg-derived viruses are used for the generation of vaccine seed viruses. Now, the National Influenza Centers can use MDCK-derived viruses for the characterization of circulating influenza viruses because of the higher isolation rate of viruses achieved with this cell line [37-39]. Previously, we showed that HA sequences of cell-derived viruses (Vero and MDCK cell lines) are more similar to that of the original circulating human viruses than the sequences of egg-derived isolates [40]. That was the reason why the Vero-derived isolates were used for the generation of vaccine reassortants.

The reassortant of 7729wt (H3N2) on the backbone of Sing/ca was immunogenic in adult volunteers, while the

vaccine strain generated with the surface proteins from 1035wt (H1N1) failed to induce any antibodies detected by the HAI test [16]. Similarly, the 6/2 reassortant of 5389wt (H1N1) virus was not immunogenic in mice. Both epidemic Vero-derived viruses 1035wt and 5389wt were found to contain the substitutions 10Ile→Val or 3F→L at the fusion peptide of HA2 subunit that led to the enhancement of the pH threshold of an HA conformational change by 0.3 pH units. Besides, virus 7729wt, which has an HA sequence identical to that of the clinical isolate, appeared to be more stable toward acidic pH than mutant virus 1035wt. This finding is consistent with our previous observations that the increased pH of HA activation is related to the decreased virus stability toward acidic pH and elevated temperature, decreased virus infectivity and immunogenicity after the intranasal



immunization of mammals [20, 21]. Therefore, the decreased immunogenicity of reassortants obtained from 1035wt and 5389wt viruses might be induced by HA mutations that occurred in the course of virus isolation in the Vero cells.

It is known that the main reason for the restricted virus growth in Vero cells is that the late endosomes of Vero cells, where the fusion of viral and cell membranes takes place, have a relatively high pH that is higher than that in MDCK cells [15]. Thus, mutations 10Ile→Val and 3Phe→Leu enable efficient virus replication in Vero cells. It is important to mention that the propagation of influenza viruses in MDCK cells as well as in eggs can also lead to the emergence of mutations which increase the pH of HA activation [19, 41] and decrease virus stability toward an acidic pH.

The human airway epithelium is known to be slightly acidic (pH 5.5 to 6.9) because of the acidic secretion of the submucosal glands [42-47]. This feature is the innate mechanism of defense against pathogens. Moreover, as a response to organic dust or inflammation, the cells of human nasal airway epithelium start to release acid [48]. Thus, strains of the live influenza vaccine require a certain level of HA stability toward acidic pH for the efficient infection of the human upper respiratory tract.

Another factor that might compromise the immunogenicity of vaccine reassortant is the incompatibility of the HA and M genes originated from different viruses. We found that the 6/2 reassortant of 5389wt (H1N1) virus on the backbone of the master strain Sing/ca induced antibodies that were detectable in ELISA but not in the HAI test, and were directed predominantly to the HA2 subunit. However, the 5/3 reassortant bearing the HA and M genes from the epidemic virus induced an equivalent amount of antibodies reacting with HA1 and HA2 subunits detectable in HAI. This result indicates that the HA of 6/2 reassortant was predominantly on the surface of virions and/or of infected cells in the low pH conformation with exposed HA2 epitopes. The incompatibility of the M gene of H2N2 virus with the HA gene of an avian virus with a polybasic cleavage site is well documented [29, 32, 49]. The authors of these publications have shown that the M2 protein of H2N2 virus lacked sufficient ion channel activity to maintain the pH of the *trans*-Golgi network above the threshold necessary to preserve the native conformation of the avian virus HA with the polybasic cleavage site. The prerequisite for the HA conformational rearrangement in the *trans*-Golgi network is the HA intracellular cleavage to HA1 and HA2 subunits, which releases the fusion peptide. The HA of avian influenza viruses with a polybasic cleavage site is known to be cleaved intracellularly by furin. Recently, it was demonstrated that the HA of human H1N1 viruses is also cleaved intracellularly by TMPRSS2 protease (reviewed in [50]). It was shown that the acid sensitive HA of the (H1N1)pdm09 virus is in the same way protected from the premature conformational changes during intracellular transport by the M2 ion channel [51]. Therefore, we can conclude that the M2 channel activity of Sing/ca virus

is too weak to keep the pH of the *trans*-Golgi network above the threshold necessary to preserve the native conformation of the acid sensitive HA of Vero-derived 5389wt virus. As a result, most of HA proteins changed the conformation to the low pH form and induced antibodies predominantly to the HA2 subunit in immunized animals.

Although the sequence of M2 protein is known to be highly conserved among different influenza viruses, there are known variations of its structure that have an impact on M2 ion channel activity [33, 34, 52]. Two changes, Leu36→Met and Leu43→Ile, were revealed in the TM domain of Sing/ca virus compared to that of 5389wt. The position 36 is in the immediate vicinity of H37 - the residue serving as the proton-selective filter of the M2 ion channel. The protonation of the H37 residue is responsible for the channel activation [53-55]. The second substitution (Leu43→Ile) is located within the H37xxxW41xxD44 motif of the channel, which is involved in the pore gating [53]. The side chain of amino acid L43 together with the side chains of Ile42 and Leu40 participates in hydrophobic interactions with the membrane [54, 56]. It is highly probable that substitutions of these amino acids would influence the structure of the H37xxxW41xxD44 motif and consequently affect the conductance of the channel [57].

It is interesting to note that the sequences of the M2 TM domains of the 7729wt (H3N2) and Sing/ca viruses are identical. Although the ion channel with this TM domain structure has low activity, the resulting pH level in the *trans*-Golgi network was probably high enough to prevent the conformational modification of the more stable HA of H3N2 virus.

Hereby, the combination of the H2N2 virus M gene and HA with the elevated pH of activation could provoke an undesirable conformational change of the HA molecules during virus maturation and affect the immunogenic properties of the vaccine strain following the intranasal immunization. Interesting results were published by O'Donnell et al. who showed that the reassortants bearing just one M gene of A/Ann Arbor/6/60 ca (H2N2) master strain (the FluMist® vaccine) were characterized by the increased pH threshold of HA activation, decreased virus stability toward acidic pH, and restricted replication in the respiratory tract of vaccinated humans [58]. Unfortunately, the authors did not elucidate the mechanism of the observed activity and explained this phenomenon as a gene constellation effect.

It is important to mention that we did not notice any significant difference in the reproduction capacity of 6/2 and 5/3 reassortants *in vitro*. This could be explained by a relatively high pH (6.45 +/- 0.03) of the *intra*-Golgi network in Vero cells [59], which do not require a highly active ion channel. This observation is in accordance with the results obtained by Watanabe et al. who showed that a virus with an impaired TM domain grew efficiently in continuous cell lines, but demonstrated restricted growth in the mouse upper respiratory tract [60].

Taken together, both the influenza virus HA stability and the compatibility of HA and M genes should be taken into consideration when constructing the reassortant vaccine strains for the live attenuated vaccines and selecting the substrate for the vaccine production. Application of reverse genetics for the construction of vaccine reassortants is of great benefit as it allows the use of the sequences of human influenza virus genes present in the clinical material without any adaptation mutations.

## REFERENCES

- Kendal AP. Cold-adapted live attenuated influenza vaccines developed in Russia: can they contribute to meeting the needs for influenza control in other countries? *Eur J Epidemiol.* 1997;13(5), 591-609.
- Rudenko L, Desheva J, Korovkin S, Mironov A, Rekstin A, Grigorieva E, Donina S, Gambaryan A, Katlinsky A. Safety and immunogenicity of live attenuated influenza reassortant H5 vaccine (phase I-II clinical trials). *Influenza Other Respi Viruses* 2008; 2(6), 203-9.
- Rudenko LG, Arden NH, Grigorieva E, Naychin A, Rekstin A, Klimov AI, Donina S, Desheva J, Holman RC, DeGuzman A, Cox NJ, Katz JM. Immunogenicity and efficacy of Russian live attenuated and US inactivated influenza vaccines used alone and in combination in nursing home residents. *Vaccine* 2000; 19(2-3), 308-18.
- Rudenko LG, Lonskaya NI, Klimov AI, Vasilieva RI, Ramirez A. Clinical and epidemiological evaluation of a live, cold-adapted influenza vaccine for 3-14-year-olds. *Bull World Health Organ.* 1996; 74(1), 77-84.
- Rudenko L, Isakova-Sivak I, Naykhin A, Kiseleva I, Stukova M, Erofeeva M, Korenkov D, Matyushenko V, Sparrow E, Kieny MP. H7N9 live attenuated influenza vaccine in healthy adults: a randomised, double-blind, placebo-controlled, phase 1 trial. *Lancet Infect Dis.* 2016; 16(3), 303-10. doi: 10.1016/S1473-3099(15)00378-3.
- Maassab HF, Heilman CA, Herlocher ML. Cold-adapted influenza viruses for use as live vaccines for man. *Adv Biotechnol Processes.* 1990; 14, 203-42.
- Ambrose CS, Luke C, Coelingh K. Current status of live attenuated influenza vaccine in the United States for seasonal and pandemic influenza. *Influenza Other Respi Viruses.* 2008; 2(6), 193-202. doi: 10.1111/j.1750-2659.2008.00056.x.
- Robertson JS. An overview of host cell selection. *Dev Biol Stand.* 1999; 98, 7-11; discussion 73-4.
- Robertson JS, Cook P, Attwell AM, Williams SP. Replicative advantage in tissue culture of egg-adapted influenza virus over tissue-culture derived virus: implications for vaccine manufacture. *Vaccine.* 1995; 13(16), 1583-8.
- Hardy CT, Young SA, Webster RG, Naeve CW, Owens RJ. Egg fluids and cells of the chorioallantoic membrane of embryonated chicken eggs can select different variants of influenza A (H3N2) viruses. *Virology.* 1995; 211(1), 302-6. doi: 10.1006/viro.1995.1405.
- Liu J, Shi X, Schwartz R, Kemble G. Use of MDCK cells for production of live attenuated influenza vaccine. *Vaccine.* 2009; 27(46), 6460-3. doi: 10.1016/j.vaccine.2009.06.024.
- Ghendon YZ, Markushin SG, Akopova II, Koptiaeva IB, Nechaeva EA, Mazurkova LA, Radaeva IF, Kolokoltseva TD. Development of cell culture (MDCK) live cold-adapted (CA) attenuated influenza vaccine. *Vaccine.* 2005; 23(38), 4678-84. doi: 10.1016/j.vaccine.2005.04.039.
- Barrett PN, Portsmouth D, Ehrlich HJ. Developing cell culture-derived pandemic vaccines. *Curr Opin Mol Ther.* 2010; 12(1), 21-30.
- Barrett PN, Portsmouth D, Ehrlich HJ. Vero cell culture-derived pandemic influenza vaccines: preclinical and clinical development. *Expert Rev Vaccines.* 2013; 12(4), 395-413. doi: 10.1586/erv.13.21.
- Murakami S, Horimoto T, Ito M, Takano R, Katsura H, Shimojima M, Kawaoka Y. Enhanced growth of influenza vaccine seed viruses in vero cells mediated by broadening the optimal pH range for virus membrane fusion. *J Virol.* 2012; 86(3), 1405-10. doi: 10.1128/JVI.06009-11.
- Romanova J, Katinger D, Ferko B, Vcelar B, Sereinig S, Kuznetsov O, Stukova M, Erofeeva M, Kiselev O, Katinger H, Egorov A. Live cold-adapted influenza A vaccine produced in Vero cell line. *Virus Res.* 2004; 103(1-2), 187-93. doi: 10.1016/j.virusres.2004.02.032.
- Noble GR, Kaye HS, Yarbrough WB, Fiedler BK, Reed CJ, Felker MB, Kendal AP, Dowdle WR. Measurement of hemagglutination-inhibiting antibody to influenza virus in the 1976 influenza vaccine program: methods and test reproducibility. *J Infect Dis.* 1977; 136 Suppl: S429-34.
- Matrosovich MN, Gambaryan AS, Tuzikov AB, Byramova NE, Mochalova IV, Golbraikh AA, Shenderovich MD, Finne J, Bovin NV. Probing of the receptor-binding sites of the H1 and H3 influenza A and influenza B virus hemagglutinins by synthetic and natural sialosides. *Virology.* 1993; 196(1), 111-21. doi: 10.1006/viro.1993.1459.
- Nakowitsch S, Waltenberger AM, Wressnigg N, Ferstl N, Triendl A, Kiefmann B, Montomoli E,

## ACKNOWLEDGEMENTS

This work was supported by Polymun Scientific GmbH, Austria.

## CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

- Lapini G, Sergeeva M, Muster T, Romanova JR. Egg- or cell culture-derived hemagglutinin mutations impair virus stability and antigen content of inactivated influenza vaccines. *Biotechnol J*. 2014; 9(3), 405-14. doi: 10.1002/biot.201300225.
20. Nakowitsch S, Wolschek M, Morokutti A, Ruthsatz T, Krenn BM, Ferko B, Ferstl N, Triendl A, Muster T, Egorov A, Romanova J. Mutations affecting the stability of the haemagglutinin molecule impair the immunogenicity of live attenuated H3N2 intranasal influenza vaccine candidates lacking NS1. *Vaccine*. 2011; 29(19), 3517-24. doi: 10.1016/j.vaccine.2011.02.100.
  21. Krenn BM, Egorov A, Romanovskaya-Romanko E, Wolschek M, Nakowitsch S, Ruthsatz T, Kiefmann B, Morokutti A, Humer J, Geiler J, Cinatl J, Michaelis M, Wressnigg N, Sturlan S, Ferko B, Batishchev OV, Indenbom AV, Zhu R, Kastner M, Hinterdorfer P, Kiselev O, Muster T, Romanova J. Single HA2 mutation increases the infectivity and immunogenicity of a live attenuated H5N1 intranasal influenza vaccine candidate lacking NS1. *PLoS ONE*. 2011; 6(4), e18577. doi: 10.1371/journal.pone.0018577.
  22. Cross KJ, Wharton SA, Skehel JJ, Wiley DC, Steinhauer DA. Studies on influenza haemagglutinin fusion peptide mutants generated by reverse genetics. *EMBO J*. 2001; 20(16), 4432-42. doi: 10.1093/emboj/20.16.4432.
  23. Clements DA, Langdon L, Bland C, Walter E. Influenza A vaccine decreases the incidence of otitis media in 6- to 30-month-old children in day care. *Arch Pediatr Adolesc Med*. 1995; 149(10), 1113-7.
  24. Klimov AI, Sokolov NI, Orlova NG, Ginzburg VP. Correlation of amino acid residues in the M1 and M2 proteins of influenza virus with high yielding properties. *Virus Res*. 1991; 19(1), 105-14.
  25. Florent G. Gene constellation of live influenza A vaccines. *Arch Virol*. 1980; 64(2), 171-3.
  26. Snyder MH, Buckler-White AJ, London WT, Tierney EL, Murphy BR. The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/79 reassortant viruses for monkeys. *J Virol*. 1987; 61(9), 2857-63.
  27. Subbarao EK, Perkins M, Treanor JJ, Murphy BR. The attenuation phenotype conferred by the M gene of the influenza A/Ann Arbor/6/60 cold-adapted virus (H2N2) on the A/Korea/82 (H3N2) reassortant virus results from a gene constellation effect. *Virus Res*. 1992; 25(1-2), 37-50.
  28. Yasuda J, Bucher DJ, Ishihama A. Growth control of influenza A virus by M1 protein: analysis of transfectant viruses carrying the chimeric M gene. *J Virol*. 1994; 68(12), 8141-6.
  29. Grambas S, Hay AJ. Maturation of influenza A virus hemagglutinin--estimates of the pH encountered during transport and its regulation by the M2 protein. *Virology*. 1992; 190(1), 11-8.
  30. Liu T, Muller J, Ye Z. Association of influenza virus matrix protein with ribonucleoproteins may control viral growth and morphology. *Virology*. 2002; 304(1), 89-96.
  31. Scholtissek C, Stech J, Krauss S, Webster RG. Cooperation between the hemagglutinin of avian viruses and the matrix protein of human influenza A viruses. *J Virol*. 2002; 76(4), 1781-6.
  32. Ciampor F, Thompson CA, Grambas S, Hay AJ. Regulation of pH by the M2 protein of influenza A viruses. *Virus Res*. 1992; 22(3), 247-58.
  33. Pinto LH, Holsinger LJ, Lamb RA. Influenza virus M2 protein has ion channel activity. *Cell*. 1992; 69(3), 517-28.
  34. Wang C, Lamb RA, Pinto LH. Direct measurement of the influenza A virus M2 protein ion channel activity in mammalian cells. *Virology*. 1994; 205(1), 133-40. doi: 10.1006/viro.1994.1628.
  35. Wang C, Lamb RA, Pinto LH. Activation of the M2 ion channel of influenza virus: a role for the transmembrane domain histidine residue. *Biophys J*. 1995; 69(4), 1363-71. doi: 10.1016/S0006-3495(95)80003-2.
  36. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem*. 2004; 25(13), 1605-12. doi: 10.1002/jcc.20084.
  37. Katz JM, Naeve CW, Webster RG. Host cell-mediated variation in H3N2 influenza viruses. *Virology*. 1987; 156(2), 386-95.
  38. Katz JM, Webster RG. Efficacy of inactivated influenza A virus (H3N2) vaccines grown in mammalian cells or embryonated eggs. *J Infect Dis*. 1989; 160(2), 191-8.
  39. Meyer WJ, Wood JM, Major D, Robertson JS, Webster RG, Katz JM. Influence of host cell-mediated variation on the international surveillance of influenza A (H3N2) viruses. *Virology*. 1993; 196(1), 130-7. doi: 10.1006/viro.1993.1461.
  40. Romanova J, Katinger D, Ferko B, Voglauer R, Mochalova L, Bovin N, Lim W, Katinger H, Egorov A. Distinct host range of influenza H3N2 virus isolates in Vero and MDCK cells is determined by cell specific glycosylation pattern. *Virology*. 2003; 307(1), 90-7.
  41. Lin YP, Wharton SA, Martin J, Skehel JJ, Wiley DC, Steinhauer DA. Adaptation of egg-grown and transfectant influenza viruses for growth in mammalian cells: selection of hemagglutinin mutants with elevated pH of membrane fusion. *Virology*. 1997; 233(2), 402-10.
  42. Washington N, Steele RJ, Jackson SJ, Bush D, Mason J, Gill DA, Pitt K, Rawlins DA. Determination of baseline human nasal pH and the effect of intranasally administered buffers. *Int J Pharm*. 2000; 198(2), 139-46.
  43. Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: soldier in the fight against

- respiratory viruses. *Clin Microbiol Rev.* 2011; 24(1), 210-29. doi: 10.1128/CMR.00014-10.
44. England RJ, Homer JJ, Knight LC, Ell SR. Nasal pH measurement: a reliable and repeatable parameter. *Clin Otolaryngol Allied Sci.* 1999; 24(1), 67-8.
  45. Arora P, Sharma S, Garg S. Permeability issues in nasal drug delivery. *Drug Discov Today.* 2002; 7(18), 967-75.
  46. Hehar SS, Mason JD, Stephen AB, Washington N, Jones NS, Jackson SJ, Bush D. Twenty-four hour ambulatory nasal pH monitoring. *Clin Otolaryngol Allied Sci.* 1999; 24(1), 24-5.
  47. McShane D, Davies JC, Davies MG, Bush A, Geddes DM, Alton EW. Airway surface pH in subjects with cystic fibrosis. *Eur Respir J.* 2003; 21(1), 37-42.
  48. Fischer H, Widdicombe JH. Mechanisms of acid and base secretion by the airway epithelium. *J Membr Biol.* 2006; 211(3), 139-50.
  49. Takeuchi K, Lamb RA. Influenza virus M2 protein ion channel activity stabilizes the native form of fowl plague virus hemagglutinin during intracellular transport. *J Virol.* 1994; 68(2), 911-9.
  50. Garten W, Braden C, Arendt A, Peitsch C, Baron J, Lu Y, Pawletko K, Hardes K, Steinmetzer T, Böttcher-Friebertshäuser E. Influenza virus activating host proteases: Identification, localization and inhibitors as potential therapeutics. *Eur J Cell Biol.* 2015; 94(7-9), 375-83. doi: 10.1016/j.ejcb.2015.05.013.
  51. Alvarado-Facundo E, Gao Y, Ribas-Aparicio RM, Jimenez-Alberto A, Weiss CD, Wang W. Influenza virus M2 protein ion channel activity helps to maintain pandemic 2009 H1N1 virus hemagglutinin fusion competence during transport to the cell surface. *J Virol.* 2015; 89(4), 1975-85. doi: 10.1128/JVI.03253-14.
  52. Holsinger LJ, Nichani D, Pinto LH, Lamb RA. Influenza A virus M2 ion channel protein: a structure-function analysis. *J Virol.* 1994; 68(3), 1551-63.
  53. Balannik V, Carnevale V, Fiorin G, Levine BG, Lamb RA, Klein ML, Degrado WF, Pinto LH. Functional studies and modeling of pore-lining residue mutants of the influenza A virus M2 ion channel. *Biochemistry.* 2010; 49(4), 696-708. doi: 10.1021/bi901799k.
  54. Schnell JR, Chou JJ. Structure and mechanism of the M2 proton channel of influenza A virus. *Nature.* 2008; 451(7178), 591-5. doi: 10.1038/nature06531.
  55. Wang J, Qiu JX, Soto C, DeGrado WF. Structural and dynamic mechanisms for the function and inhibition of the M2 proton channel from influenza A virus. *Curr Opin Struct Biol.* 2011; 21(1), 68-80. doi: 10.1016/j.sbi.2010.12.002.
  56. Pielak RM, Chou JJ. Influenza M2 proton channels. *Biochim Biophys Acta.* 2011; 1808(2), 522-9. doi: 10.1016/j.bbame.2010.04.015.
  57. Betakova T, Ciampor F, Hay AJ. Influence of residue 44 on the activity of the M2 proton channel of influenza A virus. *J Gen Virol.* 2005; 86(Pt 1), 181-4. doi: 10.1099/vir.0.80358-0.
  58. O'Donnell CD, Vogel L, Matsuoka Y, Jin H, Subbarao K. The matrix gene segment destabilizes the acid and thermal stability of the hemagglutinin of pandemic live attenuated influenza virus vaccines. *J Virol.* 2014; 88(21), 12374-84. doi: 10.1128/JVI.01107-14.
  59. Kim JH, Lingwood CA, Williams DB, Furuya W, Manolson MF, Grinstein S. Dynamic measurement of the pH of the Golgi complex in living cells using retrograde transport of the verotoxin receptor. *J Cell Biol.* 1996; 134(6), 1387-99.
  60. Watanabe T, Watanabe S, Ito H, Kida H, Kawaoka Y. Influenza A virus can undergo multiple cycles of replication without M2 ion channel activity. *J Virol.* 2001; 75(12), 5656-62. doi: 10.1128/JVI.75.12.5656-5662.2001.