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Investigation of Efficiency of Site-Specific Mutants of the Influenza Virus in Homological and Heterological Control Infection

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Abstract

The immunogenicity and protective efficacy of the site-specific mutants of A/WSN/33 (H1N1) strain of the influenza virus were compared with the similar characteristics of the cold-adapted (CA) reassortant obtained by crossing A/WSN/33 strain and CA A/Krasnodar/101/35/59 (H2N2) strain of the influenza virus under homologous and heterologous control infection. The site-specific mutants had ts-mutations localized in the genes encoding the proteins of the polymerase complex. Groups of mice were immunized intranasally with site-specific mutants and CA reassortant and then infected with specific doses of A/WSN/33 strain and A/Duck. Pennsylvania/10218/84 (H5N2) strain of avian influenza virus adapted to mice. The titer of antibodies inhibiting haemagglutination was higher in mice immunized with site-specific mutants than in mice immunized with CA-reassortant. With homologous control infection, an effective suppression of the propagation of the A/WSN/33 virulent strain was observed in the lungs of mice immunized with both one and the other vaccine preparation. In case of heterologous control of mice entering the control group, the H5N2 strain of the avian influenza virus was 78 ± 6.5% lethality. In the group of mice immunized with CA-reassortant 35 ± 4.2% mortality was observed. In mice immunized with site-specific mutants (transfectants No. 2 and No. 5), 100% survival was noted. In the group of mice immunized with transfectant No. 8 after the control heterologous infection, lethality reached 16 ± 3.0%. The data obtained indicate that the site-specific mutants of the influenza virus are not inferior to the investigated useful characteristics of CA with reassortant influenza vaccines and can be used as live influenza vaccines.

Keywords: *Site-Specific Mutants; Influenza Virus; Control Infection*

Introduction

Currently, the practice of obtaining industrial reassortants for live influenza vaccine in our country is limited to the traditional method of reassortment of parental variants in chick embryos, with subsequent selection of the variants and their selection after genomic analysis. Introduction of genetically engineered approaches to the production of influenza live vaccines makes it possible to significantly optimize the individual stages of this process. In recent years, a great interest among researchers is the method involving the direct inclusion of previously known and well characterized mutations in the genome of the epidemic variant of the influenza virus, which thus acquires the ts and att phenotype and can be used as a candidate for live influenza vaccines [1-8]. As seen from the literature, the main source of site-specific ts-mutations is the CA A/Ann Arbor/6/60 strain, obtained by Maassab more than half a century ago [9]. Three mutations in the PB1 gene (K391E, E581G, A661T), one mutation in the PB2 gene (N265S) and one mutation in the NP gene (D34G) constitute a traditional set for the modification of the genome of the overwhelming number of virulent strains of the human, animal and avian influenza virus. However, the use of this set of mutations does not allow to sufficiently attenuate the most dangerous pandemic strains of the influenza A virus and even some virulent seasonal variants of the human influenza virus. In this connection, it was quite reasonable to use ts-mutations taken from the genomes of other CA strains. In Russia for many years, CA strain A/Leningrad/134/17/57 (H2N2) [10]

is used as the donor of attenuation for live influenza vaccines. The genetic determinants responsible for the attenuation of this strain are concentrated in the PB1 genes: K265N, V591I and PB2: V478L. In another recently created donor strain A/Krasnodar/101/35/59 (H2N2), the ts mutation responsible for the ts-phenotype of this strain is localized in the PB1 gene (I147T) [11]. The potential of these mutations, as well as mutations in the genes of PA and NP of these strains, would be extremely useful in terms of modifying the phenotypic characteristics of virulent strains for the production of live influenza vaccines. Of particular interest is also the study of the possibility of including in the conserved sites genes of virulent strains of influenza virus specific attenuating mutations. Solving this problem would give an opportunity to compare the effectiveness of all these mutations, as well as expand the arsenal of sites for directed mutagenesis, which is very promising for the further design of vaccine preparations. In the Mechnikov Research Institute of Vaccines and Sera a panel of site-specific mutants of A/WSN/33 strain was obtained by including ts-mutations from the genome of CA A/Ann Arbor/6/60 (H2N2), A/Krasnodar/101/35/59 (H2N2) and A/Leningrad/134/47/57 strains into the genes of the virulent A/WSN/33 strain, which encode the proteins of the polymerase complex PB1 and PB2. The replacement of F658L was also included in the terminal conservative part of the COOH-domain of the PA-gene of the obtained site-specific mutants, which promoted their additional attenuation [12]. Some of these site-specific mutants possessed optimal phenotypic characteristics, which allowed them to be considered as possible candidates for live influenza vaccines [12]. This report presents the results of a comparative study on a model of mice of immunogenicity and the protective efficacy of a number of A/WSN/33 strain-derived site-specific mutants having ts-mutations in various genes encoding the proteins of the polymerase complex and a traditional vaccine CA reassortant having 6 "internal" Genes from CA A/Krasnodar/101/35/59 (H2N2) strain and HA and NA genes from A/WSN/33 strain, with homologous and heterologous challenge. It was of great interest to compare the immunogenic and protective potential of two types of live influenza vaccines having the same surface antigens.

Materials and Methods

Viruses

The attenuated CA A/Krasnodar/101/35/59 (H2N2) strain-donor, obtained in the Mechnikov Research Institute of Vaccines and Sera by passage at a lower temperature in chick embryos and MDCK cell culture [11]. The virulent genetically engineered r A/WSN/33 (H1N1) strain was obtained by transfection from plasmids pHW2000 with inserts of genes of A/WSN/33 (H1N1) strain, kindly provided by Dr. Webster (Memphis, USA). To perform this work, a cold-adapted (CA) reassortant was obtained by crossing CA A/Krasnodar/101/35/59 (H2N2) strain and a virulent A/WSN/33 (H1N1) strain of the influenza virus. The reassortant inherited 6 "internal" genes from the CA strain-donor and 2 genes that code for surface HA and NA proteins from A/WSN/33 strain. A virulent A/WSN/33 (H1N1) strain was used for homologous challenge. In case of heterologous challenge, A/Duck Pennsylvania/10218/1984 (H5N2) strain of the avian influenza virus was used. Adapted to mice [13].

The preparation of the CA reassortant was carried out according to the previously described standard procedure [14]. Inactivation of A/WSN/33 strain was carried out by UV irradiation (UV lamp G8W T5, Germicidal, 288 nm). During the inactivation, the titer of the virus-containing liquid decreased by 5.0 lg. A mixed infection of A/WSN/33 and A/Krasnodar/101/35/59 strains in chick embryos was carried out at a temperature of 30^0C for 18 hours. Then 2 selective passages were made in chick embryos at 25^0C in the presence of antiserum to A/Krasnodar/101/35/59 strain. The resulting reassortant was cloned by limiting dilutions, and then by plaques. Analysis of the genome of the CA reassortant was performed by PCR using differentiating primers to A/Krasnodar/101/35/59 strain. The following pairs of primers 5' to 3' were used: PB2 gene: F2-1 (CCCTGTCCATGTTAGAAACCAAGT), R2-1 (CGCTGAGTTGCCCTAGTAACGA), PB1 gene: F1-1 (AGCACAAGCAGGCAAACCAT), R2-1 (CAATCTGTGTGCTGTGG), PA- gene; F1 (AGCAAAAGCAGGTACTGATC), R2 (TGGATGTGTGTCTTCTCAGA), NP gene: F4-1 (GCCAGTGGGTACGACTTCGA), R2 (CTGATTTGACCTGCAGAG), M-gene: F1 (AGCAAAAGCAGGTAGATATTG), R2 (TGCAAGATCCCAATGATACTC), NS gene: F1 (AGCAAAAGCAGGGTGAC), R1 gene (CCCATTCTCATTACTGCTTC). HA –inhibition test with the use of antisera to serotypes H1 and H2 showed that the HA protein of the CA reassortant refers to serotype H1 (antisera to H1-1: 1024, antiserum to H2-1:20). Sequencing of the NA gene of the CA reassortant showed its belonging to serotype N1.

Site-specific mutants (transfectants) based on A/WSN/33 strain were used in the work, and a number of ts mutations were included in the genome (Table 1). Transfectant No. 2 contained in the genome of 2 ts mutations from the PB1 gene (strain L265N, V591I) of A/Leningrad/134/17/57 (H2N2) strain, the N265S ts mutation from the PB2 gene of A/Ann Arbor/6/60 (H2N2) strain and ts-mutation of F658A from the COOH domain of the PA gene of A/WSN/33 (H1N1) strain. Transfectant No. 5 contained in the genome ts-mutation Il147 Thr from the PB1 - gene of CA A/Krasnodar/101/35/59 strain, as well as the ts - mutation of F658A from the COOH domain of the PA gene of A/WSN/33 strain. Transfectant No. 8 contained in the genome a ts-mutations (K391E, E581G, E457D) from the PB1 - gene of the CA A/Ann Arbor/6/60 strain, the V290L ts-mutation from the PB2-gene of CA A/ Krasnodar/101/35/59 strain and ts-mutation F658A from the COOH-domain of the PA gene of A/WSN/33 strain.

№	Transfectants	P B 1 gene		P B 2 gene		P A gene	
		Nucleotide	Amino-acid	Nucleotide	Amino-acid	Nucleotide	Amino-acid
1	Transfectant No. 2	265 AAT 591 ATT	Asn Ile	265 AGC	Ser	658 GCT	Ala
2	Transfectant No. 5	147 ACA	Thr			658 GCT	Ala
3	Transfectant No. 8	391 GAA 581 GGG 457 GAT	Glu Gly Asp	290 UUA	Leu	658 GCT	Ala

Table 1: *Nucleotide and amino acid substitutions in the genome of transfectants No. 2, No. 5 and No. 8, incorporated by site-specific mutagenesis into genes of strain A/WSN/33 (H1N1) encoding the proteins of the polymerase complex.*

For genetic engineering works with strain A/WSN/33 of the influenza virus, an 8-plasmid transfection system based on the pHW2000 vector was used (Hoffmann et al., 2000).

Chicken embryos (CE): All viruses and site-specific mutants used in the work were maintained by passage in 10 - 11 day-old chick embryos.

The study of the ts-phenotype of viruses: The activity of reproduction of influenza viruses at different incubation temperatures was evaluated by titration results in chick embryos, incubated at 34^0C, 37^0C, 38^0C, 39^0C and expressed in RCT (reproductive capacity at different temperatures). RCT39 = (lg EID_{50}/0.2 ml at 34^0C-lg EID^{50}/0.2 ml at 39^0C). The viruses were considered temperature sensitive (ts-phenotype), if RCT39 was more than 5.0 lg EID_{50}/0.2 ml.

The study of att-phenotype of viruses: Study of the att-phenotype was carried out according to the following procedure: groups of female mongrel mice (6 - 10 heads per group) were infected intranasally under mild etheric anesthesia with viruses analyzed at various doses in a volume of 50 µl per mouse. After 72 hours, the mice were euthanized and lung tissue removed. From the lung tissue a 10% suspension was prepared in mortars with grated glass. The infectious virus titer in a 10% lung suspension was determined in chick embryos and expressed in lg EID_{50}/0.2 ml. All viruses were examined in three independent experiments.

The study of the immunogenicity of the viruses being analyzed: Mice under slight ether anesthesia were administered intranasally viral material in a volume of 50 µl twice with an interval of 21 days. 10 days after the second immunization in mice, blood was taken. The

analysis of specific inhibitory haemagglutination antibodies was performed according to MU 3.3.2.1758-03. Serum dilution was taken as the serum titer, causing a complete delay in haemagglutination.

Defining the protective efficacy of vaccine options: To determine the protective efficacy of the analyzed vaccine variants, one group of mice were immunized intranasally with light ether anesthesia with CA reassortant R A/Krasnodar/101/35xA/WSN/33 and mice of other group with transfectants No. 2, No. 5 and No. 8 in infectious titer $10^{4.0}$ EID50 in a volume of 50 µl. Then, 21 days later, the mice were immunized again. 10 days after the second immunization, the mice were infected intranasally by virulent A/WSN/33 (H1N1) strain (homologous challenge) or the virulent H5N2 strain of the avian influenza virus (heterologous challenge). After 3 days, a portion of the mice infected with strain A/WSN/33 were recovered with lung tissue and virus reproduction was determined in the lungs. In mice infected with H5N2 strain of avian influenza virus, lethality was determined within the next 10 days from the time of infection with the virulent virus.

Cell cultures: Transfection experiments used a T293 cell culture and an MDCK cell line obtained from the Pasteur Institute (France). All cells were grown at 37^0C in a CO_2 incubator. Cell cultures were passaged on MEM medium (PanEco, Moscow, Russia) containing 5% fetal bovine serum and gentamicin in an amount of 1 mg per 450 ml medium.

Recombinant DNA and reverse genetic methods

Plasmids and bacteria: For genetic engineering works with strain A/WSN/33 (H1N1) of the influenza virus, an 8-plasmid transfection system based on the pHW2000 vector was used. Each of the 8 plasmids contained the corresponding influenza virus gene flanked by the necessary regulatory elements to assemble the virus in the cell culture during transfection [15]. Plasmid pHW2000, as well as plasmids with inserts of genes of the A/WSN/33 virus strain were kindly provided by Dr. Webster (Memphis, USA). The *E. coli* DH5alpha strain was used to accumulate the plasmids.

Replication and concentration of viruses: Viruses were replicated in chick embryos by a standard procedure. For subsequent isolation of RNA, the viruses were concentrated by centrifugation. Initially, the allantoic fluid was precipitated in a Beckman J2-21 centrifuge (JA-14 rotor) at 6000 rpm for 30 minutes to precipitate the cellular debris. The virus was precipitated from the supernatant on a high-speed Beckman J2-21 centrifuge using a JA-14 rotor (14,000 rpm, 2.5 hours). The virion precipitate was resuspended in STE buffer (10 mM Tris-HCL, 100 mM NaCl, 1 mM EDTA, pH 7.4) in a Downs homogenizer.

Isolation of RNA: For the subsequent formulation of PCR, viral RNA was isolated with the help of a kit for the isolation of RNA from plasma and serum (Isogen Laboratory, Moscow).

RT-PCR: Reverse transcription was set separately from PCR, with the help of M-MuLV revertase (SibEnzyme, Novosibirsk) in accordance with the manufacturer's recommendations. PCR was placed with high-precision polymerase Tersus ("Eurogen", Moscow) in accordance with the manufacturer's recommendations. Purification of the resulting PCR products from the fusible agarose was carried out using a GeneJET Gel Extraction and DNA Cleanup Micro kit (Thermo Fisher Scientific, USA).

Mutagenesis: The mutagenesis of the PA gene was carried out using a two-step PCR. At the site of the introduction of the mutation in the gene sequence, direct and reverse mutation primers were selected with the necessary replacement. Synthesis of oligonucleotides was ordered through the company Eurogen. To control the length of the PCR product, an analytical forephus was performed in a 1.5% agarose gel using a TAE buffer. To purify the PCR product, Thermo Scientific GeneJET PCR Purification Kit (cat. No. 0702) was used.

Cloning was carried out using the so-called GoldenGate reaction (web-site: http://dx.doi.org/10.1371/journal.pone 0003647). The restriction enzyme Esp3 (BsmBI) (Fermentas/ThermoScientific Cat No. ERO451), 10x Fermentas Tango buffer, T4 DNA ligase (Sibenzyme, cat. No. E319) to 5 U/L, Dithiothreitol (DTT) to 1 mM, ATP to 1mM and linear a vector with an inset of 50 ng (vector/insertion molar ratio = 1/3). The reaction was carried out in a thermocycler with a program of 15 cycles of 5 minutes at 37^0C and 5 minutes at 17^0C.

Transformation was performed on rubidium competent bacterial cells of DH5a strain. After defrosting in ice, 1/2 part of the ligase mixture was added to the cells. Next, the cell suspension was incubated for 1 hour in ice, heat shock was performed for 2 minutes at 42°C in a water bath), incubated in ice for 2 minutes, LB medium supplemented without antibiotic and incubated in for 30 minutes at 37^0C. Cells were plated on petri dishes with 1.5% agar and LB medium with ampicillin (200 μg/ml). Petri dishes were incubated for 16 hours at 37^0C. Screening of the clones was carried out by PCR followed by electrophoretic analysis.

Sequencing: Sequencing of inserts in the resulting plasmids was carried out by Eurogen on the automatic sequencer MegaBACE-500**.**

Transfection: Transfection was performed with Lipofectamine LTX reagent (Invitrogen) either in the coculture of 293T and MDCK cells or in a one-day monolayer of 293T cells (cell monolayer density of about 70%) according to the protocol protocol attached to the Lipofectamine LTX reagent. A monolayer of cells with the addition of lipofectamine and without the addition of plasmids was used as the negative control. As a positive control, 8 plasmids containing all the genes of the original strain A/WSN/33 were added to the cell culture. In a number of experiments, 48 hours after transfection, the material was introduced into chicken embryos. Cells were removed with trypsin, and then 0.5 ml of undiluted suspension was injected into the chick embryos. After 48 hours, the hemagglutination titer of the virus in the chick embryos was determined.

All reassortants were investigated in three independent experiments.

The statistical processing of the results was carried out using the mean square deviation.

Results

Phenotypic characteristics of CA-reassortant A/Krasnodar/101/35xA/WSN/33 (H1N1) and variants of strain A/WSN/33 having site-specific mutations in the genes encoding the proteins of the polymerase complex

At the first stage of the work, a comparative study of ts-phenotype of CA-reassortant R A/Krasnodar/101/35xA/WSN/33 (H1N1) and variants of A/WSN/33 strain with site-specific mutations in genes coding proteins of the polymerase complex. As can be seen from table 2, the parent variants of the CA A/Krasnodar/101/35/59 (H2N2) strain-donor and the virulent A/ WSN/33 (H1N1) strain were significantly different in ts-phenotype. A/WSN/33 strain actively multiplied in chick embryos at both 34^0C and 38^0C. At 39^0C, strain A/WSN/33 reduced the reproductive activity very slightly. CA A/Krasnodar/101/35/59 strain actively replicated in chick embryos at 34^0C, but dramatically reduced the rate of reproduction at 38^0C and 39^0C. The CA reassortant obtained by crossing these parental variants inherited high reproductive activity from the CA strain - donor and, in contrast to A/WSN/33 strain, the inability to actively multiply at 38^0C and 39^0C. The site-specific mutants of A/ WSN/33 strain-transfectants No. 2, No. 5 and No. 8 compared to the CA reassortant and CA donor strain moderately replicated in chick embryos under permissive conditions. Under non-permissive conditions transfectants No. 5 and No. 8 sharply reduced the intensity of reproduction in chick embryos, Transfectant No. 2 under non-permissive conditions was characterized by a low level of infectious activity (3.0 ± 0.7 lg EID_{50}/0.2 ml). As can be seen from table 3, A/WSN/33 strain had relatively high reproductive activity in the lungs of mice after intranasal infection, reaching a titre of $10^{5.0}$ EID_{50}/0.2.ml. CA A/Krasnodar/101/35/59 strain-donor has lost practically the ability to reproduce in the lungs of mice. The CA reassortant obtained by crossing A/WSN/33 strain and CA A/Krasnodar/101/35/59 strain did not differ from the CA strain-donor by the att-phenotype. Transfectants No. 5 and No. 8 sharply decreased their ability to reproduce in the lungs of the mice. However, transfectant No. 2 retained residual reproductive activity in the lungs of the mouse after intranasal infection. The data obtained show a comparative similarity of the phenotypic characteristics of transfectants No. 5 and No. 8 and CA reassortant R A/Krasnodar/101/35/59 A/WSN/33.

№	The initial strains, CA-reassortant, Site-specific mutants	Titer of virus-containing liquid in chick embryos (lg EID50/0.2 mL)		
		34^{0}C	38^{0}C	39^{0}C
1	A/WSN/33**	6,5 ± 0,32	6,5 ± 0,25	6,25 ± 0,4
2	A/Krasnodar/101/35/59 (H2N2)	8,0 ± 0,4	4,0 ± 0,5	< 1,5 ± 0,4
3	CA reassortant A/Krasnodar/101/35/59 X A/WSN/33	8,0 ± 0,5	4,0 ± 1.0	< 1,5 ± 1,25
4	Tr. № 5 PB1*A/Kr$_{35}$ (Il 147 Thr) PA A/WSN/33 (F 658 A)***	6.0 ± 1.0	3.0 ± 0,8	< 1.0
5	Tr. № 2. PB1 A/Len$_{17}$ (L265N, V591I), PB2 A/AA (N265S), PA A/WSN/33 (F658 A)	6,5 ± 0,4	4,8 ± 0,6	3,0 ± 0,7
6	Tr. № 8. PB1 A/AA (K391 E, E581G, E 457D), PB2 A/Kr$_{35}$ (V290L), PA A/WSN/33 (F658A)	5.5 ± 0.35	3.5 ± 1.0	< 1.0

Table 2: *A comparative study of the ts-phenotype of the CA reassortant obtained by crossing CA strain A/Krasnodar/101/35/59 (H2N2) and strain A/WSN/33 and site-specific mutants of strain A/WSN/33, having mutations in the proteins of the polymerase complex.*

** Polymerase gene, **Name of strain: A/WSN/33, A/AA-A/Ann Arbor/6/60, A/Len17-A/Leningrad/134/17/57, A/Kr35-A/ Krasnodar/101/35/59, ***Localization of the mutation*

№	The initial strains and the resulting transfectants	Virus Titre in Mice Lungs (lg EID50/0.2 mL)
1	A/WSN/33**	5,0 ± 0,4
2	A/Krasnodar/101/35/59 (H2N2)	< 1,0
3	CA reassortant A/Krasnodar/101/35/59 X A/WSN/33	< 1,5 ± 0,5
4	Tr. № 5 PB1* A/Kr$_{35}$ (Il 147 Thr) PA A/WSN/33 (F 658 A)***	< 1,0
5	Tr. № 2. PB1 A/ Len$_{17}$ (L265N, V591I), PB2 A/AA (N265S), PA A/WSN/33 (F658 A)	2,5 ± 1.0
6	Tr. № 8. PB1 A/AA (K391 E, E581G, E 457D), PB2 A/Kr 35 (V290L), PA A/WSN/33 (F658A)	< 1.0

Table 3: *Study of the attenopathy of the CA reassortant obtained by crossing the strain A/WSN/33 (H1N1) and CA strain A/Krasnodar/101/35/59 (H2N2) and variants of the A/WSN/33 influenza strain having site-specific mutations in the genes encoding the proteins of the polymerase complex.*

**Polymerase gene, **Name of strain: A/WSN/33, A/AA-A/Ann Arbor/6/60, A/Len17-A/Leningrad/134/17/57, A/Kr35-A/ Krasnodar/101/35/59 ***Localization of the mutation*

A comparative study of the immunogenicity and protective efficacy of site-specific mutants of strain A/WSN/33 and CA R A/ Krasnodar/101/35/59 A/WSN/33 (H1N1) reassortant for homologous challenge

Groups of mice were intranasally twice immunized with $10^{4.0}$ EID$_{50}$ dose with transfectants No. 2, No. 5 and No. 8, and also with CA R A/Krasnodar/101/35/59 A/WSN/33 reassortant. Mice of the control group received saline twice intranasally. Ten days after the second immunization, the mice were taken blood and the titer of antibodies inhibiting haemagglutination was determined. As can be seen from table 4, the analyzed viruses induced a different level of humoral antibodies in immunized mice.

Serum investigated viruses	Titer of serum antibodies in RSLA when interacting with strain A/WSN/33 (H1N1) virus and strain virus A/Duck Pennsylvania/10218/1984 (H5N2) avian influenza virus (log2)			
	Antigens: strain A/WSN/33 (H1N1)**** strain A/Duck Pennsylvania/10218/1984 (H5N2)*****			
CA reassortant A/ Krasnodar/101/35/59 x A/WSN/33	6,1 ± 0,5****< 1.0*****			
Tr. №5 PB1* A/Kr_{35} (Il 147 Thr) PA A/WSN/33** (F658A)		7,2 ± 0,8 < 1,0		
Tr. №2 PB1 A/Len_{17} (K265 N, V591Il), PB2 A/AA (N265S)***, PA A/WSN/33 (F658 A)			8,5 ± 1.0 < 1.0	
Tr. №8 PB1 A/AA (K391E, E581G, E457D) PB2 A/Kr_{35} (V290 L) PA A/ WSN/33 (F658A)				8,5 ± 0,8 < 1.0
Non-immune	< 1.0 < 1.0	< 1.0 < 1.0	< 1.0 < 1.0	< 1.0 < 1.0

Table 4: *Comparative study of the immunogenicity of variants of the A/WSN/33 influenza strain with site-specific ts mutations in genes encoding the proteins of the polymerase complex and the CA reassortant obtained by crossing strain A/WSN/33 and CA strain A/Krasnodar/101/35/59 (H2N2).*

Polymerase gene, **Name of strain: A/WSN/33, A/AA-A/Ann Arbor/6/60, A/Len17-A/Leningrad/134/17/57, A/Kr35-A/ Krasnodar/101/35/59, * Localization of the mutation, ****The response of haemagglutination inhibition with strain A/WSN/33 (H1N1) and strain A/Duck Pennsylvania/10218/1984 (H5N2) was performed according to MU 3.3.2. 1758-03. Serum dilution was taken as the serum titer, causing a complete delay in haemagglutination. *****The titer of antibodies to the H5N2 virus of avian influenza*

The highest antibody titres were observed in the blood of mice immunized with transfectants No. 2 and No. 8. (log_2 8.5 ± 1.0, log_2 8.5 ± 0.8). Transfection No. 5 induced a moderate level of humoral antibodies (log_2 7.2 ± 0.8). Mice immunized with the CA reassortant were characterized by a low level of humoral antibodies (log_2 6.1 ± 0.5). At the next step mice were infected intranasally, 10 mLD_{50} ($10^{3.0}$ EID_{50}) of A/WSN/33 strain 10 days after the second immunization. After 72 hours, the mice were extracted with lungs and the virus was multiplied in the lungs of mice immunized with the viruses being analyzed. As can be seen from table 5, in nonimmunized mice from the control group, the virulent virus replication titer reached 4.25 lg EID_{50}/0.2 ml. In mice immunized with the CA reassortant, almost complete suppression of the virus multiplication in the lungs was observed, despite the low blood level of antibodies inhibiting haemagglutination. A similar result was observed in all groups of mice immunized with transfectants No. 2, No. 5 and No. 8.

№	Variants of strain A/WSN/33 of the influenza virus, used for immunization	Titre of the virus in the lungs mice (lg EID_{50}/0.2 mL)
1	CA reassortant A/WSN/33 x A/ Krasnodar/101/35/59**	< 1.0
2	Tr. №5 PB1* A/Kr_{35} (Il 147 Thr) PA A/WSN/33 (F658A)***	< 1.0
3	Tr. №2 PB1 A/Len_{17} (K 265 N, V 591 Il) PB2 A/AA (N 265 S) PA A/WSN/33 (F658 A)	< 1.0
4	Tr.№8 PB1 A/AA (K391E, E581G, E457D) PB2 A/Kr_{35} (V290L) PA A/WSN/33 (F658A)	< 1.0
5	Control: non-immune mouse	4.25 ± 0,5

Table 5: *A comparative study of the protective efficacy of variants of strain A/WSN/33 of the influenza virus containing site-specific mutations in the genes encoding the proteins of the polymerase complex and the CA reassortant, under control homologous infection.*

**Polymerase gene, **Name of strain: A/WSN/33, A/AA-A/Ann Arbor/6/60, A/Len_{17}-A/Leningrad/134/17/57, A/Kr_{35}-A/ Krasnodar/101/35/59, ***Localization of the mutation*

Comparative study of the protective efficacy of site-specific mutants of strain A/WSN/33 and and CA R A/Krasnodar/101/35/59 A/WSN/33 (H1N1) reassortant in case of heterologous challenge

It was of interest to investigate the ability of our site-specific mutants of A/WSN/33 strain and CA R A/Krasnodar/101/35/59/A/WSN/33 reassortant to cross-protect against the influenza virus of another subtype. To this end, groups of mice were intranasally twice immunized with the above viruses at a dose of 104.5 EID50. 10 days after the second immunization, the A/Mallard duck/Pennsylvania/10218/1984 (MA) (H5N2) strain was adapted to the mice. The dose of infection was 10 MLD50. A part of the mice from each group were drawn with blood and the possibility of antibodies to the avian influenza virus serotype H5N2 was investigated. As can be seen from table 4 in immunized mice and mice from the control group, there was no noticeable presence of humoral antibodies to the H5N2 avian influenza virus. In mice entering the control group, intranasal infection with the H5N2 avian influenza virus caused high mortality (78 ± 6.5%) by the 8 - 9 day after infection. The level of such lethality in the group of mice immunized with the CA reassortant after the control heterologous infection decreased to 35 ± 4.2%. ($P < 0.05$). In groups of mice immunized with site-specific mutants of strain A/WSN/33, 100% survival was observed in mice immunized with transfectants No. 2 and No. 5. In the group of mice immunized with transfectant No. 8, after control heterologous infection, a lethality of 16 ± 3.2% was observed.

№	Variants of A/WSN/33 strain of the Influenza virus, used for immunization	Antibody titer to A/Duck/ Pensilvania/10218/84 (H5N2) bird flu virus in the HI-test (log_2)	Percentage of death of mice after control heterologous infection (dose $10^{2.0}$ MLD_{50})
1	CA reassortant A/WSN/33 x A/ Krasnodar/101/35/59 **	< 1.0	35± 4,2
2	Tr. № 5. PB1* A/Kr_{35} (Il 147 Thr) PA A/WSN/33 (F658A)***	< 1.0	0
3	Tr. № 2 PB1 A/Len_{17} (K265N, V 591 Il) PB2 A/AA (N 265 S) PA A/WSN / 33 (F 658 A)	< 1.0	0
4	Tr. № 8. A/AA (K391E, E581G, E457 D) PB2 A/Kr_{35} (V 290 L) PA A/WSN/33 (F 658 A)	< 1.0	16± 3.0
5	Control: non- immune mice	< 1.0	78± 6,5

Table 6: *A comparative study of the protective efficacy of variants of strain A/WSN/33 of the influenza virus containing site-specific mutations in the genes encoding the proteins of the polymerase complex and the CA reassortant in a control heterologous infecting.*

**Polymerase gene, **Name of strain: A/WSN/33, A/AA-A/Ann Arbor/6/60, A/Len17-A/Leningrad/134/17/57, A/Kr35-A/Krasnodar/101/35/59, ***Localization of the mutation*

Study of weight characteristics of immunized mice in the process of homologous and heterologous control infection

As seen from figure 1, in mice of the control group infected with the intranasal virulent strain A/WSN/33 at a dose of 10 MLD ($10^{3.0}$ EID_{50}), a weight loss was observed, which reached 10 - 17% by the 6 - 7 day after infection. Preliminary immunization of mice with two types of live influenza vaccines prevented this phenomenon to varying degrees. Immunization of mice with transfectants No. 2, No. 5 and No. 8 prevented a significant loss of animal weight. The weight loss in mice immunized with the CA reassortant was more pronounced. By 5 - 6 days after infection, it reached 8 - 9%, but then there was a tendency to restore the weight of animals (Figure 1). With heterologous control infection, the situation was more dramatic (Figure 2). The weight loss in mice from the control group infected with the strain H5N2 of the avian influenza virus at a dose of $10^{2.0}$ MLD ($10^{4.0}$ EID_{50}) reached the level of 25 - 30% 6 - 7 days after infection. The following

days, the death of animals was observed. In mice immunized with transfectants No. 5 and No. 8, a low weight loss (7 - 8%) was observed, whereas in mice immunized with transfectant No. 2, the weight loss was quite significant (20 - 22%). Also significant was weight loss in mice immunized with CA reassortant (15 - 20%).

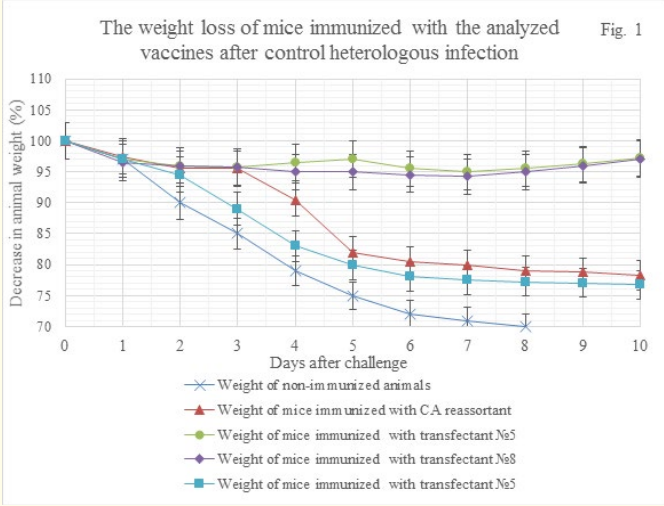

Figure 1: *The weight loss of mice immunized with the analyzed vaccines after control heterologous infection.*

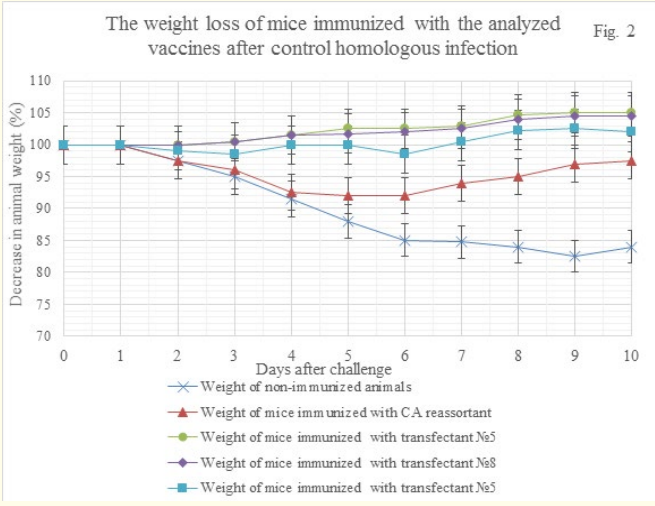

Figure 2: *The weight loss of mice immunized with the analyzed vaccines after control homologous infection.*

Discussion

Our findings indicate that the inclusion of attenuating mutations from CA A/Krasnodar/101/35/59 (H2N2) and A/Leningrad/134/17/57 (H2N2) strains-donors attenuation into genes encoding the proteins of the polymerase complex of the virulent A/WSN/33 (H1N1) strain results in the creation of effective live vaccines along with attenuating mutations taken from the genome of CA A/Ann Arbor/6/60 (H1N1) strain. The phenotypic characteristics of transfectants No. 5 and No. 8 did not differ from those of the known

CA attenuation donor strains and the CA reassortant we obtained between CA A/Krasnodar/101/35/59 (H2N2) strain and virulent A/WSN/33 (H1N1) strain. When homologous challenge was observed, it was found that the site-specific mutants of A/WSN/33 strain studied by us induced a higher level of humoral antibodies in immunized mice compared to the CA R A Krasnodar/101/35/59 A/WSN/33 reassortant. Nevertheless, both types of live vaccine preparations had high protective efficacy. Significant protective efficacy was also observed in site-specific mutants during heterologous challenge. In contrast to Zhou., *et al.* [8] who studied the protective efficacy of the site-specific mutant rNY1682-TS2 of the swine influenza virus in heterologously challenging the immunized mice with a virulent reassortant rNY1682: PR-HA/NA, (yet belonging to the same H1N1 serotype) in our case a strain belonging to another serotype A/Duck Pennsylvania/10218/84 of avian influenza virus (H5N2) was chosen as heterologous virus. Despite the high virulence of this strain for mice (lethality in the control group 78.0 ± 6.5%), in the groups of mice immunized with transfectants No. 2 and No. 5 we observed a 100% survival rate, and only in the group of mice immunized with transfection No. 2 was observed lethality 16 ± 3.0 %. Intranasal administration of the H5N2 strain of the avian influenza virus to mice immunized twice with a CA reassortant reduced the lethality to 35.0 ± 4.0%. The absence of antibodies to the H5N2 virus in immunized animals indicates the leading role of cellular immunity in defense mechanisms. The data obtained suggest that live influenza vaccines obtained by site-specific mutagenesis in homologous control infection are not inferior in effectiveness to classic CA reassortant influenza vaccines. In case of heterologous challenge, some site-specific mutants have a higher protective effect than the CA influenza reassortant vaccine, however, further studies are required for definitive withdrawal.

In mice immunized with two types of live influenza vaccines, we observed a drop in the weight of infected animals during a control infection. The intensity of this phenomenon was especially pronounced in case of heterologous control infection. However, weight loss after challenge with the H5N2 strain of the avian influenza virus in mice immunized with site-specific mutants was much less than in mice than in mice immunized with a CA reassortant. It is suggested [8] that this feature may indicate that antibodies to HA and NA proteins induced during immunization with a reassortant vaccine have little effect on the replication of an antigenically unrelated virus, especially at the initial stage of viral reproduction. However, it is possible that immunization with two types of live influenza vaccines leads to the activation of various parts of cellular immunity, which to some extent influence the metabolic processes in the organism of infected animals. It should be noted that immunization with site-specific mutants of the influenza virus leads to less pronounced disturbances in cellular metabolism in animals during the control infection with a virulent virus.

An important condition for successful practical use of site-specific mutants as live influenza vaccines is the presence of genetic stability of these drugs. An analysis of this biological property of the site-specific mutants we obtained will be part of the further task of our studies [16].

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