INTRODUCTION

Live attenuated influenza vaccine (LAIV) consists of reassortant viruses with hemagglutinin (HA) and neuraminidase (NA) gene segments inherited from the circulating wild-type (WT) parental viruses and six internal protein–encoding gene segments from cold-adapted attenuated master donor viruses (genome composition 6 : 2). In this study, we describe the difficulties in development of LAIV strains depending on the phenotypic peculiarities of the WT viruses used for reassortment. Genomic-composition analysis of 849 reassortants revealed that over 80% of reassortants based on the inhibitor-resistant WT viruses inherited WT NA as compared to 26% of reassortants based on the inhibitor-sensitive WT viruses. In addition, the highest percentage of vaccine genotype reassortants was achieved when WT parental viruses were resistant to nonspecific serum inhibitors. We show that NA may play a role in the influenza virus’ sensitivity to a nonspecific serum inhibitors. Replacing the NA of the inhibitor-sensitive WT virus with the NA of the inhibitor-resistant master donor virus significantly decreased the sensitivity of the resulting reassortant virus to nonspecific inhibitors.

MATERIALS AND METHODS

Viruses. In this work, we used 42 influenza viruses types A and B. They are the following: (1) WT influenza viruses obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, GA, United States) and (2) reassortant vaccine candidates for parenterally administered, inactivated influenza vaccine of subtypes-based inactivated vaccine type H5N1 (NIBRG-23, INDO/05, VN1203) on the basis of the high-yield donor A/PR/8/34 (H1N1) (PR8) obtained from the CDC and World Health Organization (WHO). PR8-based reassortants were prepared by the reverse genetics method with the use of H5N1 strains A/turkey/Turkey/1/05, A/Indonesia/5/05, A/Vietnam/1203/2004 as sources of HA and NA. In order to decrease pathogenicity, the cleavage site HA0 of hemagglutinin predecessor was modified by means of deleting of triplets coding four main amino acids; two cold–adapted MDVs, A/Leningrad/134/17/57 (H2N2) (L17) and B/USSR/60/69 (B60), that are used to develop the Russian LAIV are the property of the Institute of Experimental Medicine of the Russian Academy of Medical Sciences (St. Petersburg, Russia).

Viruses were propagated in 10- to 11-day old embryonated chicken eggs at a temperature of 32–33°C.

Reassortants were obtained using classical reassortment techniques [1] in the presence of polyclonal rabbit or rat antisera to donors of attenuation.

RNA isolation. RNA was isolated from virus-containing allantoic fluid with the use of a QIAamp Viral
RNA minikit in accordance with the manufacturer’s instructions (Qiagen GmbH, Germany).

**Genomic composition analysis.** The origin of segments of RNA reassortants was determined with the use of restriction fragment length polymorphism analysis (RFLP) or the mix-PCR method [3]. Sequence analysis was carried out with a BigDye Terminator v3.1 set and 3130×1 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, United States) in accordance with the manufacturer’s instructions. To analyze sequence data, the Lasergene 7.1 program package was used.

**Hemagglutination inhibition (HAI) assay** was conducted in accordance with the standard protocol [4] in a 96-well plates at room temperature with 1% suspension of erythrocytes of chickens or humans of group 0(I) Rh+. For analysis of the sensitivity of influenza viruses to nonspecific inhibitors, we used nonimmune serum of guinea pigs (Rappolovo farm, Leningrad region) heated at 80°C for 10 min for removal of thermo-sensitive inhibitors. The virus was considered resistant to nonspecific serum inhibitors if the serum titer in HAI did not exceed 40, and sensitive if it was 80 or more.

**Statistical analysis.** Pearson’s χ²-squared test (χ²) was to identify associations between inhibitor sensitivity of parental viruses used for reassortment and reassortment efficiency and the Student’s t-test for dependent samples was used to evaluate the differences in geometrical mean HAI titers between groups of viruses compared for their ability to react with normal guinea pig sera. A value of p < 0.001 was considered significant, and p < 0.0001 was considered highly significant.

**RESULTS AND DISCUSSION**

The classical method of obtaining reassortant vaccine strains of LAIV includes crossing in embryonated chicken eggs (ECE) at 32°C of parental viruses (with the donor of attenuation and WT virus taken in equal infecting doses) and selective passages with polyclonal antiserum to the donor of attenuation at a lowering of temperature to 25–26°C with further cloning by limited dilutions [1]. Theoretically, when crossing two strains of influenza virus, it is possible to form 256 different gene combinations. The use of two powerful selecting factors (antiserum to the donor of attenuation and lowered incubation temperature) causes a significant decrease in the number of undesirable combinations, but in some cases their number is still rather large.

Annually, the WHO provides recommendations regarding the contents of influenza vaccines in the current epidemic season [5]. According to Russian regulations, 5 : 3 genomic composition is also acceptable for LAIV [6] if the third WT gene of the vaccine candidate does not encode RNA polymerase protein complex components. Preparation of LAIV using the method of classical reassortment is a rather long and labor-consuming process that takes at least 2.5–3 months. Successive and fast obtaining of vaccine reassortants depends on a complex of factors, of which the phenotypic properties of the WT parental virus are decisive.

Until the end of the 1990s and beginning of the 2000s, on the basis of circulating influenza viruses A and B, it was possible to obtain vaccine strains with the desirable genome formula 6 : 2. At the same time, the HA and NA genes in most cases transferred into vaccine reassortant genome from a WT parent, while the genes coding enzymes of the nucleoproteide complex (PB2, PB1, PA, and NP) did so from the CA donor of attenuation. The amount of “cold” M and NS genes was rather large (Table 1). However, in recent years, more and more cases of separation of genes coding HA and NA have been found in process reassortment. Thus, only 13.5% of the reassortants obtained from influenza virus A circulating after 2000 contain NA from the WT parental virus. For influenza virus B, this number was 11.2%. In addition, the number of reassortants inheriting internal genes from donors of attenuation is decreasing (Table 1). In recent years, many strains currently identified by the WHO as those to include in the vaccine for the new influenza season have been highly sensitive (IS) to nonspecific inhibitors of normal blood serum. These findings also concern a wide range of modern influenza viruses A (H3N2) and the majority of influenza viruses B of the Yamagata lineage [7, 8]. We analyzed 40 WT parental viruses with respect to their sensitivity to thermostable inhibitors and obtained 849 reassortants with donors of attenuation from them.

Neuraminidase of inhibitor-resistant (IR) influenza viruses A (H3N2) and B was introduced into genome of reassortant viruses much more often than NA of IR viruses (95.9 and 87.5%, respectively). With respect to influenza viruses A (H1N1), circulation of their inhibitor-sensitive variants has not been described yet in the literature. In addition, analysis of the distribution of WT or CA of neuraminidase among 136 inhibitor-resistant reassortants of these viruses with donors of attenuation showed an advantageous inheritance of the gene coding NA from a WT parent (95.6%) (Table 2). The small number of the wild-type influenza viruses of other subtypes (two H2N2 and three H5N1) used for analysis does not allow one to draw unambiguous conclusions. Other, unknown mechanisms are probably involved in this process.

In general, the genomic analysis of reassortants prepared on the basis of 20 inhibitor-sensitive viruses showed that a third of the total number of clones (130 of 493) inherited neuraminidase of wild-type virus. On the contrary, of the 356 IR reassortants obtained on the basis of 20 IR viruses, only 59 obtained NA from the donor of attenuation (Table 2). Statistical processing of the obtained data with the usage of Pearson’s χ²-squared test showed reliability of differences at
the level of inheritance of WT-NA of IR or IS viruses by reassortants (level of significance \(p < 0.0001\)) (Table 3).

Naturally occurring inhibitors of influenza virus HA activity have been identified both in various sera (including human sera) and in fluid secretions [9–12]. Respiratory tract mucosa is not only a site of entry for infection, but also the place where primary protection of an organism from respiratory diseases starts. At this location, vaccine strains of viruses have a particular advantage in attaching to and infiltrating into sensitive cells of the host organism in comparison with vaccine strains prepared on the basis of IS of WT viruses, and they may be more appropriate for vaccination of humans. In the literature, there is no common idea regarding which WT strain is better to use for preparation of LAIV–IR or HA—but it is accepted that inhibitor-resistant strains may be preferable during preparation of a vaccine for humans [13].

The standard protocol of preparation of reassortant strains for LAIV includes treating antiserum to the donor of attenuation with the receptor-destroying enzyme (RDE) to remove nonspecific hemagglutinating inhibitors. However, even properly treated serum may still contain a residues of thermostable inhibitors [9, 14, 15], which may falsify the antibody pattern and complicates obtaining vaccine reassortants on the basis of IS epidemic viruses.

It is known that the sensitivity or resistance of influenza virus to nonspecific inhibitors of blood serum is determined by the properties of its HA [16]. This feature is inherited by reassortant viruses along with the gene coding this surface protein. Some authors pay special attention to the contribution of not only hemagglutinin, but also neuraminidase, to inhibitor-sensitivity formation. In particular, it has been established that serum inhibitors hinder the infiltration of IS virus into a cell as a result of competition for the site of binding of hemagglutinin with the receptor.

### Table 1. Distribution of inheritance of genome segments by reassortants obtained on the basis of influenza viruses A (H1N1), A (H3N2), and B circulating in different periods

| Genome segments | until 2000 year | after 2000 year |
|------------------|----------------|----------------|
|                  | from WT-parent | from donor L17 | from WT-parent | from donor L17 |
| PB2              | 2.1 (2)        | 97.9 (95)      | 9.6 (5)        | 90.4 (47)      |
| PB1              | 5.2 (5)        | 94.8 (92)      | 40.4 (21)      | 59.6 (31)      |
| PA               | 2.1 (2)        | 97.9 (95)      | 9.6 (5)        | 90.4 (47)      |
| HA               | 100.0 (97)     | 0.0 (0)        | 100.0 (52)     | 0.0 (0)        |
| NP               | 29.9 (29)      | 70.1 (68)      | 44.2 (23)      | 55.8 (29)      |
| NA               | 91.8 (89)      | 8.2 (8)        | 13.5 (7)       | 86.5 (45)      |
| M                | 42.3 (41)      | 57.7 (56)      | 65.4 (34)      | 34.6 (18)      |
| NS               | 47.4 (46)      | 52.6 (51)      | 80.8 (42)      | 19.2 (10)      |

1 A number of reassortants inheriting the specified gene from a particular parent virus; 2 donor of attenuation A/Lenin-grad/134/17/57 (H2N2); 3 donor of attenuation of B/USSR/60/69.
SA-alpha-2,6-Gal. Inhibition of neuraminidase of resistant influenza viruses caused those viruses to become inhibitor-sensitive [17]. We have likely discovered that, in many cases, the advantageous inheritance of reassortant strain HA from the wild-type virus and NA from the CA parent can be connected with peculiarities of the HA and NA of the wild-type virus.

Reassortants carrying the HA of an inhibitor-resistant donor strain derived from the wild-type virus and NA from a CA parent strain can be connected with peculiarities of the HA and NA of the wild-type virus. In order to identify the role of NA in influenza virus infection, we shall try to answer this question.

Analysis of the efficiency of obtaining strains with vaccine genome formula showed that a range of used viruses easily became involved in reassortment with donors of attenuation with the formation of a significant number of clones with a genome formula of 6:2 (for example, IR H1N1 influenza viruses). When crossing with donors of other viruses we faced certain difficulties. In particular, of the 29 reassortants based on the IS of the H2N2 influenza virus, only four possessed a genome formula of 5:3 and none had a vaccine formula of 6:2.

When obtaining an LAIV vaccine strain against two ISs of the highly pathogenic influenza viruses by crossing H5N1-PR8-reassortants (VN1203 and INDO/05) with the CA donor of attenuation L17, we found that 73.6% of isolated clones inherited the only hemagglutinin from parents VN1203 and INDO/05 (formula 7:1). In [18], it was described the impossibility of obtaining 6:2 reassortants when crossing the CA donor of attenuation with H5N1–PR8 strains for inactivated influenza vaccine (IIV) [18]. When using classical reassortment technique NIBRG–23 (H5N1) virus did not reassort with L17 at all. It is likely that gene constellation in the genome of the virus NIBRG–23 was so strong that it was impossible to break it using the class-

| Wild-type parent | Inhibitor sensitivity | Totally | Origin of NA | Genome formula2 |
|------------------|-----------------------|---------|--------------|-----------------|
|                  |                      |         | WT, %        | L17, %          | 6:2, % | 5:3, % | 7:1, % | Other, % |
| Influenza viruses A (H3N2) | Resistant | 5 | 74 | 71 (95.9%) | 3 (4.1%) | 7 (9.5%) | 25 (33.8%) | 1 (1.4%) | 41 (55.3%) |
|                  | Sensitive            | 6       | 126 | 26 (20.6%) | 100 (79.4%) | 12 (9.5%) | 13 (10.3%) | 10 (7.9%) | 91 (72.2%) |
| Influenza viruses A (H1N1) | Resistant | 7       | 136 | 130 (95.6%) | 6 (4.4%) | 71 (52.2%) | 37 (27.2%) | 0 | 28 (20.6%) |
|                  | Sensitive            | 1       | 5 | 5 (100%) | 0 | 4 (80.0%) | 1 (20.0%) | 0 | 0 |
| Influenza viruses A (H2N2) | Resistant | 2       | 53 | 14 (26.4%) | 39 (73.6%) | 0 | 0 | 39 (73.6%) | 14 (26.4%) |
|                  | Sensitive            | 1       | 12 | 12 (100%) | 0 | 0 | 0 | 12 (100%) |
| All influenza viruses A | Resistant | 14      | 268 | 220 (82.1%) | 48 (17.9%) | 82 (30.6%) | 63 (23.5%) | 40 (14.9%) | 83 (31.0%) |
|                  | Sensitive            | 9       | 167 | 67 (40.1%) | 100 (59.9%) | 12 (7.2%) | 17 (10.2%) | 10 (6%) | 128 (76.6%) |
| All influenza viruses B | Resistant | 6       | 88 | 77 (87.5%) | 11 (12.5%) | 21 (23.9%) | 30 (34.1%) | 1 (1.1%) | 36 (40.9%) |
|                  | Sensitive            | 11      | 326 | 63 (19.3%) | 263 (80.7%) | 12 (3.7%) | 12 (3.7%) | 91 (27.9%) | 211 (64.7%) |
| All influenza viruses (H1N1, H2N2, H3N2, H5N1, B) | Resistant | 20      | 356 | 297 (83.4%) | 59 (16.6%) | 103 (28.8%) | 93 (26.1%) | 41 (11.5%) | 119 (33.4%) |
|                  | Sensitive            | 20      | 493 | 130 (26.4%) | 363 (73.6%) | 24 (4.9%) | 29 (5.9%) | 101 (20.5%) | 339 (68.7%) |

1 All reassortants inherited HA of wild–type virus; 2 Genomic composition: 6:2 are reassortants containing HA and NA from WT virus; 5:3 are reassortants containing HA and NA, from the WT parent, as well as WT, NP, M, or NS gene; 7:1 are single gene reassortants containing only HA from WT virus. 4 No inhibitor–sensitive WT viruses were found among the type A (H1N1) influenza viruses tested; and 5 there were no inhibitor-sensitive variants among H1N1 viruses.
We have analyzed the frequency of obtaining reassortants with vaccine formulas of the genome and other combinations with the help of Pearson’s χ²-squared test. Reassortants with vaccine genomic composition were generated significantly more frequently from inhibitor–resistant WT influenza viruses (χ² 195.78; p < 0.0001) (Tables 2 and 3).

In the literature, scientists described some pairs of viruses which are difficult to reassort. In a wide range of cases, researchers have not been able to obtain 6 : 2 reassortments [19]. Thus, despite numerous attempts, 6 : 2 reassortments were not obtained on the basis of...
the epidemic viruses A/Kawasaki/1/86 (H1N1) or A/Texas/36/91 (H1N1). In the genome of the obtained reassortants, there were the genes PB2, NP, and NS that originated from epidemic parental viruses. It is proposed that there is a functional incompatibility of virus genes used for crossing.

There exist particular methods of enhancing reassortment efficiency—particularly, inactivation of a

| Code | Strain designation (subtype) | Gene origin (subtype) | HAI titer | Sensitivity to inhibitors |
|------|-----------------------------|-----------------------|-----------|--------------------------|
|      |                             |                       |           |                          |
| L17  | A/Leningrad/135/17/57        | H2N2                  | L17       | <10                      | Resistant^4 |
| WT2  | A/California/07/04           | H3N2                  | WT        | 2560                     | Sensitive^5 |
| R1^3 | A/California/07/04 × L17     | H3N2                  | WT        | 320                      | Sensitive (=) |
| R2   | A/California/07/04 × L17     | H3N2                  | WT        | 2560                     | Sensitive (=) |
| WT   | NIBRG-23                    | H5N1                  | WT        | 5120                     | Sensitive |
| R3   | NIBRG-23 × L17              | H5N2                  | WT        | 640                      | Sensitive (<) |
| R4   | NIBRG-23 × L17              | H5N2                  | WT        | 640                      | Sensitive (<) |
| WT   | INDO/05                     | H5N1                  | WT        | 10                       | Resistant |
| R6   | INDO/05 × L17               | H5N2                  | WT        | 10                       | Resistant (=) |
| R7   | INDO/05 × L17               | H5N2                  | WT        | 10                       | Resistant (=) |
| WT   | VN1203                      | H5N1                  | WT        | <10                      | Resistant |
| R8   | VN1203 × L17                | H5N2                  | WT        | <10                      | Resistant (=) |
| R9   | VN1203 × L17                | H5N2                  | WT        | <10                      | Resistant (=) |
| R10^6| A/Vietnam/1203/2004 × L17    | H5N2                  | WT        | <10                      | Resistant (=) |

Influenza viruses B

| Code | Strain designation (subtype) | Gene origin (subtype) | HAI titer | Sensitivity to inhibitors |
|------|-----------------------------|-----------------------|-----------|--------------------------|
| B60  | B/USSR/60/69                | B                     | B60       | 10                      | Resistant |
| WT   | B/Harbin/07/94              | B                     | WT        | 2560                     | Sensitive (<) |
| R11  | B/Harbin/07/94 × B60        | B                     | WT        | 10240                    | Sensitive |
| WT   | B/Texas/26/08               | B                     | WT        | <10                      | Resistant |
| R12  | B/Texas/26/08 × B60         | B                     | WT        | <10                      | Resistant (=) |
| R13  | B/Texas/26/08 × B60         | B                     | WT        | <10                      | Resistant (=) |
| WT   | B/Wisconsin/1/10            | B                     | WT        | 5120                     | Sensitive |
| R14  | B/Wisconsin/1/10 × B60      | B                     | WT        | 320                      | Sensitive (<) |
| R15  | B/Wisconsin/1/10 × B60      | B                     | WT        | 5120                     | Sensitive (<) |
| R16  | B/Wisconsin/1/10 × B60      | B                     | WT        | 5120                     | Sensitive (=) |
| WT   | B/Bangladesh/1994/10        | B                     | WT        | 5120                     | Sensitive |
| R17  | B/Bangladesh/1994/10 × B60  | B                     | WT        | 640                      | Sensitive (<) |
| WT   | B/Texas/06/11               | B                     | WT        | 640                      | Sensitive |
| R18  | B/Texas/06/11 × B60         | B                     | WT        | 80                       | Sensitive (<) |
| R19  | B/Texas/06/11 × B60         | B                     | WT        | 640                      | Sensitive (=) |

1 Donor of attenuation A/Leningrad/134/17/57 (H2N2) or B/USSR/60/69 (B60); 2 epidemic virus (or reassortant on the basis of PR8 with HA and NA from corresponding wild-type virus); 3 reassortants of wild-type virus with donor of attenuation; reassortants inherited HA from wild-type virus, and NA from wild-type virus (genome formula 6 : 2) or from donor of attenuation (genome formula 7 : 1); 4 virus resistant to thermostable nonspecific inhibitors of serum; 5 virus sensitive to thermostable nonspecific inhibitors of serum; 6 : 2 reassortant R10 was obtained by the method of reverse genetics described in [26]; and 7 sensitivity of reassortant virus to thermostable nonspecific inhibitors of serum in comparison with wild-type parent viruses; p < 0.0001.
wild-type parent by heating [20] or ultraviolet irradiation (UVI) [21, 22]. In our experiments, preliminary UVI inactivation of the NIBRG-23 virus (H5N1) did not lead to the formation of 6:2 or 5:3 reassortments. However, we managed to obtain strains with a genome formula of 7:1 [23].

The failed attempts to obtain H5N1 CA vaccine strains with a genome formula of 6:2 by means of the method of classical reassortment should not be a surprise. It is likely that the genome formula of 7:1 of the LAIV vaccine strain against the highly pathogenic pandemically potential avian influenza virus A (H5N1) has advantages: the presence of an additional gene from the donor of attenuation (NA) in vaccine strain is an additional guarantee of its safety. Moreover, according to a well-known belief [24], antibodies to the HA of influenza viruses are one of the most important elements of protection from influenza viruses, while anti-NA antibodies are not as crucial as anti-HA antibodies. The H5N2 avian influenza viruses are no exception [25].

The discovered selective transfer into the composition of the genome of reassortant influenza viruses strains NA from WT or CA virus, depending on the inhibitor-sensitive phenotype of the WT parent, calls the participation of NA in the development of sensitivity to nonspecific thermostable blood-serum inhibitors into question. However, it is known that it is HA that results in the sensitivity of influenza viruses to nonspecific inhibitors [17]. Some authors have called special attention to neuraminidase in the formation of this feature [17]. We have analyzed nine WT influenza A and B viruses, two CA donors of attenuation, six 6:2 reassortants containing HA and NA from WT viruses and internal genes from donors of attenuation (R2, R10, R13, R15, R16, and R19), and 13 7:1 reassortants inheriting only one gene coding HA from the WT and 7 other genes from donors of attenuation (R1, R3–R9, R11, R12, R14, R17, and R18). Table 4 shows that six of the nine WT viruses were highly IS to inhibitors of guinea pig normal blood serum. Both donors of attenuation and the viruses VN1203, INDO/05, and B/Texas/26/08 proved to be highly IR (their titer in HAI did not exceed 10).

The levels of inhibitor sensitivity of reassortant viruses with the genome formulas 6:2 and 7:1 were significantly different if IS WT virus was used as a source of surface proteins. It was also determined that an at least fourfold titer decrease in HAI with guinea pig normal serum in all 7:1 reassortants inherited HA from the IS parent and NA from the IR donor of attenuation, in comparison with the similar 6:2 reassortants and/or parental viruses (t = 11.09, p < 0.001) (Table 3). In particular, the HAI titer of highly IS virus NIBRG-23 (H5N1) was 5120. The titer of its 7:1 reassortant R3 was 640 while using the same serum.

Table 5. Sequence differences in hemagglutinin and neuraminidase of single gene reassortants influenza viruses

| Parental viruses | Reassortants | Substitutions in HA and NA compared to parental virus |
|------------------|--------------|-----------------------------------------------------|
|                  | origin of gene encoding | HA | NA | HA | NA | HA | NA |
| WT-virus or reassortant on the basis of PR8 virus | donor code | | | | | | |
| A/California/07/04 (H3N2) | L17 | R1 | WT | L17 | No change | No change | No change | No change |
| NIBRG-23 (H5N1) | L17 | R3 | WT | L17 | No change | G-765-T | No change | Arg-249-Ile |
| NIBRG-23 (H5N1) | L17 | R4 | WT | L17 | No change | No change | No change | No change |
| NIBRG-23 (H5N1) | L17 | R5 | WT | L17 | No change | No change | No change | No change |
| INDO/05 (H5N1) | L17 | R6 | WT | L17 | No change | No change | No change | No change |
| INDO/05 (H5N1) | L17 | R7 | WT | L17 | No change | No change | No change | No change |
| VN1203 (H5N1) | L17 | R8 | WT | L17 | No change | No change | No change | No change |
| VN1203 (H5N1) | L17 | R9 | WT | L17 | T-50-C | G-765-T | No | Ile-114-Thr |
| B/Harbin/07/94 | B60 | R11 | WT | B60 | No change | G-1085-A | No change | No change |
| B/Texas/26/08 | B60 | R12 | WT | B60 | No change | No change | No change | No change |
| B/Wisconsin/1/10 | B60 | R14 | WT | B60 | No change | G-1359-A | No change | Glu-436-Lys |
| B/Bangladesh/1994/10 | B60 | R17 | WT | B60 | No change | G-1085-A | No change | No change |
| B/Texas/06/11 | B60 | R18 | WT | B60 | No | G-1359-A | No | Glu-436-Lys |

1 Donor of attenuation A/Leningrad/134/17/57 (H2N2). 2 Donor of attenuation B/USSR/60/69.
To learn if there is direct a connection between the observed lowering of the inhibitor sensitivity of 7:1 reassortant viruses and changed NA functioning, we compared the nucleotides and amino acids sequences of this gene of the WT parent and the corresponding reassortants. We emphasize that we have discovered identical mutations in some reassortants of influenza viruses A (nucleotide 765 in reassortants R3 and R9) and B (nucleotide 1085 in reassortants R11 and R18) (Table 5).

These changes may be new spontaneous mutations in positions responsible for the virus’ adaptation to chicken eggs, or they may be a manifestation of the heterogeneity of the initial population of the virus used on the given positions. In any case, these mutations did not affect the phenotype of R4 virus, and none of the mutations altered the viruses’ sensitivity to thermostable inhibitors. For example, reassortant R3 was as sensitive to inhibitors as were the other two reassortants (R4 and R5) prepared on the basis of NIBRG-23 and donor L17.

Thus, changes in the level of the inhibitor sensitivity of 7:1 reassortants are likely to result from alterations in NA function rather than mutations.

The obtained data allow us to suggest that NA contributes significantly to the reassortment efficiency of WT parental influenza viruses with donors of attenuation. The highest percentage of receiving reassortant strains LAIV with the vaccine genome formulas 6:2 and 5:3 was achieved while crossing donors of attenuation with wild—type viruses that are resistant to non-specific inhibitors. In addition, it was shown that NA contributes to the inhibitor—sensitive phenotype of influenza viruses. It is clear that better realization of peculiarities and mechanisms of the nonspecific inhibitor sensitivity of influenza virus will improve the process of preparation of LAIV. Such research needs to be continued to explicate the mechanism of this phenomenon.

ACKNOWLEDGMENTS

This work was partially supported by the Program for Appropriate Technologies in Health (PATH), Washington, United States.

REFERENCES

1. Wareing, M.D., Marsh, G.A., and Tannock, G.A., Preparation and characterization of attenuated cold-adapted influenza a reassortants derived from the A/Leningrad/134/17/57 donor strain, Vaccine, 2002, vol. 20, no. 16, pp. 2082—2090.
2. Klímov, A. and Cox, N.J., PCR restriction analysis of genome composition and stability of cold-adapted reassortants live influenza vaccines, J. Virol. Methods, 1995, vol. 52, nos. 1—2, pp. 41—49.
3. Kiseleva, I.V., Vöten, J.T.M., Teley, L.C.P., Larionova, N.V., Dubrovina, I.A., Berdygulova, Zh.A., et al., Genome composition analysis of reassortant influenza viruses used in seasonal and pandemic live attenuated influenza vaccine, Mol. Gen. Microbiol. Virol, 2011, vol. 4, pp. 29—36.
4. WHO Manual on Animal Influenza Diagnosis and Surveillance, 2002. http://www.who.int/csr/resources/publications/influenza/whocdscsrms2005rev.pdf. Cited August 1, 2013.
5. WHO Recommendations on the Composition of Influenza Virus Vaccines, 2013. http://www.who.int/influenza/vaccines/virus/recommendations/en/index.html. Cited August 1, 2013.
6. Development of New Influenza Vaccine Strains and Diagnostics. Introduced by Order of the Ministry of Health of the Russian Federation no. 156/29, 1998. http://www.recipe.ru/docs/nd/print.php?id=1745. Cited August 1, 2013.
7. Larionova, N., Kiseleva, I., Isakova, I., Litvinova, O., Klímov, A., and Rudenko, L., Naturally-occurring temperature-sensitive strains of influenza B virus, in Proc. IVW—2004. Conf. Lisbon, Portugal, May 24—26, 2004, pp. 92—97.
8. Larionova, N.V., Kiseleva, I.V., Isakova, I.N., Litvinova, O.M., and Rudenko, L.G., Phenotype of epidemic influenza B virus strains isolated in different years, Vopr. Virusol., 2006, vol. 51, no. 5, pp. 38—41.
9. Krizanova, O. and Rathova, V., Serum inhibitors of mixoviruses, Curr. Top. Microbiol. Immunol., 1969, vol. 47, pp. 125—151.
10. Husseini, R.H., Sweet, C., Collie, M.H., and Smith, H., The relation of interferon and nonspecific inhibitors to virus levels in nasal washes of ferrets infected with influenza viruses of differing virulence, Br. J. Exp. Pathol., 1981, vol. 62, no. 1, pp. 87—93.
11. Ryan-Poirier, K.A. and Kawaoka, Y., Distinct glycoprotein inhibitors of influenza A virus in different animal sera, J. Virol., 1991, vol. 65, no. 1, pp. 389—395.
12. White, M.R. and Helmerhorst, E.J., Multiple components contribute to ability of saliva to inhibit influenza viruses, Oral Microbiol. Immunol., 2009, vol. 24, pp. 18—24.
13. Peetersmans, J., US Patent 3953592, 1976.
14. Choppin, P.W. and Tamm, I., Studies of two kinds of virus particles which comprise influenza A2 virus strains. II. Reactivity with virus inhibitors in normal sera, J. Exp. Med., 1960, vol. 112, pp. 921—844.
15. Cohen, A. and Belyavin, G., Hemagglutination inhibition of Asian influenza viruses: a new pattern of response, Virology, 1959, vol. 7, no. 1, pp. 59—74.
16. Matrosovich, M., Gao, P., and Kawaoka, Y., Molecular mechanisms of serum resistance of human influenza H3N2 virus and their involvement in virus adaptation in a new host, J. Virol., 1998, vol. 72, no. 8, pp. 6373—6380.
17. Gimsa, U., Grötzing, I., and Gimsa, J., Two evolutionary strategies of influenza viruses to escape host non-specific inhibitors: alteration of hemagglutinin or neuraminidase specificity, Virus Res., 1996, vol. 42, nos. 1—2, pp. 127—135.
18. Gambaryan, A.S., Lomakina, N.F., Boravleva, E.Y., Kropotkina, E.A., Mashin, V.V., Krasilnikov, I.V., et al., Comparative safety, immunogenicity, and effi-
cacy of several anti-h5n1 influenza experimental vac-
cines in a mouse and chicken models (testing of killed
and live H5 vaccine), *Influenza Other Respi. Viruses*,
2012, vol. 6, no. 3, pp. 188–195.

19. Subbarao, K., Webster, R.G., Kawaoka, Y., and Mur-
phy, B.R., Are there alternative avian influenza viruses
for generation of stable attenuated avian–human influ-
zena a reassortant viruses? *Virus Research*, 1995, vol. 39,
nos. 2–3, pp. 105–118.

20. Lind, P.E. and Burnet, F.M., Further studies of recom-
bination between heat-inactivated virus and active
virus, *Aust. J. Exp. Biol. Med. Sci.*, 1957, vol. 35, no. 6,
pp. 531–540.

21. Gotlieb, T. and Hirst, G.K., The experimental produc-
tion of combination forms of virus. vi. reactivation of
influenza viruses after inactivation by ultraviolet light,
*Virology*, 1956, vol. 2, no. 2, pp. 235–248.

22. Rudneva, I.A., Timofeeva, T.A., Shilov, A.A., Kocher-
gin-Nikitsky, K.S., Varich, N.L., Ilyushina, N.A., et al.,
Effect of gene constellation and post-reassortment
amino acid change on the phenotypic features of H5
influenza virus reassortants, *Arch. Virol.*, 2007, vol. 152,
no. 6, pp. 1139–1145.

23. Larionova, N., Kiseleva, I., Dubrovina, I., Bazhenova, E.,
and Rudenko, L., Peculiarities of reassortment of cold-
adapted influenza a master donor virus with viruses
possessed avian origin ha and h5n1, *Influenza Other
Respi. Viruses*, 2011, vol. 5, suppl. 1, pp. 346–349.

24. *Influenza*, Wilschut, J.C., McElhaney, L.E., and
Palache, A.M., Eds., Amsterdam: Elsevier, 2006.

25. Nayak, B., Kumar, S., DiNapoli, J.M., Paldurai, A.,
Perez, D.R., Collins, P.L., et al., Contributions of the
avian influenza virus HA, NA, and M2 surface proteins
to the induction of neutralizing antibodies and protec-
tive immunity, *J. Virol.*, 2010, vol. 5, pp. 2408–2420.

26. Gustin, K.M., Maines, T.R., Belser, J.A., van Hoeven, N.,
Lu, X., Dong, L., et al., Comparative immunogenicity
and cross-clade protective efficacy of mammalian cell-
grown inactivated and live-attenuated H5N1 reassor-
tant vaccines in ferrets, *J. Infect. Dis.*, 2011, vol. 204,
no. 10, pp. 1491–1499.

Translated by N. Beresteska