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Variability of nonpathogenic influenza virus H5N3 under immune pressure

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Summary.– Mutations arising in influenza viruses that have undergone immune pressure may promote a successful spread of mutants in nature. In order to evaluate the variability of nonpathogenic influenza virus A/duck/Moscow/4182-C/2010(H5N3) and to determine the common epitopes between it and highly pathogenic H5N1 avian influenza viruses (HPAIV), a set of escape mutants was selected due to action of MABs specific against A/chicken/Pennsylvania/8125/83(H5N2), A/Vietnam/1203/04(H5N1) and A/duck/Novosibirsk/56/05(H5N1) viruses. The complete genomes of escape mutants were sequenced and amino acid point mutations were determined in HA, NA, PA, PB1, PB2, M1, M2, and NP proteins. Comprehensive analysis of the acquired mutations was performed using the Influenza Research Database (https://www.fludb.org) and revealed that all mutations were located inside short linear epitopes, in positions characterized by polymorphisms. Most of the mutations found were characterized as substitutions by predominant or alternative amino acids existing in nature. Antigenic changes depended only on substitutions at positions 126, 129, 131, 145 and 156 of HA (H3 numbering). The positions 126, 145 and 156 were common for HA/H5 of different phylogenetic lineages of H5N1 HPAIV (arisen from A/goose/Guangdong/1/96) and low pathogenic American and Eurasian viruses. Additionally, mutation S145P increased the temperature of HA heat inactivation, compared to wild-type, as was proved by reverse genetics. Moreover, nonpathogenic A/duck/Moscow/4182-C/2010(H5N3) and H5N1 HPAI viruses have the same structure of short linear epitopes in HA (145-157) and internal proteins (PB2: 186-200, 406-411; PB1: 135-143, 538-546; PA: 515-523; NP: 61-68; M1: 76-84; M2: 45-53). These facts may indicate that H5 wild duck nonpathogenic virus could be used as vaccine against H5N1 HPAIV.

Keywords: avian influenza virus; H5 hemagglutinin; escape mutants; genetic analysis; phenotypic properties; site-specific mutagenesis

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Abbreviations: AIV = avian influenza virus; GsGD = A/goose/Guangdong/1/96(H5N1); HA = hemagglutinin; HPAIV = highly pathogenic avian influenza virus; IRD = Influenza Research Database; LPAIV = low pathogenic avian influenza virus; MAB = monoclonal antibody; M1 = matrix protein of influenza A virus; M2 = ion channel; NA = neuraminidase; NP = nucleoprotein; PA, PB1 and PB2 = polymerase proteins; SI = Supplementary information

Introduction

It is likely that future flu pandemics will be caused by influenza A viruses emerging due to sporadic antigenic shift and/or reassortment between viruses, which circulate in humans and birds. The most likely candidates for pandemics are viruses with hemagglutinin H1, H2, H3, H5, H6, H7, H9, and H10 and neuraminidase N1, N2, N3, N6, and N8 subtypes (Webster and Govorkova, 2014).

The H5 subtype includes the highly pathogenic (HPAIV) and the low pathogenic (LPAIV) avian influenza viruses, which belong to different genetic lineages [Influenza Research Database (IRD)]. The H5 HPAI viruses (arisen from A/goose/Guangdong/1/96(H5N1) – GsGD-lineage) have been the focus of attention since 1997, when a respiratory infection caused by this virus subtype was diagnosed in humans for the first time. The highly pathogenic avian H5Nx influenza viruses can be a severe danger for public health and the poultry industry. H5N8 HPAIV was first isolated from a domestic duck in China in 2010 and has continued to spread (Wu *et al*., 2014). Today, H5N6 has replaced H5N1 as the dominant HPAIV subtype in southern China, especially in ducks (Bi *et al*., 2016, Bulter *et al*., 2016; Lee *et al*., 2017; WHO, 2017). Two lineages of low pathogenic H5 influenza viruses have arisen from outside the GsGD lineage, Am-nonGsGD and EA-nonGsGD lineages. These lineages circulate among different species of wild ducks and other birds in American and Eurasian continents, respectively (IRD).

The surface glycoprotein, hemagglutinin (HA), is one of the main antigenic determinants, which induces antibody production in an infected organism. HA carries out two important functions in viral live cycle: (1) recognition and binding to cellular receptors and (2) mediation of the fusion of cellular and viral membranes to penetrate the host cell. The latter is dependent on the stability of a HA conformation at the pH of a given environment, termed "pH of fusion" (DuBois *et al*., 2011).

The HA of H5 influenza viruses isolated from humans, wild aquatic birds and chickens possess different properties (Matrosovich *et al*., 1999). However, immunization with a live waterfowl-origin avian influenza virus was able to effectively protect chickens against HPAIV (H5) infection (Crawford *et al*. 1998; Gambaryan *et al*., 2016). Therefore, increasing knowledge related to receptor-binding specificity, antigenic and immunogenic properties and variability of H5 viruses is essential for developing new antivirals and vaccines.

In our previous studies, we mapped the HA antigenic epitopes of an earlier mouse-adapted LPAI virus of American lineage (A/mallard/Pennsylvania/10218/84(H5N2) (Kaverin *et al*., 2002) and HPAI H5N1 human and avian viruses [(A/Vietnam/1203/04 and A/duck/Novosibirsk/56/05 (Kaverin *et al*., 2007; Rudneva *et al*., 2010)]. The present work aimed to map the HA epitopes of nonpathogenic A/duck/Moscow/4182/2010(H5N3) belonging to Eurasian nonGsGD lineage, thereby uncovering the HA antigenic epitopes for viruses of different phylogenetic lineages. For this purpose, we selected escape mutants using a set of monoclonal antibodies against HA and, for the first time, have sequenced the complete genome of escape mutants to estimate possible changes in viral proteins other than HA.

Materials and Methods

Viruses. Virus A/duck/Moscow/4182/2010(H5N3) (GenBank Acc. No. KF885672-KF885679) was kindly provided by A.S. Gambaryan (Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products, Russian Academy of Sciences, Moscow, Russia). This virus was isolated from mallard feces collected on the shore of a pond in Moscow in October 2010. Virus variant A/duck/Moscow/4182-C/2010(H5N3) was obtained from A/duck/Moscow/4182/2010(H5N3) by means limiting dilution cloning in embryonated chicken eggs according to (Kaverin *et al*., 2002) and differed from the parent by two mutations, A188E and D264N, in HA. Viruses were propagated for 48 h in the allantoic cavities of 10-day-old embryonated chicken eggs at 37°C and stored -80°C until use.

Monoclonal antibodies. A panel of virus-neutralizing MABs specific to the HA of H5 strains was used. MABs cp55, cp79, 176/26, 364/1 and 777/1 to A/chicken/Pennsylvania/8125/83(H5N2), and MABs VN04-10 and VN04-13 to the HA of A/Vietnam/1203/04(H5N1) influenza virus were generated in the Virology Department and the Department of Infectious Diseases, St Jude Children's Research Hospital (Memphis, USA). MAB 4F11 to A/duck/Novosibirsk/56/05(H5N1) was produced in N.F. Gamaleya National Research Center for Epidemiology and Microbiology D.I. Ivanovsky Instutute of Virology (Kushch *et al*., 2008).

Selection of escape mutants. Escape mutants have been obtained from A/duck/Moscow/4182-C/2010(H5N3) that was incubated with an excess of MAB for 1 h at room temperature followed by propagation in embryonated chicken eggs according to modification by Kaverin *et al*. (2002) of method by Webster and Laver (1980).

Nucleotide sequencing. During the process of selection and genetic engineering, HA gene structure was determined by Sanger sequencing as described earlier (Timofeeva *et al*., 2016). Finally, complete genome sequencing of the virus variant A/duck/Moscow/4182-C/2010(H5N3) and its escape mutants was performed by MiSeq Illumina platform as described (Voronina *et al*., 2018). Genomes were assembled using the CLC Genomics Workbench v. 9.5.2 and 12.0. Genome sequences were deposited in GenBank with Acc. Nos. MF992939-MF993018 and MK357950-MK358013 (Table 1).

Hemagglutination and hemagglutination inhibition tests (HI) were performed using 0.75% chicken erythrocytes by a standard method (Palmer *et al*., 1975).

Assessment of HA thermostability. Viruses present in clarified allantoic fluid were diluted to 128 HA units and incubated in phosphate-buffered saline (PBS) for 40 min at temperatures ranging from 45°C to 70°C in a Mastercycler nexus gradient thermostat (Eppendorf, Germany). After incubation, viruses were titrated by HA assay with 0.75% erythrocytes. The temperature, which resulted in a decrease of 6 $\log_2$ units in the HA

Table 1. Escape mutants selected from A/duck/Moscow/4182-C/2010 (H5N3) virus and effect of amino acid changes on the antigenic specificity, pH fusion and HA thermostability

Escape mutant[a]	GenBank Accession	Amino acid changes in protein		Reactivity with MAB[c]								pH optimum of fusion	Temperature of HA inactivation[d] (°C±0.2 °C)
				Site A		Site B					Site A+B		
		HA[b]	Other proteins	4F11	VN04-13	cp55	cp79	176/26	364/1	VN04-10	777/1		
dk/4182C[e]	MF992939-MF992946		-	20480	10240	20480	20480	20480	10240	5120	20480	5.50±0.07	62.9
m364/1(2)	MF992947-MF992954	K156E		20480	10240	<160	<160	<160	<160	<160	<160	5.52±0.04	62.9
m364/1(2A)	MF992971-MF992978	K156E	PA: V100I; M1: R160W	20480	10240	<160	<160	<160	<160	<160	<160	5.55±0.05	62.9
m10(14)	MK357974-MK357981	K156E	PB1: P138T	20480	10240	<160	<160	<160	<160	<160	<160	5.51±0.04	62.9
m777/1(6A)	MK357998-MK358005	S126Y, K156E	NP: R65K	20480	5120	<160	<160	<160	<160	<160	<160	5.54±0.05	62.9
m176/26(1)	MK357950-MK357957	D129N, D131N		20480	10240	<160	<160	<160	<160	<160	2560	5.60±0.05	62.9
m777/1(5)	MK357982-MK357989	S145del	PB2: D408G	<160	<160	1240	20480	20480	2560	2560	<160	5.60±0.06	60.5*
m777/1(8)	MK358006-MK358013	S145P		<160	<160	20480	20480	20480	10240	5120	<160	5.51±0.05	65.3*
m13(9)	MF993003-MF993010	S145P	PB1: D538N; PA: S522G	<160	<160	20480	20480	20480	10240	5120	<160	5.52±0.05	65.3*
m13(9A)	MF993011-MF993018	S145P	NA: I258M; M1: R78S, R101K	<160	<160	20480	20480	20480	10240	5120	<160	5.51±0.04	65.3*
m13(11)	MF992963-MF992970	S145Y	PB2: D195G	<160	<160	20480	20480	20480	10240	5120	<160	5.50±0.04	60.5*
m13(11A)	MF992979-MF992986	S145Y	PB2: D195G; PB1: S694N	<160	<160	20480	20480	20480	10240	5120	<160	5.50±0.04	60.5*
m4F11(15A)	MF992987-MF992994	S145P	M2: C50Y	<160	<160	20480	20480	20480	10240	5120	<160	5.50±0.05	65.3*

[a]There is only one representative from the mutants with identical structure and properties in the Table. [b]HA/H3 numbering (Nobusawa *et al.*, 1991). The HI titers of MABs in reactions with escape mutants. The HI titer is the dilution of antibody that inhibits 8 hemagglutination units of virus. The MABs were produced against the HA of A/Chicken/Pennsylvania/8125/83(H5N2) (cp 55, cp79, 176/26, 364/1 and 777/1), A/Vietnam/1203/04(H5N1) (VN04-10 and VN04-13) and A/duck/Novosibirsk/56/05(H5N1) (4F11). [d]The temperature of HA inactivation was determined as the temperature at which HA titer was decreased by 5–6 logs, compared to unheated respective virus. [e]The original virus A/duck/Moscow/4182-C/2010(H5N3). *P <0.05 compared with value for wild-type virus (one-way ANOVA).

titer, was taken as the temperature of HA heat inactivation (Rudneva *et al*., 2013).

Assessment of pH optimum of fusion. Viruses present in the clarified allantoic fluid (250 µl) were diluted to 128 HA units in PBS, were mixed with 50 µl of 2.5% suspension of chicken erythrocytes and incubated on ice for 1 h to allow virus binding (Krenn *et al*., 2011). After centrifugation at 72 g, pellets were resuspended in 250 µl of 2-(N-morpholino)ethanesulfonic acid (MES) buffer at various pH values (from 5.0 to 6.5) and incubated at 37°C for 1 h. Then, samples were precipitated (72 g) and supernatants (170 µl) were transferred to 96-well plates to determine the optical density (at 450 nm) which correlates with the erythrocyte hemolysis induced by virus and cell membrane fusion. The lowest pH value that resulted in the highest optical density was taken as the optimal pH. Reported results are the means of pH of fusion ± SD measured in 3 replicative experiments (Rudneva *et al*., 2013).

Virus growth kinetics in embryonated chicken eggs. 10-day-old embryonated chicken eggs were infected with 1000 50% egg infectious dose (EID_{50}) of tested viruses and incubated for 12, 24, 36 and 48 h at 37°C. After each time interval, a group of 5 eggs were inoculated with the same virus, then transferred to 4°C to be chilled overnight. Allantois fluids were titrated by hemagglutination assay and the data are expressed as mean $\log_2$ reciprocal HA titer ± SD.

Mutant virus recovery. Viruses with definite mutations in HA were created on the base of VN-H5N1-PR8/CDC-RG virus by reverse genetics, as described earlier (Hoffmann *et al*., 2000; Timofeeva *et al*., 2016). Briefly, nucleotide substitutions were introduced into plasmids carrying the HA gene using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, United States). Plasmid transfection was performed in a mixed culture of HEK-293T–MDCK for virus recovery. Virus was subsequently propagated in 10-day-old chicken embryos for 48 h at 37°C.

Statistical analysis. Analysis of variance (ANOVA) was used for comparing the values of pH of fusion, temperatures of HA inactivation, and HI assay. A probability value equal to 0.05 was used to indicate that the received data were not a result of chance.

Results

Selection and antigenic characterization of H5N3 escape mutants

Virus A/duck/Moscow/4182-C/2010(H5N3) was used as the wild-type virus for the selection of escape mutants. Primarily, antigenic properties were tested in cross-reaction with MAB panels against HA of strains A/chicken/Pennsylvania/8125/83(H5N2), A/Vietnam/1203/04(H5N1) and A/duck/Novosibirsk/56/05(H5N1) (Kaverin *et al*., 2002, 2007; Kushch *et al*., 2008). Five positive reactions were observed from 7 MABs against A/chicken/Pennsylvania/8125/83(H5N2), 2 from 7 MABs against A/Vietnam/1203/04(H5N1) and only 1 from 6 MABs against A/duck/Novosibirsk/56/05(H5N1) (data not shown). The MABs, which positively reacted with A/duck/Moscow/4182-C/2010(H5N3), were used to obtain escape mutants (Table 1, Fig. 1). These previously characterized MABs could be divided based on their specificity for HA antigenic sites: MABs recognizing site A (4F11, VN04-13), MABs specific for site B (cp55, cp79, 176/26, 364/1, and VN04-10) and MAB 777/1 that recognizes overlapping A and B sites.

17 escape mutants were selected due to the neutralizing ability of MABs and their antigenic properties were tested by HI assay (Table 1). Results suggest that no escape mutants cross-reacted with MAB 777/1, except mutant m176/26(1). Three escape mutants (m364/1(2), m364/1(2A) and m10(14)) retained reactivity with site A-specific MABs and 7 others (m777/1(5), m777/1(8), m13(9), m13(9A), m13(11), m13(11A) and m4F11(15A)) with site B-specific MABs.

Sequence analysis of H5N3 escape mutants

Complete genomes of 17 escape mutants were sequenced. All mutants contained 1 or 2 amino acid changes in HA. Nine escape mutants carried additional mutations in viral proteins other than HA (NA, PA, PB1, PB2, M1, M2 and NP, Table 1).

Disruption of immune reactivity with site A-specific MABs correlated with amino acid changes in position 145 of HA by polymorphisms (S, P, Y, del). Mutation K156E was observed in escape mutants, which displayed altered reactivity with site B-specific MABs. One of those mutants – m777/1(6A) – contained an additional mutation in HA (K156E+S126Y).

Mutant m176/26(1) acquired two mutations, HA: D129N+D131N, and retained reactivity with site A-specific MABs (4F11 and VN04-13) and 777/1 recognizing both antigenic sites A and B.

Since antigenically important mutations might possess pleiotropic effects that would give mutants the advantage for predominant spread (Rudneva *et al*., 2013), we tested some phenotypic features of escape mutants.

Estimation of pH of fusion and HA thermostability of H5N3 escape mutants

The erythrocyte hemolysis test showed that the optimal pH of fusion for escape mutants was not different than that of wild-type virus (Table 1).

Differences in HA thermostability were observed for mutants with varying amino acids at position 145. Viruses

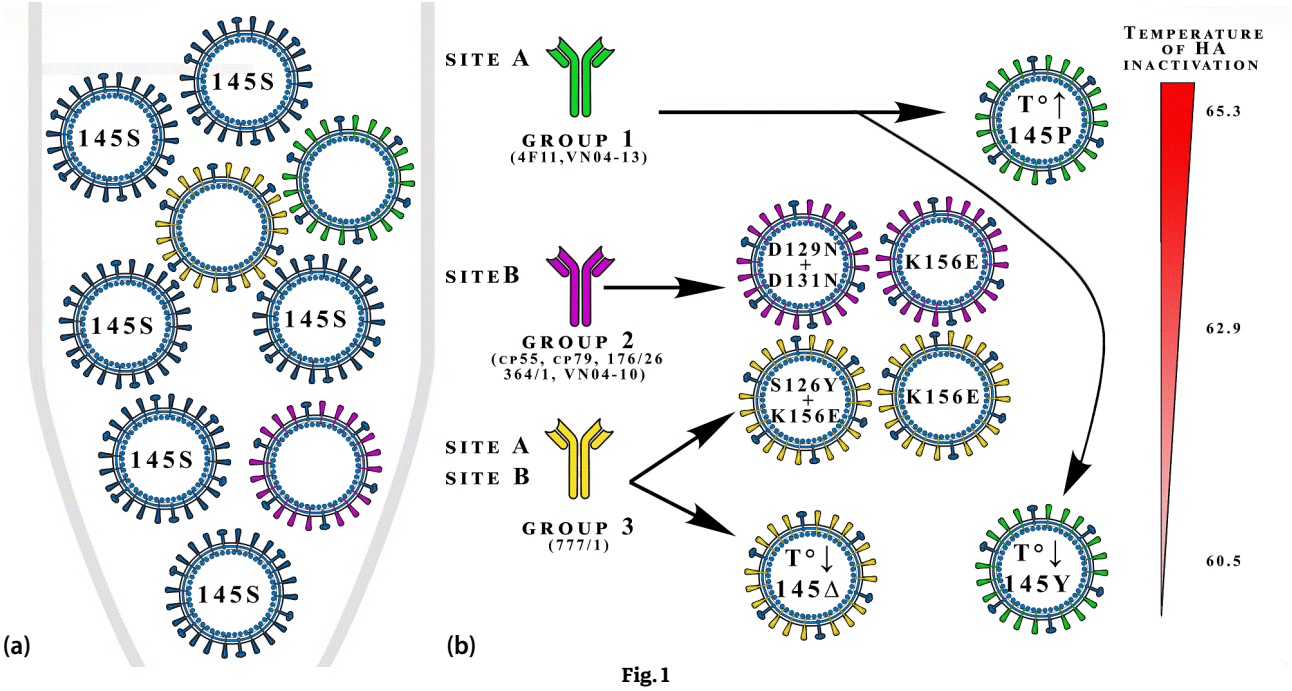


Fig. 1

Selection of LPAIV A/duck/Moscow/4182-C/2010 (H5N3) escape mutants by specific MABs

(a) Due to spontaneous mutations during replication, existing heterogenic population of H5 influenza virus contains predominant 145S in HA and minor polymorphic mutations (not marked). HA specific MABs neutralize the virions with appropriate epitope except for virions with minor mutations that changed epitope and allowed virus to survive. Mutations are designated inside virions. The MABs and complementary HA antigenic sites on the viral surface are colored - site A (green), site B (purple), and site A+B (yellow). **(b)** Point mutation at position 145 in HA promotes an escape from MABs action and changes the virus thermostability.

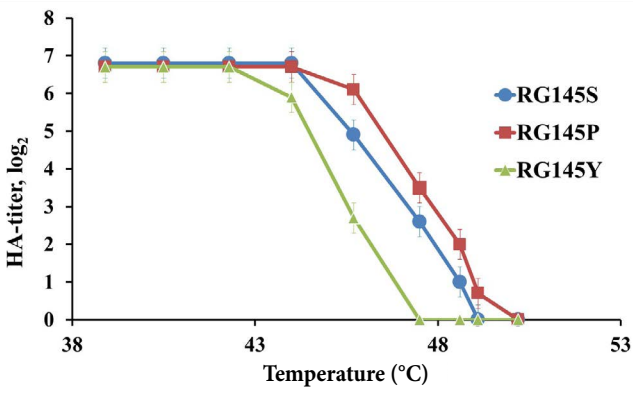


Fig. 2

Thermostability of recombinant VN-H5N1-PR8/CDC-RG viruses with mutation in position 145 of HA after incubation at the indicated temperatures for 40 min

Hemagglutination titers are shown on the *Y* axis.

carrying mutation 145del (m777/1(5)) or 145Y (m13(11) and m13(11A)) demonstrated statistically significant decreases in HA heat inactivation (60.5±0.2°C, P <0.05) compared to wild-type. In contrast, escape mutants containing 145P (m777/8, m13(9), m13(9A), and m4F11(15A)) exhibited a significantly elevated temperature (65.3±0.2°C, P <0.05) of HA heat inactivation compared to the wild-type (62.9±0.2°C, P <0.05). The other escape mutants did not have remarkable changes relative to the initial virus A/duck/Moscow/4182-C/2010(H5N3).

Generation of viruses by reverse genetics and analysis of their thermostability

To uncover the role of amino acid 145 in maintenance of HA thermostability, 3 virus variants were created by reverse genetics of VN-H5N1-PR8/CDC-RG vaccine strain. They differed only by the single mutation in HA: RG145S, RG145P and RG145Y. As shown in Fig. 2, mutant RG145P possessed the highest thermostability of the mutants, while the other 2 mutants remained comparable to escape mutants.

Surprisingly, we observed remarkable differences (about 12°C) in average temperature of HA thermostability between initial avian virus A/duck/Moscow/4182-C/2010(H5N3) and human VN-H5N1-PR8/CDC-RG. This result can be explained by host-dependent origin and the specific properties of these viruses, which are caused by viral structure.

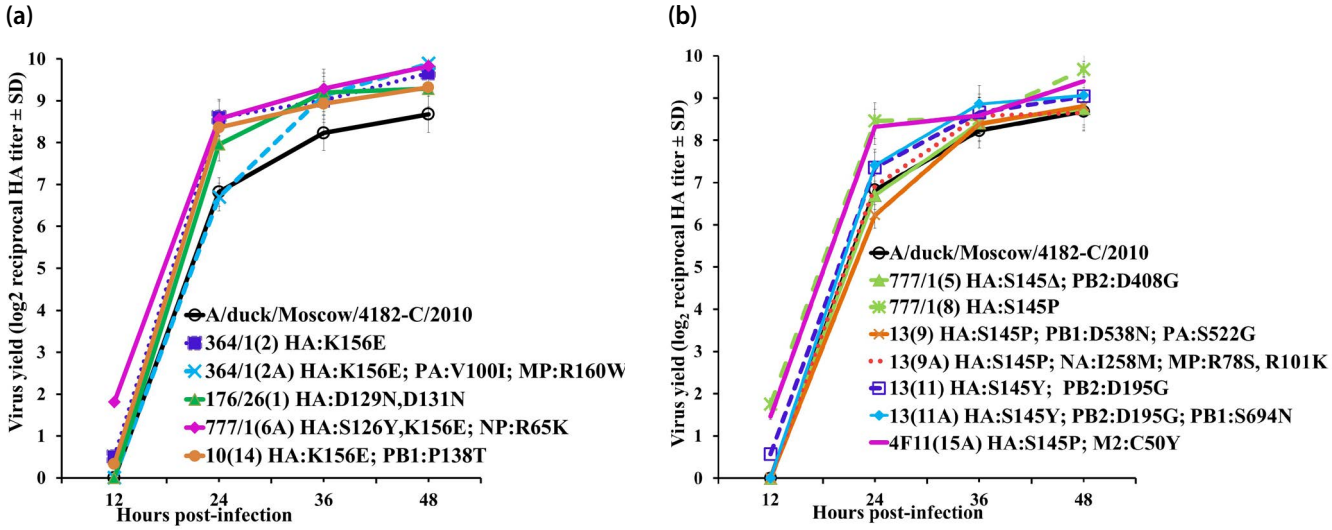


Fig. 3

Replication kinetics of H5N3 escape mutants in comparison with wild-type virus A/duck/Moscow/4182-C/2010 in embryonated chicken eggs

Eggs were infected with viruses at 1000 EID_{50}. Virus yield (log_2 reciprocal HA titer) was titrated by HA assay 12, 24, 36 and 48 h post-infection. Each data point represents the mean value from 3 independent experiments.

Virus replication in embryonated chicken eggs

All escape mutants carrying HA: K156E substitution had a similar replication pattern and gave 2–4 times greater virus yield after 36 and 48 h post-infection (h.p.i.) than the parent strain. The exception was mutant m777/1(6A) (HA: K156E+S126Y, NP: R65K). This mutant possessed additional mutations in HA and NP and demonstrated increased virus replication in the first 12 h.p.i (Fig. 3a).

Similar increased replication, in the first 12 h.p.i., was observed for mutants with a single substitution, HA: S145P (m777/1(8)) and those with an additional mutation in the M2 protein (m4F11(15A); HA: S145P+M2: C50Y). Other mutants carrying HA: 145P and additional mutations in internal proteins did not have this property but had growth curves similar to that of the parent virus, A/duck/Moscow/4182-C/2010(H5N3) (Fig. 3b).

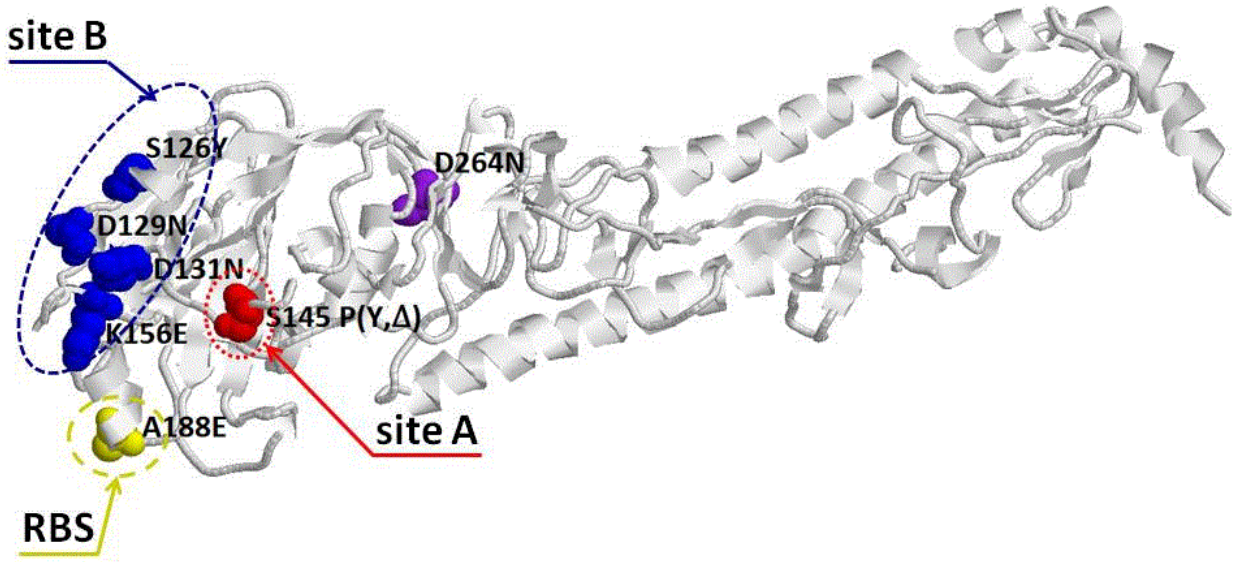
Comprehensive analysis of mutations

Comprehensive analysis was performed for each position, in which mutations had occurred. The technical tools from the Influenza Research Database website (https://www.fludb.org) were applied. We explored the occurrence frequency of substituted amino acids in natural isolates, their polymorphisms and locations relative to functional sites and epitopes that were predicted or experimentally determined (Supplementary information (SI) S1-S6).

Antigenically significant mutations at 126, 129, 131, 145 and 156 positions of HA coincided with short linear epitopes (SI S1-S3) and were mapped to the 3-dimensional structure of HA/H5 (Fig. 4) in our previous studies (Kaverin *et al.*, 2002, 2007, Rudneva *et al.*, 2010). There was some amino acid variety in the positions mentioned above where, among natural H5 isolates, 126S and 156K were the prevailing mutations observed. Two amino acids, Serine and Proline, dominated in position 145. Predominance of amino acids depends on viral host species specificity or the influence of immune pressure. Thus, differences in prevailing amino acids at positions 129 and 131 were observed between H5N1 viruses of the GsGD lineage (129D and 131E) and non-pathogenic wild duck viruses of other lineages, Am-nonGsGD and EA-nonGsGD (129N and 131D).

Among the original viruses (A/mallard/Pennsylvania/10218/84, A/Vietnam/1203/04, A/duck/Novosibirsk/56/05 and A/duck/Moscow/4182-C/2010) that were used to obtain escape mutants (Kaverin *et al.*, 2002, 2007; Rudneva *et al.*, 2010), the common identical immunodominant amino acids in HA were 126S, 145S, and 156K (SI S3). Moreover, those viruses possessed identical short linear epitopes (145-SSFFRNVVWLIKK-157 (H3 numbering); corresponds to 157-169 HA/H5 precursor numbering).

Escape mutants that survived the addition of HA-specific MABs possessed substitutions with an alternative amino acid in the corresponding positions (SI S1, S2), except the m777/1(5), which contained a 145del (HA: S145del, PB2: D408G). No viruses with this deletion have



Location of amino acid changes in the HA of H5 escape mutants in the 3-dimensional structure of A/Vietnam/1203/2004 (Stevens *et al.*, 2006)

Antigenic site A (in HA/H3) is colored in red; antigenic site B (in HA/H3) is colored in blue. Mutations differing between cloned variant A/duck/Moscow/4182-C/10 and the original strain A/duck/Moscow/4182/2010(H5N3 are colored in yellow and purple. RBS – receptor binding site. Image was created with RasMol (www.rasmol.org) and the HA1 structure was obtained from the RCSB Protein Data Bank (PDB ID 2FKO) using pdb-tools software (https://doi.org/https:doi.org/10.2210/pdb2FKO/pdb). Amino acid positions are designated in mature H3 numbering.

been found in nature and reverse genetics failed to produce a viable 145del virus.

Proline, at position 145, has a conformationally inflexible structure, which may increase the HA protein conformational stability at elevated temperatures (Peralvarez-Martin *et al.*, 2008), as was shown by reverse genetics in this work.

The location of residue 145 coincides with antigenic site A in the lateral loop near the receptor binding site (Fig. 4). A mutation in this position may lead to conformational changes and may affect receptor specificity of those mutated influenza viruses. Indeed, the mutation S145P resulted in increased affinity towards sialyl receptors, as was shown earlier (Ilyushina *et al.*, 2004; Santos *et al.*, 2019). Resistance to high temperatures and increased affinity for cell receptors can give viruses an advantage of more successful environmental spread. In fact, it should be noted that the occurrence frequency of the S145P substitution among H5 viruses increased from 8.9% to 87.8% between 2010–2019 (SI S4). However, this effect may be due to vaccination against HPAIV H5N1.

Residue 156 lies at the tip of the globular head of the HA molecule (Fig. 4). Mutation K156E did not affect HA thermostability but increased virus replicative capacity in comparison with wild-type (Fig. 3b). The involvement of the polymorphism at residue 156 in altering host-dependent antigenic properties and reproductive capacities was described earlier (Katz *et al.*, 1987; Kilbourne *et al.*, 1988; Gambaryan *et al.*, 1998). The charge of amino acids in this position influence electrostatic interactions between the globular part of viral HA and the negatively charged cell surface. Replacement K156E resulted in the wild-type amino acid charge to flip from positive to negative, thereby reducing the binding affinity between the virus and the cell membrane. Thus, newly generated viral particles could be released more easily from the cell surface and spread with fluid.

In addition to mutations in HA, escape mutants have been found to have concomitant mutations in internal proteins. All mutations are located in experimentally determined epitopes in positions that have also been characterized by polymorphisms. Most of these epitopes are highly conservative. We studied 12 short linear epitopes (8–15 a.a. in length) of PB2, PB1, PA, NP, M1, M2, and NA proteins for A/duck/Moscow/4182-C/2010, A/Vietnam/1203/04 and A/duck/Novosibirsk/56/05. Among them, 8 epitopes of these viruses have the same structure (PB2: 186–200, 406–411; PB1: 135–143, 538–546; PA: 515–523; NP: 61–68; M1: 76–84; M2: 45–53) (SI S5, S6).

Here, escape mutants acquired point mutations, in which amino acids were substituted by predominant (PB1: S694N, M1: R101K) or alternative ones (PB2: D195G,

M2: C50Y), which have been seen previously in natural isolates, apart from M1: R160W, which was not found in nature. Some mutations are located on a linear sequence within functional domains that provide protein-protein or RNA-protein interaction. Judging by the lack of significant changes in the reproduction of escape mutants relative to the wild-type virus (Fig. 3a,b), it is unlikely that these substitutions have a functional significance. Additionally, as is known, substitutions found in internal proteins of escape mutants are less frequent.

Discussion

Viruses circulating in natural habitats undergo immune pressure from host organisms that have had previous contact with those influenza viruses. However, some viral particles can escape this immune pressure and continue their existence and spread due to advantageous mutations. We have simulated this immune selection process in the laboratory by using monoclonal antibodies against HA to produce escape mutants. Additionally, a similar approach allowed us to map the fine antigenic structure of H5N1 HPAIV (Kaverin *et al*., 2007; Rudneva *et al*., 2010). In this study, we investigated nonpathogenic virus A/duck/Moscow/4182-C/2010 to determine the common epitopes between LPAI and H5N1 HPAI viruses.

Influenza viruses can spontaneously mutate during replication and produce heterogenic populations with mutations in different genome segments and viral proteins. These events can explain polymorphisms existing at some positions of viral proteins. However, only a restricted number of mutants can predominantly spread in particular conditions. In our case, all escape mutants with HA substitutions arose from immune pressure mediated by the action of monoclonal antibodies, which were specific against HA (Fig. 1). Therefore, depending on existing heterogenic population and MAB specificity, varying amino acids at immunodominant positions of escape mutants may occur.

For the first time, the complete genomes of escape mutants were sequenced in this work. The character of mutations that were found in proteins other than HA suggests their sporadic occurrence. Mainly, the mutations that occurred were represented by substitutions with predominant or alternative amino acids existing in the nature.

However, we hypothesized that some concomitant mutations in internal proteins were induced by defective changes in HA, such as deletion 145del or the rare mutation 145Y, in order to compensate this defect. For example, mutants m777/1(5) (HA: S145del; PB2: D408G), m13(11) (HA: S145Y; PB2: D195G) and m13(11A) (HA: S145Y; PB2: D195G; PB1: S694N) had a level of virus accumulation that did not differ from wild-type virus even with defective mutations in HA.

Therefore, we determined that the general immunodominant positions 126, 145 and 156 of HA in H5 viruses belong to the 3 different phylogenetic lineages GsGD, Am-nonGsGD, and EA-nonGsGD. These are located in the antigenic sites A (145) and B (126 and 156) on the 3-dimensional structure of HA.

Moreover, representatives of the highly pathogenic gs-GD- and low pathogenic EA-nonGD- lineages have the same structure of short linear epitopes in HA (145-157) and internal proteins (PB2: 186–200, 406–411; PB1: 135–143, 538–546; PA: 515–523; NP: 61–68; M1: 76–84; M2: 45–53). This information contributes an explanation of the protective effect of the mallard's non-pathogenic virus A/duck/Moscow/4182/2010(H5N3) (EA-nonGD) against challenge with the highly pathogenic H5N1 virus gs-GD, as was shown in chickens [Gambaryan *et al*, 2016].

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Supplementary information is available in the online version of the paper.

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