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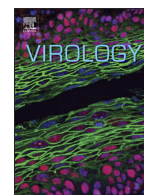

Pleiotropic effects of hemagglutinin amino acid substitutions of H5 influenza escape mutants

Irina A. Rudneva [a], Tatiana A. Timofeeva [a], Anna V. Ignatieva [a], Aleksandr A. Shilov [a], Petr S. Krylov [a], Natalia A. Ilyushina [b,*], Nikolai V. Kaverin [a,**]

[a] D.I. Ivanovsky Institute of Virology, 123098 Moscow, Russia
[b] FDA CDER, 29 Lincoln Drive, Bethesda, MD 20892, USA

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ABSTRACT

In the present study we assessed pleiotropic characteristics of the antibody-selected mutations. We examined pH optimum of fusion, temperatures of HA heat inactivation, and *in vitro* and *in vivo* replication kinetics of the previously obtained influenza H5 escape mutants. Our results showed that HA1 N142K mutation significantly lowered the pH of fusion optimum. Mutations of the escape mutants located in the HA lateral loop significantly affected H5 HA thermostability ($P<0.05$). HA changes at positions 131, 144, 145, and 156 and substitutions at positions 131, 142, 145, and 156 affected the replicative ability of H5 escape mutants *in vitro* and *in vivo*, respectively. Overall, a co-variation between antigenic specificity and different HA phenotypic properties has been demonstrated. We believe that the monitoring of pleiotropic effects of the HA mutations found in H5 escape mutants is essential for accurate prediction of mutants with pandemic potential.

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Introduction

The three-dimensional mapping of the antigenic sites recognized in the hemagglutinin (HA) by influenza virus-neutralizing antibodies was performed both with the use of drift virus variants and escape mutants resistant to monoclonal antibodies (MAbs). Both approaches were applied for the first mapping of influenza virus of H3 HA subtype (Wiley et al., 1981) using the X-ray three-dimensional model of the HA molecule (Wilson et al., 1981). The same approach was used for influenza of H1 (Caton et al., 1982) and H2 HA subtypes (Tsuchiya et al., 2001) with the use of the H3 three-dimensional structure.

Since the antigenic drift is considered to occur in human, but not in avian influenza A viruses, the escape mutants were mostly used for the mapping of antibody-binding sites in H5 and H9 avian influenza strains with pandemic potential (Kaverin et al., 2002, 2004, 2007; Philpott et al., 1990; Rudneva et al., 2010). However, a process that resembles an antigenic drift has been revealed in natural H5N1 isolates in Asia produced by vaccination of fowl (Smith et al., 2006). Amino acid changes in drift variants and escape mutants often coincide, but like in human viruses, a coincidence is not a general rule, since the mutations in some escape mutants differ from those in natural variants (Stevens et al., 2006). Moreover, a coinciding position does not necessary mean the same amino acid substitution, as the change at the same residue may differ between influenza drift isolate and escape mutant.

The absence or infrequent occurrence of similar mutations either in human or avian drift variants as compared to escape mutants is not easy to explain. One possible explanation could be concomitant pleiotropic features caused by an amino acid change inducing the neutralizing effect of an antibody. At least two phenotypic characteristics variations have been detected in escape mutants: a loss of virulence of avian influenza strains (Kaverin et al., 2002, 2004; Philpott et al., 1989) and changes in the affinity for sialic receptors in avian and human viruses (Hensley et al., 2009; Ilyushina et al., 2004; Kaverin et al., 2004; Rudneva et al., 2012). Importantly, a positive correlation has been recently demonstrated between receptor binding avidity and escape from polyclonal antibodies of H1 influenza viruses, indicating that the presence of variations in receptor binding favors the maintenance of circulation of antibody-selected mutants (Hensley et al., 2009). However, other possible phenotypic features of escape mutants, such as pH optimum of fusion, HA thermostability, viral replication in different models, have never been explored and their variations in connection to immune selection are unknown. Therefore, one can hardly rely on the influence of antibody-selected mutations on the virulence or receptor affinity as the only possible pleiotropic effect causing the limitation of the selection of antigenic drift variants.

* Corresponding author. Fax: +1 301 480 3256.
** Correspondence to: Laboratory of Virus Physiology, D.I. Ivanovsky Institute of Virology RAMS, Gamaleya Street 16, 123098 Moscow, Russia.
E-mail addresses: Natalia.Ilyushina@fda.hhs.gov, natasha.ilyushina@gmail.com (N.A. Ilyushina), nik.kaverin@gmail.com (N.V. Kaverin).

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Our previous studies on the antigenic mapping of the HAs of H5N1 and H5N2 influenza A viruses (Kaverin et al., 2002, 2007; Rudneva et al., 2010) allowed us to assay a broad collection of the H5 escape mutants containing amino acid substitutions in several parts of the HA molecule. To assess pleiotropic effects of the antibody-selected mutations located mainly in the HA lateral loop, we examined here pH optimum of fusion, temperatures of H5 HA inactivation, and *in vitro* and *in vivo* replication kinetics of the previously generated H5 escape mutants.

Results

HA amino acid substitutions of H5 escape mutants

Seventeen escape mutants (selected with ten different MAbs) were chosen for this study, including twelve mutants generated from mouse-adapted A/Mallard/Pennsylvania/10218/84 (H5N2) virus (Mld/PA/84-MA) and five H5 mutants selected from influenza virus containing the HA and NA genes of A/Vietnam/1203/04 (H5N1) virus in the genetic background of A/Puerto Rico/8/34 (H1N1) strain (VNH5N1-PR8/CDC-RG). The escape mutants carried either a single HA1 substitution, or two/three mutations in HA1 and HA2 subunits acquired either in the course of one-step selection or resulting from successive selection with two/three MAbs (Tables 1 and 2). Nine escape mutants of Mld/PA/84-MA virus contained changes in the HA lateral loop at positions 140, 142–145 (H3 numbering here and throughout the text). Six single-step escape mutants carried only one HA1 mutation; but two mutants, m4F11(1) and m4G10(10), selected with the MAbs against A/Duck/Novosibirsk/56/2005 (H5N1), contained additional amino acid changes in HA2 (Tables 1 and 2). Since three escape mutants, m58(1)-24B9, m55(2)-24B9 and m46(7)-55-24B9, that were selected in a two- or three-step selection procedure carried additional changes to the substitutions in the HA lateral loop, we included three initial single-step mutants (m58(1), m55(2), and m46(7)-55) having substitutions at positions 129, 131, and 156 in order to distinguish the effect of different single HA mutations. Furthermore, 5 escape mutants, m8(9), m13(13), m15(17), m15 (20), and m777/1(4), selected from VNH5N1-PR8/CDC-RG virus contained single amino acid substitutions in the lateral loop at residues 143–145 (Tables 1 and 2).

To investigate whether the mutations observed in generated H5 escape mutants might occur in H5 isolates circulating from 2003 to 2012, we analyzed the H5 HA sequences deposited in GenBank (Table 1). Our analysis revealed that mutation S145P was found in HA1 of 4.6–41.3% of contemporary H5 isolates, whereas other changes at position 145 were rare (S145T, ~0.2%) or not present in any H5 influenza strains examined (S145Y/F). HA1 mutations at residue 129, N129D, and at residue 144, K144E and R144G, were found with frequency of emergence 65.8% and ~5.0%, respectively (Table 1). Furthermore, the remaining 7 mutations in HA1 (D131N, P140L, N142K, G143D/E, and K156N/T) and 2 mutations in HA2 (L124F and A198T) were found in 0.1% to 2.5% of contemporary H5 influenza isolates.

Effect of HA amino acid substitutions of H5 escape mutants on pH of fusion

We first studied the effect of HA amino acid substitutions of the selected H5 escape mutants on pH of fusion. Our data showed that the optimal pH of fusion of the most escape mutants, including two mutants, m4F11(1) and m4G10(10), that contained changes in HA2 subunit did not differ from that of the respective wild-type viruses (Table 2). The only exception that exhibited a significant decrease of pH value was m55(2)-24B9 with 2 amino acid changes,

Table 1
Frequency of emergence of amino acid changes in the H5 HA of influenza viruses during 2003–2012.

Amino acid changes[a]	Escape mutants with mutations in HA1 (1) and HA2 (2)	Year of isolation									
		2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
$N129D_1$	m46(7)-55, m46(7)-55-24B9	42/118 (35.6)[b]	71/405 (17.5)	407/673 (60.5)	771/989 (78.0)	525/711 (73.8)	270/358 (75.4)	213/270 (78.9)	205/272 (75.4)	248/326 (76.1)	54/62 (87.1)
$D131N_1$	m58(1), m58(1)-24B9	2/118 (1.7)	1/405 (0.2)	17/673 (2.5)	1/989 (0.1)	3/711 (0.4)	2/358 (0.6)	0/270 (0)	0/272 (0)	0/326 (0)	0/62 (0)
$P140L_1$	m46(7)-55-24B9	0/118 (0)	2/405 (0.5)	1/673 (0.2)	0 (0)	0/711 (0)	0/358 (0)	1/270 (0.4)	4/272 (1.5)	0/326 (0)	0/62 (0)
$N142K_1$	m55(2)-24B9	0/118 (0)	0/405 (0)	0/673 (0)	0/989 (0)	0/711 (0)	1/358 (0.3)	0/270 (0)	1/272 (0.4)	0/326 (0)	0/62 (0)
$G143D_1$	m4F11(1)	0/118 (0)	0/405 (0)	0/673 (0)	0/989 (0)	0/711 (0)	0/358 (0)	0/270 (0)	1/272 (0.4)	0/326 (0)	0/62 (0)
$G143E_1$	m15(20)	0/118 (0)	0/405 (0)	16/673 (2.4)	3/989 (0.3)	0/711 (0)	8/358 (2.2)	3/270 (1.1)	2/272 (0.7)	0/326 (0)	0/62 (0)
$K144E_1$	m8(9)	0/118 (0)	0/405 (0)	1/673 (0.2)	16/989 (1.6)	0/711 (0)	16/358 (44.7)	5/270 (1.9)	1/272 (0.4)	1/326 (0.3)	0/62 (0)
$R144G_1$	m24B(9)	0/118 (0)	0/405 (0)	0/673 (0)	0/989 (0)	0/711 (0)	70/358 (19.6)	41/270 (15.2)	35/272 (12.9)	2/326 (0.6)	1/62 (1.6)
$S145P_1$	m4F11(4), m15(17), m4G10(10), m58(1)-24B9	6/118 (5.1)	39/405 (9.6)	146/673 (21.7)	319/989 (32.3)	296/711 (41.6)	148/358 (41.3)	82/270 (30.4)	75/272 (27.6)	15/326 (4.6)	8/62 (12.9)
$S145Y_1$	mPA/777/1(5)	0/118 (0)	0/405 (0)	0/673 (0)	0/989 (0)	0/711 (0)	0/358 (0)	0/270 (0)	0/272 (0)	0/326 (0)	0/62 (0)
$S145F_1$	mPA/777/1(7), m13(13)	0/118 (0)	0/405 (0)	0/673 (0)	0/989 (0)	0/711 (0)	0/358 (0)	0/270 (0)	0/272 (0)	0/326 (0)	0/62 (0)
$S145T_1$	m777/1(4)	0/118 (0)	0/405 (0)	0/673 (0)	1/989 (0.1)	0/711 (0)	2/358 (0.6)	2/270 (0.7)	1/272 (0.4)	0/326 (0)	0/62 (0)
$K156N_1$	m55(2), m55(2)-24B9	0/118 (0)	0/405 (0)	0/673 (0)	2/989 (0.2)	0/711 (0)	0/358 (0)	1/270 (0.4)	0/272 (0)	0/326 (0)	0/62 (0)
$K156T_1$	m46(7)-55, m46(7)-55-24B9	0/118 (0)	2/405 (0.5)	0/673 (0)	0/989 (0)	0/711 (0)	0/358 (0)	0/270 (0)	0/272 (0)	0/326 (0)	0/62 (0)
$L124F_2$	m4G10(10)	0114 (0)	0/299 (0)	0/530 (0)	2/777 (0.3)	0/663 (0)	0/317 (0)	0/238 (0)	0/249 (0)	0/312 (0)	0/62 (0)
$A198T_2$	m4F11(1)	0114 (0)	0/299 (0)	1/530 (0.2)	5/777 (0.6)	1/663 (0.2)	0/317 (0)	0/238 (0)	2/249 (0.8)	0/312 (0)	0/62 (0)

[a] H3 HA numbering (Nobusawa et al., 1991).
[b] Total number of H5 influenza viruses submitted to GenBank. In parenthesis, the percentage of isolates with identified amino acid substitution.

Table 2
Effect of HA amino acid substitutions of H5 escape mutants on pH of fusion, HA thermostability and virulence in mice.

Virus	Amino acid changes[a]	pH optimum of fusion	Temperature of HA inactivation[b] (°C ± 0.2 °C)	Virulence[c]	Source for virulence data
Mld/PA/84	–	5.63 ± 0.03	63.0	7.25 ± 0.25	Smirnov et al., 2000
Mld/PA/84-MA	–[d]	6.05 ± 0.07	57.6	3.52 ± 0.68	Kaverin et al., 2002
m58(1)	D131N	6.00 ± 0.08	55.7*	6.38 ± 0.82*	Kaverin et al., 2002
m24B9	R144G	5.96 ± 0.10	57.6	4.00 ± 0.77	Kaverin et al., 2002
m4F11(4)	S145P	6.15 ± 0.08	61.4*	3.83 ± 0.34	This study
mPA/777/1(5)	S145Y	6.10 ± 0.03	55.7*	3.81 ± 0.48	Krylov et al., 2009
mPA/777/1(7)	S145F	6.13 ± 0.02	55.7*	6.38 ± 0.36*	Krylov et al., 2009
m55(2)	K156N	6.13 ± 0.09	57.6	6.13 ± 0.81*	Kaverin et al., 2002
m46(7)-55	N129D, K156T	6.00 ± 0.10	57.6	3.50 ± 0.67	Kaverin et al., 2002
m58(1)-24B9	D131N, S145P	6.20 ± 0.04	57.6	3.80 ± 0.34	This study
m55(2)-24B9	N142K, K156N	5.77 ± 0.08*	57.6	3.86 ± 0.24	This study
m4F11(1)	G143D; A198T in HA2	5.93 ± 0.03	57.6	4.66 ± 0.48	This study
m4G10(10)	S145P; L124F in HA2	6.06 ± 0.06	59.5*	3.86 ± 0.30	This study
m46(7)-55-24B9	N129D, P140L, K156T	6.05 ± 0.08	57.6	5.00 ± 0.64*	Kaverin et al., 2002
VNH5N1-PR8/CDC-RG	–	5.80 ± 0.01	54.0	4.04 ± 0.58	Krylov et al., 2009
m15(20)	G143E	5.83 ± 0.03	52.6*	4.63 ± 0.54	This study
m8(9)	K144E	5.80 ± 0.03	52.6*	4.32 ± 0.34	This study
m13(13)	S145F	5.79 ± 0.01	51.5*	6.10 ± 0.28*	Krylov et al., 2009
m15(17)	S145P	5.85 ± 0.02	57.6*	4.38 ± 0.30	This study
m777/1(4)	S145T	5.85 ± 0.01	51.5*	4.16 ± 0.24	This study

[a] H3 numbering (Nobusawa et al., 1991).
[b] The temperature of HA inactivation was determined as a temperature at which HA titer was decreased by 5–6 logs compared to unheated respective virus.
[c] Expressed as $\log_{10} EID_{50}/MLD_{50} \pm SE \times t_{\alpha,n-2}$, where t_α is a Student's coefficient with probability α (α=0.95).
[d] The mouse-adapted variant Mld/PA/84-MA differed from the original Mld/PA/84 strain by four HA mutations S203F, A263T, E273G, and L320P (Smirnov et al., 2000).
* $P < 0.05$ compared with value for respective wild-type virus (one-way ANOVA).

N142K and K156N ($P < 0.05$). Since single-step escape mutant m55 (2) that carried mutation K156N exhibited no changes in pH optimum, our data indicated that the decrease of pH value fusion of double-step mutant m55(2)-24B9 is associated with the rise of electrostatic positive charge in the HA lateral loop at position 142 (Table 2).

Effect of HA amino acid substitutions of H5 escape mutants on HA thermostability

To determine whether HA mutations of the H5 escape mutants affect the HA thermostability, we next measured their temperatures of HA heat inactivation. We observed that the temperature of HA stability of the escape mutants carrying amino acid substitutions at position 145 differed (Table 2). Mutation S145P in mutants m4F11(4) and m15(17) selected from the wild-type viruses Mld/PA/84-MA and VNH5N1-PR8/CDC-RG, respectively, resulted in an increase of HA heat inactivation ($P < 0.05$). In contrast, the other changes at this position, S145Y in mPA/777/1(5), S145T in m777/1(4), and S145F in mPA/777/1(7) and m13(13), significantly decreased the HA temperature stability ($P < 0.05$). Our results showed that additional HA2 mutation L124F did not alter the effect of S145P substitution on H5 HA thermostability because similar increase in the temperature of HA heat inactivation was observed in both mutants m4F11(4) and m4G10(10) in comparison with Mld/PA/84-MA virus (Table 2). However, D131N change that results in acquisition of glycosylation site (Kaverin et al., 2002) lowered the effect of S145P on increase of HA temperature stability in double escape mutant m58(1)-24B9. Since single-step mutant m58(1) having D131N exhibited a significant decrease in heat stability compared to the wild-type virus Mld/PA/84-MA ($P < 0.05$), our data suggested that glycosylation could counteract the effect of the S145P substitution on increase of temperature inactivation of H5 HA protein. Our data also demonstrated that mutations G143E and K144E resulted in a significant decrease of HA temperature inactivation of escape-mutants of VNH5N1-PR8/CDC-RG virus ($P < 0.05$, Table 2). All other changes (N129D, P140L, N142K, G143D, R144G, K156N, and A198T in HA2) had no effect on H5 HA thermostability.

Further, in order to assess the effect of high temperature on H5 HA activity we performed a polyacrylamide gel electrophoresis (PAGE) analysis of purified influenza viruses after the heating at different temperatures (Fig. 1). Namely, we analyzed the wild-type virus Mld/PA/84-MA, escape mutant m4F11(4) with amino acid change S145P resulting in increase of HA heat stability and mutant mPA/777/1(7) with mutation S145F having a low level of HA heat inactivation by PAGE in non-reducing conditions. Our results revealed that the levels of the heating conditions ensuring the destruction of HA1+HA2 protein (Fig. 1) and the temperatures for the loss of HA activity (Table 2) were identical. Additionally, binding of these three H5 viruses with two MAbs, cp46 and VN04-2 against A/Chicken/Pennsylvania/1370/83 (H5N2) and A/Vietnam/1203/04 (H5N1) viruses, respectively, was also assayed at different temperatures by enzyme-linked immunosorbent assay (ELISA). We found that the loss of antibody binding strongly correlated with the temperature of HA inactivation of all three viruses studied (data not shown).

Effect of HA amino acid substitutions of H5 escape mutants on viral growth in chicken embryonated eggs

To evaluate the effect of HA amino acid substitutions of H5 escape mutants on viral growth *in vitro*, we assayed their viral yields in comparison with respective wild-type strains in embryonated chicken eggs after infection with 1000 EID_{50}. Although most of the escape mutants grew to similar titers as compared to their respective wild-type viruses, we observed that mutations at positions 131, 144, 145, and 156 affected the replicative ability of several H5 escape mutants in eggs (Fig. 2). Single-step escape mutant m4F11(4) having mutation S145P replicated to significantly lower titers than did Mld/PA/84-MA at 18 and 24 h post-infection (Fig. 2A, $P < 0.01$). The yield of the mutant m13(13) with mutation S145F was 2.9 logs lower than that of its parental wild-type virus VNH5N1-PR8/CDC-RG at 18 h post-infection

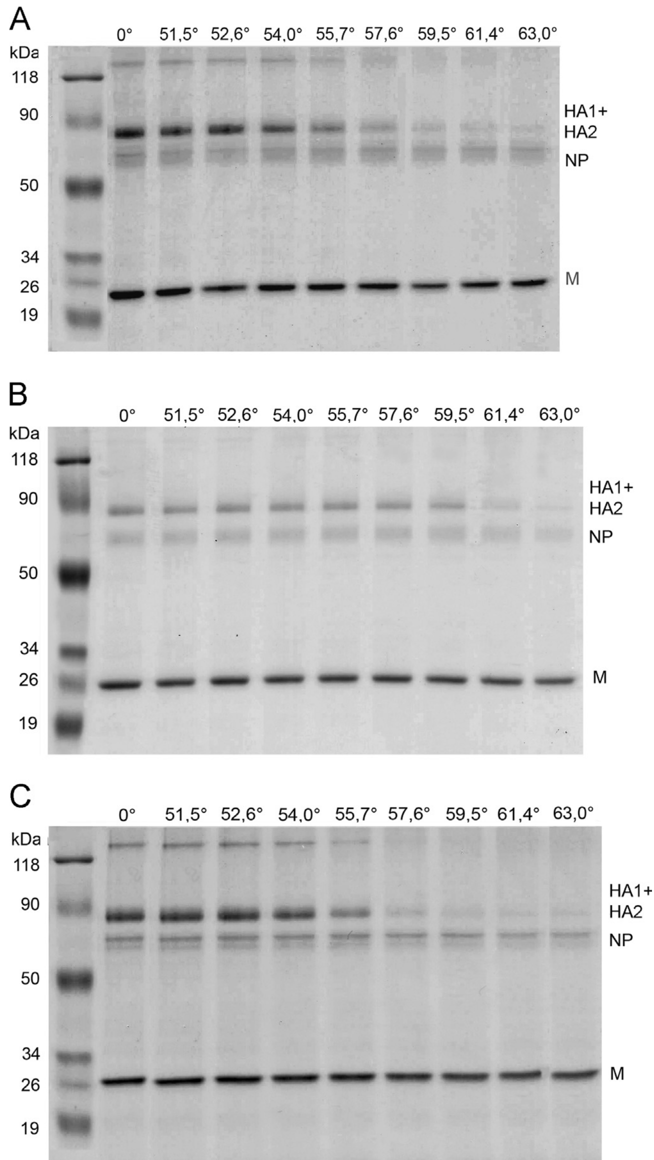

Fig. 1. PAGE analysis of purified H5 influenza viruses, (A) Mld/PA/84-MA, (B) m4F11(4), and (C) mPA/777/1(7), after the heating at different temperatures (°C).

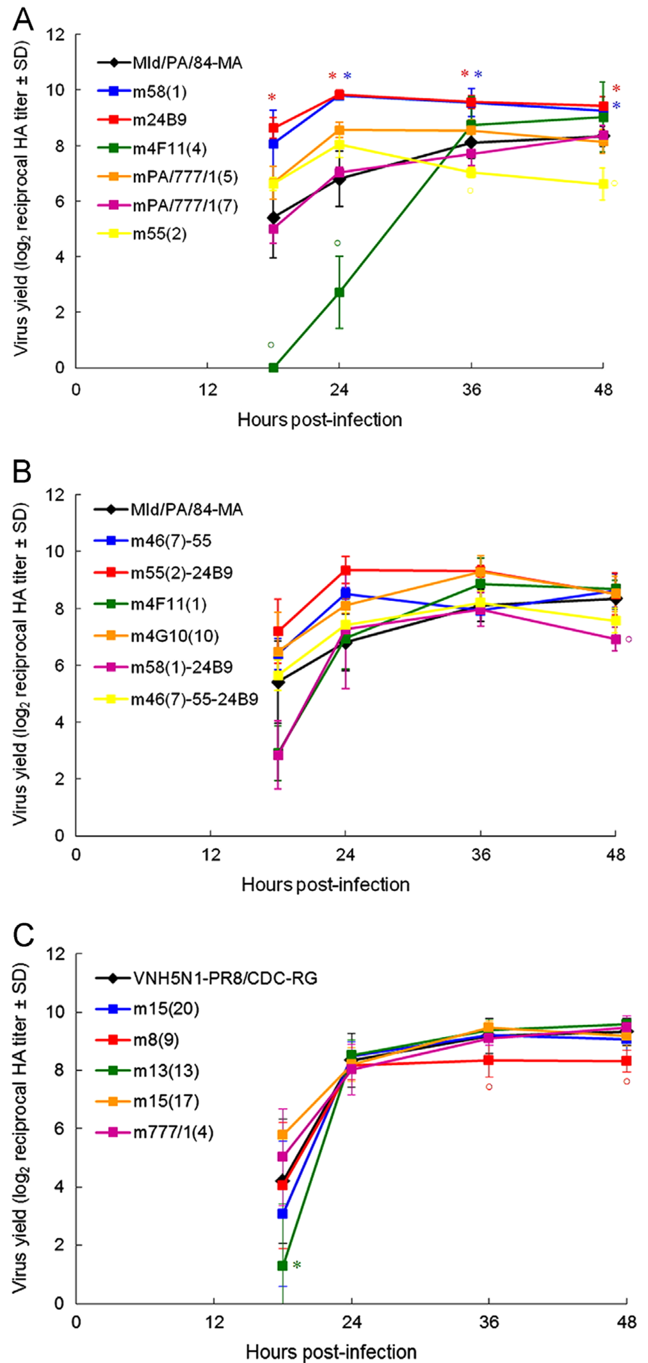

Fig. 2. Replication kinetics of H5 escape mutants in comparison with their respective wild-type viruses, (A, B) Mld/PA/84-MA and (C) VNH5N1-PR8/CDC-RG, in embryonated chicken eggs. Eggs were infected with viruses at a multiplicity of infection of 1000 EID_{50}. Virus yield (log_2 reciprocal HA titer) was titrated by HA assay 18, 24, 36, and 48 h post-infection. Each data point represents the mean value from two-three independent experiments. $^*P<0.05$, $^\circ P<0.01$ compared with value for respective wild-type virus (one-way ANOVA).

(Fig. 2C, $P<0.05$). The replication abilities of the escape mutants m55(2) having K156N and m8(9) having K144E were significantly lower (~0.8–1.7 logs) than those of their respective wild-type viruses at 36 and 48 h post-infection (Fig. 2A and C, $P<0.01$).

Further, comparison of viral yields demonstrated that single escape mutants m58(1) with D131N and 24B9 with R144G grew significantly better than their wild-type virus Mld/PA/84-MA at all post-infection time points (Fig. 2A, $P<0.05$). In contrast, double escape mutant m58(1)-24B9 that contained two changes in HA1, D131N and S145P, grew to the same extent as the respective wild-type virus. Taken together, our data demonstrated that H5 HA mutation S145P could lead to the delayed virus accumulation of the escape mutants of the wild-type virus Mld/PA/84-MA *in vitro*, but not of those of the wild-type virus VNH5N1-PR8/CDC-RG. In addition, since mutant m4G10(10) containing S145P together with substitution L124F in HA2 subunit grew to the same level as Mld/PA/84-MA, L124F was associated with restoring of the replicative ability of H5 influenza mutants to the wild-type pattern in eggs (Fig. 2B).

Effect of HA amino acid substitutions of H5 escape mutants on virulence in mice

To assess the connection between antibody-selected mutations in H5 HA and virulence, we compared the lethality of the H5 escape mutants with that of the respective wild-type viruses in a mouse model (Table 2). Two amino acid changes D131N and K156N were found to be associated with significant decrease in virulence ($P<0.05$; Kaverin et al., 2002). However, the double

mutants m58(1)-24B9 and m55(2)-24B9 having either D131N or K156N, respectively, had the same degree of virulence as the wild-type virus Mld/PA/84-MA. This finding could be ascribed to a reversal of the effects of these HA1 changes by S145P and N142K mutations (Table 2). The amino acid substitution S145F led to a significantly decreased virulence of the single mutants generated from both wild-type viruses, mPA/777/1(7) and m13(13) ($P<0.05$; Krylov et al., 2009). Our results also showed that the triple mutant m46(7)-55-24B9 was slightly but statistically significantly less virulent than was m46(5)-55 due to the additional mutation P140L (Table 2). All other amino acid changes present in selected H5 escape mutants did not significantly influence their virulence, or, at least, their effect was too low to be revealed as statistically significant.

Discussion

Selection of escape mutants by monoclonal antibodies is a powerful method for the mapping of antibody-binding sites on influenza virus HA protein recognized by virus-neutralizing antibodies. Besides the identification of antibody-binding sites, an extensive characterization of escape mutants revealed a co-variation of antigenic specificity with other phenotypic features. For example, a loss of virulence for birds was described for H5 escape mutant (Philpott et al., 1989). A variation in the affinity for sialic receptors, as well as a decrease of virulence was shown in our earlier studies for H5 and H9 escape mutants selected from respective mouse-adapted strains (Kaverin et al., 2002, 2004). Recently, a detailed characterization of H1 escape mutants has been performed for classical A/Puerto Rico/8/34 (H1N1) strain (Hensley et al., 2009) and for pandemic A/Moscow/IIV01/2009 (H1N1) virus (Rudneva et al., 2012) with respect to changes in their receptor binding affinity. Notably, a positive correlation between increase in cellular receptor binding avidity and diminishing antigenicity has been demonstrated (Hensley et al., 2009). However, in the present study we revealed no statistically significant changes in the affinity of the escape mutants to “avian-type” sialyl receptors (data not shown). Furthermore, the measurement of pH of fusion optimum as well as the assessment of HA heating stability has been recently applied to study the impact of both HA1 and HA2 mutations on influenza virus evolution in the course of attenuation (Nakowitsch et al., 2011) and on transmission of H5N1 variants in mammals (Imai et al., 2012; Negovetich and Webster, 2010). However, to our knowledge, these phenotypic features and their possible pleiotropic effects have never been explored in influenza escape mutants.

In our previous studies we generated a broad collection of the escape mutants containing amino acid substitutions in several parts of H5 HA protein (Kaverin et al., 2002, 2007; Rudneva et al., 2010). Namely, the relevant amino acid changes of the mutant viruses selected for this study were grouped in three regions of the HA1 polypeptide chain. The region 140, 142–145 corresponds to site A in the H3 structure (Wiley et al., 1981) and to site Ca2 in H1 (Caton et al., 1982), forming a protruding loop at the side of the HA molecule. In the H5 HA structure, this area is designated as site 5 (Philpott et al., 1990), site II (Kaverin et al., 2002) or site 1 (Stevens et al., 2006). This lateral loop is a loose structure, suggesting that substitutions in its area do not cause strict rearrangements of the HA three-dimensional surface. Further, the amino acid change observed in H5 escape mutants at position 156 corresponds to the area involved in the formation of site B and subsite Iab in the H3 (Wiley et al., 1981) and H5 (Kaverin et al., 2002) molecules, respectively. The H5 area containing residues 129 and 131 partially overlaps a region involved in the antigenic site Sa in H1 HA (Caton et al., 1982) and corresponds to site B in H3 and subsite Ia in H5 HA (Kaverin et al., 2002). In the present study, we assessed whether mutations in the mentioned above regions could affect not only the interactions of H5 HA with MAbs, but also other functional properties of the HA protein.

Analysis of pH of fusion optimum of the H5 escape mutants provided limited data. This finding seems to be anticipated, as the changes in the pH optimum is expected to be associated mostly with the changes in the HA stalk, although the mutations in the HA1 globular head affecting the pH of fusion can occur (DuBois et al., 2011; Imai et al., 2012). The only amino acid substitution that significantly lowered the pH of fusion optimum was N142K in the HA1 lateral loop observed in the double escape mutant m55(2)-24B9 ($P<0.05$), since K156N as a single substitution did not affect the pH optimum. The N142K results in a decrease of positive electrostatic charge of the HA1 surface, which may be regarded as a possible cause for the lowering of the pH of fusion. However, its rare occurrence ($<0.5\%$) in recent H5 viruses suggests that HA1 N142K could be a harmful substitution for maintaining of influenza viral fitness.

Our results showed that mutations of the H5 escape mutants located mainly in the HA lateral loop significantly influenced the temperature of HA heat inactivation ($P<0.05$). The temperature of HA thermostability proved to be the same as the temperature for destruction of HA1+HA2 protein in non-reducing conditions. It is obvious, that differences in temperatures of HA activity between different escape mutants reflected the effect of heating on HA conformation accompanied by the loss of disulfide bonds. We found that the S145P amino acid change produced a significant rise in the level of HA heat inactivation in the H5 escape mutants selected from both wild-type viruses, whereas G143E, K144E, and S145Y/F/T substitutions resulted in decreased HA thermostability ($P<0.05$). In addition, our results showed that D131N change that results in acquisition of glycosylation site (Kaverin et al., 2002) significantly lowered the HA heat stability ($P<0.05$), suggesting that the attachment of a carbohydrate residue at the top of the HA protein could affect its thermostability.

A correlation between virus replication and immunogenic features resulting from amino acid substitutions in antigenic sites have been previously described (Both et al., 1983; Chen et al., 2010). In this study we observed a significant inverse correlation between pH of fusion of H5 escape mutants and the amount of virus shed in embryonated chicken eggs (Spearman correlation coefficient -0.62, $P=0.004$). Our data showed that HA mutations at positions 131, 144, 145, and 156 and substitutions at positions 131, 142, 145, and 156 affected the replicative ability of H5 escape mutants *in vitro* and *in vivo*, respectively. Interestingly, the amino acid substitution S145P, unlike its effect on the HA heat stability, caused the opposite effect on the replication of the wild-type virus Mld/PA/84-MA in the allantoic cavities of embryonated chicken eggs, but not in a mouse model. In contrast, mutations K144E, S145F, and K156N were associated both with significant decrease in virulence in eggs and/or mice and with lowering of HA thermostability ($P<0.05$). HA1 amino acid changes N142K, R144G, and L124F in HA2 subunit increased virus replication ability either in eggs or in mice, but did not exhibit any effect on the temperature of HA heat inactivation. Noteworthy, K144E, R144G, and S145P changes associated with different pleiotropic effects were the only mutations that are abundant among H5 natural isolates ($\sim10.9\%$), whereas other changes are extremely rare ($\sim0.2\%$). No one can ascribe the frequency of emergence of these three mutations in natural isolates as a result of widespread of neutralizing antibodies in nature. However, their additional phenotypic features, such as the effect on HA heat stability and/or on viral replication *in vitro* and *in vivo*, may be one of the plausible causes of their preponderance during natural selection as compared with the other amino acid changes observed in H5 escape

mutants. It would be of interest to study the effect of amino acid changes in H5 escape mutants on virus replication in various cell culture systems, as well as on the ability to adapt to mammalian cells. Overall, our results suggested that the resistance to heating as a loss of sensitivity to neutralizing antibodies might be more advantageous for circulating virus than the changes in multicycle accumulation.

Further, in the present study we demonstrated the pleiotropic effects of the antibody-selected mutations located not only in the HA lateral loop, but also at the distant end of HA globule corresponding to site B in H3 HA (Wilson et al., 1981). Interestingly, D131N resulted in significant enhancement of multicycle virus replication in embryonated chicken eggs, but in decreased virulence in mice, which paralleled its lowering effect on HA thermostability ($P < 0.05$). Our previous study revealed that this change was associated with increased hemagglutination inhibition titers with normal mouse serum inhibitors (Kaverin et al., 2002) and with decreases in the levels of neutralizing antibody and the immune protection afforded by vaccination with the respective H5 escape mutant (Smirnov et al., 2004). Taken together, our results suggested that the acquisition of an oligosaccharide attachment next to the tip of the HA1 subunit could lead to the emergence of multiple pleiotropic characteristics of the antibody-selected mutation, which, however, do not provide any selective advantage during natural selection.

In conclusion, the data presented here can be regarded as the first step on the phenotypic characterization of the antibody-selected mutations in H5 HA protein. A co-variation between antigenic specificity and different HA phenotypic properties has been demonstrated. We believe that the monitoring of pleiotropic effects of the HA mutations found in H5 escape mutants is essential for accurate prediction of mutants with pandemic potential and of all possible outcomes of immune selection in H5 influenza A viruses.

Materials and methods

Viruses

The avian non-pathogenic H5N2 influenza virus A/Mallard/Pennsylvania/10218/84 (Mld/PA/84, GenBank accession no. AF100180) was obtained from the virus repository of the Virology Department of St. Jude Children's Research Hospital (Memphis, TN, USA). The virus was adapted to mice by lung-to-lung passage, as described previously (Smirnov et al., 2000). The mouse-adapted variant was designated Mld/PA/84-MA (GenBank accession no. AF512925) and this virus differed from the original Mld/PA/84 by four HA mutations S203F, A263T, E273G, and L320P (Smirnov et al., 2000). Eleven escape mutants (m55(2), m58(1), m24B9, m55(2)-24B9, m58(1)-24B9, m46(7)-55-24B9, m46(7)-55, m4F11(1), m4F11(4), mPA/777/1(5), and mPA/777/1(7); GenBank accession nos. AF512927, AF512928, AF5 12932, AF512933, AF512936, AF512937, AF512941, GU183554, GU18 4555, KF006997, and KF006998, respectively) were selected with panel of anti-H5 HA MAbs, as previously described (Kaverin et al., 2002, Krylov et al., 2009, Rudneva et al., 2010). Escape mutant of Mld/PA/84-MA, m4G10(10), was selected in this study.

The reverse genetics-derived influenza virus containing the HA and NA genes of A/Vietnam/1203/04 (H5N1) in the genetic background of the high-growth master strain A/Puerto Rico/8/34 (H1N1) (VNH5N1-PR8/CDC-RG) was kindly provided by R. Donis (Centers for Disease Control and Prevention, Atlanta, GA). The HA gene of this virus had been modified by site-specific mutagenesis to delete the multibasic amino acids at the HA cleavage site. Of 13 escape mutants (Kaverin et al., 2007), we used 5 mutants, m8(9), m13(13), m15(17), m15(20), and m777/1(4) (GenBank accession nos. EU122396, EU122399, EU122402, EU122403, and EU122406, respectively).

Viruses were propagated by growth for 48 h in the allantoic cavities of 10-day-old embryonated chicken eggs at 37 °C and were stored at −80 °C until used.

Selection of escape mutants

As described previously (Kaverin et al., 2002), Mld/PA/84-MA influenza virus was incubated with an excess of MAb 4G10 produced against A/Duck/Novosibirsk/56/05 (H5N1) (Rudneva et al., 2010) for 1 h at room temperature. The mixture was inoculated into 10-day-old embryonated chicken eggs and incubated for 48 h at 37 °C. Virus was harvested and used for limiting-dilution cloning in embryonated chicken eggs.

Virus purification

Viruses were concentrated and purified by clarification of the allantoic fluid at low speed and then by centrifuging through a 20% (w/v) sucrose dissolved in ST buffer (0.1 M NaCl, 0.01 M Tris–HCl pH=7.4) in a SW 27-1 rotor for 90 min at 23.000 rpm at 4 °C. The pellet was resuspended in ST buffer and clarified by low-speed centrifugation.

Assessment of pH optimum of fusion

Clarified viruses (250 μl) standardized to 128 HA units in phosphate-buffered saline (PBS) were mixed with 50 μl of 2% suspension of chicken red blood cells (CRBC) and incubated on ice for 1 h to allow virus binding (Krenn et al., 2011). Then, the mixtures were pelleted at 72 g and the supernatants were removed. 250 μl of MES buffer at various pH values (from 5.0 to 6.0) were added, followed by incubating at 37 °C for 1 h. After centrifugation (72 g), 170 μl of supernatant were transferred to 96-well plates and the amount of hemoglobin released by virus-cell fusion induced hemolysis was determined by the measurement of optical density at 450 nm. 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 ± standard deviations (SD) measured by 3 replicative experiments.

Assessment of HA thermostability

Clarified allantoic viruses standardized to 128 HA units in PBS or the purified viruses standardized to 2048 HA units were incubated for 40 min at different temperatures ranging from 50 °C to 63 °C in Master-cycler Gradient thermostate (Eppendorf, Germany). After incubation, viruses were either titrated by HA assay with 0.5% CRBC or analyzed by PAGE (4.0 μg of total HA protein) with Coomassie blue staining as described previously (Carr et al., 1997). The temperature that resulted in 6 $\log_2$ decrease of HA titer (when the HA1 + HA2 band is more intense compared to the residual bands by PAGE) was taken as the temperature of HA heat inactivation.

Virus growth kinetics in chicken embryonated eggs

To determine the growth kinetics, 10-day old embryonated chicken eggs were infected with 1000 50% egg infectious dose (EID_{50}) of each H5 influenza virus and incubated for 18, 24, 36, and 48 h at 37 °C. After each time interval, a group of 5 eggs inoculated with the virus was transferred to 4 °C, chilled overnight, and titrated by HA assay with 1% CRBC. The data are expressed as mean $\log_2$ reciprocal HA titer ± SD.

Elisa

Purified H5 viruses standardized to 2048 HA units were aliquoted and incubated for 40 min at different temperatures ranging from 50 °C to 63 °C in Master-cycler Gradient thermostate (Eppendorf, Germany). Viral aliquots were diluted 1:100 in PBS and then used for coating of the wells in 96-well microtitre plates (Corning Inc., USA). Binding of H5 viruses with the MAbs cp46 and VN04-2 against A/Chicken/Pennsylvania/1370/83 (H5N2) and A/Vietnam/1203/04 (H5N1) viruses, respectively, was assessed by ELISA using peroxidase-labelled anti-mouse immunoglobulin antiserum and TMB as a substrate. A_{450} was determined and expressed as a percentage of the values obtained at 0 °C.

Infection of mice

White outbred female mice weighing 6–8 g (purchased from the Laboratory Animal Breeding Institution of the Russian Academy of Medical Sciences, Andreevka, Moscow Region, Russia) were lightly anaesthetized with diethyl ether and inoculated intranasally. For the assessment of virulence, mice were inoculated with 100 µl of serial 10-fold dilutions of virus-containing allantoic fluid (six/seven mice per dilution). Mouse deaths were registered during the 10 days after inoculation and the mortality rate was expressed in terms of 50% mouse lethal dose (MLD_{50}), as calculated by the method of Reed and Muench (1938).

Statistical analysis

Values of pH of fusion, temperatures of HA inactivation, virus yields in embryonated chicken eggs and in mice were compared by analysis of variance (ANOVA). A probability value of 0.05 was prospectively chosen to indicate that the findings were not the result of chance alone.

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