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Escape mutants of pandemic influenza A/H1N1 2009 virus: Variations in antigenic specificity and receptor affinity of the hemagglutinin

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ABSTRACT

A panel of 6 neutralizing monoclonal antibodies (MAbs) raised against A/Moscow/IIV01/2009 (H1N1) virus isolated during the 2009 pandemic was used for the selection of 26 escape mutants. The mutants were characterized in immune cross-reactions with the panel of MAbs. The sequencing of the mutant HA genes revealed 5 amino acid positions recognized by monoclonal antibodies: 129, 156, 158, 159, and 190 (H3 numbering). The amino acid positions were distributed in two epitopes belonging to antigenic sites Sa and Sb. The mutant HAs exhibited variations in the affinity to synthetic high molecular mass sialic acid-containing receptor analogues. Results are discussed in connection with the antigenic drift potential of the "swine-like" pandemic 2009 influenza virus.

1. Introduction

The targets of anti-virus immunity in influenza are mostly the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The leading role in this respect belongs to the HA. For this reason it is important to reveal the antigenically relevant parts of the HA molecule, where the amino acid substitutions lead to the acquisition of resistance to the neutralizing antibodies. Few of influenza A virus 16 HA subtypes have been characterized with respect to the location and structure of their antigenic sites on the three-dimensional (3D) model of the HA molecule. Three decades ago the H3 HA was characterized by sequencing the HA molecules of antigenic drift variants and escape mutants, and the antigenic epitopes were mapped (Wiley et al., 1981) in the 3D structure of the molecule (Wilson et al., 1981). For many years the 3D structure of HA was available only for the H3 subtype. The H3 structure was used for the antigenic mapping of the H1 (Caton et al., 1982) and H2 (Tsuchiya et al., 2001) HA molecules, and to perform initial studies on the mapping of the H5 HA molecule (Philpott et al., 1990). After the X-ray crystallographic structures of H5 and H9 HA were reported (Ha et al., 2001, 2002), we performed the analysis of the antigenic sites of the HA molecule of H5 and H9 HA subtypes (Kaverin et al., 2002, 2004, 2007; Rudneva et al., 2010). Besides the mapping of the antigenic sites on 3D structure, we revealed a pleiotropic character of mutations conferring resistance to monoclonal antibodies (MAbs). In several cases, the amino acid substitution in the HA of an escape mutant resulted not only in the escape from the neutralizing effect of the MAb, but also in a change in virus virulence, and/or a change in the affinity to sialic acid-containing polymers mimicking the cellular receptors used by the influenza viruses for the attachment to cells (Kaverin et al., 2004; Rudneva et al., 2005).

The 2009 influenza pandemic was caused by a novel "swine-like" H1N1 influenza A virus (Neumann et al., 2009; Smith et al., 2009) that resulted from a reassortment of two previously circulating swine viruses: an American "triple reassortant" strain and a Euroasiatic swine virus (Smith et al., 2009; Garten et al., 2009). The 2009 pandemic raised a concern about the future appearance of drift variants and the new outbreaks caused by the influenza virus of H1N1 subtype. The first publication on the antigenic mapping of the HA molecule of H1 subtype appeared as early as 1982 (Caton et al., 1982). However, since there exists a broad variability within the H1 subtype, the peculiarities of the antigenic structure of the HA of the new pandemic 2009 strain are of interest.

Abbreviations: HA, hemagglutinin; NA, neuraminidase; MAb, monoclonal antibody; HI, hemagglutination inhibition; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; PAA, polyacrylamide; RBC, red blood cells; 3′SL, Neu5Acα2-3Galβ1-4Glcβ; 3′SLN, Neu5Acα2-3Galβ1-4GlcNAcβ; 6-su-3′SLN, Neu5Acα2-3Galβ1-4-(6-O-su)GlcNAcβ; 6′SL, Neu5Acα2-6Galβ1-4Glcβ; 6′SLN, Neu5Acα2-6Galβ1-4GlcNAcβ; 6-su-6′SLN, Neu5Acα2-6Galβ1-4-(6-O-su)GlcNAcβ.

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In the present studies we performed selection of escape mutants using influenza A/Moscow/IIV01/2009 (H1N1) strain isolated during the 2009 pandemic. The strain was isolated in parallel in Madin–Darby canine kidney (MDCK) cell culture and in embryonated chicken eggs (Lvov et al., 2009). The variant isolated in eggs was used in our studies. We used a reassortant having HA and NA genes of A/Moscow/IIV01/2009 (H1N1) virus and the other genes of A/Puerto Rico/8/34 (H1N1) virus (Ignatieva et al., 2011) as the wild-type virus for the selection of escape mutants. The escape mutants were characterized by their cross-reactions with MAbs, by the amino acid changes in their HA molecules, and by their affinity to high molecular mass synthetic sialosides.

2. Materials and methods

2.1. Viruses

The variant of the virus strain A/Moscow/IIV01/2009 (H1N1) isolated in the embryonated chicken eggs (designated as A/Moscow/IIV01/2009-E), as well as strain X-31 (H3N2), were obtained from the Virus Collection of D. I. Ivanovsky Institute of Virology, Moscow. The reassortant ReM8 containing HA and NA genes of the A/Moscow/IIV01/2009-E strain and the other 6 genes of A/Puerto Rico/8/34 (H1N1) virus was obtained by crossing of A/Moscow/IIV01/2009-E and X-31 (H3N2) viruses and characterized in our previous publication (Ignatieva et al., 2011). The protocol used for reassortment was similar to the one described by Schulman and Palese (1976) with modifications described in our earlier publications (Kaverin et al., 1988, 1998). The A/Moscow/IIV01/2009 (H1N1)-E virus was UV-irradiated to lower the infectious titer by 5 $\log_{10}$, mixed with an equal amount of non-irradiated X-31 (H3N2) and used for a one-cycle infection of the embryonated chicken eggs. The mixed infection yield was treated with polyclonal guinea pig serum against X-31 (H3N2) virus and cloned by 6 limiting dilution passages in the embryonated chicken eggs. The reassortant clones were genotyped by partial sequencing. The reassortant ReM8 containing HA and NA genes of the A/Moscow/IIV01/2009-E strain and the other 6 genes of A/Puerto Rico/8/34 (H1N1) virus was chosen for the selection of escape mutants. The viruses were propagated in 10-day old embryonated chicken eggs. The virus-containing allantoic and cultural fluids were aliquoted, and stored at −80 °C.

2.2. Monoclonal antibodies

The preparation of MAbs used in these studies was performed with the use of the technique described in our earlier publications (Masalova et al., 2002; Klimova et al., 2011). Briefly, BALB/c female mice were immunized intraperitoneally four times at 2-week intervals with purified β-propiolactone-inactivated A/Moscow/IIV01/2009-E virus mixed with an equal volume of Freund's adjuvant. Hybridization of the mouse splenocytes with the Sp2/0 myeloma cells was performed, and 69 hybridomas producing antiviral antibodies were selected. Seven hybridomas were used to obtain MAb-containing mouse ascites fluids. The MAbs belonged to IgG class. Six MAbs (3D9, 5F7, 6A3, 1E7, 3A3, 10G2) were shown to react with HA and to have virus-neutralizing activity (Klimova et al., 2011). The reciprocal titers of the MAb-containing ascites fluids in hemagglutination-inhibition (HI) reaction with homologous virus or with ReM8 varied from 1:51,200 to 1:102,400.

2.3. Selection of escape mutants

The selection was performed in embryonated chicken eggs (Webster and Laver, 1980) in the modification described in our earlier work (Kaverin et al., 2002). Virus was treated with an excess of MAb and the mixture was inoculated into the allantoic cavity of embryonated chicken eggs. The virus yield was used for limiting dilution cloning in embryonated chicken eggs. The first-generation mutants were selected from ReM8 virus. Second-generation mutants were obtained by further selection with the MAbs to which a first-generation mutant retained sensitivity.

2.4. Serologic methods

HI reaction was performed by conventional technique (Palmer et al., 1975). Enzyme-linked immunosorbent assay (ELISA) was performed essentially as described by Philpott et al. (1989) with modifications described in our earlier publications (Kaverin et al., 2002; Varich et al., 2011).

2.5. Assay of virus binding to sialic acid-containing polymers

Two methods were used to assess the affinity of the HA to sialooligosaccharides coupled to high molecular weight polyacrylamide (PAA). The direct assay of the affinity to the sialosides (Matrosovich et al., 2000; Mochalova et al., 2003; Tuzikov et al., 2000) was performed with the use of biotinylated synthetic sialoglycoconjugates with PAA synthesized as described earlier (Shilova et al., 2005). The other technique used to assess the affinity of the HA to similar biotin-free conjugates involved the assay based on the ability to inhibit the virus hemagglutination (Matrosovich et al., 1990; Mammen et al., 1995; Mochalova et al., 2003).

2.6. Assay of virus elution from red blood cells (RBCs)

Efficiency of elution from chicken RBC was measured as described by Imai et al. (2010). The twofold dilutions of virus were incubated with 0.5% suspension of RBC at 4 °C, the plates were transferred to 37 °C, and the reversal of hemagglutination was recorded after 4 h incubation.

2.7. Polymerase chain reaction (PCR) amplification and sequencing

Viral RNA was isolated from virus-containing allantoic fluid. Reverse transcription and subsequent PCR was performed using primers specific for the HA gene segment (primer sequences are available upon request). PCR products were purified with the QIAquick PCR purification kit (Qiagen). The DNA template was sequenced by using a DNA ABI Prism 3130 sequencer (Applied Biosystems) and BigDye Terminator v3.1 kit; DNA sequences were completed and edited by using DNASTAR sequence analysis software (DNASTAR Inc.). The nucleotide sequences obtained in this study have been deposited in the GenBank database (accession numbers JQ858372 to JQ858398).

3. Results

3.1. Sequential selection and sequence analysis of escape mutants

The reassortant strain used as the wild-type virus for the selection of escape mutants, ReM8, was obtained by crossing of A/Moscow/IIV01/2009 (H1N1)-E strain with reassortant X-31 (H3N2) virus containing HA and NA genes of A/Aichi/2/68 (H3N2) virus and the other genes of A/Puerto Rico/8/34 (H1N1) virus (Baez et al., 1980). The virus produced higher yields in the embryonated chicken eggs as compared to the parent A/Moscow/IIV01/2009 (H1N1)-E strain (Ignatieva et al., 2011), which allowed us to use appropriate virus concentrations in the selection studies.

The escape mutants were selected in two successive steps. In the first step the reassortant virus ReM8 having HA and NA

Table 1
Amino acid substitutions in the HA of escape mutants selected by MAbs against influenza A/Moscow/IIV01/2009 (H1N1) virus.

MAbs	Escape mutants	Amino acid substitutions[a]
3D9	m3D9(1); m3D9(4); m3D9(5); m3D9(9)	K156E
5F7	m5F7(10); m5F7(11)	G158E
6A3	m6A3(1); m6A3(5)	G158E
3A3	m3A3(3); m3A3(5)	N159D
1E7	m1E7(3); m1E7(4)	N159D
10G2	m10G2(2); m10G2(4);	D190N
	m10G2(6)	D190N; S210N
	m10G2(7)	D190E; G228E; K285M
	m10G2(12)	
3D9, 5F7	m3D9(9)-5F7(14)	K156E;G158E
	m3D9(9)-5F7(17)	K156E;N129D
5F7, 3A3	m5F7(10)-3A3(1); m5F7(10)-3A3(4)	G158E; N159D
5F7, 10G2	m5F7(10)-10G2	G158E; D190N
3A3, 5F7	m3A3(3)-5F7(28)	N159D; N129S;
	m3A3(3)-5F7(29)	N159D; G158E
3A3, 10G2	m3A3(3)-10G2	N159D; D190N
6A3, 10G2	m6A3(5)-10G2	G158E; D190E

[a] Amino acid positions (H3 numbering).

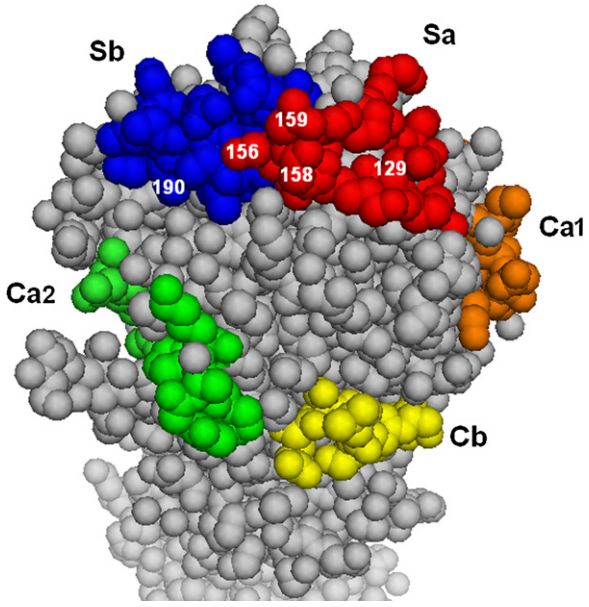

Fig. 1. Positions of the amino acid changes in the antigenic sites on the globular head of HA of A/Moscow/IIV01/2009 (H1N1) influenza virus. Images were created with PyMOL 0.99, and the HA structure was obtained from the Protein Data Bank (PDB accession number 3LZG). Amino acid positions are designated in mature H3 numbering.

of A/Moscow/IIV01/2009-E strain was neutralized with one of the MAbs against A/Moscow/IIV01/2009-E virus, and the escape mutants were isolated by limited dilution cloning. Four mutants were selected with the MAb 3D9, two mutants with each of the MAbs 3A3, 6A3, 5F7 and 1E7, and five mutants with the MAb 10G2 (Table 1). The designations of the mutants included the MAb used for selection and the number of cloned mutant. In the second step, four first-generation mutants, m3D9(9), m5F7(10), m3A3(3) and m6A3(5), were chosen for the use in the selection of second-generation mutants. Overall, 26 mutants were generated. The HA genes of the mutants were sequenced. The sequencing revealed that 15 out of 17 first-generation mutants were single mutants. The amino acid changes in the HA of the single mutants were located in 4 positions: 156, 158, 159, and 190 (H3 numbering here and throughout the text). Two first-generation mutants with a substitution in position 190 had additional amino acid substitutions. The sequencing of the HA of double mutants revealed an additional amino acid change in position 129 (Table 1).

3.2. Antigenic epitopes revealed by cross-reactions in HI and ELISA

The cross-reactions of first-generation mutants in HI and ELISA tests with the panel of MAbs are presented in Table 2. For each series of mutants with identical amino acid substitutions, only one mutant was included in the table. The reactions revealed that the amino acid substitutions in escape mutants recognized by the MAbs were distributed in two operational epitopes. One monoclonal antibody, 10G2, recognized amino acid residue in position 190, but not the amino acid positions 156, 158 and 159. Additional amino acid changes S210N, G228E, and K285M in the mutants carrying the substitution in position 190 did not affect the reaction with MAbs. The other four antibodies recognized positions 156, 158 and 159, either all three, like the antibodies 3D9, 6A3 and 1E7, or two of them, like the MAb 3A3, or only the position 158, like the antibody 5F7. The results of ELISA in most cases corresponded to the results of HI test. The mutants having amino acid changes in positions 129, 156, and 158 retained the ability to react with the MAb 1E7, although they were resistant in HI test. Occasional discrepancies between the results of HI and ELISA had been observed in escape mutants earlier (Philpott et al., 1989; Kaverin et al., 2004).

The cross-reactions of the double mutants with the MAbs revealed additional relevant amino acid substitutions, N129D and N129S, selected and recognized by the MAb 5F7 (Table 3). Interestingly, the double mutant m6A3(5)-10G2 carrying amino acid substitutions G158E and D190E failed to react with the MAb 3A3, although the first-generation mutants carrying these mutations separately, as well as the double mutant m5F7(10)-10G2 having the substitutions G158E and D190N, retained the reaction with the MAb 3A3. Our result suggested that MAb 3A3 felt the combined effect of the substitutions G158E and D190E, but not their separate effects, and not the effect of G158E and D190N.

Two epitopes recognized by the MAbs used in our studies belong to different antigenic sites. The positions recognized by the MAb 10G2 belongs to site Sb, whereas the other antibodies recognize amino acid positions belonging to site Sa (Fig. 1). However, the double mutant m6A3(5)-10G2 carrying amino acid changes G158E and D190E did not react with the antibody 3A3, although this antibody does not recognize the G158E amino acid substitution in single mutants. It seems plausible that the MAb 3A3, although it reacts with the amino acid residues in positions 156 and 159 belonging to site Sa (Fig. 1), recognizes also the amino acid change D190E belonging to site Sb, that is, it overlaps two antigenic sites. Such overlapping had been observed for MAbs to H5 HA in our earlier studies (Kaverin et al., 2007).

3.3. Affinity of escape mutants for sialyl substrates

In our earlier studies (Kaverin et al., 2002, 2004; Rudneva et al., 2005) we observed pleiotropic effects of the amino acid substitutions in the HA of H5 and H9 escape mutants, including the changes in the affinity to synthetic high-molecular-mass analogues of sialic receptors of influenza virus. The amino acid substitutions at positions 156, 158 and 190 present in our escape mutants were shown to be associated with the changes in the HA receptor-binding specificity (Gambaryan et al., 1998; Matrosovich et al., 2000; Stevens et al., 2004). This prompted us to perform a

Table 2
HI and ELISA reaction of MAbs with first-generation escape mutants.

MAbs	Escape mutants											
	m3D9(9) K156E[a]		m5F7(10) G158E		m3A3(3) N159D		m10G2(6) D190N		m10G2(7) D190N; S210N		m10G2(12) D190E; G228E; K285M	
	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA
3D9	<	–	<	–	<	–	0	+	0	+	0	+
5F7	0	+	<	–	0	+	0	+	0	+	0	+
6A3	<	–	<	–	<	–	0	+	0	+	0	+
3A3	<	–	0	+	<	–	0	+	0	+	0	+
1E7	<	+	<	+	<	–	0	+	0	+	0	+
10G2	0	+	0	+	0	+	<	–	<	–	<	±

<, HI titer at least 32-fold (5 $\log_2$) less than the titer with the wild-type virus.
0, HI titer of the MAb does not differ from the titer with the wild-type virus.
+, over 75% binding.
–, less than 25% binding.
±, binding from 25% to 75%.
Percentage binding calculated by the equation $A = 100 \times (B_{xv}/B_{pv})/(B_{xw}/B_{pw})$, where A is the percentage binding as compared with that of the wild-type virus, B_{xv} is of the MAb to test virus, B_{pv} is binding of pooled MAbs to test virus, B_{xw} is binding of the MAb to wild-type virus, and B_{pw} is binding of pooled MAbs to wild-type virus.
[a] Amino acid substitutions (H3 numbering).

measurement of the affinity of the escape mutants of the 2009 virus to sialic acid-containing receptor analogues in the form of biotinylated sialoglycopolymers. The assay revealed that all single amino acid changes leading to a decrease of the positive electrostatic charge of the HA molecule surface led to an increase of K_d, that is, to a decrease of the affinity to both alpha-2-3- and alpha-2-6-sialic receptors. The only amino acid substitution increasing the positive charge, D190N, increased the affinity as compared to the wild-type virus or, as the second mutation in a double mutant, restored the binding lowered by the first mutation. The results were confirmed in the assay based on the inhibition of virus hemagglutination by sialylglycopolymers similar to the conventional HI test (data not shown).

3.4. Elution of escape mutants from RBC

The differences in the affinity to sialylglycopolymers were shown earlier to correlate with the differences in the efficiency of the virus elution from red blood cells (Wagner et al., 2000). We measured the kinetics of the elution of the escape mutants from chicken RBCs. The efficiency of elution varied widely among the escape mutants. There was an inverse correlation between the efficiency of elution and the affinity to sialylglycopolymers: the escape mutants with a lowered affinity had an increased ability to elute, that is, they needed a lower virus concentration for a complete elution in 4 h at 37 °C. The mutants with an enhanced or restored affinity to sialosides exhibited a low elution efficiency (Table 4).

3.5. Incidence of the amino acid substitutions registered in the escape mutants among recent H1N1 isolates

The search in GenBank revealed that among over 6500 isolates only 160 strains had the substitutions identical to the ones present in our escape mutants. The substitution N129D was encountered with an increasing frequency, whereas the other substitutions were infrequent and quickly disappeared from circulation (Table 5).

4. Discussion

The studies on the antigenic epitopes recognized by MAbs in the HA of the H1N1 pandemic 2009 strain are scarce, and only 4 amino acids at positions, 125, 157, 158, and 166, have been revealed as antigenically relevant in the previously described antigenic sites (Manicassamy et al., 2010; Krause et al., 2010). Recently 3 amino acid substitutions in a novel low-variable site were identified (Krause et al., 2011). Overall, the number of amino acid changes in the HA of the escape mutants of the H1N1 2009 pandemic virus, both in the present studies (Table 1) and in the previously published reports is relatively low, especially as compared to the overall number of antigenically important positions reported for the H1 HA in

Table 3
HI and ELISA reactions of MAbs with second-generation escape mutants.

Monoclonal antibodies	Escape mutants													
	m3D9(9)-5F7(14) K156E[a]; G158E		m3D9(9)-5F7(17) K156E; N129D		m5F7(10)-3A3(1) G158E; N159D		m5F7(10)-10G2 G158E; D190N		m3A3(3)-10G2 N159D; D190N		m3A3(3)-5F7(28) N159D; N129S		m6A3(5)-10G2 G158E; D190E	
	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA	HI	ELISA
3D9	<	–	<	–	<	–	<	–	<	–	<	–	<	–
5F7	<	–	<	–	<	–	<	–	0	+	<	+	<	–
6A3	<	–	<	–	<	–	<	–	<	–	<	–	<	–
3A3	<	–	<	–	<	–	0	+	<	–	<	–	<	+
1E7	<	–	<	+	<	–	<	+	<	–	<	–	<	+
10G2	0	+	0	+	0	+	<	–	<	–	0	+	<	±

<, HI titer at least 32-fold (5 $\log_2$) less than the titer with the wild-type virus.
0, HI titer of the MAb does not differ from the titer with the wild-type virus.
+, over 75% binding.
–, less than 25% binding.
±, binding from 25% to 75%.
[a] Amino acid positions (H3 numbering).

Table 4
Affinity of wild-type virus and escape mutants to synthetic sialooligosaccarides and elution from RBC.

Virus	Amino acid change	Sialoglycopolymers						Elution efficiency[b]
		3′SL	3′SLN	6-su-3′SLN	6′SL	6′SLN	6-su-6′SLN	
ReM8	–	0.58 ± 0.08[a]	0.37 ± 0.07	0.77 ± 0.11	1.49 ± 0.45	0.85 ± 0.08	1.20 ± 0.45	64
m3D9(9)	K156E	0.94 ± 0.01	0.82 ± 0.16	1.02 ± 0.16	>4	>4	>4	8
m5F7(10)	G158E	1.26 ± 0.12	0.44 ± 0.05	0.44 ± 0.10	>4	2.66 ± 0.3	>4	16
m3A3(3)	N159D	0.87 ± 0.10	0.63 ± 0.09	0.62 ± 0.09	2.49 ± 0.39	3.40 ± 0.14	>4	16
m10G2(6)	D190N	0.58 ± 0.08	0.18 ± 0.07	0.64 ± 0.09	0.74 ± 0.19	0.52 ± 0.09	0.20 ± 0.08	64
m2D9(9)-5F7(14)	K156E G158E	1.08 ± 0.06	0.74 ± 0.08	1.24 ± 0.18	>4	>4	>4	2
m2D9(9)-5F7(17)	K156E N129D	1.72 ± 0.28	1.03 ± 0.18	0.65 ± 0.11	>4	>4	>4	4
m5F7(10)-10G2	G158E D190N	0.40 ± 0.08	0.30 ± 0.02	0.30 ± 0.10	0.67 ± 0.14	0.65 ± 0.08	0.78 ± 0.10	64
m3A3(3)-5F7(28)	N159D N129S	1.01 ± 0.15	1.60 ± 0.17	0.60 ± 0.10	2.54 ± 0.34	3.28 ± 0.30	>4	4
m3A3(3)-5F7(29)	N159D G158E	1.64 ± 0.15	1.99 ± 0.18	0.64 ± 0.2	>4	>4	>4	2
m3A3(3)-10G2	N159D D190N	0.50 ± 0.11	0.51 ± 0.02	0.76 ± 0.04	1.24 ± 0.27	1.05 ± 0.12	1.10 ± 0.14	64
6A3(5)-10G2	G158E D190E	0.27 ± 0.02	0.26 ± 0.04	0.25 ± 0.03	>4	>4	>4	16

[a] K_d = mean ± SE × $t_{\alpha,n-1}$ (μM sialic acid), where t_α is Student's coefficient with probability $\alpha = 0.90$, from four independent experiments. The lower K_d value corresponds to the higher affinity.
[b] The concentration of virus in HAU necessary for the complete elution from RBC in 4 h at 37 °C.

earlier studies (Caton et al., 1982). This may indicate to a decreased ability of the 2009 virus HA to induce the formation of highly potent neutralizing antibodies or to a predominant role of a limited region of the 2009 HA molecule in the induction of the antibodies.

In several studies the amino acid substitutions in HA changing its antigenic specificity were shown to produce additional effects. The amino acid substitution in the HA of a pathogenic avian virus was shown to correlate with the loss of virulence for birds (Philpott et al., 1990). In our earlier studies the effect of amino acid changes in the HA of escape mutants of mouse-adapted influenza H5 and H9 viruses was shown to produce a decrease in virulence for mice (Kaverin et al., 2002, 2004; Rudneva et al., 2005). The concomitant effects of amino acid substitutions, including the substitution G158E revealed in the escape mutants in our studies, on the antigenicity and receptor binding was shown for swine H1N1 influenza virus (Both et al., 1983; Gambaryan et al., 1998). In our studies on H9 viruses we registered a decrease in the binding of low-virulent escape mutants to biotinylated sialoglycopolymers (Rudneva et al., 2005). In the present studies the major part of amino acid substitutions in the HA of the escape mutants decreased the ability to bind to sialooligosaccharides. The substitutions in 3 amino acid positions among 5 positions recognized by the MAbs led to a decrease in the binding to sialosides used (Table 4). The effect of the substitutions in position 129 could not be measured, since we failed to select single mutants with a change in this position. However, as the second mutation in double mutants, the substitutions N129D and N129S had no effect on the affinity (Table 4). Noteworthy, the amino acid changes decreasing the positive electrostatic charge of the HA molecule surface, except N129D, lowered the affinity to sialooligosaccharides, whereas the amino acid substitution increasing the positive charge, D190N, increased the affinity as compared to the wild-type virus or, as the second mutation in a double mutant, restored the binding lowered by the first mutation. Position 190 is located very close to the receptor-binding site of HA. Two sialooligosaccharides (Table 4) have sulfate group at position 6 of GlcNAc residue, and, therefore, possess double negative charge. Nevertheless, D190N substitution causes similar effect on binding sulfated and sulfate-free sialosides. Thus, the charge effect acts at the level of direct receptor recognition rather than due to total negative charge of sialoglycopolymer macromolecule.

The amino acid changes in the HA of escape mutants not only can reveal the amino acid positions important for the binding of neutralizing antibodies. They also may be relevant as amino acid substitutions involved in the antigenic drift of the circulating virus. However, as revealed by the search in GenBank, only one amino acid change, N129D, was encountered in the new H1N1 isolates with increased frequency. Recently the substitution N129D in an H1N1 isolate was shown to change the HA specificity in the reaction with polyclonal sera as compared to the specificity of the initial 2009 pandemic strain (Strengell et al., 2011). However, all the other substitutions selected by MAbs in our studies were exceptional in 2010–2011 isolates (Table 5). One may surmise that this is due to an adverse effect of the decrease in the affinity to sialyl-terminated cell receptors produced by amino acid changes lowering the electrostatic charge of the HA (Table 4). One may suggest that the advantage conferred by rendering the virus resistant to an antibody may not be enough to overcome the disadvantage resulting from the damage to the ability of the virus to bind the cell receptors. However, it has to be taken into account that the wild-type virus used in our studies was an egg-grown variant differing from the virus isolated in MDCK cells by an amino acid change R226Q. The MDCK-isolated virus had a higher affinity to alpha-2-6-sialooligosaccharides as compared to the HA of the egg variant used as the wild-type virus, whereas the affinity of MDCK-isolated A/Moscow/IIV01/2009 virus to alpha-2-3-sialosides was low (data not shown). For this reason the data on the effect of the amino acid changes on the affinity to sialic receptors have to be regarded with caution: it would be premature to come to any definite conclusions concerning the effect of similar mutations in the circulating swine-like H1N1 virus. Still, a low frequency of the amino acid changes identical to the ones observed in escape mutants indicates to a limitation imposed on the antigenic drift of the H1N1 pandemic virus by the lowering of the electrostatic charge in the vicinity of the receptor-binding pocket. It seems likely that the limitation is implemented through affinity change.

It seems likely that in future the polyclonal response to the amino acid changes registered in the escape mutants generated in our studies will be revealed by HI tests with convalescent sera. Such data were shown recently to be informative in the studies with H3N2 influenza viruses (Nobusawa et al., 2012). However, as

Table 5
Amino acid changes in the HA of influenza 2009-2011 H1N1 strains identical to the changes encountered in the HA of escape mutants.

Amino acid changes	Year		
	2009	2010	2011
N129D	6	78	30
N129S	1	0	1
K156E	11	3	0
G158E	15	1	1
N159D	3	1	2
D190N	5	0	0

the 2009 H1N1 pandemic strains have been remarkably stable antigenically since their initial appearance, the use of convalescent sera in the studies with 2009 virus seems to be premature.

We believe that further analysis of the phenotypic features of antibody-resistant mutants may have some prognostic value with respect to the antigenic drift of the pandemic H1N1 virus.

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