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Virology 329 (2004) 33–39

VIROLOGY

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Monoclonal antibodies differentially affect the interaction between the hemagglutinin of H9 influenza virus escape mutants and sialic receptors

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Received 14 March 2004; returned to author for revision 30 April 2004; accepted 3 August 2004

Available online 11 September 2004

Abstract

To determine the receptor binding properties of various H9 influenza virus escape mutants in the presence and absence of antibody, sialyloligosaccharides conjugated with biotinylated polyacrylamide were used. A mutant virus with a L226Q substitution showed an increased affinity for the Neu5Acα2-3Galβ1-4Glc. Several escape mutants viruses carrying the mutation N193D bound to Neu5Acα2-6Galβ1-4GlcNAc considerably stronger than to Neu5Acα2-6Galβ1-4Glc. Several monoclonal antibodies unable to neutralize the escape mutants preserved the ability to bind to the hemagglutinin as revealed by enzyme-linked immunosorbent assay. In each case, the bound monoclonal antibodies did not prevent the binding of the mutant HA to high affinity substrates and did not displace them from the virus binding sites. Together, these data suggest that amino acid changes selected by antibody pressure may be involved in the specificity of host-cell recognition by H9 hemagglutinin and in the ability of viruses with these mutations to escape the neutralizing effect of antibodies in a differential way, depending on the specificity of the host cell receptor. It may be important in the natural evolution of the H9 subtype, a plausible candidate for the agent likely to cause a future pandemic.

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Keywords: Influenza virus; Hemagglutinin; Monoclonal antibodies; Receptor specificity; Sialyloligosaccharides

Introduction

The hemagglutinin (HA) and neuraminidase (NA) of influenza viruses circulating in humans accumulate amino acid changes that can confer resistance to the neutralizing effect of antibodies. This process, called antigenic drift, has been extensively studied, especially in influenza viruses of the H1 and H3 subtypes (Cox and Bender, 1995; Jackson and Nestorowicz, 1985; Raymond et al., 1986). The amino acid substitutions that confer resistance to antibodies can be artificially selected by treatment with monoclonal antibodies (Mabs). Using escape mutants derived through this selection process together with naturally occurring drift variants, the antigenic epitopes have been mapped in the three-dimensional structure of several HA subtypes (Caton et al., 1982; Kaverin et al., 2002, 2004; Philpott et al., 1990; Tsuchiya et al., 2001; Wiley et al., 1981).

The amino acid changes in the HA of escape mutants and drift variants may have pleiotropic effects on the biologic traits of the virus. It has been previously reported that the amino acid substitutions can affect the affinity of HA for sialic receptors (Daniels et al., 1987; Fleury et al., 1998; Reading et al., 1997). Our previous data indicate that such phenomena are frequent in escape mutants of H9 subtype virus (Kaverin et al., 2004).

Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; 3′SL, Neu5Acα2-3-Galβ1-4Glc-, 3′-sialyllactose; 6′SL, Neu5Acα2-6-Galβ1-4Glc, 6′-sialyllactose; 6′SLN, Neu5Acα2-6-Galβ1-4GlcNAc, 6′-sialyl-(*N*-acetyllactosamine); PAA, polyacrylamide; biot, biotin; Sug-PAA-biot, biotinylated polyacrylamide glycoconjugates (detail structural formulas see Mochalova et al., 2003).

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doi:10.1016/j.virol.2004.08.002

The mechanisms of the neutralizing effect of anti-HA antibodies on virus infectivity are not completely understood. Several different neutralization mechanisms have been described for IgM, IgA, and IgG antibodies (Knossow et al., 2002; Outlaw and Dimmock, 1990; Taylor and Dimmock, 1985). In our previous studies on H5 (Kaverin et al., 2002) and H9 (Kaverin et al., 2004) escape mutants, we encountered discrepancies between the ability of certain Mabs to bind HA and their ability to neutralize infection and inhibit hemagglutination. In addition to these discrepancies, we frequently observed differences in the affinity of H9 escape mutants for sialic receptors (Kaverin et al., 2004). These observations prompted us to analyze the effect of the binding of monoclonal antibodies to the mutant H9 HAs on their ability to recognize different sialyl substrates. To reveal the host-specific differences in the blocking of HA-receptor interaction by the Mab, we used different types of sialic receptors. The sialyl substrates contained either Neu5Acα2-3Gal-, which is more typical for avian species (Katz et al., 1987), or Neu5Acα2-6Gal-, which is more typical for human and swine species (Katz et al., 1990), or amine-containing sialyloligosaccharides, which are considered to be the most similar to the natural receptors present on human cells (Eisen et al., 1997).

In our earlier studies (Kaverin et al., 2004), a panel of 10 anti-H9 Mabs was used to obtain 18 escape mutants of a mouse-adapted variant of the H9N2 influenza virus A/Swine/Hong Kong/9/98 (Sw/HK/9/98-MA). The escape mutants were selected with a two-step protocol, essentially as described previously (Webster and Laver, 1980). The passage of the wild-type mouse adapted strain in eggs did not result in the selection of receptor-binding mutants. All of the escape mutants and the wild-type virus were tested by enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) with the panel of anti-H9 Mabs, and the hemagglutinin genes were sequenced. The results of HI assay and ELISA coincided in most cases, except those for MAbs 19A10 and 7B10, which reacted with the mutant virus m19A10 in ELISA but not in HI assay, and those for MAb 8C4, which behaved in this way with four mutants that had a common amino acid change. These findings prompted us to investigate the receptor-binding and the antibody-binding properties of this set of H9 influenza virus escape mutants.

Results

To investigate the fine mechanisms of competition between antibody and receptor molecules we used a novel approach based on the inhibition of binding of labeled receptor analogs to viruses by monoclonal antibodies.

Affinity of H9 influenza virus escape mutants toward biotinylated polyvalent synthetic sialoglycoconjugates

As a preliminary experiment, we estimated the affinity of biotinylated sialoglycoconjugates to H9 escape mutants in direct enzyme-linked assay. Namely, viruses were directly coated onto 96-well plates and their receptor-binding activities were measured using a set of Sug-PAA-biot conjugates (3′SL-, 6′SL-, and 6′SLN-PAA-biot) (Matrosovich et al., 2000; Mochalova et al., 2003) followed by streptavidin-peroxidase staining (Table 1).

In good agreement with our previous data obtained in a competitive assay (Kaverin et al., 2004), the affinity of the wild-type virus and all escape mutants except one with a L226Q change was negligible for the 3′SL-bearing polymer, which more closely resembles avian virus receptors (H3 numbering here and throughout text). This L226Q mutant exhibited an increased affinity for 3′SL-PAA-biot (Table 1). The amino acid substitution L226Q usually correlates with a shift in the affinity of HA from the “human-type” sialic receptors to the “avian-type” sialic receptors, that is, from preference for a 2′–6′ link to 2′–3′ link between the sialic acid residue and galactose. The four escape mutants carrying a N193D mutation exhibited the decreased affinity for 6′SL-PAA-biot (Table 1).

Competitive binding of monoclonal antibodies and sialic substrates to H9 influenza virus escape mutants

To calculate the dissociation constants (K_d) of the virus–antibody complexes we performed direct enzyme-linked immunosorbent assays using the set of Mabs that bind in ELISA but do not react in HI assay (Kaverin et al., 2004). For each mutant virus, we used positive and negative controls, that is, the Mabs that bound the escape mutants and those that did not bind them, respectively (Table 2). We used the Mab 15F1 as positive control for

Table 1
Receptor-binding activity of H9 influenza virus escape mutants

Viruses	Amino acid substitutions	K_d (μM of Neu5Ac)[a]		
		6SLN-PAA-biot	6SL-PAA-biot	3SL-PAA-biot
Sw/HK/9/98-MA	–	0.19 ± 0.03	0.21 ± 0.02	4.36 ± 0.35
m19A10	L226Q	1.02 ± 0.08	0.95 ± 0.06	0.45 ± 0.06
m7B10	N193D	0.19 ± 0.03	0.51 ± 0.05	4.25 ± 0.29
m3D11	N193D	0.18 ± 0.02	0.55 ± 0.05	4.41 ± 0.48
m7B10-18G4	S116I, T135K, N193D	0.18 ± 0.03	0.56 ± 0.03	4.50 ± 0.23
m7B10-18G4′	T135K, N193D	0.19 ± 0.07	0.50 ± 0.02	4.59 ± 0.28

[a] In each case, K_d = mean ± SE × $t_{\alpha,\ n-1}$, where t_α—Student’s coefficient with probability α (α = 0.90) was obtained in four independent experiments.

Table 2
Antibody-binding activity of H9 influenza virus escape mutants

Viruses	Amino acid substitutions	$K_d \times 10^{-5}$ (μM of Mabs)[a]				
		19A10 (145)[b]	7B10 (145, 193)	3D11 (193, 226)	15F1 (162, 198)	8C4 (198, 189)
Sw/HK/9/98-MA	–	1.98 ± 0.18	1.87 ± 0.21	0.90 ± 0.34	1.18 ± 0.13	2.24 ± 0.46
m19A10	L226Q	3.61 ± 0.54	4.67 ± 0.39	>50	1.39 ± 0.18	2.30 ± 0.11
m7B10	N193D	1.94 ± 0.18	>50	>50	1.14 ± 0.22	5.02 ± 0.31
m3D11	N193D	2.03 ± 0.13	>50	>50	1.81 ± 0.50	5.18 ± 0.37
m7B10-18G4	S116I, T135K, N193D	1.85 ± 0.21	>50	>50	1.12 ± 0.14	5.07 ± 0.51
m7B10-18G4′	T135K, N193D	1.89 ± 0.17	>50	>50	1.03 ± 0.15	4.72 ± 0.42

[a] In each case, K_d = mean ± SE × $t_{\alpha,\ n-1}$, where t_α— Student's coefficient with probability α (α = 0.90) was obtained in four independent experiments.
[b] Mutations in the H9 HA preventing HA–Mab interaction.

the escape mutants m7B10, m3D11, m7B10-18G4, and m7B10-18G4′, and the Mab 8C4 for the m19A10 virus. The Mab 3D11 was used as negative control for the mutants m19A10 and m3D11, and the Mab 7B10 for the others. All the viruses under study exhibited a high affinity for all the Mabs except for the negative controls as expected.

Our earlier studies (Kaverin et al., 2004) and the data of the direct ELISA test have shown that only one amino acid substitution D145E completely prevented the interaction between H9 HA and the Mab 19A10, and therefore, this position is within the area that forms the antigenic epitope of this monoclonal antibody (Table 2). The mutations L226Q, N193D, and N193T prevented the interaction between the H9 escape mutants and the Mab 3D11, whereas the Mab 7B10 recognized an antigenic epitope including N193D, N193T, and D145E amino acids. Therefore, the epitopes of these two Mabs did not coincide, although two identical escape mutants m7B10 and m3D11 with N193D change were selected by the Mabs 7B10 and 3D11 (Kaverin et al., 2004).

Fig. 1 shows that antigenic epitope of the Mab 7B10 is in the right part of the receptor-binding site, whereas the epitope of the Mab 3D11 overlaps with the upper and the left parts of the receptor-binding site of the HA molecule, that is, the place where the moieties of the receptor adjacent to the sialic acid are located. The amino acids 198 and 189 are in the same place where the asialic parts of 3′SL-bearing polymer protrude. The mutations in these positions prevent the interaction between HA H9 and the Mab 8C4. The Mab 15F1 used as a positive control in this study recognizes the epitope containing the amino acid positions 162 and 198 (Kaverin et al., 2004) in the upper part of the HA molecule over the receptor-binding site (Fig. 1).

The competitive assay was based on the competition for binding sites on the viral particle between nonlabeled Mab and biotinylated polyvalent synthetic sialoglycoconjugates or enzyme-labeled sialylglycoprotein fetuin compounds. Serial dilutions of the Mabs were added to the labeled receptor analogs and incubated with the viruses. The concentration of Mabs that result in 50% inhibition of receptor binding were calculated, and the values of $K_{50\%\mathrm{Ing}}$ were compared with the K_d measured in direct ELISA (Table 3). As a rule, these values were closely related. If the Mab weakly bound to viruses ($K_d > 50 \times 10^{-5}$ μM), it did not compete with the receptor. The Mabs possessing high affinity for viruses efficiently displaced the receptor molecule from the binding sites. Notably, significant differences between the dissociation constants measured in direct and competitive ELISAs were seen in only three cases (Table 3). The Mabs 19A10 and 7B10 bound to the escape mutant m19A10 with high affinity equal to the affinity for the parent virus, and successfully competed with 6′SL- and 6′SLN-bearing polymers. However, it did not displace the 3′SL-bearing polymer and fetuin. The Mab 8C4 interacted with the four escape mutants containing the N193D mutation in the opposite way, namely did not compete with 6′SLN trisaccharide and

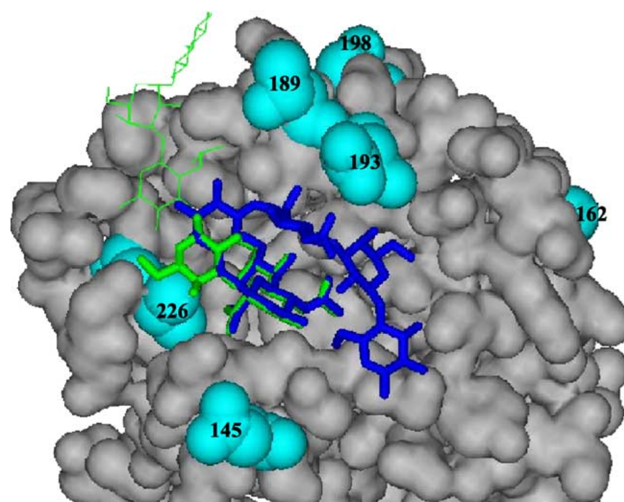

Fig. 1. Three-dimensional model of the H9 HA complexed with receptor molecules (Ha et al., 2001). LSTc is shown in blue (pdb.1JSI), sialylgalactose of LSTa is shown in green (pdb.1JSH), the other residues of LSTa were transferred from three-dimensional data of the H3 HA complexed with LSTa (Eisen et al., 1997) (were kindly provided by Dr. Eisen) and are shown in thin green lines. The amino acid substitutions are shown in pale blue (DS Viewer Pro 5.0, Accelrys Inc. software). The overlapping antigenic epitopes contain positions 145 and 193 (the epitope of Mab 7B10), 193 and 226 (Mab 3D11), 198 and 189 (Mab 8C4), 162 and 198 (Mab 15F1), and 145 (Mab 19A10).

Table 3
Receptor-binding activity of H9 influenza virus escape mutants in competitive reactions with monoclonal antibodies and biotinylated polyvalent synthetic sialoglyconjugates

Viruses	Competitive assay with	$K_{50\%Ing} \times 10^{-5}$ (μM of Mabs)[a]				
		19A10 (145)[b]	7B10 (145, 193)	3D11 (193, 226)	8C4 (198, 189)	15F1 (162, 198)
Sw/HK/9/98-MA	fetuin	4.85 ± 0.32	1.52 ± 0.41	2.75 ± 0.23	2.15 ± 0.25	2.57 ± 0.21
	6SL-PAA-biot	4.57 ± 0.28	2.98 ± 0.23	2.47 ± 0.31	2.79 ± 0.22	2.39 ± 0.15
	6SLN-PAA-biot	4.32 ± 0.22	2.44 ± 0.37	2.38 ± 0.20	2.34 ± 0.19	2.48 ± 0.32
	3SL-PAA-biot	4.69 ± 0.37	2.68 ± 0.28	2.51 ± 0.37	2.53 ± 0.28	2.13 ± 0.25
m19A10 (L226Q)	fetuin	>100	>100	>100	2.17 ± 0.24	1.87 ± 0.26
	6SL-PAA-biot	5.87 ± 0.43	4.22 ± 0.29	>100	2.29 ± 0.17	1.49 ± 0.13
	6SLN-PAA-biot	6.08 ± 0.46	4.69 ± 0.38	>100	2.42 ± 0.12	1.68 ± 0.31
	3SL-PAA-biot	>50	>50	>100	2.12 ± 0.14	1.93 ± 0.27
m7B10 (N193D)	fetuin	4.76 ± 0.30	>100	>100	1.88 ± 0.26	1.62 ± 0.35
	6SL-PAA-biot	4.64 ± 0.25	>100	>100	2.02 ± 0.20	1.38 ± 0.24
	6SLN-PAA-biot	4.42 ± 0.22	>100	>100	>50	1.43 ± 0.28
	3SL-PAA-biot	4.58 ± 0.36	>100	>100	2.28 ± 0.35	1.67 ± 0.21
m3D11 (N193D)	fetuin	4.49 ± 0.31	>100	>100	1.86 ± 0.18	1.93 ± 0.27
	6SL-PAA-biot	4.52 ± 0.25	>100	>100	2.06 ± 0.33	1.77 ± 0.31
	6SLN-PAA-biot	4.82 ± 0.36	>100	>100	>50	1.56 ± 0.44
	3SL-PAA-biot	4.61 ± 0.33	>100	>100	2.38 ± 0.37	1.84 ± 0.19
m7B10-18G4	fetuin	4.75 ± 0.22	>100	>100	2.21 ± 0.16	1.27 ± 0.15
(S116I, T135K, N193D)	6SL-PAA-biot	4.67 ± 0.18	>100	>100	2.35 ± 0.35	1.84 ± 0.28
	6SLN-PAA-biot	4.40 ± 0.32	>100	>100	>50	1.44 ± 0.31
	3SL-PAA-biot	4.53 ± 0.29	>100	>100	2.55 ± 0.22	1.56 ± 0.24
m7B10-18G4′	fetuin	4.37 ± 0.25	>100	>100	1.90 ± 0.14	1.06 ± 0.07
(T135K, N193D)	6SL-PAA-biot	4.55 ± 0.28	>100	>100	2.26 ± 0.31	1.43 ± 0.19
	6SLN-PAA-biot	4.48 ± 0.22	>100	>100	>50	1.19 ± 0.25
	3SL-PAA-biot	4.68 ± 0.35	>100	>100	1.89 ± 0.27	1.77 ± 0.28

[a] In each case, $K_{50\%Ing} \pm$ SE $\times\, t_{\alpha,\, n-1}$, where t_α—Student's coefficient with probability α (α = 0.90) was obtained in four independent experiments.
[b] Mutations in the H9 HA preventing HA–Mab interaction.

competed with other receptors. The Mab 3D11, that recognizes the epitope overlapping with the receptor-binding site, and the Mab 15F1, that interacts with the area located directly over the receptor-binding site, displaced all the types of receptors.

Discussion

Data on the receptor-binding specificity of influenza viruses are important for understanding the mechanisms of virus evolution and interspecies transfer. To mimic the natural process of polyvalent cooperative interactions between the influenza virus and its receptor on the surface of the host cell, we studied the binding of H9 escape mutants to sialyloligosaccharides in the presence and absence of monoclonal antibodies.

Among the amino acid substitutions in H9 HA, the change L226Q in the receptor-binding site is of special interest. This replacement plays a prominent role in the receptor binding specificity. It has been shown that residue 226 of HA is the key amino acid in the recognition of Neu5Acα2-3Gal by H2, H3, and H9 influenza viruses (Connor et al., 1994; Matrosovich et al., 2000, 2001). The selection of escape mutants with substitutions in position 226 and a subsequent change in receptor affinity has been described for H3 subtype viruses (Daniels et al., 1987).

Competitive ELISAs demonstrated that this L226Q replacement, which leads to a dramatic increase in affinity for 3′SL, allows the virus–Mab complexes to react with 3′SL-PAA-biot, but not with 6′SL- or 6′SLN-PAA-biot (Table 3). This disparity could not be explained by different affinity of virus to 3′SL- and 6′SL-bearing polymers, since the affinity values were nearly equal (Table 1). So, the only explanation was the different orientation of distal parts of 3′SL- and 6′SL receptors' molecules that resulted in the competition of 19A10 and 7B10 antibody for 6′SL receptors and the absence of competition for 3′SL-groups. This is in agreement with the data of X-ray analysis of the complexes of H9 hemagglutinin with receptor molecules (Ha et al., 2001). The third saccharide and more proximal moieties of 3′sialo-sugar receptors contact the amino acids 187, 189, and 198 situated in the upper left corner of receptor-binding site. For this reason, the antibody attached to 145 amino acid in the lower right part of the receptor-binding site fails to compete with receptor molecule. On the contrary, the stalk of 6′SLN receptors contacts the epitopes of the Mabs 19A10 and 7B10 and competes with these antibodies.

It is not surprising that the m19A10 mutant–antibody complexes also bind to fetuin because the latter has both Neu5Acα2-3Gal- and Neu5Acα2-6Gal-containing receptors. The hemagglutinin is the component of influenza virus membrane against which infectivity-neutralizing antibodies are directed, and the previous results indicated that

neutralization of infectivity by the antibody involves the inhibition of receptor binding (Bizebard et al., 1995). Moreover, the inhibition of receptor binding is an obligatory component of neutralization (Fleury et al., 1999). The amino acid change L226Q in the H9 HA receptor-binding site may provide an advantage in overcoming the neutralizing effects of antibodies by allowing the recognition of host cell receptors despite the presence of antibody.

Mutants containing the N193D replacement have a reduced affinity to 6′SL-PAA-biot but not to 6′SLN-PAA-biot. Thus, they are able to distinguish 6′SL from 6′SLN (Table 1). The preferential binding of early H1N1 human influenza viruses to 6′SLN-receptor has been shown (Gambaryan et al., 1997, 1999; Matrosovich et al., 1997), and such receptor-binding specificity is also typical for contemporary H3N2 human viruses (Mochalova et al., 2003).

The 6′-SLN has been proposed by Eisen et al. (1997) to be more similar to the human receptor than is 6′SL. The folded conformation of 6′SLN, which is stabilized by a hydrogen bond and van der Waals contacts between Neu5Ac and GlcNAc moieties, suits the architecture of the hemagglutinin receptor-binding site much better than the extended conformation of 6′SL trisaccharide. In the H9 HA complex with pentasaccharide LSTc, terminated 6′SLN-group, the three proximal saccharides (GlcNAc-3, Gal-4, Glc-5) fold back over the top of the sialic acid and exit from the site at the front-right and contact the binding site at the loop formed by residues 125–137, at the 150 loop, and at Asn-193 (Ha et al., 2001).

The results of competitive ELISAs between Mab 8C4 and biotinylated sialyloligosaccharides suggest that the increased affinity toward 6′SLN-PAA-biot caused by the N193D mutation confers an ability to bind 6′SLN in the presence of the bound 8C4 antibody (Table 3). As the epitope of this antibody is in region of 189 and 198 amino acids, it is not surprising that it competes with 3′SL receptor molecules, but not with 6′SLN.

The presented data demonstrate that residues 226 and 193 of the H9 HA are involved not only in the specificity of host-cell recognition but also in the ability of viruses carrying these mutations to escape the neutralizing effects of antibodies that neutralize infectivity by preventing virus from binding to cells (Knossow et al., 2002). The HA changes that allow H9 viruses to escape the neutralizing effects of bound antibody can therefore also result in the switching of receptor specificities and represent an alternative to previously described mechanisms of escape from neutralization by antibodies (Fleury et al., 1998). The co-selection of antigenic and biologic variants has implications for the evolution of influenza viruses in the face of immunological pressures. These data suggest that in natural conditions immune selection can produce variants having both altered host specificity and an ability to escape a host's immune defense mechanism, factors important in considering the natural evolution of H9 influenza viruses, a plausible candidate for the next pandemic agent (Webby and Webster, 2001).

Materials and methods

Materials

For receptor-binding assays, 96-well PV-microtiter plates from Costar, USA, and horseradish peroxidase-streptavidin conjugate and *o*-phenylenediamnine from Boehringer Mannheim, Germany were used. The synthesis of biotinylated polyvalent synthetic sialoglycoconjugates was described earlier (Bovin et al., 1993; Mochalova et al., 2003; Tuzikov et al., 2000). According to monosaccharide analysis data, the molar content of Sug in all conjugates is 20%, and the molar content of biotin in Sug-PAA-biot is 5%.

Viruses

The influenza virus A/Swine/Hong Kong/9/98 (H9N2) (GenBank accession no. AF222810), from the virus repository of the Department of Infectious Diseases at St. Jude Children's Research Hospital, was adapted to mice by serial lung-to-lung passage (Kaverin et al., 2004). Escape mutants of the mouse-adapted variant, which was designated Sw/HK/9/98-MA, were selected with a panel of anti-H9 Mabs (Kaverin et al., 2004). The viruses were propagated in 9- to 10-day-old embryonated chicken eggs. For studies on virus binding, the virus-containing allantoic fluids were clarified from cellular debris by low-speed centrifugation and used without further purification. Nucleotide sequences used in this study are available in the GenBank database under the accession numbers AY428485, AY428494, AY428495, AY428497, AY428499, AY428500 (Kaverin et al., 2004).

Monoclonal antibodies

A panel of virus-neutralizing monoclonal antibodies to the HA of H9 strains was used. This panel included six MAbs to A/Duck/Hong Kong/Y280/97 (H9N2). The MAbs were produced by the Department of Infectious Diseases, St. Jude Children's Research Hospital, by the method of Kohler and Milstein (1976).

Solid-phase direct binding assay for measuring receptor-binding activity

The binding specificity of H9 influenza virus escape mutants was investigated in a direct binding assay. Plates were coated with viruses at a titer of 4–8 hemagglutination units (50 μl/well) at 4 °C for 16 h followed by washing with 0.05% Tween 20 in phosphate-buffered saline (PBS-T). After the addition of Sug-PAA-biot, 30 μl/well in PBS supplemented with 0.02% of Tween 20;

0.02% of bovine serum albumin, and 3 μM of the neuraminidase inhibitor (2,3-didehydro-2,4-dideoxy-4-amino-*N*-acetyl-D-neuraminic acid) (working buffer) plates were incubated at 4 °C for 1 h. The starting concentration of Sug-PAA-biot was 20 μM on sialic acid; 2-fold serial dilutions were used. Plates were washed with a cold PBS-T and incubated with streptavidin–peroxidase in the working buffer at 4 °C for 1 h. After washing, 100 μl/well of substrate solution (0.1 M sodium acetate, pH 5.0, containing 4 mM *o*-phenylendiamine and 0.004% H_2O_2) was added and the reaction was stopped with 2 M H_2SO_4. Optical density was determined at 492 nm with a Multiscan plate reader (Labsystems, Finland). The dissociation constants (K_d) were determined as Neu5Ac concentration at the point $A_{max}/2$ of Scatchard plots. The reported data represent the mean of at least four individual experiments for each mutant virus.

The binding of H9 escape mutants to fetuin was also performed in a direct solid-phase assay with the immobilized virus and horseradish peroxidase-conjugated fetuin (Gambaryan and Matrosovich, 1992).

Serologic methods

Direct enzyme-linked immunosorbent assay (ELISA) was performed essentially as described by Philpott et al. (1989) with some modifications. Briefly, 4-8 hemagglutinating units of virus were bound to each well at 4 °C in PBS pH 7.4. Twofold serial dilutions of MAbs in PBS containing 0.05% Tween 20 and 5% bovine serum were added, and plates were incubated for 1 h at room temperature, washed five times with PBS-T, and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse goat immunoglobulin (Sigma). The enzymatic activity was detected by hydrolysis with 0.004% hydrogen peroxide in the presence of 4 mM *o*-phenylenediamine in 0.1 M citrate-phosphate buffer, pH 5.0 and was quantified by measuring the absorbance at a wavelength of 492 nm. The K_d was determined as the concentration of Mab at the point $A_{max}/2$ of Scatchard plots. The reported data represent the mean of dissociation constants of virus–Mab complexes calculated in three or four individual experiments for each escape mutant.

The competitive assay was based on the competition for binding sites on the viral particle between nonlabeled monoclonal antibody and enzyme-labeled sialylglycoprotein fetuin (Matrosovich et al., 1993) or biotinylated polyvalent synthetic sialoglycoconjugates. Briefly, influenza viruses diluted to the titer of 4–8 hemagglutination units were adsorbed onto the wells of 96-well PV-microtiter plates from Costar, USA (50 μl/well) at 4 °C for 16 h. After unbound virus was washed off with PBS-T, 50 μl of solutions containing a fixed amount of fetuin labeled with horseradish peroxidase or biotinylated polyvalent synthetic sialoglycoconjugates and a variable amount of nonlabeled monoclonal antibody were added to the plate, which was then incubated for 1 h at 4 °C. The solutions were prepared in PBS supplemented with 0.02% bovine serum albumin, 0.02% Tween 20, and 3 μM of the neuraminidase inhibitor (2,3-didehydro-2,4-dideoxy-4-amino-*N*-acetyl-D-neuraminic acid). After this incubation, the plates were washed with PBS-T, and the amount of labeled fetuin or biotinylated polyvalent synthetic sialoglycoconjugates was determined by using the standard *o*-phenylenediamine chromogenic substrate.

The dissociation constants of the virus complexes with Mabs were calculated for each concentrations of the compound used in the competitive reaction, and the results were averaged.

Acknowledgments

These studies were supported by NATO Collaborative Linkage Grant 979155 and by grants 02-04-48109, 03-04-48353, and 04-04-48819 from the Russian Foundation for Basic Research (RFBR). We thank Richard J. Webby for critical comments and for editorial assistance.

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