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Influence of single amino acid substitutions in the hemagglutinin on the antigenic and receptor-binding properties of influenza virus B/Florida/04/2006 of Yamagata-like evolutionary lineage

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ABSTRACT

Influenza A and B viruses use sialylated oligosaccharide chains expressed on the surface of a host cell as the cell entry receptors. The type of the bond between sialic acid (SA) and the neighboring galactose residue (Gal) is one of the main characteristics that define the type of receptor. Influenza viruses recognize SAα2-3Gal- or SAα2-6Gal-structures on the surface of the cells. Influenza A viruses of avian origin bind α2-3-sialylated glycans, while the human strains bind preferentially α2-6-sialylated ones. However, the receptor-binding specificity of influenza B viruses has not been characterized sufficiently so far. In this study, we selected the escape mutants of influenza B/Florida/04/2006 strain (Yamagata-like lineage) using monoclonal antibodies (mAb) to hemagglutinin (HA). The analysis of the amino acid sequences of mAb-induced escape mutants revealed the single amino acid substitutions 40Tyr→His, 85His→Tyr, 202Asn→Lys and 242Ser→Arg in 10F4-, 8H11-, 8H3- and 9A3-induced HA variants, correspondingly. It was shown that the single amino acid substitutions 202Asn→Lys and 242Ser→Arg alter the receptor-binding specificity of the influenza B virus. These findings are important for the understanding of the influence of individual amino acid residues in HA on the receptor-binding properties of influenza B Yamagata-like lineage viruses and allow us to predict the possible ways of their evolution.

INTRODUCTION

The influenza viruses B are circulating mostly in the human population and cause epidemics every 2-3 years. The disease usually has a severe form and could have a lethal outcome. The first step in the development of infection is the interaction of the influenza virus glycoprotein – hemagglutinin (HA) – with neuraminic acid – the top residue of carbohydrate chains located on the surface of the host cell. The N-acetyl-neuraminic acid (Neu5Ac) is the predominant form of sialic acids (SA) found in mammalian cells. It is known that the avian influenza A viruses have an affinity predominantly for hydrocarbons with the *a*2-3 glycoside bond between SA and the neighboring galactose (GA) residue while the human influenza A viruses interact mostly with the SA*a*2-6Gal chains [1-4]. The receptor-binding specificity of the influenza A viruses had been studied in detail and it is considered to be one of the factors that influence virus pathogenicity [5, 6]. As regards the receptor-binding specificity of influenza B viruses, there is rather limited data available [7-9]. Although all of the influenza B viruses belong to the same type, from the beginning of 1980s they are divided to two antigenically different evolutionary branches. The reference-viruses B/Victoria/2/1987 and B/Yamagata/16/1988 are considered to be the first representatives of these two branches [10]. At present, it is known that virus strains of the B/Yamagata lineage predominantly bind to the SA*a*2-6Gal oligosaccharides, while the virus strains of B/Victoria lineage recognize both SA*a*2-6Gal- and SA*a*2-3Gal- chains [11].

This paper is focused on the analysis of the influence of the single amino acid substitutions in the HA heavy chain on the receptor-binding properties of the influenza virus B/Florida/04/2006 of the Yamagata evolutionary lineage.

MATERIALS AND METHODS

Monoclonal antibodies

Monoclonal antibodies (mAb) to the influenza B viruses were obtained in the laboratory of Biotechnology of the diagnostic preparations of Research Institute of Influenza according the method [12] modified as follows.

The Balb/c mice were intraperitoneally immunized with 70 μg of the influenza virus B/Florida/04/2006 which was purified by ultracentrifugation. After 3 months, the mice were boosted by the purified fraction of the surface glycoproteins of the same virus (15 μg). After

72 h the splenocytes of immune animals were hybridized with the mouse myeloma cells of the PxAg.653 line in 10 : 1 ratio in the presence of 50% PEG-2000 solution in DMEM. The traditional ELISA was used in the course of the primary clones testing using the fraction of the surface glycoproteins of the influenza virus B/Florida/04/2006 and peroxidase labeled anti-mouse IgG in dilution 1:10,000 (Sigma, USA). Clones with the highest specific activity were selected, cultivated in HAT media and exposed to five-fold re-cloning. The obtained stable mAb producing clones were frozen.

The hemagglutination assay

The hemagglutination assay (HA) was performed according to the WHO recommendations. The 50 µl aliquots of 0.5% suspension of chicken erythrocytes were added to the twofold diluted samples of virus in 50 µl of 0.1 M phosphate buffered saline (PSB). Virus titer was determined as the reciprocal of the maximum dilution at which the agglutination of erythrocytes was observed. The 1% suspension of guinea pig and horse erythrocytes were used for receptor-binding analysis.

The hemagglutination inhibition assay

The hemagglutination inhibition assay (HAI) was performed according to the recommendations of the WHO. Twofold dilutions of mAbs were prepared in 50 µl of 0.1 M PBS. Then, 50 µl of the virus suspension containing 4 HA units were added to each well. After 1 h incubation, 100 µl of 0.5% chicken erythrocytes were added to the microtiter wells. The reciprocal of the maximum dilution of mAb at which the inhibition of the hemagglutination was observed was defined as HAI titer.

The selection of the escape mutants

The previously described method was used to obtain the escape mutants [13]. The samples of the B/Florida/04/2006 virus were incubated with the virus specific mAbs for 1h at 37°C following by injection into the chicken embryos. Virus yield was harvested and cloned by the limiting dilution method. Virus antigenic properties were investigated by HAI with the panel of mAbs.

Sequencing

RNA of the influenza B virus was isolated using the QIAamp Viral RNA MiniKit (Qiagen) reagents. The reverse transcription of RNA was performed for 40 min at 37°C using the random hexamer primers and commercial kit Reverta-L (Interlabservice, Russia). Polymerase DiaTaq (Interlabservice, Russia) was used for the amplification step. The direct sequencing was performed by means of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) using the genetic analyzer GA3130 (Applied Biosystems, USA).

RESULTS AND DISCUSSION

The characteristic of mAbs used in the study

In order to identify the virus-neutralizing epitopes in the HA molecule of influenza B viruses of the Yamagata lineage, we have developed four mAbs to the influenza virus B/Florida/04/2006, namely 8H3, 8H11, 9A3, and 10F4. The obtained mAbs specifically inhibited viruses of the Yamagata lineage while they did not have any virus-neutralizing activity regarding the influenza B viruses of Victorian lineage. According to the western blot analysis, all of the mAbs were binding to the HA1 subunit (unpublished data). Due to the high virus-neutralizing activity of these mAbs, it was possible to generate the escape mutants of B/Florida/04/2006 virus.

Selected escape mutants and their antigenic properties

Four escape mutants of the influenza virus B/Florida/04/2006 corresponding to each of four described above mAbs were obtained. In order to identify the specific amino acid substitutions in HA of escape mutants the sequencing of HA gene was performed. It was found that all of the obtained escape mutants have the single amino acid substitutions. The amino acid substitutions His→Tyr at position 85 (88 according to the conventional numbering system for H3N2 influenza A virus, shown further in parentheses), and Tyr→His at position 40 (50) were identified in the escape mutants selected by using mAbs 8H11 and 10F4, respectively. Both substitutions are located in HA1 subunit close to the HA stem region of the influenza B virus (Fig. 1).

The escape mutant produced by interaction with the 8H3 mAb has the amino acid substitution Asn→Lys at position 202 (193). This position is located in the HA

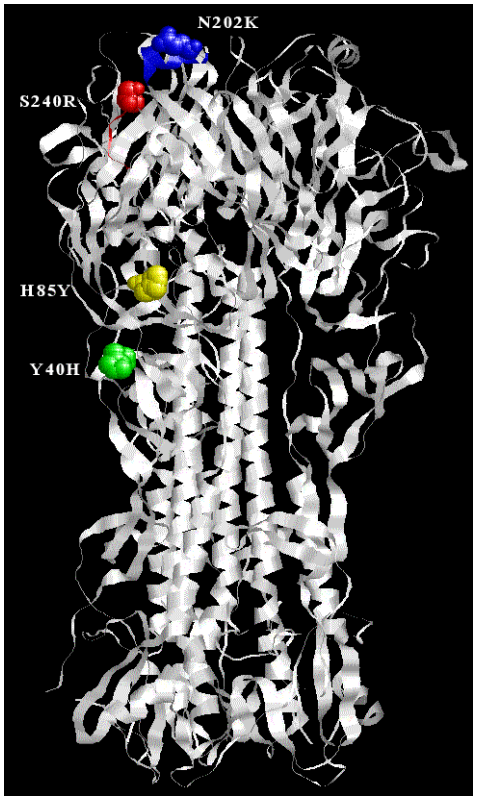

Fig. 1. The localization of the amino acid substitutions in the HA molecule of escape mutants of influenza B/Florida/04/2006 virus of the Yamagata lineage (the helix 190 is shown in blue, the loop 240 is shown in red).

helix 190 [14] which forms the antigenic site BB1 [15]. The escape mutant, selected by using the mAb 9A3, has the amino acid substitution 242 (227) Ser→Arg. This amino acid residue is located in the loop 240 and contributes to the formation of the receptor-binding pocket of the type B influenza virus [16]. The three-dimensional model of HA which shows the localization of the immunodominant epitopes was created based on the identification of the variable epitopes in escape mutants with the use of published crystal structure data of the HA molecule of the influenza virus B/Yamanashi/166/1998 (PDB ID: 4M40) [16] and the RasMol software (version 2.7.4.2).

The obtained escape mutants were analyzed by HAI with mAbs (Table 1). It was shown that all of the produced escape mutants completely lost their ability to interact with the homologous mAb. It should be mentioned that escape mutants 8H3, 8H11, and 10F4 retained the parent phenotype of binding with the heterologous mAbs (Table 2). At the same time, the escape mutant 9A3, in addition to the acquired resistance to the homologous mAb lost the ability to interact with the heterologous mAb 8H3. The analogous phenomenon has been described in the literature [17]. The authors showed that from the eight different escape mutants (V1 – V8) of virus B/Osaka/983/97 of Victorian lineage, selected with mAb 10B8, only two (V3 and V8), which have the amino acid substitution Lys→Thr at position 203 (202 according to the Yamagata numbering and 193 according to the H3 numbering), lost their ability to interact with the heterologous mAb 8E6. At the same time, the escape mutants M1 and M2 selected with mAb 8E6 that have the substitution of 241(240/225) Pro→Ser were interacting with the heterologous mAb 10B8. This suggested that the amino acid residue at position 203 (202/193) is part of the 8E6 mAb epitope or that it is located in close proximity to this epitope.

Escape mutants receptor-binding specificity

As it was shown recently, the influenza B virus strains of the Yamagata lineage interact predominantly with the SA*a*2-6Gal-containing carbohydrate chains while the Victorian isolates recognize both types of sialosides [11]. In order to explore the effect of the different HA amino acid substitutions on the virus receptor-binding specificity, we analyzed the relative activity of escape mutants with respect to the erythrocytes of different animal species (Table 2). It is known that the structures of the sialylated oligosaccharide chains, expressed on the surface of erythrocytes of chicken, guinea pigs, and horses primarily differ by the bond type between the SA and Gal [18].

As is shown in Table 2, the wild type virus B/Florida/04/2006 did not show any binding with the horse erythrocytes in contrast to the guinea pig and chicken erythrocytes. That allows to assume that this virus strain predominantly interacts with the *a*2-6-sialylated chains. Interaction patterns of the escape mutants 8H11 (85His→Tyr) and 10F4 (40Tyr→His) with the corresponding erythrocytes were similar to those of the wild type virus. However, the mutant 8H3 with the substitution 202Asn→Lys acquired the capability to interact with the horse erythrocytes, which have mostly SA*a*2-3Gal-terminated oligosaccharides expressed on their surface. As it was shown previously [19], the majority of the amino acid residues surrounding the influenza B virus HA receptor-binding pocket are identical for the virus strains of the Yamagata and Victorian lineages with the exception of the several amino acids at positions 163, 198, 202, and 203 (numbering for the Victorian lineage). In addition, it should be mentioned that there is no potential N-glycosylation site at 163Asn in the HA of the Yamagata-like viruses. Furthermore, while the Victorian lineage viruses have the negatively charged

Table 1. The interaction of the escape mutants with the monoclonal antibodies 8H3, 8H11, 9A3, and 10F4 in HAI

Viruses	The titer of the mAb in HAI				Amino acid substitutions in HA
	8H3	8H11	9A3	10F4	
B/Florida/04/2006	20480	160	5120	320	-
Escape mutant 8H3	20	80	2560	160	202Asn→Lys
Escape mutant 8H11	20480	≤20	2560	160	85His→Tyr
Escape mutant 9A3	<20	80	≤20	160	242Ser→Arg
Escape mutant 10F4	20480	160	2560	≤20	40Tyr→His

Table 2. The receptor-binding properties of the escape mutants of the influenza B/Florida/04/2006 virus

Viruses	The HA titer			Amino acid substitutions in HA
	Chicken erythrocytes[a]	Guinea pig erythrocytes[b]	Horse erythrocytes[c]	
B/Florida/04/2006	128	128	⩽ 2	-
Escape mutant 8H3	128	64	64	202Asn→Lys
Escape mutant 8H11	128	128	<2	85His→Tyr
Escape mutant 9A3	128	64	8-16	242Ser→Arg
Escape mutant 10F4	64	64	<2	40Tyr→His

[a] Carbohydrate chains are terminated with both SA*a*2-3Gal and SA*a*2-6Gal.
[b] Carbohydrate chains are predominantly terminated with the SA*a*2-6Gal.
[c] Predominantly contain SA*a*2-3Gal-terminated carbohydrate chains.

198Glu, neutral 202Ala, and positively charged 203Lys residues in the helix 190, Yamagata-like viruses contain positively charged 197Lys, 201Lys, and neutral 202Asn, respectively. It is assumed that these four amino acid residues can play an important role in the recognition of the sialylated chains [11]. As is shown in Table 2, the substitution of the Asn to Lys at position 202 (203 according to the Victorian lineage numbering) affected the HA properties apparently by broadening its binding capabilities. Thus, the escape mutant 8H3, in contrast to the wild type virus, binds to the horse erythrocytes (with mostly *a*2-3-sialylated chains) preserving at the same time the parent phenotype of the agglutination of the guinea pig erythrocytes (with mostly *a*2-6-sialylated chains). It should be mentioned that the wild type B/Florida/04/2006 as well as all the escape mutants contain Lys at positions 197 (198 for the Victorian lineage) and 201 (202 for the Victorian lineage), which is typical for the Yamagata-like viruses. As was shown previously, the virus B/Victoria/504/2000, which interacts with the receptors with oligosaccharides terminated with SA*a*2-6Gal, gained substitutions 141Gly→Glu, 162Arg→Met, and 196Asp→Tyr in the course of adaptation to growth in chicken embryos. As a result, the receptor specificity of the adapted virus HA has extended by inclusion of the SA*a*2-3Gal containing oligosaccharides [7]. The analysis of the virus B/Florida/04/2006 HA sequence and all the escape mutants showed that there were no substitutions at these positions – all the obtained escape mutants had 141Gly, 162Arg, and 196Asp residues as well as the wild type virus, which was used for selection. Furthermore, we discovered that neither the virus B/Florida/04/2006 nor any of the obtained escape mutants had substitution at position 95Phe. It is known that the amino acid residue at this position plays an important role in the formation of the receptor-binding pocket and defines the lower influenza virus B affinity for the receptors in comparison with the influenza virus A (98Tyr) [20].

One more escape mutant – 9A3 – gained the ability to interact with horse erythrocytes as well as 8H3. Escape mutant 9A3 has the amino acid substitution at 242(227) Ser→Arg that is the part of the loop 240. The loop 240 and helix 190 are located in the distance from the membrane and form the top and left edge of the HA receptor-binding pocket of the influenza B viruses. The rearrangement of the loop 240 side chains could significantly change the HA antigenic properties [16]. There is a hydrogen bond between the residues 242(227) Ser and 240(225) Pro. The substitution of Ser by Arg at position 242 probably breaks this hydrogen bond that has an impact not only on the HA antigenic properties but also on its receptor-binding specificity. The assumption about the mutual influence of the amino acid residues located in the loop 240 and helix 190 is also proven by the fact that escape mutant 9A3 lost its ability to interact with mAb 8H3.

CONCLUSION

The results of this investigation showed that the amino acid substitutions in the HA molecule that appear due to the escape of the influenza virus B/Florida/04/2006 from the neutralizing action of mAbs 8H3, 8H11, 9A3, or 10F4 not only influence the virus antigenic properties but also could change its receptor specificity. Thus, the substitution of Asn at position 202 by Lys in HA molecule probably broadens the spectrum of the ligands recognized by this virus - in addition to the erythrocytes containing mostly the SAα2-6Gal- chains (guinea pig), the virus began to interact with the erythrocytes having mostly the SAα2-3Gal- chains. The amino acid residue at position 242 (substitution of Ser by Arg) could also influence the influenza virus B/Florida/04/2006 receptor-binding properties.

It is necessary to point out that it is very important to monitor the receptor-binding characteristics of the circulating and emerging influenza A and B viruses because the repertoire of HA receptors defines tissue tropism and, consequently, virulence of the virus [5, 6]. Thus, the patients infected with the influenza B virus strains that bind not only to the SAa2-6Gal-chains but also SAa2-3Gal ones (avian type) are prone to developing pneumonia and more often have the symptoms of gastrointestinal tract damage [11]. In general, the knowledge of the mechanisms of the virus interaction with the host cell, which is directly associated with the HA receptor-binding properties, gives the key to understanding the possible trends of the virus evolution.

CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

CITATION

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