POST-REASSORTMENT AMINO ACID CHANGE IN THE HEMAGGLUTININ OF A HUMAN-AVIAN INFLUENZA H5N1 REASSORTANT VIRUS ALTERS ITS ANTIGENIC SPECIFICITY

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Received July 11, 2008, accepted August 19, 2008

Summary. – It was shown earlier that the reassortant influenza virus having hemagglutinin (HA) gene of A/Duck/Primorie/2621/2001 (H5N2) virus and 7 genes of A/Puerto Rico/8/34 (H1N1) virus produced low yields in embryonated chicken eggs. We found that a variant reassortant selected by serial passages in eggs produced higher yields than the initial reassortant. The variant reassortant had an amino acid substitution in the hemagglutinin N244D (H3 numbering). In this report we demonstrated that the post-reassortment amino acid substitution N244D altered the antigenic specificity of HA as revealed by the loss of reactivity with an anti-H5 monoclonal antibody in hemagglutination-inhibition (HI) test. The results are discussed in association with the evolution of H5 hemagglutinin.

Key words: influenza virus; reassortment; amino acid change; antigenic specificity

The necessity for a functional balance between the affinity of influenza virus HA to sialyl receptors and the functional activity of viral neuraminidase (NA) was shown by the analysis of deletion mutants (Liu and Air, 1993), mutants resistant to NA inhibitors (Gubareva *et al*., 1996), and with the use of reverse genetics (Wagner *et al*., 2000). In our previous studies we observed that influenza A virus reassortants possessing HA derived from avian viruses and low-functional N1 NA of a human parental virus displayed a tendency for virion clustering and produced low yields (Rudneva *et al*., 1993). The high-yield replication could be partially restored by serial passaging of the reassortants (Rudneva *et al*., 1996). The passage variants usually had amino acid substitution in the HA that increased the negative local charge in the vicinity of the receptor-binding pocket, what contributed to a decreased affinity of HA to sialic acid receptors (Kaverin *et al*., 2000, 1998). Recently we demonstrated that an H5N1 reassortant obtained by crossing of a low-virulent avian H5N2 virus and the high-yield human H1N1 strain produced low yields in chicken embryos, whereas a passage variant having an amino acid change N244D in HA accumulated to a higher level (Rudneva *et al*., 2007).

In the present communication we report that this passage variant has a lower affinity to sialic acid-containing substrates and also has an unexpected change in the antigenic specificity of HA as revealed by HI test using a monoclonal antibody (MAb) VN04-13 against the HA of A/Vietnam/1203/04 (H5N1) virus.

The reassortant R22/II was obtained by the reassortment of low pathogenic avian influenza A/Duck/Primorie/2621/2001 (H5N2) virus and human influenza A/Puerto Rico/8/34 (H1N1) virus producing high yields (Rudneva *et al*., 2007). The low-yield H5N1 reassortant R22/II containing HA gene of A/Duck/Primorie/2621/2001 (H5N2) virus and 7 genes

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Abbreviations: HA = hemagglutinin; HI = hemagglutination-inhibition; MAb = monoclonal antibody; NA = neuraminidase

of A/Puerto Rico/8/34 (H1N1) virus was passaged 10 times in embryonated chicken eggs and cloned by limiting dilution passages. The passage variant R22/II-10 produced higher yields than the initial reassortant R22/II, but lower yields than parental A/Puerto Rico/8/34 (H1N1) virus. The sequencing revealed that the HA of R22/II-10 variant had an amino acid change N244D (Rudneva *et al*., 2007).

We measured the affinity of both H5N1 reassortant viruses to high molecular weight sialyl receptors (Fig. 1). The affinity to high-molecular weight sialic acid-containing substrates was measured for fetuin in a direct assay and for 3'-sialylglycopolymers in a competitive assay based on the inhibition of binding of the peroxidase-conjugated fetuin (Matrosovich *et al*., 1993). 3'-sialylglycopolymers were attached to a polyacrylic carrier.

The quantitative competition assay revealed 1.5–2-fold decrease of binding of the passage variant R22/II-10 as compared to the initial reassortant R22/II (Fig. 1). The indicated decrease was relatively moderate when compared to the values obtained in our previous studies with post-reassortment passage variants of H2N1, H3N1, and H4N1 human-avian reassortants (Kaverin *et al*., 2000, 1998). However, the extent of the affinity drop to the sialic substrate measured for R22/II-10 variant corresponded roughly to the enhancement of growth that also was about 1.5-fold (Rudneva *et al*., 2007).

In our earlier studies we showed that amino acid changes in HA that decreased the negative electrostatic charge of the HA protein also decreased the affinity of HA to sialic acid-containing receptors. Subsequently, the tendency to the virion aggregation was reduced and at the same time the virus yield of the human-avian reassortants increased (Kaverin *et al*., 2000, 1998). However, in our studies dealing with H3N2 human-avian influenza virus reassortants, we observed that the post-reassortment changes could occur not only in HA, but also in the NA gene (Ilyushina *et al*., 2005). In order to verify whether any amino acid substitutions occurred in NA during the passaging of the R22/II reassortant, we performed sequencing of the NA genes of R22/II and R22/II-10 reassortants. Sequence analysis did not reveal any change in the NA gene after serial passaging.

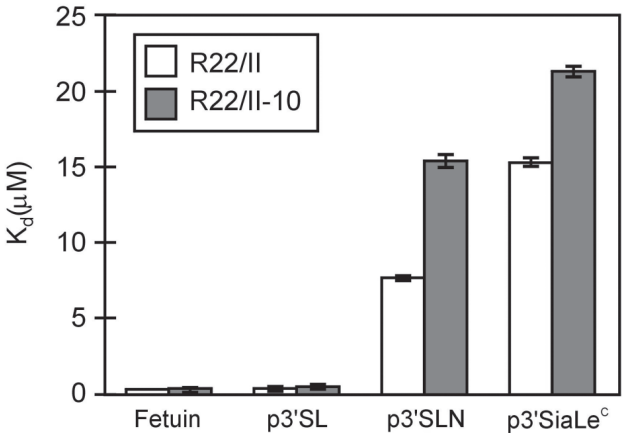

Fig. 1

Affinity of H5N1 reassortant R22/II and its passage variant R22/II-10 to the sialic acid-containing substrates

K_d (mean ± SE×$t_{\alpha, n-1}$, where t_γ = Student's coefficient at the level of significance $\alpha = 0.95$) was measured in four independent experiments. p3'SL: 3'-sialyllactose (Neu5Acα2-3Galß1-4Glcß); p3'SLN: 3'-sialyllactosamine (Neu5Acα2-3Galß1-4GlcNAcß); p3'SiaLe^C: 3'-sialyllactosamine (Neu5Acα2-3Galß1-3GlcNAcß).

Table 1. Reactivity of the reassortant R22/II and its passage variant R22/II-10 in HI test and ELISA with the MAb VN04-13

Virus	HI test*	ELISA**
R22/II	12,800	1,165 ± 71
R22/II-10	200	1,102 ± 64

*HI titer against 8 hemagglutination units.**Absorbance (mean ± SE×$t_{\alpha, n-1}$, where t_α = Student's coefficient with probability $\alpha = 0.95$) was obtained in four independent experiments.

Preliminary results of HI test with a set of anti-H5 MAbs did not reveal any effect of the N244D amino acid change in R22/II-10 variant on the antigenic specificity of HA (Rudneva *et al*., 2007). Nevertheless, we attempted to analyze the antigenic specificity of this variant in more detail by the MAbs against HA of A/Vietnam/1203/04 (H5N1) strain that were characterized in our previous report (Kaverin *et al*., 2007). However, the majority of the tested MAbs (VN04-2, VN04-8, VN04-9, VN04-10, VN04-15, and VN04-16) did not react with the HA of A/Duck/Primorie/2621/2001 (H5N2) virus in HI test and could not be used for the analysis of R22/II and R22/II-10 H5N1 reassortants (data not shown). The only MAb reacting with the wild-type HA of A/Duck/Primorie/2621/2001 (H5N2) virus in HI test was MAb VN04-13, which reacted with the parental virus till the dilution 1:12,800. Thus, we could use this MAb in HI test and ELISA for characterization of R22/II and R22/II-10 viruses (Kaverin *et al*., 2002). In HI test we found a dramatic loss of the sensitivity of variant R22/II-10 in comparison to the initial reassortant R22/II. However, the HA of R22/II-10 retained its ability to bind to the MAb VN04-13 as revealed in ELISA (Table 1). We could not analyze the effect of passaging on the infectivity neutralization, because the MAb VN04-13 did not neutralize the infectivity of A/Duck/Primorie/2621/01 (H5N2) virus and the reassortants contained the same HA gene (data not shown).

In our previous studies was shown that MAb VN04-13 had an unusually broad epitope comprising a part of the antigenic site A and a part of antigenic site B (Kaverin *et al*., 2007). The position 244 is located outside of these antigenic sites and belongs to the area that corresponds to the site D in H3 subtype (Wiley *et al*., 1981). It seemed unlikely that the MAb VN04-13 reacted with three epitopes situated so widely apart. However, site D is located largely at the interface of monomers in HA trimer and the effect of amino acid changes in site D may be affected through a structural modification of an area distant from the position of the amino acid change (Wiley *et al*., 1981). It was noteworthy that the N244D substitution in HA did not preclude the binding of the MAb VN04-13 as revealed in ELISA. It seemed plausible that the MAb did not block the interaction of the receptor-binding pocket with cell receptor while bound to HA of R22/II-10 (Table 1).

The highly pathogenic H5N1 avian influenza virus caused an outbreak of pneumonia in humans in Hong Kong in 1997 (de Jong *et al*., 1997). Since 2003, H5N1 viruses have been spreading in several countries in South-East Asia causing occasional disease in humans (Smith *et al*., 2006). A variant of H5N1 virus spreads to the Middle East, Africa, Siberia, and Western Europe (World Health Organization, 2008). The H5N1 viruses undergo an expressed genetic divergence including the antigenic variability of the HA (Stevens *et al*., 2006). Unlike human viruses, avian influenza viruses are generally considered to be evolutionary static (Webster *et al*., 1992). The antigenic variation of HA in H5N1 viruses was ascribed to a vaccination of birds producing immune background and instigating a process similar to the antigenic drift in human strains (Horimoto *et al*., 2004). Host-dependent selection associated with the crossing of species barrier was also considered as a possible cause of antigenic variation of H5N1 viruses (Huang *et al*., 2007). The observation described in this report may be considered as an indication of existence of yet another mechanism, which involves a post-reassortment amino acid substitution producing pleiotropic effect including a change of the antigenic specificity. We observed this effect in a model system, but we could not measure the effect of the post-reassortment amino acid change on the infectivity neutralization. However, since the reassortment events are suggested to play a role in the evolution of H5 viruses (Duan *et al*., 2007), the phenomenon described in this report should be taken into consideration as a plausible factor in the antigenic evolution of highly pathogenic H5N1 viruses.

Acknowledgments. We thank Dr. Elena A. Govorkova for the kind gift of the monoclonal antibodies. The work was supported by the grants 06-04-48085-a and 07-04-00005-a from the Russian Foundation for Basic Research.

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