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Changes in the hemagglutinin of H5N1 viruses during human infection – Influence on receptor binding ☆

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ABSTRACT

As avian influenza A(H5N1) viruses continue to circulate in Asia and Africa, global concerns of an imminent pandemic persist. Recent experimental studies suggest that efficient transmission between humans of current H5N1 viruses only requires a few genetic changes. An essential step is alteration of the virus hemagglutinin from preferential binding to avian receptors for the recognition of human receptors present in the upper airway. We have identified receptor-binding changes which emerged during H5N1 infection of humans, due to single amino acid substitutions, Ala134Val and Ile151Phe, in the hemagglutinin. Detailed biological, receptor-binding, and structural analyses revealed reduced binding of the mutated viruses to avian-like receptors, but without commensurate increased binding to the human-like receptors investigated, possibly reflecting a receptor-binding phenotype intermediate in adaptation to more human-like characteristics. These observations emphasize that evolution in nature of avian H5N1 viruses to efficient binding of human receptors is a complex multistep process.

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Introduction

Since 1997, highly pathogenic avian influenza A(H5N1) viruses have spread among poultry and wild birds in Asia, the Middle-East, Europe and Africa and caused over 600 reported human infections in 15 countries with a case-fatality ratio of approximately 60% (WHO, 2012). Sporadic human infections continue to occur in countries where A(H5N1) viruses have become endemic in birds, providing a persistent threat to global health due to the possibility of virus adaptation towards efficient transmission among humans and ensuing pandemic spread. Recent evidence from experiments with ferrets, a widely accepted animal model for influenza in humans, suggest that only a limited number of genetic changes is needed for airborne transmission of H5N1 viruses (Imai et al., 2012; Herfst et al., 2012). Thus, monitoring of genetic changes, especially during human infections, and studying the relevance of such changes for human adaptation remains essential.

The molecular mechanisms that allow avian influenza viruses to cross the species barrier from birds to humans are incompletely understood. However, a prerequisite for efficient transmission of avian viruses between humans is a change from preferential recognition by the virus hemagglutinin (HA) of α2-3-sialyl-galactose-terminating host cell receptors, predominant in avian respiratory and gastrointestinal epithelia, to those terminating in α2-6-sialyl-galactose, which predominate in the human upper respiratory tract (Rogers and Paulson, 1983; Rogers and D'Souza, 1989). Accordingly, HAs of the 20th century pandemic viruses (H1N1 in 1918, H2N2 in 1957 and H3N2 in 1968) evolved from their original recognition of avian α2-3-sialyl- to preferential α2-6-sialyl-receptor binding (Connor et al., 1994; Matrosovich et al., 2000; Glaser et al., 2005; Stevens et al., 2006b; Tumpey et al., 2007). Thus to better understand the nature and significance of such adaptive changes, it is essential to monitor HA changes that may affect receptor specificity of avian influenza viruses, particularly during human infection.

In this study, we screened receptor-binding preferences of thirty H5N1 viruses, isolated in MDCK cells, from poultry and humans in Vietnam during 2004 and 2005, by comparing hemagglutination patterns using horse and guinea pig red blood cells (RBCs), which differ in sialic acid receptor distribution (Ito et al., 1997b; Medeiros et al., 2001). We detected patterns indicative of changes in receptor specificity in three human H5N1 isolates. Our analyses of these viruses and their egg-passaged counterparts indicated a rapid emergence of adaptive receptor-binding variants of H5N1 virus, and also demonstrated that marked discrepancies can occur in quasispecies distributions between clinical specimens and cell culture- or egg-grown viruses, thus emphasizing the need for genetic monitoring directly in clinical specimens.

Results

Agglutination of horse RBCs

To screen for the receptor-binding preferences of avian and human influenza H5N1 viruses isolated in MDCK cells, we compared the relative agglutination of horse RBCs, which express predominantly α2-3-sialyl sequences (Ito et al., 1997a) and guinea pig RBCs, which express both α2-3- and α2-6-sialyl sequences (Medeiros et al., 2001). As predicted, seasonal human H1N1 and H3N2 influenza A viruses ($n=10$) did not agglutinate horse RBCs, whereas avian influenza viruses ($n=15$; H5N1, H4N6, H6N1) isolated from poultry agglutinated efficiently both horse and guinea pig RBCs (Table 1). Out of 11 influenza H5N1 viruses isolated from upper respiratory tract specimens of humans, eight agglutinated efficiently both types of RBCs, similar to poultry viruses. However, two of the human H5N1 virus isolates (A/Vietnam/CL1/2004 and A/Vietnam/CL105/2005) resembled seasonal human influenza A viruses and did not agglutinate horse RBCs (Table 1). A third isolate (A/Vietnam/CL2009C/2005) showed a 15-fold lower hemagglutination titer with horse RBCs than with guinea pig RBCs (Table 1 and Supplementary Table S1). These results suggested that three of the 11 human H5N1 viruses had reduced binding to α2-3-sialyl receptors.

Table 1
Hemagglutination titers of MDCK cell- and egg-cultured Influenza A viruses.

Virus	Subtype	Origin	MDCK GMT[a]		ω5 GMT[a]	
			Horse	Guinea pig	Horse	Guinea pig
A/VN/HTD33/04	H1N1	Human	0	75	0	24
A/VN/HTD34/04	H1N1	Human	0	192	0	32
A/VN/HTD566/04	H3N2	Human	0	53	0	4
A/VN/HTD567/04	H3N2	Human	0	21	0	8
A/Ck/VLC13/06	H5N1	Chicken	267	144	12	32
A/Ck/VLC15/06	H5N1	Chicken	491	416	64	24
A/Dk/LAD404/06	H4N6	Duck	341	352	48	128
A/Dk/DTD516/06	H6N1	Duck	224	160	32	32
A/VN/CL1/04	H5N1	Human	0	100	512	192
A/VN/CL2/04	H5N1	Human	52	72	64	48
A/VNCL26/04	H5N1	Human	64	192	256	192
A/VN/CL36/04	H5N1	Human	64	68	64	64
A/VN/PEV016/04	H5N1	Human	32	44	128	96
A/VN/CL100/05	H5N1	Human	112	352	192	384
A/VN/CL105/05	H5N1	Human	0	120	128	320
A/VN/CL107/05	H5N1	Human	112	464	NA[b]	NA[b]
A/VN/CL115/05	H5N1	Human	112	200	8	20
A/VN/CL119/05	H5N1	Human	80	136	384	384
A/VN/CL2009C/05	H5N1	Human	27	405	128	96

ω5: five serial passages in the allantois of embryonated hen eggs.
[a] Geometric mean titers of triplicates.
[b] Not analyzed due to technical difficulties.

We investigated whether the hemagglutination properties of these three human H5N1 viruses would 'revert' to efficient horse RBC agglutination when replicating in the presence of predominantly α2-3-sialyl receptors. Indeed, after passaging the viruses five times in the allantois of embryonated chicken eggs, which contain only α2-3-sialyl receptors (Ito et al., 1997b), all three viruses (re)gained efficient agglutination of horse RBCs (Table 1). Passage in eggs did not, however, alter the agglutination patterns of other avian influenza viruses isolated from humans or poultry or seasonal human influenza viruses (Table 1). Plaque assays showed that the MDCK cell isolate and egg passaged variant of A/Vietnam/CL1/2004 and A/Vietnam/CL105/2005 exhibited similar growth characteristics in MDCK cells.

Amino acid changes in HA

To identify amino acid changes in HA associated with the hemagglutination patterns of A/Vietnam/CL1/2004, A/Vietnam/CL105/2005, and A/Vietnam/CL2009C/2005, the sequences of HA1 of the MDCK cell isolates were compared with those following passage in eggs. A single amino acid difference was observed in the HA of each egg-grown virus (Table 2): Asp186Glu in A/Vietnam/CL1/2004, Val134Ala in A/Vietnam/CL105/2005, and Phe151Leu in A/Vietnam/CL2009C/2005 (H5 numbering). Changes in the first two viruses represented reversions to the avian H5 consensus sequence following egg passage. The consensus residue at position 151 is Ile rather than Leu. Interestingly, sequence analysis in the original clinical specimen revealed a subpopulation of 151Ile and not Leu (see below).

Quasispecies distributions in clinical specimens and virus isolates

To determine whether the sequences in the MDCK cell isolates were representative of those present during the human infection

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