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Selection of variant viruses during replication and transmission of H7N1 viruses in chickens and turkeys

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ABSTRACT

The influence of different glycosylation patterns of the haemagglutinin glycoprotein of H7N1 avian influenza viruses on virus replication *in vivo* was examined. Experimental infection of chickens and turkeys was carried out with H7N1 avian influenza viruses with alternative sites of glycosylation in the haemagglutinin and infected birds were sampled daily by swabbing the buccal and cloacal cavities. cDNAs of the HA1 coding region of the HA gene were prepared from the swabs and cloned into plasmids. Sequencing multiple plasmids made from individual swabs taken over the period of virus shedding showed that viruses with specific patterns of glycosylation near the receptor binding site were stable when birds were infected with a single variant, but when presented with a mixed population of viruses encoding differing patterns of glycosylation a specific variant was rapidly selected in the infected host.

Introduction

Avian influenza (AI) viruses naturally circulate in wild aquatic birds as viruses of low pathogenicity (LP) for poultry but transmission to other avian species can result in more serious sporadic or sustained infections. Transmission of highly pathogenic (HP) AI viruses to domestic poultry or mutation of LP viruses to HP after infection of poultry results in serious disease in which mortality approaching 100% can be observed. However, LP avian influenza viruses (LPAI) can cause significant disease when exacerbated by other infections or unfavourable environmental conditions (Brown et al., 2006; Capua and Alexander, 2009).

Sixteen haemagglutinin (HA) and nine neuraminidase (NA) subtypes of the virus surface glycoproteins have been recognised in viruses collected from birds in the order Anseriformes or in the order Charariiformes; a sub-set of which circulate in mammals, but recently a new sub-type (H17N10) has been reported to be present exclusively in the mammalian order Chiroptera (Olsen et al., 2006; Tong et al., 2012; Webster et al., 1992). Transmission of viruses from water birds to a new host species does not always result in a successful persistence and spread of the virus within the population, and replication in a new species will impose pressures on the virus to adapt to the new host. Transmission from wild bird to domestic poultry species is seen frequently (Alexander and Brown, 2000; Webster et al., 1997) and can cause mild to severe disease (Alexander, 2007; Xu et al., 2007). Infection by HPAI and LPAI viruses in domesticated poultry represents a substantial threat to poultry production and can have a serious economic impact. Avian influenza viruses also can pose a risk to public health through their ability to cause zoonotic infections (Butt et al., 2005; Guo et al., 1999; Koopmans et al., 2004).

A number of distinct molecular changes in all gene segments have been shown to play a pivotal role in adaptation, persistence and increased virulence in both poultry and mammalian hosts (Baigent and McCauley, 2003; de Wit et al., 2010; Hatta et al., 2001; Reading et al., 2009; Sorrell et al., 2010; Wu et al., 2009; Yen et al., 2009). Adaptation within a new host species has been notably observed with respect to the two virus glycoproteins which are responsible for virus binding to sialic acid receptors on the host cell surface, fusion of the virus with the cell (both mediated by HA) and release of assembled progeny virus from the infected cell (mediated by NA). During interspecies transfer of LPAI viruses from wild birds to poultry and circulation within a new host species, the HA genes of H5 and H7 viruses can acquire (1) additional glycosylation sites adjacent to the receptor binding site (Banks and Plowright, 2003; Banks et al., 2001; Matrosovich et al., 1999) and (2) multiple basic amino acid residues (arginine and lysine) at the HA cleavage site, which is usually a prerequisite for high pathogenicity. These changes can also be associated with alterations to the NA glycoprotein in which a deletion of several

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amino acids within the stalk domain is observed (Gulati et al., 2009; Matrosovich et al., 1999). The combination of these adaptive changes in the HA and the NA are thought to modify the complex interactions between the virus receptor (sialic acid), the receptor binding protein (HA) and the NA, the protein responsible for release of the virus from its receptor, therefore altering the balance between the three factors that can result in differences in replication characteristics. These differences alter virus replication in cultured cells (Baigent and McCauley, 2001; Mitnaul et al., 2000; Wagner et al., 2002) and can result in changes in virulence for chickens and mice (Matsuoka et al., 2009; Munier et al., 2010; Reading et al., 2009).

The N-linked carbohydrate side chains at certain positions on the HA of influenza A viruses isolated from various animals and humans are highly conserved and therefore appear to be essential for the formation or maintenance of functional HA (Schulze, 1997). However, oligosaccharide diversity might have a major selective effect and the presence or absence of oligosaccharides on glycosylation sites can determine the fitness of the virus for growth by modulating the biological properties of the HA by interfering with antibody binding (Munk et al., 1992; Schulze, 1997; Skehel et al., 1984), receptor binding (Klenk et al., 2002; Marinina et al., 2003; Wagner et al., 2000), proteolytic activation (Deshpande et al., 1987) and trimer assembly (Roberts et al., 1993).

Among H5 subtype viruses two potential N-linked glycosylation sites at asparagine residues 131 and 158 (H3 numbering) located at the tip of the globular head of HA1 close to the receptor binding site are highly variable (Banks and Plowright, 2003; Matrosovich et al., 1999). An equally striking variability in glycosylation has been documented in H7 subtype viruses (Banks and Plowright, 2003), notably including LPAI H7N1 viruses isolated during circulation in poultry over a nine month period in Italy during 1999–2000. From this epizootic, of 45 HA sequences of LPAI viruses retrieved from public databases, 7 isolates contained a signal for glycosylation at position 123 (133 in H3 numbering) and lacked a motif at position 149 (158 in H3 numbering); the HA from 17 isolates had a glycosylation motif at 149 but not at residue 123, and the remaining 21 viruses had no glycosylation sequon at either site. In contrast, later in the epizootic, following the emergence of HPAI isolates, of 35 HPAI viruses sequences available, the HA of 34 viruses had a glycosylation signal at the 123 site but not at 149 and the remaining isolate lacked a glycosylation motif at either site. The evolutionary drive that led these viruses to acquire additional carbohydrate moieties on the HA and become established in poultry is not clear but modulation of receptor binding might provide these viruses with a greater potential for infection and transmission, as suggested by Matrosovich et al. (1999); alternatively, masking of an antigenic site might make it possible for re-infection of poultry previously infected with viruses of the same sub-type.

In the present study, we sought to investigate the evolution of glycosylation sequons in HA1 of H7N1 viruses in poultry infections and how the virus might change and adapt in an individual poultry host during the course of a single infection. In particular, we asked: (1) are distinct glycosylated variants equally selected and shed from an infected bird and (2) are the differently glycosylated viruses equally well transmitted to contact birds? We established experimental infections of chickens and turkeys with selected H7N1 (LPAI) viruses isolated early during the outbreak in poultry in Italy in 1999 that had conserved glycosylation sites at Asn residues located at positions 12, 28 and 231 in HA1 and residues 82 and 154 in HA2, but the glycosylation at residues 123 and 149 of HA1 was variable. Of the three selected model viruses examined (1) A/turkey/Italy/3466/99 (Italy/3466) encoded a glycosylation site at 123 but not at position 149, (2) A/turkey/Italy/4042/99 (Italy/4042) had glycosylation sites at residue 149 and lacked the site at 123 and (3) A/chicken/Italy/1279/99 (Italy/1279) was found to represent a mixed population with viruses showing four patterns of glycosylation at the two sites with the HA gene coding either no glycosylation at either site, glycosylation at one or the other of the two sites and with glycosylation at each of the two sites. Chickens or turkeys were infected with these viruses and sequence analysis of the HA gene was carried out on multiple cDNA clones prepared from swab samples taken from infected birds to determine the degree of variation or selection that might occur in the virus during infection.

Results

Genetic differences within virus samples used as inocula

To investigate the extent of variation of viruses within infected chicken and turkey hosts during the course of infection both species were infected experimentally with three H7N1 (LPAI) viruses, Italy/3466, Italy/4042 and Italy/1279. These viruses had been chosen on the basis of variation at the N-linked potential glycosylation sequons, as described above, in the vicinity of the receptor binding site of the HA. The reported sequences for Italy/3466 had a potential glycosylation site at residue Asn 123 but lacked a site at residue Asn 149 (mature H7 HA1 numbering, open reading frame numbers Asn 141 and Asn 167 respectively). Italy/4042 contained a potential glycosylation motif at residue 149 but not at position 123. The reported sequence for Italy/1279 virus lacked glycosylation motifs at both positions. Sequences of the remaining gene segments of these viruses were not available in public databases.

To analyse the genetic difference between these viruses, sequence analysis of all eight gene segments per virus was undertaken. cDNA amplicons covering the complete open reading frames of PB2, PB1, PA, HA, NP, NA, M and NS genes were prepared from allantoic fluid made for inoculum stocks; cDNAs were cloned and between 6 and 10 individual cDNA clones prepared from each segment for each virus were sequenced. Sequence analysis based on this number of cDNA clones analysed showed very limited polymorphism within each gene segment for each virus (Table 1) with the exception of the HA gene of Italy/1279 virus which showed marked polymorphism. This heterogeneity was observed within the inoculum at nucleotide positions 427 and 505 resulting in amino acid changes (A→T or vice versa) at residues 125 and 151 which alters the potential glycosylation sequon at amino acid residues Asn 123 and Asn 149. The level of heterogeneity of the HA1 coding region of each virus was examined in greater detail using a ‘deep amplicon’ sequencing approach.

Identification of glycosylation status at residues Asn 123 and Asn 149

In order to determine whether potential glycosylation motifs at position 123 and 149 were able to be glycosylated, four recombinant reverse genetics (RG) viruses containing HA and NA gene of Italy/1279 virus were generated. The HA gene of RG viruses encoded the four different potential glycosylation patterns observed in the Italy/1279 inoculum stock: (1) glycosylation at residue 149 and not at residue 123, (2) glycosylation at residue 123 and not at residue 149, (3) glycosylation at both residues 123 and 149 and (4) no glycosylation at either site. SDS-PAGE analysis of HA1 of these RG viruses showed differential mobility depending on the potential glycosylation status (Fig. 1A). The electrophoretic mobility of the HA1 polypeptide of viruses with potential glycosylation at residue 149 (Fig. 1A, lane 1) or at residue 123