Journal of General Virology (2004), **85**, 1001–1005 DOI 10.1099/vir.0.19519-0

Short Communication

The index influenza A virus subtype H5N1 isolated from a human in 1997 differs in its receptor-binding properties from a virulent avian influenza virus

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Received 21 July 2003
Accepted 23 December 2003

To gain insight into the events that occur when avian influenza viruses are transmitted to humans, the receptor-binding properties of the index H5N1 influenza virus isolated from a human in 1997 and the A/turkey/Ontario/7732/66 (H5N9) virus were compared, by using a haemadsorption assay. Cells expressing the haemagglutinin (HA) of the human isolate were adsorbed by both chicken red blood cells (RBCs) and human RBCs; those expressing the avian virus HA were only adsorbed by chicken RBCs. These results indicate that human and avian influenza virus H5 HAs differ in their recognition of sialyloligosaccharides on the RBCs of different animal species. Mutational analyses indicated that differences in both the oligosaccharide chains and in the amino acid sequences around the HA receptor-binding site were responsible for this difference in receptor binding. These data further support the concept that alteration in receptor recognition is important for replication of avian viruses in humans.

Influenza A viruses infect a variety of animals, including humans, pigs, horses, marine mammals and birds. There are a number of influenza virus A subtypes, haemagglutinins (HAs), H1 to H15, and neuraminidases (NAs), N1 to N9, all of which are found in birds (Wright & Webster, 2001). Virus subtypes that are not found in humans have the potential to cross the species barrier and possibly cause pandemics (Shortridge, 1995). In 1997, for example, an H5N1 virus was transmitted from birds to humans in Hong Kong, killing six of the 18 people it infected (Claas *et al.*, 1998; Subbarao *et al.*, 1998). The mechanism by which this avian virus infected and caused disease in humans, while other avian viruses have not, remains unknown. However, high HA cleavability, a mutation in the PB2 gene and cytokine dysregulation imposed by the NS1 have been implicated in the virulence of this virus (Hatta *et al.*, 2001; Seo *et al.*, 2002; Cheung *et al.*, 2003).

A figure showing the haemadsorption of the wild-type and selected mutant HAs is available as supplementary data in JGV Online.

The receptor-binding preference of influenza viruses correlates with the animal species from which the viruses are isolated; human isolates preferentially bind to the terminal sialic acid of glycoprotein and glycolipid receptors with α2,6 linkages to galactose (SAα2,6Gal), whereas avian isolates prefer α2,3 linkages (SAα2,3Gal) (Connor *et al.*, 1994; Couceiro *et al.*, 1993; Ito *et al.*, 1998; Rogers & D'Souza, 1989). Alteration in receptor specificity may have occurred when the HA gene of an avian virus was introduced into humans, resulting in the 1957 (Asian influenza) and in the 1968 (Hong Kong influenza) pandemics, since the earliest viruses available from these pandemics recognize SAα2,6Gal (Matrosovich *et al.*, 2000). Interestingly, the index H5N1 human isolate (A/Hong Kong/156/97) from the 1997 outbreak preferentially recognizes the SAα2,3Gal receptor (Matrosovich *et al.*, 1999; Ha *et al.*, 2001), suggesting that the change in receptor preference may not be required for primary human infection by avian influenza viruses.

To understand further the bird-to-human transmission of influenza virus that occurred in 1997, we compared

receptor specificity between the Hong Kong virus and a virulent avian H5 virus by using an assay that has not previously been used to study the 1997 human isolate.

We cloned the cDNA of the HA genes from the index human isolate in the Hong Kong 1997 outbreak, A/Hong Kong/156/97 (HK/97; H5N1) (Claas *et al.*, 1998; Subbarao *et al.*, 1998), and from the virulent avian virus, A/turkey/Ontario/7732/66 (Ty/Ont; H5N9) (Horimoto & Kawaoka, 1994), into the pCAGGS/MCS expression vector, which contains the chicken β-actin promoter (Kobasa *et al.*, 1997; Niwa *et al.*, 1991). When COS-7 cells were transfected with these plasmids (1 μg plasmid per well of a six-well plate) HAs were expressed on the cell surface as detected by immunostaining with a pool of anti-H5 monoclonal antibodies (61B2, 61E2, 81E5; the latter two react with the HA2 portion of the HA; Horimoto *et al.*, 2004) (see supplementary figure in JGV Online). A haemadsorption assay was performed by using human and chicken red blood cells (RBCs). We found that both human and chicken RBCs adsorbed cells expressing HK/97 HA, but only chicken RBCs adsorbed cells expressing Ty/Ont HA (Fig. 3). These data suggest that receptor recognition differs between these two viruses. However, both RBCs bound to cells expressing HAs from other virulent avian viruses including A/turkey/Ireland/1378/85 (H5N8) HA (data not shown). We therefore focused on Ty/Ont HA to analyse further the properties of the HK/97 HA. Human and chicken RBCs contain both SAα2,3Gal and SAα2,6Gal glycoconjugates (Ito *et al.*, 1997; Medeiros *et al.*, 2001); however, our results indicate a quantitative and/or qualitative difference in the relative proportions of these cell surface molecules in the RBCs of humans compared to chickens.

To understand the molecular basis for the species-specific difference in haemadsorption between the two HAs, we compared their amino acid sequences and found that Ty/Ont HA contained two additional *N*-linked potential glycosylation sites at positions 131 and 158 (using the H3 numbering system; 142 and 170 in H5, respectively) that were not present in HK/97 HA. Both of these additional sites are located around the receptor-binding site (Weis *et al.*, 1988) (Fig. 1). Since terminal sialic acids on oligosaccharides near the receptor-binding site are known to affect receptor recognition (Ohuchi *et al.*, 1995), Ty/Ont HA-expressing cells were treated with *Vibrio cholerae* sialidase (10 mU ml^{-1} at 37 °C for 1 h). Following this treatment, human RBCs were able to adsorb Ty/Ont HA-expressing cells (data not shown), indicating that desialylation of the oligosaccharides in Ty/Ont HA is essential for its interaction with human RBCs, but not with chicken RBCs.

To obtain direct evidence that receptor recognition is affected by oligosaccharide side chains near the HA receptor-binding site, we constructed Ty/Ont HA mutants, Ty/Ont HA.131− (Asn-131→Asp) and Ty/Ont HA.158− (Ser-160→Ala), lacking the potential glycosylation sites at positions 131 and 158, respectively (QuikChange XL site-directed mutagenesis kit, Stratagene). We were unable to generate a double mutant lacking both sites, therefore we substituted the sequence that encodes amino acids 123–133 of Ty/Ont HA.131− with the corresponding sequence from HK/97-HA to create Ty/Ont-HA.131/158−, which lacks both potential glycosylation sites. This substitution resulted in the alteration of three amino acids at positions 124, 125 and 129. Although these amino acids do not interact directly with receptor molecules (Weis *et al.*, 1988), we are unable to predict the indirect effects of these amino acid differences on receptor binding. The mobilities of these mutant HAs in SDS-PAGE were faster than that of the wild-type HA, confirming loss of glycosylation (Fig. 2A). Both Ty/Ont HA mutants that lacked only one glycosylation site were haemadsorbed by chicken, but not human, RBCs analogous to wild-type Ty/Ont HA; however, the mutant that lacked both glycosylation sites was adsorbed by human

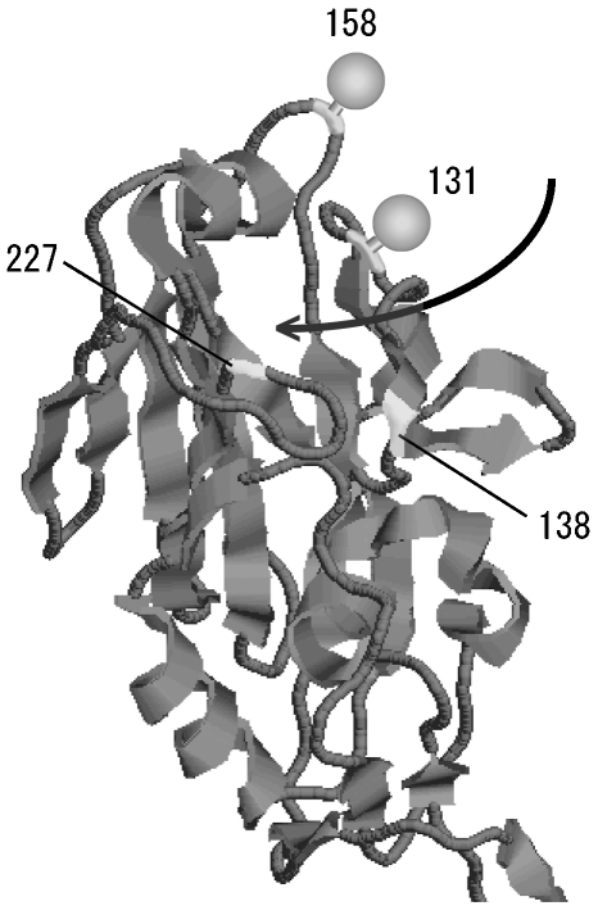

Fig. 1. Globular head portion of H5 HA (Ha *et al.*, 2002; Protein DataBank 1JSM) illustrating the amino acid residues examined in this study. The arrow points to the receptor-binding site. Oligosaccharide side chains of Ty/Ont HA near the receptor-binding site at positions 131 and 158 are shown schematically. Amino acids at positions 138 and 227 are highlighted in white.

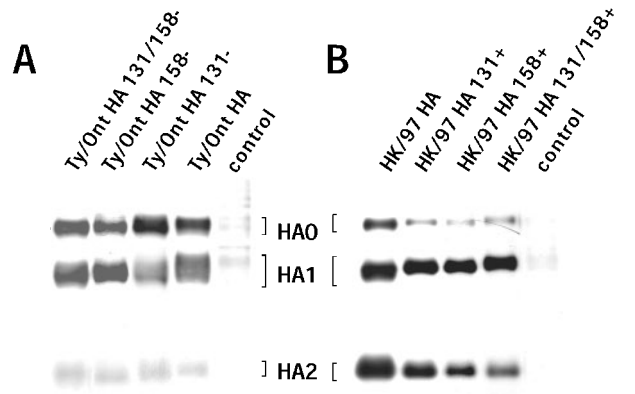

Fig. 2. Confirmation of the deletion or addition of glycosylation sites in the HA mutants. The HAs on the surface of plasmid-transfected COS-7 cells were biotin-labelled using a commercial kit (Amersham Pharmacia). The cells were lysed, reacted with anti-H5 antibodies and then incubated with protein A beads. The immunoprecipitates were resolved by 10 % SDS-PAGE under reducing conditions.

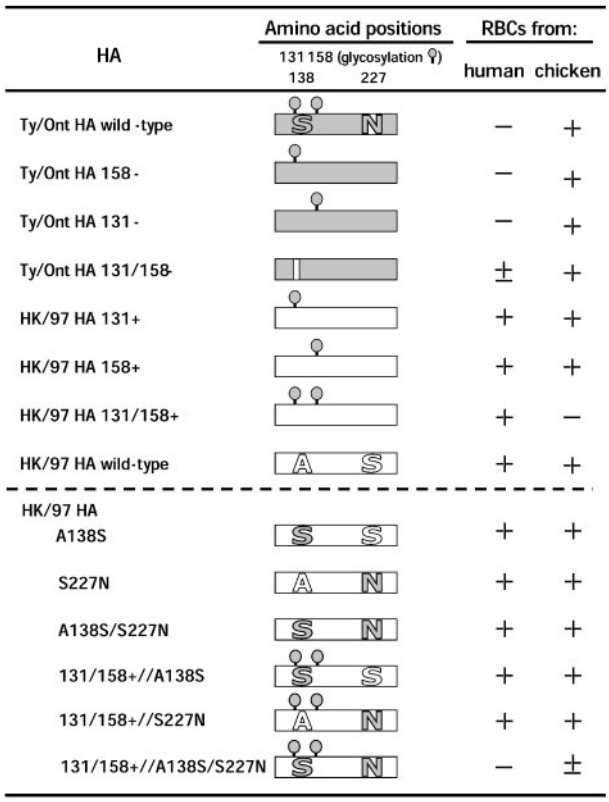

Fig. 3. The effect of glycosylation and amino acid substitution on haemadsorption activity. The result is assessed by counting numbers of haemadsorption-positive and -negative cells in five randomly selected microscopic fields; − represent no haemadsorption; + represent more than 50 % haemadsorption-positive cells; ± represent ~ 10 % haemadsorption-positive cells (for details see supplementary figure in JGV Online).

RBCs, albeit to a more limited extent than wild-type HK/97 HA (Fig. 3). All of the mutants bound to human RBCs after sialidase treatment. We also constructed HK/97 HA mutants (HK/97 HA.131+, 158+ and 131/158+) that contained additional glycosylation sites (Asp-131→Asn and/or Ala-160→Thr substitutions) (Fig. 2B). Two of these mutants, HK/97 HA.131+ and 158+, both of which contained one additional glycosylation site, were haemadsorbed by both human and chicken RBCs, whereas mutant HK/97 HA.131/158+, which possessed two additional glycosylation sites, was not adsorbed by chicken RBCs (Fig. 3). HK/97 HA.158+ resembled A/Hong Kong/486 HA, which contains a potential glycosylation site at position 158. We therefore tested the haemadsorption properties of this strain and found no difference between its HA and that of HK/156. Overall, these data suggest that differences in glycosylation near the HA receptor-binding site do not completely explain the difference in receptor recognition between HAs, although oligosaccharides near the receptor-binding site can alter binding affinity and/or specificity, as revealed by the HK/97 HA mutants.

We then focused on the amino acid sequence in the HA receptor-binding site (amino acids 98, 134–138, 153, 155, 183, 190, 194, 195 and 224–229 in the H3 numbering system; Garcia *et al.*, 1996; Claas *et al.*, 1998) and found differences in the site between the two HA species. Recent reports have shown that a single amino acid change in this site can alter the haemagglutination phenotype with chicken RBCs among H3 human viruses (Nobusawa *et al.*, 2003). We found two amino acid differences in the receptor-binding site between HK/97 HA and Ty/Ont HA at positions 138 and 227 (150 and 239 in the H5 numbering system, respectively). The amino acid at position 227 can alter pathogenicity of H5N1 virus, possibly affecting receptor binding (Hatta *et al.*, 2001). We therefore substituted one or both amino acids at these positions from HK/97 HA to Ty/Ont HA (from Ala to Ser at 138, and/or from Ser to Asn at 227, respectively). However, these substitutions did not alter the haemadsorption phenotype (Fig. 3; HK/97 HA.A138S, S227N and A138S/S227N).

We next tested whether changing the oligosaccharide side chains and making the amino acid substitutions in the receptor-binding site affected HA receptor-binding specificity. We constructed HK/97 HA mutants that had the two additional glycosylation sites (HK/97 HA.131/158+) as well as one or both of the amino acid substitutions at positions 138 and/or 227 (Fig. 3; 131/158+//A138S, 131/158+//S227N and 131/158+//A138S/S227N). HK/97 HA mutants with the glycosylation sites did not exhibit the haemadsorption phenotype shown by Ty/Ont HA when only one of the amino acid substitutions was introduced (HK/97 HA.131/158+//A138S and 131/158+//S227N). However, when both amino acid substitutions were made, the HK/97 HA with the additional glycosylation sites (HK/97 HA.131/158+//A138S/S227N) was adsorbed by chicken

(to a limited extent probably due to suboptimal binding affinity) but not human RBCs, a haemadsorption phenotype similar to that of Ty/Ont HA. This mutant bound to human RBCs after sialidase treatment. Thus, we conclude that both glycosylation and the amino acid sequence in the receptor-binding site cooperatively determine receptor-binding specificity of HA.

Here we have shown a difference in the haemadsorption phenotype with human RBCs between the index H5N1 human isolate in 1997 and a virulent avian virus. This difference relates to the affinity of these two viruses for SAα2,3Gal-containing receptors on human cells, and may be explained by a mechanism similar to that recently reported for H3N2 viruses (Nobusawa *et al.*, 2003). The H3N2 human viruses, isolated after 1992, do not agglutinate chicken RBCs and also have reduced binding activity to SAα2,6Gal-containing sialyloligosaccharides in MDCK cells. However, these viruses bind tightly to MDCK cells that have been desialylated and then resialylated with *N*-acetyl-D-neuraminyl-(α2,6)-D-galactopyranosyl-(β1,4)-*N*-acetyl-D-glucosamine, suggesting that the asialo portion of the sialyloligosaccharides may be responsible for receptor differentiation. Identification and characterization of SAα2,3Gal-containing receptors on human cells together with binding analysis of human and avian isolates to these molecules would further our understanding of the receptor specificity changes that occur when viruses are transmitted from birds to humans.

Acknowledgements

We thank Sue Watson for scientific editing. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministries of Education, Culture, Sports, Science and Technology, Japan, by CREST (Japan Science and Technology Corporation) and by Public Health Service research grants from the National Institute of Allergy and Infectious Diseases. K. I.-H. was supported by a JSPS Research Fellowship for Young Scientists.

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