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A Single Amino Acid Substitution in 1918 Influenza Virus Hemagglutinin Changes Receptor Binding Specificity

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The receptor binding specificity of influenza viruses may be important for host restriction of human and avian viruses. Here, we show that the hemagglutinin (HA) of the virus that caused the 1918 influenza pandemic has strain-specific differences in its receptor binding specificity. The A/South Carolina/1/18 HA preferentially binds the α2,6 sialic acid (human) cellular receptor, whereas the A/New York/1/18 HA, which differs by only one amino acid, binds both the α2,6 and the α2,3 sialic acid (avian) cellular receptors. Compared to the conserved consensus sequence in the receptor binding site of avian HAs, only a single amino acid at position 190 was changed in the A/New York/1/18 HA. Mutation of this single amino acid back to the avian consensus resulted in a preference for the avian receptor.

The hemagglutinin (HA) of influenza virus is a surface protein involved in binding host cell sialic acid in the early stages of infection. In general, human viruses preferentially interact with an *N*-acetylsialic acid attached to galactose with an α2,6 linkage (SAα2,6Gal), whereas avian viruses mostly bind to *N*-acetylsialic acid attached to galactose with an α2,3 linkage (SAα2,3Gal) (2, 14–16, 25). The receptor binding specificity of HA has been implicated in determining the host range of a given influenza virus. In agreement with this hypothesis, human tracheal epithelium was shown to contain mostly SAα2,6Gal (3), whereas duck gut epithelium contains mostly SAα2,3Gal (9). In addition, as viruses adapt to a new host, the receptor binding can become more restricted to one type of sialic acid linkage (5, 13, 17, 25). These adaptations suggest the existence of selective pressure on receptor utilization depending on the host animal. However, it should be noted that all avian and human cells do not exclusively contain SAα2,3Gal and SAα2,6Gal, respectively. For example, chicken lung and intestinal epithelial cells contain both types of sialic acid linkages (6).

The 1918 influenza pandemic killed an estimated 50 million people, and the origin of this virulent strain continues to be of interest (10). One hypothesis suggests that the virus crossed the species barrier from birds to humans in part due to critical mutations in the HA (H1 subtype) that changed the binding preference from the avian sialic acid (α2,3) linkage to the human (α2,6) form (1, 12, 23). Six residues are conserved in the HA1 domains of most avian H1 HAs, implicating these residues in the ability to bind to the avian SAα2,3Gal receptor (7, 14). Comparison of the avian HA consensus sequence with HA sequences from the 1918 influenza virus strains shows that only one or two of these conserved residues, depending on the viral isolate, are different (Table 1) (23, 24). An earlier study of H1 HAs from swine influenza viruses shows that these amino acids, 190 and 225, are important for determining the receptor binding specificity (13). Here, we report the receptor binding capability of the A/South Carolina/1/18 virus HA and the A/New York/1/18 virus HA (23), which differ by one amino acid, and assess the importance of the amino acids at positions 190 and 225 in this specificity (amino acid residues have H3 numbering) (28).

We used hemadsorption of erythrocytes on cells expressing influenza virus HA as an assay for receptor binding specificity. The binding depends on the interaction of HA with sialic acid-containing molecules on the surface of red blood cells. In order to study the receptor binding properties of the 1918 HAs, we enzymatically modified chicken red blood cells (CRBCs) to express either SAα2,6Gal or SAα2,3Gal, as previously described (19, 22). All of the sialic acid was removed from 100 μl of 10% CRBCs using 50 mU *Vibrio cholerae* neuraminidase (VCNA; Roche) at 37°C for 1 h. Removal of sialic acid was confirmed by loss of hemagglutination of virus-like particles (VLPs) by chicken red blood cells (Fig. 2A). Subsequently, resialylation was performed using 0.5 mU α2,3-(*N*)-sialyltransferase (Calbiochem) or 2.5 mU α2,6-(*N*)-sialyltransferase (Calbiochem) and 1.5 mM CMP-sialic acid (Sigma) at 37°C in 50 μl for 30 min or in 125 μl for 60 min, respectively. Confirmation of successful resialylation was assayed using hemagglutination of well-defined human and avian viruses (data not shown). The A/South Carolina/1/18 HA gene was subcloned into the mammalian expression vector pCAGGS (23, 27). The HA corresponding to A/New York/1/18 virus was made by mutation of A716G in the A/South Carolina/1/18 HA

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TABLE 1. Critical amino acids for the receptor binding specificity of the influenza HA[a]

Viral HA	Amino acid position (H3 numbering)					
	77	138	186	190	194	225
A/South Carolina/1/1918	D	A	P	D	L	D
A/New York/1/1918	D	A	P	D	L	**G**
Avian H1 consensus	D	A	P	**E**	L	**G**

[a] The avian consensus sequence of the H1 HA was determined by comparing human and avian HA sequences (14). The above amino acids are conserved in most avian H1 HAs. Boldface type indicates a change from the A/South Carolina/1/18 HA sequence.

that coded for a D225G mutation in the protein (Table 1). Human embryonic kidney cells (293T) were transfected with the A/South Carolina/1/18 HA or the A/New York/1/18 HA using Lipofectamine 2000 (Invitrogen). After 24 h, the 293T cells were treated with 5 mU VCNA to remove sialic acids on the HA which could interfere with the assay, and cells were incubated with 0.5% CRBCs for 30 min at 4°C (20). After a thorough washing, the remaining red blood cells were lysed in 150 μl 20 mM Tris-HCl (pH 7.5), 177 mM NH_4Cl for 30 min at 25°C. Absorbance of released hemoglobin at 540 nm was used to quantify the amount of red blood cells bound (18). Controls include HAs cloned from a human H3 virus, A/Moscow/10/99, and an avian H3 virus, A/duck/Ukraine/1/63.

All the HAs tested in the hemadsorption assay bind CRBCs, which have been described to express both α2,3- and α2,6-linked sialic acid (19). The A/duck/Ukraine/1/63 virus binds preferentially to SAα2,3Gal, whereas the human virus, A/Moscow/10/99, preferentially binds to the SAα2,6Gal-resialylated CRBCs (Fig. 1A). Like the A/Moscow/10/99 HA, the HA from A/South Carolina/1/18 virus binds better to SAα2,6Gal-resialylated CRBCs than to SAα2,3Gal-resialylated CRBCs. The receptor binding specificity of the A/South Carolina/1/18 HA for SAα2,6Gal is in agreement with a previous report (11). However, the A/New York/1/18 HA which differs from the A/South Carolina/1/18 HA only at position 225 (G225 versus D225) binds equally well to SAα2,6Gal- and SAα2,3Gal-resialylated CRBCs. The reduced binding to the resialylated CRBCs by all HA constructs in comparison to unmodified blood cells is probably due to the inability to completely replace the density of sialic acid on red blood cells with the purified enzymes (19).

To understand the importance of residue 190 that differs (Table 1) from the avian consensus in the receptor binding pocket of the 1918 HA, we mutated the aspartic acid at this position in the A/New York/1/18 HA to a glutamic acid (1918 "avian" HA). In the hemadsorption assay, this construct had reduced binding to the SAα2,6Gal-resialylated CRBCs, as shown in Fig. 1A. The 1918 "avian" HA bound approximately

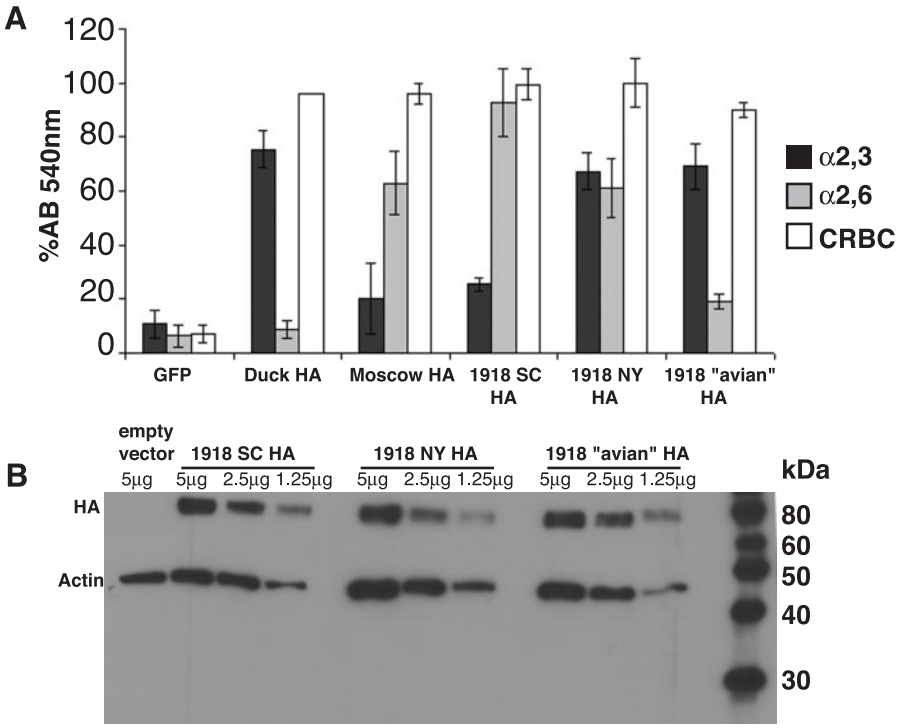

FIG. 1. (A) Release of hemoglobin measured by absorbance at 540 nm of untreated and resialylated CRBCs lysed after hemadsorption on 293T cells transfected with 3 μg each of plasmids expressing A/duck/Ukraine/1/63 HA, A/Moscow/10/99 HA, A/South Carolina/1/18 HA, A/New York/1/18 HA, or an HA containing a D190E mutation in the A/New York/1/18 HA ("avian" HA). Each bar represents the average of three separate experiments. Values are reported as percent maximal absorbance. CRBCs were resialylated with α2,3 or α2,6 sialic acid as described in the text. (B) Representative Western blot of 293T cells transfected with 3 μg pCAGGS 1918 HA constructs. The blot was probed with an anti-1918 HA monoclonal antibody (kindly provided by Tom Moran) and an anti-actin monoclonal antibody (Sigma). Total cellular protein of 5 μg, 2.5 μg, or 1.25 μg was loaded as indicated. Quantification of three Western blots was performed using the public domain NIH Image program, ImageJ. HA expression was normalized to actin. AB, absorbance; GFP, green fluorescent protein.

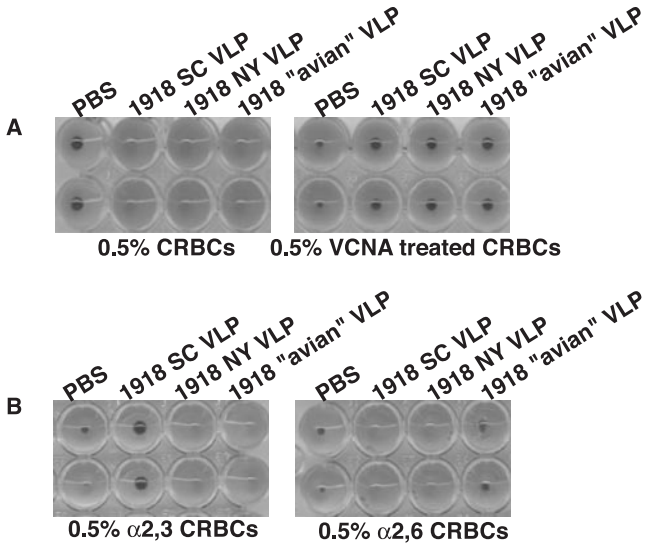

FIG. 2. (A) Hemagglutination assay of virus-like particles (VLPs) using unmodified (left) and VCNA-treated CRBCs (right). In both panels the top row corresponds to two hemagglutination units and the bottom row is a 1:2 dilution. (B) Hemagglutination assay of virus-like particles (VLPs) using CRBCs. The left panel shows SAα2,3Gal-resialylated CRBCs hemagglutinate VLPs expressing the A/New York/1/18 and the 1918 "avian" HAs but not A/South Carolina/1/18 HA. The right panel shows SAα2,6Gal-resialylated CRBCs hemagglutinate VLPs expressing the A/South Carolina/1/18 and A/New York/1/18 HAs but not 1918 "avian" HA. The amount of VLPs expressing the respective HAs was normalized with untreated CRBCs. In both panels the top row corresponds to two hemagglutination units and the bottom row is a 1:2 dilution. Production of SAα2,3Gal CRBCs was carried out in a 10% suspension with 0.5 mU of α2,3-(*N*)-sialyltransferase (Calbiochem) using 1.5 mM CMP-sialic acid in 50 μl for 2 h at 37°C. Production of SAα2,6Gal CRBCs was carried out in a 10% suspension with 1.0 mU of α2,6-(*N*)-sialyltransferase using 1.5 mM CMP-sialic acid in 125 μl for 2 h at 37°C.

70% less SAα2,6Gal-resialylated CRBCs than the A/South Carolina/1/18 HA and had a binding profile most similar to that of the avian A/duck/Ukraine/1/63 HA. We do not believe this was solely due to reduced protein expression or a nonfunctional mutant, because the 1918 "avian" HA bound well to unmodified CRBCs and to SAα2,3Gal-resialylated CRBCs. In addition, quantification by Western blot showed a less than 1.3-fold difference in protein expression (Fig. 1B).

Virus-like particles (VLPs) afforded another method for studying the receptor binding properties of the 1918 HA. Production of VLPs was carried out by transfection of 293T cells with 1 μg each of the pCAGGS plasmid expressing B/Yamagata/1/73 neuraminidase, matrix, and nonstructural protein 1 with A/New York/1/18 HA, A/South Carolina/1/18 HA, or 1918 "avian" HA (21). The B/Yamagata/1/73 viral proteins were used because they gave the highest titer for released VLPs. The VLPs produced equal hemagglutination units in assays with untreated CRBCs. In hemagglutination assays with α2,3Gal-resialylated CRBCs, the A/New York/1/18 HA and the 1918 "avian" HA VLPs hemagglutinated the CRBCs (Fig. 2B, left panel), whereas only the A/New York/1/18 HA and A/South Carolina/1/18 HA VLPs hemagglutinated the α2,6Gal-resialylated CRBCs (Fig. 2B, right panel). These data emphasize the fact that the consensus 1918 "avian" HA preferentially binds to α2,3Gal-resialylated CRBCs, whereas the A/South Carolina/1/18 HA has a low affinity for these CRBCs. Clearly, these data show the importance of positions 190 and 225 in the 1918 HA in determining its receptor binding specificity.

In conclusion, using hemadsorption and hemagglutination assays, we show that the HA surface glycoprotein from the influenza virus causing the 1918 pandemic preferentially binds SAα2,6Gal sialic acid corresponding to the human receptor and that one strain, A/New York/1/18, also binds the SAα2,3Gal sialic acid of the avian receptor. This experimentally confirms the prediction made by Reid et al. based on sequence comparisons of the 1918 HA sequence with other HAs (24). Only a single amino acid change in the HA (D190E) from the A/New York/1/18 strain to the avian consensus sequence is required to reduce binding to the human receptor. Two structures of the 1918 HA (cleaved [HA1/HA2] and uncleaved [HA0]) were recently determined by X-ray crystallography (8, 26). The structure of the 1918 HA in complex with receptor analogs has not yet been solved, but the structure of a similar viral HA from A/swine/Iowa/30, in complex with both the avian and human receptor analogs, is available (8). By analogy to the crystal structure of the human receptor in complex with the swine HA, the authors propose that the aspartic acid at position 190 in the 1918 influenza HA makes contacts with the GlcNAc and galactose of the SAα2,6Gal sugar moiety stabilizing the receptor-HA interaction. Our results support this notion and indicate that the presence of the larger amino acid, glutamate, in the avian HA receptor-binding pocket may be too bulky to accommodate the α2,6 sugar moiety.

Although sequence information for an avian H1 influenza virus that may have been the direct precursor of the 1918 pandemic strain does not exist, partial sequence of an avian H1 subtype HA from 1917 shows conservation over the receptor binding domain (4). The present work suggests that minimal changes in the receptor binding domain of an avian HA may have been enough to broaden its binding targets to include the major sialic acid receptor expressed on human respiratory epithelium.

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