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Short Communication

Protease-Dependent Hemagglutinin Cleavage Contributes to Alteration in Chicken Hemagglutination by the H3N2 Influenza A Virus

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SUMMARY: The human influenza A virus (H3N2) has been the predominant influenza strain since 1992, and one property of this virus is non-agglutination of chicken erythrocytes [Ch(−) virus]. The Ch(−) virus in our study was able to acquire chicken hemagglutination [Ch(+)] by trypsin passage but not by chymotrypsin passage. Moreover, the trypsin-passaged Ch(+) viruses reacquired the Ch(−) property after a further chymotrypsin passage. In particular, genetic analysis showed no evidence of mutations in the hemagglutinin (HA) gene during either trypsin or chymotrypsin passages: the only differences found were in the HA cleavage sites between the trypsin-passaged virus and the chymotrypsin-passaged virus as determined by the N-terminal amino acid sequence. These results suggested that protease-dependent differences at the viral HA cleavage site, rather than genetic mutations, are likely to have a significant effect on the viral ability to produce chicken hemagglutination.

Influenza A virus enters a host cell when the viral hemagglutinin (HA) proteins bind to sialic acid expressed on the surface membrane of the host cell (1–3). The binding of HA to erythrocyte sialic acid leads to hemagglutination, and the subsequent characteristic cellular clumping that occurs has been widely used in the diagnosis and monitoring of influenza (4,5). The human influenza A virus generally binds to sialic acid containing an α2,6-galactose linkage (SAα2,6Gal), while avian viruses bind to SAα2,3Gal (6). Therefore, guinea pig erythrocytes expressing more SAα2,6Gal than SAα2,3Gal and chicken erythrocytes expressing more SAα2,3Gal than SAα2,6Gal are often employed to study receptor specificity of the influenza virus (7,8).

The human influenza A virus (H3N2) has been identified as the causative agent of periodic flu epidemics since 1968 (9). In earlier epidemics, the H3N2 virus was found to agglutinate both chicken and guinea pig erythrocytes. However, the viral strain that became prominent during the 1992/1993 flu season lacked the chicken hemagglutination [Ch(−) virus] (8,10,11). In our study, the trypsin- or chymotrypsin-passaged Ch(−) virus produced lines with differing hemagglutination properties. Trypsin passage altered the Ch(−) virus and enabled it to agglutinate chicken erythrocytes, while chymotrypsin passage did not. Genetic and amino acid sequence analyses were performed on the protease-passaged viruses in order to elucidate the mechanism responsible for these different hemagglutination properties.

Influenza A/Hyogo/36/2004 (H3N2), isolated in Madin–Darby canine kidney (MDCK) cells, was used as a representative strain among the 4 Ch(−) viral strains tested. The viruses were passaged with 5 μg/ml of trypsin from porcine pancreas type II-S (Sigma-Aldrich Japan, Ltd., Tokyo, Japan) or 100 μg/ml of α-chymotrypsin from bovine pancreas type II (Sigma) 4 times in MDCK cells using a 100-fold dilution of the former virus for inoculation. To evaluate the properties of this protease-passaged virus, hemagglutination assays were performed using 0.75% guinea pig erythrocytes and 0.5% chicken erythrocytes. In total, 50 μl of a 2-fold dilution of each viral fraction was incubated with guinea pig and chicken erythrocytes for 1 h at room temperature and for 30 min at 4°C, respectively. Titers of hemagglutination in trypsin- and chymotrypsin-passaged viruses were high in guinea pig erythrocytes (titer, 64–128) (Table 1). In chicken erythrocytes, hemagglutination titers were found to increase in the trypsin-passaged viruses (titer, 32–64), while no hemagglutination was detected in the chymotrypsin passaged group (Table 1), suggesting that trypsin passage contributes to the acquisition of ability to cause chicken hemagglutination by the Ch(−) virus, while chymotrypsin passage has no such effect. In addition, the Ch(+) virus, which acquired chicken hemagglutination ability after 3 trypsin passages, lost this property after only a single further chymotrypsin passage. These data, therefore, indicate that viral chicken hemagglutination is protease

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Table 1. Hemagglutination property of trypsin- or chymotrypsin-passaged A/Hyogo/36/2004 (H3N2) virus

Passage no.	Hemagglutination titer			
	Trypsin passage		Chymotrypsin passage	
	Guinea pig	Chicken	Guinea pig	Chicken
1	128	<2	128	<2
2	128	64	64	<2
3	128	64[1)]	128	<2
4	128	32	64	<2

[1)]: This virus lost this Ch(+) property after only a single further chymoptrypsin passage.

dependent.

To identify genetic modifications in the chicken hemagglutination-revertant Ch(−) virus, sequence analyses of all genes were performed for both the trypsin- and chymotrypsin-passaged viruses. Viral RNA was extracted from single- and quadruple-passaged viruses using the QIAamp Viral RNA Mini Kit (Qiagen K.K.-Japan, Tokyo, Japan) and analyzed using the SuperScript® III One-Step RT-PCR System with Platinum® Taq (Life Technologies Japan, Tokyo, Japan). The RT-PCR products of all segments were directly sequenced by the Dragon Genomics Center (Takara Bio Co. Ltd., Mie, Japan) using the Applied Biosystems 3730xl DNA Analyzer (Life Technologies Japan). The primers used for the RT-PCR and sequence reactions are listed in Table 2. When compared with the single-passage group, no genetic mutations were observed in either the trypsin or chymotrypsin quadruple-passaged viruses [DDBJ: AB761203 (PB2), AB761204 (PB1), AB761205 (PA), AB761206 (HA), AB761207 (NP), AB761208 (NA), AB761209 (M), and AB761210 (NS)], except for the D151E change on the NA protein of the trypsin quadruple-passaged virus. However, this NA mutation was unique for the A/Hyogo/36/2004 strain among the 4 equally tested Ch(−) strains (data not shown). Therefore, we inferred that the mutation was incidental and non-instrumental in the hemagglutination change. These results indicated that the ability of trypsin-passaged Ch(−) virus to produce chicken hemagglutination was altered without any genetic mutation in the HA gene.

The influenza A virus becomes infectious when cellular proteases cleave the pro-peptide HA0 into HA1 and HA2 (12–14). The HA protein of the formerly predominant H3N2 strain A/Aichi/2/68, which causes chicken cell agglutination, is cleaved at Arg345 (15) (Fig. 1A). In order to evaluate potential changes in the HA cleavage site due to protease passage, trypsin or chymotrypsin quadruple-passaged viruses were stained using a glycoprotein staining kit. The viruses were pelleted via ultracentrifugation at 100,000 × *g* for 1 h, and the pellets were suspended in phosphate-buffered saline. The concentrated viruses were then ultracentrifuged at 100,000 × *g* with a 20% sucrose cushion for additional 90 min. These purified viruses were also subjected to SDS-PAGE, Coomassie brilliant blue staining, and glycoprotein staining (Thermo Scientific Pierce Protein Research Products, Rockford, Ill., USA). As shown in Fig. 2, multiple glycoprotein fragments were observed. Among these, fragments 1, 2, and 3 were deduced by their molecular weights to be HA1, NA, and HA2, respectively. However, the additional HA fragments, which had lower molecular weights than HA2 (fragments 4 and 5 in Fig. 2), were unexpected findings. Therefore, to analyze the amino acid sequences of these fragments, the N-terminal amino acids of fragments 3, 4, and 5 were sequenced (APRO Life Science Institute, Inc., Tokushima, Japan). The N-terminal sequence of fragment 3 was GIFGA, identifying it as HA2 (Table 3). Fragment 4 was under the threshold required to iden-

Table 2. Primers used in genes sequence analysis

Primer name	Sequence 5′–3′	Application
PB2_1F	AGCAAAAGCAGGTCAATTATATTC	RTPCR/Seq
PB2_2F	ATGAGGGGTATGAGGAGTTCACAA	Seq
PB2_3F	TGGTCTCAGAATCCTGCAATGTTG	Seq
PB2_1R	AGTAGAAACAAGGTCGTTTTTAAA	Seq
PB2_2R	TTCGCATCTTTCTGAAAATGCCTT	RTPCR/Seq
PB2_3R	AACTTCAATGTATATACTGCTTGT	Seq
PB1_1F	AGCGAAAGCAGGCAAACCATTTGA	RTPCR/Seq
PB1_2F	AAGGCTAATAGATTTCCTCAAGGA	Seq
PB1_3F	AAATCAACCTGAGTGGTTCAGAAA	Seq
PB1_4F	ACATGAGCAAAAAGAAGTCCTATA	Seq
PB1_5F	GAATCCCTTTGTCAGCCATAAAGA	Seq
PB1_1R	AGTAGAAACAAGGCATTTTTTCAT	Seq
PB1_3R	GATTTCTGTTTAATGTCCAATCAT	Seq
PB1_4R	GATTCGTTGAAATACTTCAGGTCG	RTPCR/Seq
PA_1F	AGCAAAAGCAGGTACTGATTCGAA	RTPCR/Seq
PA_2F	AGACTACACTCTCGACGAGGAAAG	Seq
PA_3F	GGACTAAAAACATGAAGAAAACGA	Seq
PA_4F	AAGGCGAAAAACCAATTTATATGG	Seq
PA_5F	ATTGGGGAGTCCCCCAAGGGAGTG	Seq
PA_1R	AGTAGAAACAAGGTACTTTTTTGG	Seq
PA_2R	TCAGTTAGCTCGCAGGCCTTGTTG	RTPCR/Seq
PA_3R	TGGCCTTTTCAAGGTAATATATGT	Seq
HA_1F	AGCAAAAGCAGGGGATATTT	RTPCR/Seq
HA_2F	CTTTCAAAATGTAAACAGGA	Seq
HA_3R	AGTAGAAACAAGGGTGTTTT	RTPCR/Seq
HA_4R	TCCTGAATTCTCCCTTCTAC	Seq
HA_5R	AGTATGTCTCCCGGTTTTAC	Seq
NP_1F	AGCAAAAGCAGGGTTAATAATCAC	RTPCR/Seq
NP_2F	CAGAGGACAAGAGCTCTTGTTCGA	Seq
NP_3F	AGCACACAAGAGTCAGCTGGTGTG	Seq
NP_1R	AGTAGAAACAAGGGTATTTTTTCC	RTPCR/Seq
NP_2R	GCCAGATTCGCCTTATTTCTTCTT	Seq
NA_1F	AGCAAAAGCAGGAGTAAAGATGAA	RTPCR/Seq
NA_2F	ACCGGACCCTATTGATGAATGAAT	Seq
NA_3F	GCCATTGCCTAGATCCTAACAATG	Seq
NA_1R	AGTAGAAACAAGGAGTTTTTTCTA	RTPCR/Seq
NA_2R	CATTCATCAATAGGGTCCGGTAAG	Seq
M_1F	AGCAAAAGCAGGTAGATATTGAAA	RTPCR/Seq
M_2F	ACAGGTCCCATAGGCAAATGGTGG	Seq
M_1R	AGTAGAAACAAGGTAGTTTTTTAC	RTPCR/Seq
NS_1F	AGCAAAAGCAGGGTGACAAAGACA	RTPCR/Seq
NS_2F	ATGTTGAAAGCGAATTTCAGTGTG	Seq
NS_1R	AGTAGAAACAAGGGTGTTTTTTA	RTPCR/Seq
NS_2R	TTTCGCCAACAATTGCTCCCTCTT	Seq

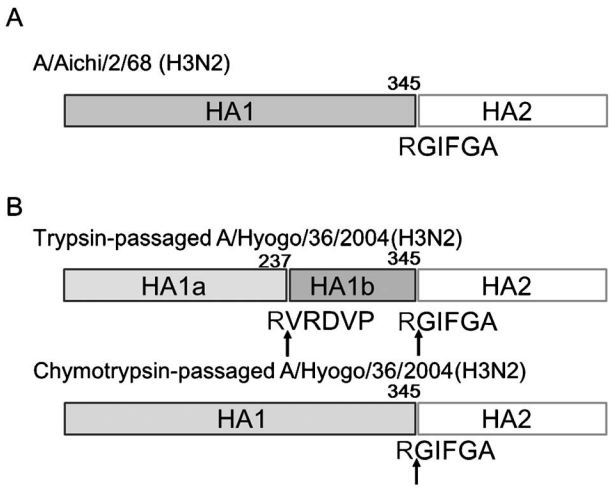

Fig. 1. Schematic diagram of HA cleavage site. (A) HA cleavage site of A/Aichi/2/68 (H3N2) virus. HA0 is cleaved at Arg345 into HA1 and HA2. (B) HA cleave sites of A/Hyogo/36/2004 (H3N2) virus quadruple-passaged with trypsin or chymotrypsin. In trypsin-passaged virus, HA0 is cleaved at Arg345 into HA1 and HA2, and HA1 is cleaved at Arg237 into HA1a and HA1b. In chymotrypsin-passaged virus, HA0 is cleaved at Arg345 into HA1 and HA2.

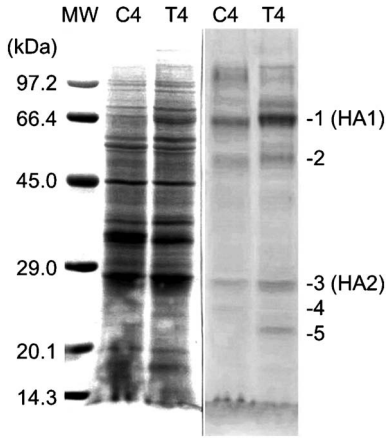

Fig. 2. Glycoprotein staining of A/Hyogo/36/2004 (H3N2) virus quadruple-passaged with trypsin or chymotrypsin. SDS-PAGE of the purified quadruple-passaged A/Hyogo/36/2004 (H3N2) virus with trypsin (T4) or chymotrypsin (C4) was performed. Viral proteins were stained with Coomassie brilliant blue staining (left column) and a glycoprotein staining kit (right column).

tify the sequences; however, fragment 5 featured VRDVP N-terminal sequences, which indicated that further cleavage of HA1 occurred in this protease-passaged virus (Table 3). Supplemental Western blotting analysis using anti-H3N2 goat serum (PA1-7222; ABR Affinity Bioresearch Inc., Golden, Colo., USA) revealed that the triple trypsin-passaged viruses also contained fragment 5, while single trypsin-passaged viruses did not (data not shown). Although cell-dependent glycosylation changes in the viruses during passages may have affected on the HA cleavage (16,17), the actual mechanisms for HA cleavage by chymotrypsin and gradual acquisition of chicken hemagglutination by trypsin remain unknown. However, taken together, these findings suggest that trypsin passage of the Ch(−)

Table 3. N-terminal amino acid sequence of fragments in 4-times passaged virus with trypsin or chymotrypsin

Fragment	N-terminal amino acid sequence	
	T4[1)]	C4[1)]
3[2)]	GIFGA	GIFGA
4[2)]	—	n[3)]
5[2)]	VRDVP	—

[1)]: Four-times passaged virus with tyrpsin (T) or chymotrypsin (C).
[2)]: Number represents the bands shown in Fig. 2.
[3)]: n, not identified.

virus causes additional cleavage of HA1 at Arg237 and contributes to changes in the chicken hemagglutination properties of the virus, without genetic mutation (Fig. 1B).

According to database analysis, the newly found HA cleavage site (the amino acid arginine [R] at position 237) first appeared in isolates obtained in 2002 and has since been retained (data not shown). These results demonstrate the likelihood that the cell surface binding affinity of the newer H3N2 viruses has changed as a result of host protease activity; however, detailed analyses to confirm this finding are required to completely elucidate the mechanisms of how the introduction of an additional cleavage site in the HA molecule can affect such a change in hemagglutination.

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Conflict of interest None to declare.

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