Short Communication

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

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(Received November 19, 2012. Accepted July 1, 2013)

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 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-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.

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These results indicated that the ability of trypsin- and chymotrypsin-passaged virus to produce chicken hemagglutination (Ch) was different. Therefore, we inferred that the mutation was incidental and not 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., 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 without any genetic mutation in the NA gene.

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

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>Trypsin passage</th>
<th>Chymotrypsin passage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Guinea pig</td>
<td>Chicken</td>
</tr>
<tr>
<td>1</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
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<td>128</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>32</td>
</tr>
</tbody>
</table>

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

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) (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 was 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 identify 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 identify...
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 cleavage 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).

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

<table>
<thead>
<tr>
<th>Fragment</th>
<th>N-terminal amino acid sequence</th>
</tr>
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<tbody>
<tr>
<td>3&lt;sup&gt;1)&lt;/sup&gt;</td>
<td>GIFGA</td>
</tr>
<tr>
<td>4&lt;sup&gt;2)&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td>5&lt;sup&gt;2)&lt;/sup&gt;</td>
<td>VRDVP</td>
</tr>
</tbody>
</table>

<sup>1)</sup> Four-times passaged virus with trypsin (T) or chymotrypsin (C).
<sup>2)</sup> Number represents the bands shown in Fig. 2.
<sup>3)</sup> 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).

Acknowledgments This work was supported by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), The Global Center of Excellence for Education and Research on Signal Transduction Medicine in the Coming Generation, and by ERATO (The Japan Science and Technology Agency). We thank Bruce Collins for English editing.

Conflict of interest None to declare.

REFERENCES


