Differences in the Hemagglutinin Amino Acids of Japanese and US Influenza A/H3N2 Viral Isolates and their Relationship with Vaccine Strain Differences

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Abstract

The effects of vaccination on the dynamics of influenza virus remain largely unknown. Different A/H3N2 vaccine strains were used in Japan and the United States (US) in the 2014-2015 season. To examine how different vaccine strains affect the selection of surviving variants, we compared hemagglutinin (HA) sequences in Japan with those in the US. A total of 85 A/H3N2 samples from 38 vaccinated and 47 unvaccinated Japanese patients (33 from the 2013-2014 and 52 from the 2014-2015 Japanese seasons) were isolated and genetically analyzed using a next-generation sequencer for comparison with 113 US isolates (30 from the 2013-2014 season and 83 from the 2014-2015 season) referenced from the GenBank database. HA1 amino acid (AA) differences between Japan and the US for the 2014-2015 vaccine strains were found at sites 128 (epitope B), 142 (A), 145 (A) and 198 (B). 145S and 198S in Japanese isolates, which matched with those of the 2014-2015 vaccine strain, significantly decreased as observed while matching these AAs with those of the 2014-2015 vaccine strain (72.7% vs. 19.2% for 128A, P<0.0001; 75.8% vs. 15.4% for 142G, P<0.0001). Our data suggest that vaccine selection might be associated with the emergence of influenza variants genetically distant from vaccine strains.

Keywords: Influenza; Vaccination; Vaccine selection; Mutation; Hemagglutinin

Introduction

Annual outbreaks of influenza viruses result in a high morbidity and mortality in humans. A/H3N2 viruses are the most common and virulent of the influenza subtypes [1,2]. Antigenic drift, changes in antigenicity through the accumulation of mutations in the hemagglutinin (HA) gene, which encodes a major surface protein, is chiefly responsible for the continuing circulation of these viruses [1,2]. As a result, influenza vaccines must be frequently updated based on new variant analyses. In recent seasons, however, the protection by influenza vaccines has been suboptimal [3-5].

It is possible that the immune pressure elicited by vaccination (vaccine pressure) has an effect on the selection of influenza variants that become epidemic in the following season; thus, understanding the mechanisms would contribute to more accurate HA prediction, which would be helpful for counteracting future epidemic viruses. Based on this hypothesis, we have been investigating the influence of vaccine pressure on the epidemic dynamics of influenza viruses.

In Japan, recent influenza vaccine strains have been selected according to the World Health Organization (WHO) recommendations; however, in the 2014-2015 Japanese season, an A/H3N2 vaccine strain different from that recommended by the WHO was chosen in order to more efficiently avoid a decrease in antigenicity due to “egg-adaptation”, as explained by the National Institute of Infectious Diseases [6]. This circumstance presented us an opportunity to analyze how different vaccine strains affect the selection of surviving variants in different regions, based on the assumption that vaccine pressure has a certain effect on influenza epidemics.

The necessary data was gathered through our network of physicians throughout Japan who routinely collect influenza virus samples, along with patient information that includes vaccination history [7-10]. In this study, we determined the full-length sequence of HA genes of influenza A/H3N2 viruses isolated from both vaccinated and unvaccinated patients in the 2013-2014 and 2014-2015 seasons, and compared amino acid (AA) mutations at different HA1 AA sites between Japanese and the United States (US) vaccine strains, using these sequences for the Japanese isolates and the reference sequences for the US isolates.

Materials and Methods

Sample collection

Nasopharyngeal swabs for influenza virus isolation were collected and vaccination history was determined from patients who had a positive result on a rapid influenza antigen test, given at one of the member clinics of our nation-wide study network of general practitioners [7-10]. Informed consent was obtained from all patients. All patients were outpatients, and this study did not include any patients with severe chronic respiratory diseases, renal diseases, liver diseases, or heart failure. All viral samples were collected before the initiation of neuraminidase inhibitors (NAIs). Background
information on these patients, including their vaccination history, was also collected. In this study, the isolates of 85 samples (33 from the patients were collected more than four weeks
segments [12] were as follows: forward primer (Uni-12), 5´-ACGCGTGATCAGTAGAAACAAGG-3´.

Next generation sequencing

Texas/50/2012 (X-223) and A/New York/39/2012 (X-233A) were used for 30 sec, and an extension step at 72
medium and 75 µL of the medium was cultured using Madin-Darby
determined by PCR [11]. RT-PCR was performed using the H3N2
season and 83 from the 2014-2015 season) were referenced from the
30-2014 season) were retrieved from all but thirteen
states.

Viral RNA extraction and RT-PCR

Nasopharyngeal swabs from patients were soaked in virus transport medium and 75 µL of the medium was cultured using Madin-Darby canine kidney (MDCK) cells. Viral RNA was extracted from infected MDCK cell culture supernatants using the Maxwell 16 LEV simply RNA Cells Kit (Promega, Madison, WI). The A/H3N2 subtype was determined by PCR [11]. RT-PCR was performed using the H3N2 RNA samples. PCR primers, synthesized based on the 3´ and 5´ terminal nucleotides that are common to all human influenza A virus segments [12] were as follows: forward primer (Uni-12), 5´-ACGCGTGATCAGCAAAGCAGG-3´ and reverse primer (Uni-13), 5´-ACGCGTGATCAGTGAACCAAAGG-3´. The PCR consisted of 31 cycles of a denaturing step at 94°C for 30 sec, an annealing step at 57°C for 30 sec, and an extension step at 72°C for 2 min.

Next generation sequencing

A DNA library for Illumina sequencing was prepared using the Nextera XT DNA Sample Prep kit (Illumina, San Diego, CA). Sequencing was conducted via a paired-end, 2 × 250 bp cycle run, using the Illumina MiSeq sequencing system and MiSeq Reagent Kit version 2 (300 Cycle) (Illumina) [13].

Bioinformatic analysis

Data processing was performed using the pipeline prepared by Amelieff Co [13]. The reference sequence was A/New York/396/2005 (H3N2) (GenBank accession numbers for the eight gene segments: CY002079, CY121123, CY121122, CY121117, CY121120, CY121119, CY121118, and CY121121). The HA amino acid (AA) sequence was deduced from the obtained nucleotide sequence.

Nucleotide sequence accession number

The sequence data from this study were deposited into the DDBJ/EMBL/GenBank nucleotide sequence databases under the following accession numbers: LC111574-LC111606, and LC155845-LC155896.

Statistical analysis

Categorical variables between groups were tested using the Fisher’s exact test. P<0.05 was considered to be statistically significant. All statistical analyses were performed using the JMP Pro software, version 11 (SAS Institute, Inc., Cary, NC, USA).

Results

Table 1 shows the influenza A/H3N2 vaccine strains selected in the US and Japan from the 2011-2012 to the 2015-2016 season. Different vaccine strains were used only in the 2014-2015 season during this five year period. In the US, A/Texas/50/2012 was chosen for the 2014-2015 season as well as in the previous season (2013-2014). In parallel, A/New York/39/2012 was selected for the 2014-2015 season in Japan (Table 1).

Table 1: Influenza A/H3N2 vaccine selection in Japan and the United States.

Of the AA sites known to be susceptible to mutation induced by egg-adaptation (sites 156, 186, and 219) [14], sites 186 and 219 have the possibility of different AAs between the Japanese and US vaccine strains of the 2014-2015 season, but these AA differences were not

<table>
<thead>
<tr>
<th>Season (country)</th>
<th>Vaccine strain</th>
<th>AA (epitope)</th>
<th>AA128 (epitope B)</th>
<th>AA142 (epitope A)</th>
<th>AA145 (epitope A)</th>
<th>AA198 (epitope B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012-2013 (US and JPN)</td>
<td>A/Victoria/361/2011</td>
<td>T</td>
<td>R</td>
<td>N</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>2013-2014 (US and JPN)</td>
<td>A/Texas/50/2012</td>
<td>N</td>
<td>R</td>
<td>N</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>2014-2015 (US)</td>
<td>A/Texas/50/2012</td>
<td>N</td>
<td>R</td>
<td>N</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>2015-2016 (US and JPN)</td>
<td>A/Switzerland/9715293/2013</td>
<td>A</td>
<td>G</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

AA: Amino Acid; US: United States; JPN: Japan

Table 1: Influenza A/H3N2 vaccine selection in Japan and the United States.

The AA sites known to be susceptible to mutation induced by egg-adaptation (sites 156, 186, and 219) [14], sites 186 and 219 have the possibility of different AAs between the Japanese and US vaccine strains of the 2014-2015 season, but these AA differences were not


definite based on the result of reference sequencing. Identical AAs (156H, 186G, and 219S) were detected in all of the isolates from Japan and the US in both the 2013-2014 and 2014-2015 seasons. Based on these results, these three AA sites were excluded from our analysis.
Table 2: Amino acid mutation at Hemagglutinin 1 (HA1) sites 145 and 198 of influenza A/H3N2 viruses isolated in Japan and the United States during the 2013-2014 and 2014-2015 seasons.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>US</td>
<td>S</td>
<td>100 (30/30)</td>
<td>98.8 (82/83)</td>
<td>1</td>
<td>S</td>
<td>100 (30/30)</td>
<td>96.4 (80/83)</td>
<td>0.564</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0 (0/30)</td>
<td>1.2 (1/83)</td>
<td>1</td>
<td>A</td>
<td>0 (0/30)</td>
<td>0 (0/83)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>JPN</td>
<td>S</td>
<td>57.6 (19/33)</td>
<td>5.8 (3/52)</td>
<td>&lt;0.0001</td>
<td>S</td>
<td>100 (33/33)</td>
<td>75 (39/52)</td>
<td>0.0012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>42.4 (14/33)</td>
<td>94.2 (49/52)</td>
<td>&lt;0.0001</td>
<td>A</td>
<td>0 (0/33)</td>
<td>25 (13/52)</td>
<td>0.0012</td>
<td></td>
</tr>
<tr>
<td>Vaccinated</td>
<td>S</td>
<td>43.8 (7/16)</td>
<td>4.5 (1/22)</td>
<td>0.0054</td>
<td>S</td>
<td>100 (16/16)</td>
<td>63.6 (14/22)</td>
<td>0.0119</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>56.2 (9/16)</td>
<td>95.5 (21/22)</td>
<td>0.0054</td>
<td>A</td>
<td>0 (0/16)</td>
<td>36.4 (8/22)</td>
<td>0.0119</td>
<td></td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>S</td>
<td>70.6 (12/17)</td>
<td>6.7 (2/30)</td>
<td>&lt;0.0001</td>
<td>S</td>
<td>100 (17/17)</td>
<td>83.3 (25/30)</td>
<td>0.1435</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>29.4 (5/17)</td>
<td>93.3 (28/30)</td>
<td>&lt;0.0001</td>
<td>A</td>
<td>0 (0/17)</td>
<td>16.7 (5/30)</td>
<td>0.1435</td>
<td></td>
</tr>
</tbody>
</table>

aThis indicates AAs of vaccine strains in each season.  
bThis indicates a comparison of the frequency of isolates displaying the indicated AA between the 2013-2014 and 2014-2015 seasons.

cThis indicated 198P in two isolates and 198L in one isolate.

AA: Amino Acid; US: United States; JPN: Japan

We examined definitely different HA1 AA sites between the two vaccine strains selected in the 2014-2015 season and found four AA sites (sites 128, 142, 145, and 198) (Table 1). The frequencies of AA mutations at sites 145 and 198 were compared for the isolates from Japan and the US (Table 2). In the vaccine strains of the 2013-2014 season, 145N was present in both countries in the 2013-2014 season. In the 2014-2015 season, AA145 changed from N to S in the Japanese vaccine strain. In the 2013-2014 season, the dominant AA145 in the US isolates (10/52 isolates) (P<0.0001). In addition, the rate of 145S in the 2014-2015 season was lower in the isolates from vaccinated than from unvaccinated patients (4.5% vs. 16.7%). In the 2014-2015 season, AA145 changed from N to S (145N in US and 145S in JPN), and the predominance of 145S, similar to that of 128S, was lost in the Japanese 2014-2015 season. In addition, the rate of 145S in the 2014-2015 season was lower in the isolates from vaccinated than from unvaccinated patients (13.6% vs. 16.7%), although this difference was not statistically significant.

The analysis of sites 128 and 142 is shown in Table 3. In the 2013-2014 season, 128N was present in the vaccine strains of both countries. In the 2014-2015 season, AA128 changed from N to A only in the Japanese vaccine strain. In the 2013-2014 season, the dominant AAAs at site 128 were 128T (63.3%) in the US and 128A (72.7%) in Japan. In the 2014-2015 season, the 128A found in the isolates from Japan decreased in frequency from 72.7% (24/33 isolates) to 19.2% (10/52 isolates) (P<0.0001). In addition, 128A dominance was lost in both the isolates from vaccinated and unvaccinated patients (from 93.8% to 22.7% in the vaccinated; from 52.9% to 16.7% in the unvaccinated); however, the rate of 128A in the 2014-2015 season was higher in the isolates from vaccinated than from unvaccinated patients (22.7% vs. 16.7%). In the 2014-2015 season, AA142 changed from R to S in the Japanese vaccine strain. AA combination at sites 128 and 142, 128A-142G, was found, as reported in our previous study [13]. Therefore, the predominance of 124G, similar to that of 128A, was lost in the Japanese 2014-2015 season. In addition, the rate of 142G in the 2014-2015 season was lower in the isolates from vaccinated than from unvaccinated patients (13.6% vs. 16.7%), although this difference was not statistically significant.
### Table 3: Amino acid mutation at Hemagglutinin 1 (HA1) sites 128 and 142 of influenza A/H3N2 viruses isolated in Japan and the United States during the 2013-2014 and 2014-2015 seasons.

<table>
<thead>
<tr>
<th>Country</th>
<th>AA</th>
<th>% (No. of isolates/total isolates)</th>
<th>% (No. of isolates/total isolates)</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AA</th>
<th>% (No. of isolates/total isolates)</th>
<th>% (No. of isolates/total isolates)</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>JPN</td>
<td>A</td>
<td>72.7 (24/33)</td>
<td>19.2 (10/52)</td>
<td>&lt;0.0001</td>
<td>G</td>
<td>75.8 (25/33)</td>
<td>15.4 (8/52)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>24.2 (8/33)</td>
<td>80.8 (42/52)</td>
<td>&lt;0.0001</td>
<td>R</td>
<td>24.2 (8/33)</td>
<td>84.6 (44/52)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>A</td>
<td>93.8 (15/16)</td>
<td>22.7 (15/22)</td>
<td>&lt;0.0001</td>
<td>G</td>
<td>93.8 (15/16)</td>
<td>13.6 (3/22)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>6.2 (1/16)</td>
<td>77.3 (17/22)</td>
<td>&lt;0.0001</td>
<td>R</td>
<td>6.2 (1/16)</td>
<td>86.4 (19/22)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>A</td>
<td>52.9 (9/17)</td>
<td>16.7 (5/30)</td>
<td>0.0182</td>
<td>G</td>
<td>58.8 (10/17)</td>
<td>16.7 (5/30)</td>
<td>0.0077</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>41.2 (7/17)</td>
<td>83.3 (25/30)</td>
<td>0.0182</td>
<td>R</td>
<td>41.2 (7/17)</td>
<td>83.3 (25/30)</td>
<td>0.0077</td>
</tr>
</tbody>
</table>

<sup>a</sup>This indicates AAs of vaccine strains in each season.

<sup>b</sup>This indicates a comparison of the frequency of isolates displaying the indicated AA between the 2013-2014 and 2014-2015 seasons.

<sup>c</sup>This indicated 142K in seven isolates.

**AA:** Amino Acid; **US:** United States; **JPN:** Japan

As shown in Table 2, 198A was detected in 13 of 52 isolates (25.0%) in the Japanese 2014-2015 season. Sites 198 and 128 were both within antigenic epitope B. 128A remained detectable in 10 of 52 isolates (19.2%), as observed when including the isolates with 198A (Table 4). On the other hand, 128A was detected in only one isolate (2.6%, P=0.021), when excluding the isolates with 198A (Table 4). In addition, none of 128A was detected in the vaccinated patients under the condition of 198A exclusion.
Table 4: Amino acid mutation at Hemagglutinin 1 (HA1) site 128 of influenza A/H3N2 isolates with or without 198A in the Japanese 2014-2015 season.

Discussion

Vaccine mismatch was of concern because of the antigenic change of vaccine strains due to egg-adaptation; however, the indicated adaptation sites, particularly sites 156,186 and 219, exhibited identical AAs in all of the Japanese and US isolates. Thus, these egg-adaptation sites, included within known antigenic epitopes, did not seem to function as the main antigenic sites recognized by the host during the 2013-2014 and 2014-2015 seasons.

In the field of avian influenza, antigenic drift-related HA mutation due to escape from vaccine-induced immunity has been reported to cause vaccine failure [15,16]; however, the presence of vaccine-induced antigenic drift has not been demonstrated in humans. We recently reported evidence showing that vaccine pressure works to select influenza variants genetically distant from vaccine strains and affects the dynamics of the epidemic variants for humans [13]. At the beginning of the previous study, the phylogenetic method did not segregate viruses isolated from vaccinated and unvaccinated patients, as indicated in the report [17]. One possible explanation for the vaccine pressure not being reflected in the phylogenetic tree is that viruses that are able to infect vaccinated persons will become epidemic, irrespective of vaccination status. Viruses containing mutations that evade host immunity will be isolated from both the vaccinated and unvaccinated patients, resulting in no segregation in the phylogenetic tree of the viruses isolated from the vaccinated patients. We were first able to suggest the possible effect of vaccine pressure on HA mutation and its relation to the emergence of epidemic variants by directly comparing AA differences from the corresponding vaccine strains between isolates from vaccinated and unvaccinated patients. As shown in the phylogenetic tree in the previous study [13], the epidemic viruses in the Japanese 2013-2014 and 2014-2015 seasons formed clades, which were segregated by AA mutations other than the four AA sites that were examined in this study. Thus, if we had based this study only on the results of phylogenetic tree analysis, we would have missed HA mutations possibly associated with the emergence of local variants.

Our present study allowed the examination of the influence of different vaccine strains on the HA mutations of influenza epidemic viruses, because different vaccine strains were selected between Japan and the US in the 2014-2015 season, in contrast to the 2013-2014 season. In the four different AA sites between Japanese and the US vaccine strains, the isolates from Japan showed more predominant AA mutations that were genetically distant from the corresponding vaccine strain, as compared to those from the US (Tables 2 and 3). Particularly, 145N, which was not detected in the US, was extremely predominant in Japan. 198A was observed in the isolates from Japan but was not detected in those from the US. HA AA site 145 is within antigenic epitope A and 198 within epitope B. Thus, genetic variation was generated in important antigenic sites, such as epitopes A and B. This variation may be attributed to AA differences in the vaccine strains used in the two countries. As a result, these findings suggest the influence of vaccination on drift mutation.

AA sites 128 and 198 are both within antigenic epitope B [18]. In our analysis (Table 4), 128A was detected in 10 (19.2%) of 52 Japanese isolates, when including the isolates with 198A. On the other hand, 128A was detected in only one isolate (2.6%, 1/39 isolates), when excluding the isolates with 198A. Thus, 128A was detected in 9 (69.2%) of 13 Japanese isolates with 198A. In contrast, almost all isolates without 198A exhibited 128T (97.4%, 38/39 isolates). The acquisition of 198A or 128T appears to be alternative. These mutations within epitope B may have a similar effect as the structural change for antigenic drift.

The mechanisms responsible for HA mutation in influenza viruses seem to be very complicated, with many potentially contributing factors. Therefore, it was anticipated that not all HA mutations would be explained only by the immune pressure elicited by vaccination. The global epidemiological situation of H3N2 might also be associated with HA mutations, as indicated by global surveillance [19]. Thus, in the analysis of HA mutation in each region, the presence of local variants itself is not included as a prerequisite. In this study, we have obtained findings suggesting that there are local variants within epidemic H3N2 viruses in Japan. The emergence of 145N and 198A is especially intriguing (Table 2). The AA patterns of sites 128 and 142 appear to be similar in both the Japan and US samples, irrespective of AA differences at these sites between the samples of the two countries in the 2014-2015 seasonal vaccine (Table 3). Indeed, this result might be associated with the global spread of seasonal epidemic viruses. On the other hand, in Japan the emergence of 198A appears to be related to the mutation of site 128 (Table 4). Japan-specific selection of a vaccine strain in the 2014-2015 season might have accelerated the emergence of 198A and also affected the mutation pattern of site 128. Thus, our approach to the analysis of HA mutation revealed the presence of local variants that were not reflected by a phylogenetic analysis, and the emergence of these variants may be attributed, at least in part, to vaccine pressure.

The vaccine strain (A/Switzerland/9715293/2013) was chosen for the 2015-2016 season in both Japan and the US (Table 1). Interestingly, the A/NewYork/39/2012 strain used in the 2014-2015 season in Japan matched the four AA sites (128A, 142G, 145S, and 198S) with those of the 2015-2016 vaccine strain. As mentioned above, these four sites appeared to exhibit AA mutations that were genetically distant from the vaccine strain in the Japanese 2014-2015 season. These mutations, particularly 145N and 198A, would be anticipated to emerge along with the match of the vaccine strain. Although our results are not conclusive, we are looking forward to investigating the HA AA sequences of the Japanese and US isolates in the 2015-2016 season, for confirmation.

A limitation of this study is that viral isolation could cause AA mutations during cultivation (MDCK passage). Direct sequencing from clinical samples would resolve this issue, although the direct use of respiratory samples involves the risk of detecting defective viruses with no growth activity potential.

It is unlikely that epidemic viruses in the 2014-2015 season were formed solely by AA mutations at the four sites that were different in the Japanese and US vaccine strains. It is unknown to what extent AA mutations at the four sites would be associated with the selection of
epidemic variants in the two countries. Further study of the antigenic changes caused by AA mutations at these four sites will be necessary to determine the precise mechanisms of this challenging issue. However, the difference in the composition of epidemic viruses between Japan and the US is apparent. It would be natural to attribute this variation to AA differences in the vaccine strains, which might have induced different vaccine pressure.

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Authors’ Contribution

Yong Chong: Designed the study, carried out data analysis and drafted the manuscript.

Hideyuki Ikematsu: Designed the study, carried out data analysis and drafted the manuscript.

Conflicts of Interest

The authors indicate no potential conflicts of interest.

References