Cocirculation of Antigenic Variants and the Vaccine-Type Virus during the 2004–2005 Influenza B Virus Epidemics in Japan

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In the 2004–2005 season, there was a large epidemic of the influenza B virus Yamagata group in Kobe, Japan. In hemagglutination inhibition tests, most of the clinical isolates from Kobe showed antigenicities similar to those of previous isolates (the vaccine-type virus). Only a few antigenic variants were isolated around the peak of the epidemic; however, Kobe residents developed antibodies against the variants during the season. The antigenic variants showed a one-point mutation of a nucleotide in the HA1 gene (C440A or G421A), which resulted in the substitution of one amino acid in the 150 loop of the HA molecule (T147N or G141R). The 150 loop is one of four epitopes of the hemagglutinin molecule of the influenza B virus. We established a system to detect one-point differences in the nucleotides of the 150 loop by means of high-resolution melting curve analysis with LCGreen. With this system, the isolates were determined to be the vaccine-type virus, antigenic variants, or a mixture of both. Some isolates were shown to be mixtures although they had been recognized as the vaccine-type virus with the hemagglutination inhibition tests. Thus, the antigenic variants appeared in the early period of the epidemic and were cocirculating with the vaccine-type virus during the epidemic.

Influenza epidemics occur every winter in Japan, as in European and North American countries. Over the past 20 years, the influenza B virus has caused epidemics in humans, as have the H1 and H3 subtypes of the influenza A virus. Recent isolates of influenza B virus strains are divided into two large lineages in a phylogenetic tree: one group is represented by B/Victoria/2/87 and the other by B/Yamagata/16/88 (5). B/Victoria group strains were dominant in the 1980s, whereas B/Yamagata strains became dominant in the early 1990s (5, 10, 11, 20, 24, 29). In 1994, B/Victoria strains reemerged in southeastern China. In Japan, in the 1996–1997 season, the first epidemic of B/Victoria occurred after the reemergence, and both B/Victoria and B/Yamagata strains were isolated in the same season (11). Since then, the strains of both lineages have caused epidemics in turn: B/Yamagata strains in the 1998–1999, 2000–2001, and 2004–2005 seasons and B/Victoria in the 2002–2003 and 2006–2007 seasons (12–14, 16–18).

We have been studying the antigenicities of the influenza B virus with monoclonal antibodies (MAbs). By analyzing the amino acid sequences of the hemagglutinin (HA) molecule, we have previously reported that the antigenic variants of the influenza B virus appeared with a point nucleotide mutation of the HA1 gene which caused the substitution of an amino acid in the HA molecule (12–14, 16–18). Since a three-dimensional model of the influenza A virus HA molecule was reported in the early 1980s (26), the immunodominant antigenic sites of the influenza B virus HA have been determined by comparing its amino acid sequences with those of the influenza A virus HA (1, 6). We have previously reported neutralizing epitope sites detected with MAbs 5H4 and 3A12 in the prominent region of HA (15), which corresponds to “site A” of the influenza A virus HA. The recently reported crystal structure of the HA molecule of the influenza B virus shares an overall similarity and domain organization with that of the influenza A virus. There are four major epitopes, the 120 loop (amino acids aa 116 to 137), the 150 loop (aa 141 to 150), the 160 loop (aa 162 to 167), and the 190 helix (aa 194 to 202) (25). The epitope sites of 5H4 and 3A12 have been determined to be in the 150 loop. The epitope sites are specific for B/Yamagata strains and were conserved from the late 1980s until the isolates that did not react to 5H4 by hemagglutination inhibition (HI) tests appeared in the 1998–1999 season. A single nucleotide mutation that created an amino acid substitution in the 150 loop (R149K) was responsible for modulating the 5H4 epitope (13), and the R149K virus became a major isolate in the following B/Yamagata epidemic in the 2000–2001 season (14). In contrast to 5H4, 3A12 possessed HI activities against R149K variants as well, and all of the 2000–2001 isolates in Kobe, Japan, reacted well to 3A12 on HI tests. On the other hand, the antigenic variants that appeared in the 2000–2001 epidemics revealed a point mutation in the 120 loop (D126N). With the plaque cloning method, one of the clinical isolates was shown to be a mixture of the vaccine-type virus and the antigenic variant (14). This finding was confirmed by means of high-resolution melting curve analysis with LCGreen (19). This new technique clearly demonstrated that the vaccine-type virus and the antigenic variant were circulating together during the epidemic and that humans were exposed to the mixture.

Melting curve analysis is a recently introduced automated, high-throughput method for detecting single-nucleotide polymorphisms (SNPs). The importance of routine detection of genetic SNPs has been emphasized to identify drug responders or non-responders and patients at increased risk for drug toxicity (4). Therefore, a simple and rapid method of analyzing SNPs is
needed. At the end of the 20th century, the fluorescent melting analysis of PCR in conjunction with real-time PCR was introduced (23, 27) and was followed by melting techniques using fluorescently labeled oligonucleotide probes (2, 9). Then, high-resolution melting curve analysis was reported as a convenient technique (3). This technique is performed with closed tubes and can be completed in less than 15 min. It does not require real-time PCR instruments, allele-specific PCR, or fluorescently labeled oligonucleotides. The process is made possible by heteroduplex-detecting DNA dyes that can be used at saturating concentrations without inhibiting PCR. In particular, LCGreen was reported to be superior to other dyes, such as SYBR green I, ethidium bromide, SUBR gold, Pico green, TOTO-1, and YOYO-1 (28). Wild-type and homozymous-mutant samples are distinguished by melting temperature ($T_m$) shifts.

As mentioned above, the antigenic variants of influenza B virus appear during the epidemic season and have been isolated from clinical specimens along with the vaccine-type strains (12, 14, 16). With the virus plaque cloning method, it was demonstrated that the vaccine-type strains and the variants were present in isolates from individual patients (14, 16). When the proportions of the two viruses with distinct antigenicities were not equal, the less prevalent virus was not always detected with the HI test or direct sequencing. Therefore, it was necessary to perform virus plaque cloning in order to detect the mixture-type isolates (16, 19). However, the virus plaque cloning method requires considerable time and labor.

The fact that the antigenic variants of the influenza B virus often appear with one-point mutations in the HA1 gene (12–14, 16–18) gave us the idea of applying the melting curve analysis with LCGreen to distinguish antigenic variants from vaccine-type strains. By this easy method, it became possible to determine in a short time a number of isolates to be vaccine-type strains, the antigenic variants, or a mixture of both. In addition, the proportions of the antigenic variants in the mixture-type isolates were estimated (19).

In the 2004–2005 season, a large epidemic of B/Yamagata occurred in Japan. This epidemic was as large as the epidemics in the late 1980s, when B/Yamagata caused the first epidemic in Japan. However, most of the isolates were vaccine-type viruses. Only a small number of antigenic variants were obtained around the peak of the epidemics. By means of melting curve analysis with LCGreen, we studied whether the variants were cocirculating with the vaccine-type virus from the earlier period of the epidemic.

**MATERIALS AND METHODS**

**Viruses.** A total of 87 influenza virus B/Yamagata isolates in Kobe, Japan. 85 isolated in the 2004–2005 season and 2 isolated in previous seasons, were utilized. Propagation was carried out in Madin-Darby canine kidney (MDCK) cells.

**Antibodies.** MAB 3A12 was obtained from mice immunized with the B/Yamagata group strain B/Kadoma/506/1999. The ascitic fluid from mice injected with hybridoma cells was used as a source of Mabs. Every year, standard sera are provided by the National Institute of Infectious Diseases (NIID), Tokyo, Japan: for B/Yamagata, hyperimmune sheep serum against B/Johannesburg/5/1999 for the 2004–2005 season and immune ferret serum against B/Shanghai/361/2002 for the 2005–2006 season. Human serum samples were collected before and after the epidemic from patients aged 31 to 59 years.

**HI tests.** HI tests were performed with antibodies treated with a receptor-destroying enzyme (Takeda Chemical Industries Ltd., Osaka, Japan) and guinea pig red blood cells. The HI titer is the reciprocal of the antibody dilution (21).

**RESULTS**

**Characteristics of the 2004–2005 Kobe isolates.** In the 2004–2005 season, a total of 125 strains were isolated from clinical specimens in Kobe, Japan. With the HI tests, 40 strains were identified to be AH3, and 85 strains were identified to be B/Yamagata. Among the 85 B/Yamagata strains, 53 strains were isolated from patients younger than 16 years, 9 strains from patients aged 17 to 29 years, 17 strains from patients aged 31 to 59 years, and 6 strains from patients older than 61 years. All 85 strains reacted well to the hyperimmune sheep serum against B/Johannesburg/5/1999, the standard serum for B/Yamagata provided by NIID Japan for the 2004–2005 season. When the HI tests were performed with 3A12, 82 of 85 strains reacted well. B/Kobe/103/2005 did not react to 3A12 at all, whereas the other two strains, B/Kobe/113/2005 and B/Kobe/115/2005, reacted to 3A12 at lower titers (Fig. 1). These three strains were isolated at the peak of the influenza epidemic from patients aged 31 to 59 years. The immune ferret serum against the vaccine strain B/Shanghai/361/2002 was provided by NIID Japan as the standard serum for B/Yamagata for the 2005–2006 season. The viral antigenicities of the isolates were studied retrospectively (Fig. 1). B/Kobe/87/2001 is a representative B/Yamagata strain from the previous epidemic, and B/Kobe/3/2004 is a representative B/Yamagata strain from...
the previous season. The HI titer of the ferret serum to B/Kobe/103/2005 was 1/16 that for the vaccine strains and 1/4 that for the control strains. In contrast, the HI titers to B/Kobe/113/2005 and B/Kobe/115/2005 were similar to those for the control strains.

The HI tests were performed with the human serum samples collected before and after the 2004–2005 season (Table 1). Two serum samples collected before the 2004–2005 season reacted to B/Kobe/67/2005 with titers similar to those for B/Kobe/87/2001. However, they reacted at titers 1/4 to 1/16 that for B/Kobe/103/2005, B/Kobe/113/2005, and B/Kobe/115/2005. In contrast, eight of nine serum samples collected after the season reacted to the 2004–2005 isolates equally whether they were the vaccine-type strains or the variants; the HI titers of three serum samples were 80 to 160 and those of five serum samples were 20 to 40. Among these serum samples, two were taken from the same persons who donated the serum samples before the season (donors A and B). The low HI titers against B/Kobe/103/2005, B/Kobe/113/2005, and B/Kobe/115/2005 increased after the season. From the results shown in Fig. 1 and Table 1, B/Kobe/103/2005 was determined to be an antigenic variant. As for B/Kobe/113/2005 and B/Kobe/115/2005, the results with ferret serum and human serum were inconsistent. This might be due to a difference in the antigenicities of the vaccine strain and the previous Kobe strains. Here, we examine the difference in viral antigenicity between the Kobe isolates in the 2004–2005 season and those in the previous seasons. Therefore, we focused on the results with human serum samples and determined B/Kobe/113/2005 and B/Kobe/115/2005 to be antigenic variants. Conclusively, two kinds of the antigenic variants were isolated, and the residents of Kobe developed antibodies against them during the 2004–2005 epidemic.

**Genetic analysis.** Further analyses were performed with 17 strains isolated from patients aged 31 to 59 years. First, the nucleotide sequence (nt 6 to 1008) and the deduced amino acid sequence of the HA1 gene were analyzed and were compared with those of strains isolated in the previous seasons (Fig. 1). The nucleotide sequences in the 150 loop (nt 421 to 447) of 14 isolates (DDBJ accession no. AB385603, AB385605, and AB426532 to AB426543) were exactly the same as those of the previous 2 isolates, B/Kobe/87/2001 and B/Kobe/3/2004 (AB071620 and AB381880), whereas three antigenic variants showed single nucleotide mutations that corresponded to amino acid substitutions. B/Kobe/103/2005 (AB385604), which did not react to 3A12, had a nucleotide mutation, C440A, corresponding to an amino acid substitution, T147N. B/Kobe/113/2005 and B/Kobe/115/2005 (AB385607 and AB385608), which reacted with 3A12 at lower titers, had a nucleotide mutation, G421A, corresponding to an amino acid substitution, G141R.

### Table 1. Results of the HI tests done with human serum samples donated before and after the 2004–2005 season

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sample donated before the season</th>
<th>Sample donated after the season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>B/Kobe/87/2001</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>B/Kobe/3/2004</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>B/Kobe/39/2005</td>
<td>80</td>
<td>ND</td>
</tr>
<tr>
<td>B/Kobe/67/2005</td>
<td>320</td>
<td>160</td>
</tr>
<tr>
<td>B/Kobe/105/2005</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>B/Kobe/113/2005</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>B/Kobe/115/2005</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

*a* The HI titer is shown as the reciprocal of the antibody dilution.

*b* ND, not done.
Treatment with high-dose 3A12. Next, 17 strains were treated with 3A12 by means of a method similar to that for inducing laboratory mutants (15) by modifying the ratios of the virus and the MAb. The isolates were treated with a 10-times-higher dose of the MAb and inoculated in MDCK cells in 24-well plates. As a control, virus treated with medium only was inoculated in MDCK cells. Inoculated-virus titers were adjusted so that the control wells were filled with cells showing cytopathic effects on the next day (the second day). (The days in parentheses show when the wells were filled with the cells showing cytopathic effects.) The replication of B/Kobe/103/2005 was not affected by treatment with 3A12 (the second day). The replication of B/Kobe/113/2005 and B/Kobe/115/2005 occurred only a day later (the third day). With the above-mentioned three antigenic variants, the HI titers to 3A12 were not affected by whether the strains had been treated with 3A12. The other 14 strains reacted differently. The cytopathic effects of four strains were observed on the fourth day, and the harvested virus did not react to 3A12 in the HI tests. The experiments were repeated with these four strains, and the same result was observed with one strain, B/Kobe/39/2005. With 10 other strains, no cytopathic effects were observed as late as the sixth day.

Genetic analysis was performed with the 3A12-treated strains of B/Kobe/115/2005 and B/Kobe/39/2005 (B/Kobe/115/2005-T and B/Kobe/39/2005-T, respectively) (Fig. 1). The nucleotide sequence of the HA1 gene (nt 6 to 1008) of B/Kobe/115/2005-T (AB385609) was exactly the same as that of B/Kobe/115/2005, whereas that of B/Kobe/39/2005-T differed by a nucleotide (C440A) from that of B/Kobe/39/2005, which caused a difference in an amino acid (T147N) (AB385606). Therefore, it was considered that a nucleotide mutation, C440A, was responsible for the alteration of the virus reactivity to 3A12 in the HI tests. Consequently, the nucleotide sequence of the 150 loop of B/Kobe/39/2005-T was exactly the same as that of B/Kobe/103/2005, and B/Kobe/39/2005-T was determined to be an antigenic variant.

High-resolution melting curve analysis with LCGreen. Finally, the isolates were analyzed by means of high-resolution melting curve analysis with LCGreen. Figure 2 shows the normalized, high-resolution melting curves of B/Kobe/103/2005, B/Kobe/67/2005, B/Kobe/39/2005, B/Kobe/39/2005-T, and B/Kobe/3/2004. The curve of B/Kobe/67/2005 was very close to that of B/Kobe/3/2004. In contrast, the curve of an antigenic variant, B/Kobe/103/2005, with a point mutation, C440A, showed the \( T_m \) shift. The curve of B/Kobe/39/2005 was between the curves of B/Kobe/67/2005 and B/Kobe/103/2005, whereas that of B/Kobe/39/2005-T was at the same location as that of B/Kobe/103/2005. Therefore, B/Kobe/39/2005 was shown to be a mixture of the vaccine-type virus and the antigenic variant.

Figure 3 shows the curves of the other antigenic variants, B/Kobe/113/2005 and B/Kobe/115/2005. Their curves showed \( T_m \) shifts from the curve of B/Kobe/67/2005; however, they were not colocalated. The curve of B/Kobe/115/2005 was shifted slightly toward that of B/Kobe/67/2005. The experiments were repeated three times, and the results were consistent. On the other hand, the curve of B/Kobe/115/2005-T was at the same location as that of B/Kobe/113/2005. Therefore, we concluded that B/Kobe/115/2005 was a mixture of the antigenic variant and a small amount of the vaccine-type virus, although the HI tests showed that it was an antigenic variant.

Figure 4 shows the curves of other isolates that the HI tests had shown to be vaccine-type viruses. The curves of the majority of the isolates were very close to the curve of B/Kobe/67/2005, suggesting that they comprised mostly vaccine-type virus. However, the curves of a few isolates were rather close to the curve of B/Kobe/39/2005, suggesting that a small amount of the antigenic variants was present in the isolates.

DISCUSSION

For the past 10 years in Japan, an epidemic of the influenza A virus, either subtype H1 or subtype H3, has occurred every year, and an epidemic of the influenza B virus, either the
Victoria or the Yamagata group, has occurred every other year. One major reason for the repeated epidemics is that the different types of virus cause them in turn. In addition, the viral antigenicities vary from season to season. In particular, the antigenicities of the influenza A virus vary frequently. In contrast, the antigenicities of the influenza B virus are relatively stable (10, 11). This stability might be due to the fact that the influenza B virus does not possess the abundant reservoirs that the influenza A virus does. The influenza B virus has been isolated primarily from humans, except for a few isolates from seals (22). Therefore, the antigenic variants of the influenza B virus are suggested to be created in humans. We have been studying the antigenicities of field isolates of the influenza B virus in Japan for the past 10 years. The antigenic variants appeared during the epidemic season, usually with a point mutation of a nucleotide in the HA1 gene, which resulted in an amino acid substitution. Then, some of the variants became major isolates in subsequent seasons (12, 14, 15, 17). As mentioned in the introduction, the neutralizing epitope of 3A12 specific for B/Yamagata has been conserved since the late 1980s. We speculate that the established and accumulated human immunities began to induce the naturally occurring antigenic variants by modulating the 3A12 epitope. The 2004–2005 epidemic of B/Yamagata in Japan was one of the largest epidemics in the past 20 years. However, in Kobe, the majority of clinical isolates showed antigenicities similar to those of isolates from the B/Yamagata epidemic of the 2000–2001 season (Fig. 1; Table 1). The isolates reacted to 3A12 as well. A similar tendency was observed throughout Japan in that 97% of all Japanese isolates were vaccine-type viruses (7). In spite of the fact that only a few antigenic variants were isolated, the residents of Kobe acquired antibodies against them during the season. How can these discrepancies be explained? We postulate three reasons for the discrepancies. First, the 2004–2005 epidemics started almost a month late in Japan. The number of cases of influenza began to increase in the beginning of February and peaked in the middle of March. Therefore, 80% of the influenza B isolates were obtained before the peak of the epidemics. It is possible that the antigenic variants were isolated in greater numbers in the latter half of the epidemic. Second, most of the clinical specimens were obtained from children younger than 16 years. The reason is that the majority of the surveillance point clinics were pediatric offices. As explained in the introduction, B/Yamagata epidemics occurred in the 1998–1999, 2000–2001, and 2004–2005 seasons. Because the 2000–2001 epidemic was not so large, children younger than 6 years might not have been exposed to the B/Yamagata strains. They were naïve for the vaccine-type virus. Thus, it is possible that these children were infected with the vaccine-type virus, even when both the vaccine-type virus and the antigenic variants were circulating. Finally, the HI tests have a technical problem. When the viruses with distinct antigenicities are present in the isolates of single patients and the proportions of the two viruses are not equal, the less prevalent virus is not always detected with the HI test (12, 14). For example, two strains in the 2000–2001 season were found to be mixtures of the antigenic variants and the vaccine-type virus at a ratio of 2:1 by means of melting curve analysis with LCGreen, although they had been identified as antigenic variants with the HI test and direct sequencing (19). In these cases, the presence of the vaccine-type virus was not detected with the HI test. Therefore, in the case of the 2004–2005 isolates, the presence of the antigenic variants may not have been detected with the HI test with 3A12. For these reasons, we selected 17 strains isolated from patients aged 31 to 59 years and analyzed them further. First, the strains were treated with a high dose of 3A12. The fact that B/Kobe/39/2005 was not completely neutralized with 3A12 suggested that antigenic variants were present. However, the escaping mutants might have been induced by treatment with 3A12. Therefore, B/Kobe/39/2005 was analyzed further by means of melting curve analysis with LCGreen and was found to be a mixture of the vaccine-type virus and the antigenic variant (Fig. 2). Furthermore, some other strains were found to be mixtures, although the proportions of the variants were lower (Fig. 4). B/Kobe/103/2005, an antigenic variant, was isolated at the peak of the epidemic; however, B/Kobe/39/2005, a mixture strain, was obtained in the beginning of the season. Therefore, the antigenic variants had already appeared in the early period of the epidemic and were circulating with the vaccine-type virus. We speculate that the antigenic variants were selected under human immunity, when persons with acquired antibodies were infected with the mixture virus. This might explain why the B/Yamagata epidemic in the 2004–2005 season was so large.

We have speculated that the antigenic variants appeared during the epidemic season and that they became major isolates in subsequent seasons (12, 16, 17). By means of melting curve analysis with LCGreen, the appearance of the antigenic variants was detected dynamically. This novel technique allows us to analyze the antigenic shifts more precisely. The information will benefit public health management, especially from the perspective of selecting suitable strains for vaccines and predicting the scale of future epidemics.
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