Neutralizing epitopes specific for influenza B virus Yamagata group strains are in the ‘loop’

Naoko Nakagawa,1 Ritsuko Kubota,2 Toshimasa Nakagawa3 and Yoshinobu Okuno2

1Department of Parasitic Agents, Kobe Institute of Health, 4-6, Minatojima-nakamachi, Chuo-ku, Kobe 650-0046, Japan
2Division of Virology, Department of Public Health, Osaka Prefectural Institute of Public Health, 3-69, 1-Chome, Nakamichi, Higashinari-ku, Osaka 537-0025, Japan
3Department of Clinical Pathology, Osaka Medical College, 2-7 Daigaku-cho, Takatsuki 569-8686, Japan

To study the neutralizing epitopes of influenza B virus Yamagata group strains, two monoclonal antibodies (mAbs) were used to select escape mutants of the virus. mAbs 5H4 and 3A12 were found to react with B/Yamagata group strains in haemagglutination inhibition and neutralization tests; no reactivity with B/Victoria group strains was observed. Most of the mutants reacted poorly to polyclonal ferret antibody against the 1998 isolate. Analysis of the deduced amino acid sequences identified a single amino acid substitution at residue 141 (Gly→Arg) or 149 (Arg→Gly) in 5H4-escape mutants and 141 (Gly→Arg), 147 (Thr→Ile) or 148 (Ser→Gly) in 3A12-escape mutants. These residues are situated in close proximity in the ‘loop’ of the haemagglutinin molecule. These epitopes have been conserved in B/Yamagata group strains for almost 10 years in Japan but amino acid substitutions in the loop have been observed in clinical isolates only since 1999.

There are influenza epidemics every year. Influenza B virus strains cause epidemics in humans as H1 and H3 subtype strains of influenza A virus. In contrast to the antigenicities of influenza A virus, those of influenza B virus are relatively stable (Lindstrom et al., 1999; McCullers et al., 1999). Recent isolates of influenza B virus strains are divided into two large phylogenetic trees: one group is represented by B/Victoria/2/87 and the other is represented by B/Yamagata/16/88 (Kanegae et al., 1990). B/Victoria group strains were dominant in the 1980s, while B/Yamagata strains became dominant in the early 1990s (Kanegae et al., 1998; Lindstrom et al., 1999; McCullers et al., 1999; Nerome et al., 1998; Rota et al., 1999; Yamashita et al., 1988). Group-specific antigens have been conserved. Monoclonal antibody (mAb) 10B8 reacted with all B/Victoria group strains in haemagglutination inhibition (HI) and neutralization (NT) tests, while no reactivity was shown with B/Yamagata strains (Nakagawa et al., 1999). The B/Victoria group neutralizing epitope is situated at the ‘tip’ of the haemagglutinin (HA) molecule. This observation is based on the phenomenon that amino acid residues of 10B8-induced escape mutants are either substituted or deleted in this region. B/Yamagata strains lack one to three amino acid residues in the tip; therefore, the epitope for 10B8 is specific for B/Victoria group strains. Furthermore, early B/Yamagata group strains, such as those isolated in 1984, have identities as high as 95% with the B/Victoria group strains isolated at that time. This fact suggested that neutralizing antibodies, such as mAb 10B8, induced the B/Victoria group strains to create antigenic variants with amino acid deletions for survival and consequently, Yamagata group strains appeared (Nakagawa et al., 2001a). In contrast to mAb 10B8, mAbs 5H4 and 3A12 reacted with all B/Yamagata strains in HI and NT tests. However, in Japan, 5H4-nonreacting strains appeared in the 1998–1999 season and have been increasing in number ever since. A single amino acid substitution at position 149 (Arg→Lys) in the HA gene created these natural variants (Nakagawa et al., 2001b).

We studied the neutralizing epitope sites specific for B/Yamagata group strains.

Virus strains B/Kadoma/122/1999, B/Kadoma/409/2000, B/Kobe/69/2001 and B/Kobe/5/2002 were isolated from clinical specimens. mAb 3A12 was obtained by immunizing mice with the 5H4-nonreacting strain B/Kadoma/506/1999. Ascites fluid of mice injected with hybridoma cells were used as the source of all mAbs. The standard ferret polyclonal...
antibody was provided by the National Institute of Infectious Diseases, Japan: the sera against B/Yamanashi/166/1998 for B/Yamagata strains. Human post-vaccination sera were collected at Baba Children’s Clinic in Kadoma City, Osaka Prefecture, Japan. Results of HI and NT tests are expressed as the reciprocal of antibody dilution (Okuno et al., 1990). Competitive assays were performed by modifying the method reported previously (Waxham & Wolinsky, 1985). Escape mutants were induced by incubating B/Kadoma/122/1999 with mAb 3A12 by modifying the method described previously (Berton et al., 1984; Lambkin et al., 1994; Nakagawa et al., 2001a). Briefly, 1 × 10⁷ f.f.u. ml⁻¹ virus were incubated for 1 h at 30°C in the presence of 10 μl mAb 3A12. The virus/mAb mixture was inoculated to Madin–Darby canine kidney cells in 24-well plates and were analysed as described previously (Nakagawa et al., 1994). Nucleotide sequences of the escape mutants (positions 1194–1171) for the first half and (nt 774–751) for the second half.

mAb 3A12 was obtained by immunizing mice with a 5H4-nonreacting strain, as described above. Therefore, the epitopes of mAbs 3A12 and 5H4 should be distinct. However, in the competitive assay, excess amount of one mAb blocked almost 50% of the binding of the other mAb to the virus (data not shown). To analyse the epitope sites precisely, further experiments with escape mutants were performed. We reported previously that two escape mutants (B/Kadoma/122/1999-V1 and -V2) were induced with mAb 5H4 and that they showed a single amino acid substitution at residue 149 (Arg→Gly) and 141 (Gly→Arg) of the HA1 protein, respectively (Nakagawa et al., 2001b). In addition, nine mutants (B/Kadoma/122/1999-V3 to -V11) were induced with mAb 3A12 by incubating the same strain, B/Kadoma/122/1999. Table 1 shows the results of the HI and NT tests. 5H4-escape mutants did not react with either 5H4 or 3A12. Among 122-escape mutants, six (122-V4, -V7, -V8, -V9, -V10 and -V11) did not react with mAb 5H4. In contrast, three 122-escape mutants (122-V3, -V5 and -V6) reacted well with mAb 5H4. Analysis of nucleotide sequences clarified that all escape mutants had single point mutations in the HA gene that correspond to single amino acid substitutions. All of the 5H4-nonreacting mutants showed the same substitution observed with B/Kadoma/122/1999-V2 (Gly141→Arg). Two of the 5H4-reacting mutants (B/Kadoma/122/1999-V3 and -V6) showed the substitution at residue 148 (Ser→Gly) and the other (B/Kadoma/122/1999-V5) at residue 147 (Thr→Ile) (Table 1).

Virus reactivities to the polyclonal ferret antibody against B/Yamanashi/166/1998 are shown in Table 1. The clinical isolates, both 5H4-reacting B/Kadoma/122/1999 and 5H4-nonreacting B/Kadoma/409/2000 (Arg149→Lys), reacted well with this polyclonal antibody. In contrast, 10 of 11 escape mutants reacted poorly with it; however, the degree of reactivity varied. Only B/Kadoma/122/1999-V5 reacted with the polyclonal ferret antibody in HI and NT tests at levels similar to those found for a parental strain, B/Kadoma/122/1999. It is worth mentioning that B/Kadoma/122/1999-V1 (Arg149→Gly) hardly reacted with the polyclonal antisera, although B/Kadoma/409/2000 (Arg149→Lys) reacted well. The alternation of virus reactivity to polyclonal antibody depends greatly on which amino acid is substituted for the Arg residue at position 149. It is suggested that the

### Table 1. Results of HI and HI tests and deduced amino acid residues of laboratory-induced escape mutants

<table>
<thead>
<tr>
<th>Strains</th>
<th>5H4 NT test</th>
<th>3A12 NT test</th>
<th>Ferret sera*</th>
<th>5H4 HI test</th>
<th>3A12 HI test</th>
<th>Ferret sera*</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/Kadoma/122/1999</td>
<td>25 600</td>
<td>12 800</td>
<td>2 560</td>
<td>25 600</td>
<td>6 400</td>
<td>320</td>
<td>Arg¹⁴⁹→Lys</td>
</tr>
<tr>
<td>B/Kadoma/409/2000</td>
<td>&lt;100</td>
<td>12 800</td>
<td>2 560</td>
<td>&lt;100</td>
<td>6 400</td>
<td>320</td>
<td>Arg¹⁴⁹→Gly Gly¹⁴¹→Arg</td>
</tr>
<tr>
<td>5H4-escape mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V1</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>20</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;10</td>
<td>Arg¹⁴⁹→Gly Gly¹⁴¹→Arg</td>
</tr>
<tr>
<td>V2</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>80</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>3A12-escape mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3, V6</td>
<td>12 800</td>
<td>&lt;100</td>
<td>160</td>
<td>51 200</td>
<td>&lt;100</td>
<td>20</td>
<td>Ser¹⁴⁸→Gly Gly¹⁴¹→Arg</td>
</tr>
<tr>
<td>V4, V7–V11</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>80</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>V5</td>
<td>12 800</td>
<td>&lt;100</td>
<td>5 120</td>
<td>51 200</td>
<td>&lt;100</td>
<td>320</td>
<td>Thr¹⁴⁷→Ile</td>
</tr>
</tbody>
</table>

*The ferret sera against B/Yamanashi/166/1998 was utilized.
structure of the HA protein is influenced more when Arg is substituted by a Gly residue rather than a Lys residue.

Amino acid sequences of the HA1 polypeptides of the influenza B virus strains were compared to those of A/Aichi/2/68 and numbered according to the A/Aichi sequence for ease of reference to the structure of the H3 HA molecule of influenza A virus (Berton et al., 1984; Krystal et al., 1982). With this method, amino acid 141 is referred to as 137, 142, 143 and 144. Fig. 1 shows the three-dimensional structure of the influenza A virus H3 HA molecule, as determined by Wilson et al. (1981). Though the identified amino acid substitutions of influenza B virus are only predicted on the basis of sequence alignments, all of the amino acid residues above are suggested to be close to each other in the HA loop. The neutralizing epitopes of mAbs 5H4 and 3A12 are either overlapping or situated close enough to affect each other. The data of the competitive assay that the binding of one mAb was partially blocked by the other supported this idea. The loop, as well as the tip, has been reported as one of the most antigenic sites for the influenza virus HA protein. The variation in these regions is often observed in naturally occurring or laboratory-induced antigenic variants (Berton et al., 1984; Berton & Webster, 1985; Cleveland et al., 1997; Daniels et al., 1987; Lambkin et al., 1994; Luoh et al., 1992; Wiley et al., 1981). In contrast to the B/Victoria group-specific epitope site being situated in the tip (Nakagawa et al., 2001a), those of B/Yamagata strains are located in the loop. In addition, there exist more than two independent neutralizing sites in the loop. This is based on the fact that 5H4-nonreacting strains isolated from clinical specimens reacted well with mAb 3A12, as did three of the 3A12-escape mutants react with mAb 5H4 (Table 1). Eventually, it is of note that most 5H4- or 3A12-escape mutants failed to react well with polyclonal ferret antibody. Therefore, the loop plays an important role(s) in the neutralization of B/Yamagata group strains.

Group-specific antigens have been conserved for 10 years of B/Yamagata group strain epidemics. Actually, in Japan, the standard ferret sera were prepared using B/Mie/1/93 strain for the epidemic season in 1994–1995 through 1998–1999. All 1998–1999 B/Yamagata isolates in Osaka Prefecture reacted well with it in HI tests. However, the established human immunity began to induce naturally occurring antigenic variants (Fig. 2). At first, in the 1998–1999 season, 5H4-nonreacting strains appeared with the amino acid substitution at residue 149 (Arg→Lys) (Nakagawa et al., 2001b). Then, in the 2000–2001 season, isolates were found to react only poorly to the polyclonal ferret antibody against the previous isolate.

<table>
<thead>
<tr>
<th>Fig. 1. Three-dimensional model of the influenza A virus HA molecule. Data on the secondary structure of the HA molecule of A/Aichi/2/68 (code: 2VIU) was obtained through the internet using RASMOL software. A view of the HA molecule from the front is shown. Residues 137, 142, 143 and 144 are marked. Numbers in parentheses show the residues of influenza B virus that refer to the above residues of influenza A virus, respectively.</th>
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<tr>
<th>Fig. 2. Comparison of deduced amino acid sequences of the HA1 region and the HI titres of the clinical isolates. Sequences of amino acid residues 12–336 of the strains shown compared with those of B/Kadoma/122/1999. For a polyclonal antibody, the ferret sera against B/Yamanashi/166/1998 was utilized. For human post-vaccination sera, those of 24-month-old children were utilized.</th>
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</table>
phenomenon resulted from the amino acid substitution at residue 126 (Asn→Asp), which is situated outside of the loop (Nakagawa et al., 2002). All strains reacted with mAb 3A12 and escape mutants induced from the 2000–2001 isolates with mAb 3A12 showed the substitution at position 147 (Thr→Ile). In the 2001–2002 season, a strain was isolated with another substitution in the loop at residue 148 (Ser→Asp). Though this amino acid constitutes the neutralizing epitope site for mAb 3A12 (Fig. 1), this substitution did not alter virus responses to mAb 3A12. Instead, reactivity with the polyclonal antibody was reduced further (Fig. 2). This substitution might have affected other neutralizing sites in the loop. The idea was confirmed further with data from human post-vaccination sera taken from 24-month-old children who had no history of influenza virus infection. These children were vaccinated with B/Yamanashi/166/1998 in the first year and with B/Johannesburg/5/1999 in the second year of their lives. The antigenic characters of these vaccine strains are similar to those of B/Kadoma/122/1999 and B/Kadoma/409/2000 strains, respectively. In HI tests, both sera showed HI titres of 80–160 to B/Kadoma/122/1999 and B/Kadoma/409/2000, which were as high as those to the vaccine strains. However, the sera reacted less strongly with B/Kobe/69/2001. Finally, they did not react with B/Kobe/5/2002. Therefore, it is suspected that these vaccinations did not successfully prevent the children from the infection of the recent influenza B virus strains with amino acid substitutions at positions 126 and/or 147. The phenomenon confirms the idea that multiple neutralizing epitopes are situated in the loop.

When new amino acid substitutions or deletions take place in the loop, the virus gains distinct antigenicities from the existing ones and a new group of influenza B virus may appear from the variants. This information is of benefit to the management of public health; for example, for speculation on the scale of future epidemics and especially from the point of view of selecting suitable strains for vaccines.

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