Antigenic variants with amino acid deletions clarify a neutralizing epitope specific for influenza B virus Victoria group strains

Naoko Nakagawa,1 Ritsuko Kubota,1 Toshimasa Nakagawa2 and Yoshinobu Okuno1

1 Division of Virology, Department Public Health, Osaka Prefectural Institute of Public Health, 3-69 1-Chome, Nakamichi, Higashinari-ku, Osaka 537-0025, Japan
2 Department of Clinical Pathology, Osaka Medical College, 2-7 Daigaku-cho, Takatsuki 569-8686, Japan

To study the neutralizing epitopes of influenza B virus Victoria group strains, two monoclonal antibodies (MAbs) were used to select antigenic variants of the virus. MAbs 10B8 and 8E6 were found to react with B/Victoria group strains in three tests, peroxidase–antiperoxidase staining, haemagglutination inhibition and neutralization tests; no reactivity with B/Yamagata group strains was observed. Analysis of the deduced amino acid sequences of 10B8-induced variants identified a single amino acid deletion at residue 165 or 170, as well as a single amino acid substitution at residues 164 (Asp → Tyr), 165 (Asn → Ser or Thr) or 203 (Lys → Thr or Asn). A single amino acid substitution at residue 241 (Pro → Ser) was observed in 8E6-induced variants. Three-dimensional analysis showed that the epitopes for both MAbs were situated in close proximity to each other. Since B/Yamagata group strains are characterized by amino acid deletions at residues 164–166, the epitope for MAb 10B8 is strictly specific for B/Victoria group strains.

Influenza is one of the most important infectious diseases in both industrial and developing countries. Influenza B virus strains cause epidemics in humans as H1 and H3 subtype strains of influenza A virus. Compared with influenza A virus, influenza B virus is characterized by a low rate of antigenic change; genetic reassortment, insertion and deletion are responsible for its evolution (Lindstrom et al., 1999; McCullers et al., 1999). 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). Virus strains of the B/Victoria group were dominant all over the world in the 1980s, while the B/Yamagata group strains became dominant in the early 1990s. However, in the mid 1990s, B/Victoria group strains re-emerged in South China. Since the 1996–1997 epidemic season in Japan, two distinctly different antigenic groups of influenza B viruses have been isolated from clinical specimens during the same epidemic season (Nakagawa et al., 1999; Nerome et al., 1998). We obtained monoclonal antibodies (MAbs) specific for influenza B virus and used them for peroxidase–antiperoxidase (PAP) staining. When MAbs specific for either the nucleoprotein or the matrix protein were used, both the B/Victoria and B/Yamagata group strains were detected equally well. When MAb 10B8, which is specific for the haemagglutinin (HA) molecule, was used for PAP staining, the two strains were clearly distinguishable (Nakagawa et al., 1999). MAb 10B8 not only reacted with all B/Victoria group strains in PAP staining, but also showed strong activity in haemagglutination inhibition (HI) and neutralization (NT) tests. MAb 8E6 reacted with about 70% of the B/Victoria group strains isolated during the 1996–1997 season in PAP staining and showed HI and NT test reactivity with 45%. Neither MAb reacted with the B/Yamagata group strains (Nakagawa et al., 2000).

In order to study neutralizing epitopes that are specific for the B/Victoria group strains, antigenic variants were induced by incubating B/Osaka/983/97 with MAb 10B8 or 8E6. B/Osaka/983/97 is a B/Victoria group strain with which MAbs 10B8 and 8E6 show strong reactivity in HI and NT tests (Nakagawa et al., 2000). B/Osaka/983/97 was cloned by the plaque method and then diluted to $1 \times 10^6$ f.f.u./ml. As the source of MAbs, ascitic fluids of mice injected with hybridoma cells were utilized. Table 1 shows the HI and NT test results expressed as a reciprocal of the antibody dilution (Okuno et al.,...
Table 1. Results of PAP staining, HI and NT tests and deduced amino acid residues

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>MAb 10B8</th>
<th>MAb 8E6</th>
<th>MAb 10B8</th>
<th>MAb 8E6</th>
<th>MAb 10B8</th>
<th>MAb 8E6</th>
<th>Amino acid substitution or deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/Osaka/983/97</td>
<td>+</td>
<td>+</td>
<td>25,600</td>
<td>12,800</td>
<td>25,600</td>
<td>12,800</td>
<td>Asn&lt;sup&gt;165&lt;/sup&gt; → Ser</td>
</tr>
<tr>
<td>V1</td>
<td>–</td>
<td>+</td>
<td>&lt;100</td>
<td>12,800</td>
<td>&lt;100</td>
<td>12,800</td>
<td>Asn&lt;sup&gt;165&lt;/sup&gt; → Tyr</td>
</tr>
<tr>
<td>V2</td>
<td>–</td>
<td>+</td>
<td>&lt;100</td>
<td>6,400</td>
<td>&lt;100</td>
<td>6,400</td>
<td>Lys&lt;sup&gt;803&lt;/sup&gt; → Thr</td>
</tr>
<tr>
<td>V3 and V6</td>
<td>–</td>
<td>–</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>Asp&lt;sup&gt;164&lt;/sup&gt; → Tyr</td>
</tr>
<tr>
<td>V4</td>
<td>–</td>
<td>+</td>
<td>&lt;100</td>
<td>1,600</td>
<td>&lt;100</td>
<td>3,200</td>
<td>Asp&lt;sup&gt;164&lt;/sup&gt; → Tyr, Lys&lt;sup&gt;803&lt;/sup&gt; → Asn</td>
</tr>
<tr>
<td>V5</td>
<td>–</td>
<td>+</td>
<td>&lt;100</td>
<td>1,600</td>
<td>&lt;100</td>
<td>3,200</td>
<td>Thr&lt;sup&gt;170&lt;/sup&gt; → X*</td>
</tr>
<tr>
<td>V6</td>
<td>–</td>
<td>+</td>
<td>&lt;100</td>
<td>3,200</td>
<td>&lt;100</td>
<td>3,200</td>
<td>Asn&lt;sup&gt;165&lt;/sup&gt; → X*</td>
</tr>
<tr>
<td>V7</td>
<td>–</td>
<td>+</td>
<td>&lt;100</td>
<td>1,600</td>
<td>&lt;100</td>
<td>3,200</td>
<td>Pro&lt;sup&gt;241&lt;/sup&gt; → Ser</td>
</tr>
<tr>
<td>M1 and M2</td>
<td>+</td>
<td>–</td>
<td>25,600</td>
<td>&lt;100</td>
<td>25,600</td>
<td>&lt;100</td>
<td></td>
</tr>
</tbody>
</table>

* X, Amino acid deletion.

1990). A 2 ml sample of virus was incubated for 1 h at 30 °C in the presence of either 125 µl of MAb 8E6 or 62.5 µl of MAb 10B8. The virus–MAb mixture was inoculated into 10-day-old embryonated chicken eggs (100 µl per egg) and the allantoic fluid was harvested after 60 h of incubation at 35 °C (Berton et al., 1984; Lambkin et al., 1994). HI and NT tests on the allantoic fluid of each egg were performed separately with each MAb in order to identify antigenic variants. Finally, eight variants (V1–8) of B/Osaka/983/97 were obtained in the presence of MAb 10B8 and two variants (M1 and M2) were obtained in the presence of MAb 8E6. Table 1 shows a summary of the three tests. None of the eight 10B8-induced variants reacted with MAb 10B8 in subsequent PAP staining, HI or NT tests. Although MAb 8E6 did not react with V3 and V8 in any of the three tests, reactivity with the other six variants was evident. The degree of the reactivity was, however, varied. Similarly, MAb 8E6 did not react with 8E6-induced variants M1 and M2, but, in contrast, MAb 10B8 did react with M1 and M2 in each of the three tests at levels similar to those found for parental strain B/Osaka/983/97 (Table 1).

The nucleotide sequences of the antigenic variants were analysed as reported previously (Nakagawa et al., 2000). RNA was obtained from virus-infected Madin–Darby canine kidney cells and cDNA synthesis was carried out. Influenza B virus-specific DNA was amplified by PCR and products were sequenced with the ABI Prism Big Dye Terminator Cycle Ready Reaction kit (Perkin Elmer). Samples were analysed using the ABI Prism 310 automatic sequencer (Perkin Elmer). As sequencing of the HA1 region was carried out in two sections, two sets of primers were prepared. 5’ CTACTCTGTTAGTACATCC (nt 52–72) and 3’ TGGGAAGCCACCAAATCTGAGAAAC (nt 774–751) were used for the first half and 5’ ACCTCAGGATCTTGCCCTAAGG (nt 493–514) and 3’ TGTCTATCCGTGCCAACCCTGCAAT (nt 1194–1171) were used for the second half. When antigenic variants V1–8 were analysed, a single point mutation, resulting in a single amino acid substitution at residues 164 (Asp → Tyr; V4), 165 (Asn → Ser or Tyr; V1 and V2) or 203 (Lys → Thr; V3 and V8), was observed in five of the variants. V3 and V8, which did not react with either MAb 8E6 or 10B8, showed an amino acid substitution at residue 203, whereas V5 contained a double point mutation, resulting in single amino acid substitutions at residues 164 (Asp → Tyr) and 203 (Lys → Asn). V6 and V7 lacked three continuous nucleic acids, resulting in a single amino acid deletion at residue 165 (Asp) or 170 (Thr). When M1 and M2 were analysed, a single point mutation, resulting in a single amino acid substitution at residue 241 (Pro → Ser), was observed (Table 1). Therefore, amino acid substitutions or deletions at residues 164, 165, 170 and 203 affected the epitope for MAb 10B8, while amino acid substitutions at residues 203 and 241 affected the epitope for MAb 8E6.

The 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 164 is referred to as 158, 165 as 159, 170 as 160, 203 as 193 and 241 as 225, respectively. Fig. 1 shows the three-dimensional structure of the influenza A virus H3 HA molecule, as determined by Wilson et al. (1981). Residues 158, 159, 160, 193 and 225 are shown. In contrast to influenza A virus, influenza B virus strains possess four extra amino acids at the top of the HA molecule, which correspond to residues 166–169. Therefore, residues 165 and 170 are referred to as residues 159 and 160 and are situated next to each other. In our previous study with naturally occurring antigenic variants of the B/Victoria group strains (Nakagawa...
et al., 2000), we reported that substitutions of amino acids at residues 197 (Ser → Lys or Asn) and 199 (Ala → Asn or Thr) modulated the neutralizing epitope for MAb 8E6. Therefore, in Fig. 1, we marked both amino acid residues and referred to them as residues 187 and 189, respectively. However, certain amino acid substitutions at residues 197 (Asp → Ser) and 199 (Ala → Thr or Ile) did not alter MAb 8E6 neutralization. The same phenomenon is observed with the amino acid substitution at residue 203, in that V3 and V8 (Lys203 → Thr) are not recognized by MAb 8E6 in the three tests, while V5 (Lys203 → Asn) is recognized and neutralized by MAb 8E6, similar to the parental strain (Table 1). Therefore, it is unclear whether these residues constitute an epitope for the MAb or whether only some of the residues constitute an epitope and the remainder influence it. Furthermore, differences in the three-dimensional structure of the influenza A and B virus HA molecules should be considered. However, in Fig. 1(B), it is clear that the epitopes for the two MAbs are situated close to each other, with the amino acid residue 203 (pink) in the middle. Residues 164, 165, 170 and 203 constitute or influence the epitope for MAb 10B8 and residues 197, 199, 203 and 241 constitute or influence the epitope for MAb 8E6. The epitope for MAb 10B8 is located at the very tip of the HA molecule, while that for MAb 8E6 is in the proximity of the proposed receptor-binding pocket (Weis et al., 1988). These sites are close enough to affect each other and, therefore, it is understandable that MAb 8E6 reacted with 10B8-induced variants in the HI and NT tests (Table 1).

Several researchers have studied the antigenic structure of influenza virus with antigenic variants selected with MAbs or polyclonal antibodies (Berton et al., 1984; Berton & Webster, 1985; Cleveland et al., 1997; Lambkin et al., 1994; Luoh et al., 1992; Okuno et al., 1993). Antigenic variants often show a single point mutation resulting in a single amino acid substitution and it was reported, therefore, that single amino acid substitutions are sufficient to alter the antigenicity of the HA molecule of influenza virus (Berton et al., 1984). Actually, most of our antigenic variants, as shown in Table 1, have single amino acid substitutions. However, V6 and V7 showed single amino acid deletions. An amino acid deletion mutant of influenza A virus H3 subtype strain lacking seven amino acids between residues 224 and 230 has been described previously (Daniels et al., 1987), and with influenza B virus strains it is the first report. In contrast to the B/Victoria group strains, the B/Yamagata group strains lack one to three amino acids between residues 164 and 166. Therefore, the epitope for MAb 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 the same time. This fact suggests that
neutralizing antibodies, such as MAb 10B8, induce the B/Victoria group strains to create antigenic variants with amino acid deletions for survival and, consequently, the B/Yamagata group strains appeared.

The neutralizing epitopes of influenza B virus Victoria group strains were analysed. The fact that single amino acid deletions altered the antigenicity of the HA molecule of influenza virus clarified that the epitope for MAb 10B8 is strictly specific for the B/Victoria group strains.

The work is supported by grants from the Ministry of Education, Science and Culture of Japan (#12670386). We thank Ms Yumiko Yamamoto for her excellent technical assistance.

References


Received 20 February 2001; Accepted 15 May 2001