Development of Monoclonal Antibodies to West Nile Virus and Their Application in Immunohistochemistry

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West Nile virus (WNV) is endemic throughout Africa, Eurasia, America, and Australia and has important implications for avian, horse, and human health. In these regions, dead birds are monitored for the presence of WNV through immunohistochemistry (IHC) and PCR. However, a number of the tools for IHC are inadequate owing to their cross-reactivity to other Japanese encephalitis serogroup viruses. Here we have established eight monoclonal antibodies (MAbs) to WNV. Four of them bound to the envelope protein, three of them bound to nonstructural protein 1 (NS1), and one bound to precursor membrane protein (prM), as shown by Western blot analysis. The anti-NS1 MAbs and the anti-prM MAb did not cross-react with Japanese encephalitis virus (JEV), Murray valley encephalitis virus, or St. Louis encephalitis virus in an indirect enzyme-linked immunosorbent assay. One NS1-specific MAb, SHW-32B1, and the previously reported NS1-specific MAb, SHW-7A11, were shown by IHC to specifically detect the cytoplasm of degenerated cells in the heart and brain of a WNV-infected goose. Neither of these MAbs were shown by IHC to cross-react with degenerated cells in the brain of a JEV-infected pig. These MAbs are the first reported anti-NS1 MAbs that can be used for WNV-specific IHC using formalin-fixed, paraffin-embedded sections. They may be useful for WNV research and surveillance.

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ELISA, and Western blotting. All experiments using infectious viruses were approved by the Biosafety Committee of the National Institute of Animal Health in Japan and were performed in a biosafety level 3 laboratory.

Monoclonal antibody production. The methods for MAb production and antibody purification have been previously described (7).

Western blot analysis. Western blot analysis was performed as described previously (7). The anti-WNV NS1 antibody SHW-7A11 (7), the anti-WNV E antibody HB112 (ATCC, Manassas, VA), the anti-WNV prM antibody ab25888 (Abcam, Cambridge, MA), and WNV-infected chicken serum (plaque reduction neutralization test [PRNT] titer of 90% reduction; 1:800; kept in our laboratory) were used as reference antibodies.

Antibody reactivity in Table 1 are indicated in Fig. 1. ND, not done.

IHC result for a:

- 0, no reactivity.
- 1, weak reactivity.
- 2, strong reactivity.

The selection criteria of antibody reactivity are indicated in Fig. 1.

Animal welfare. All experiments using living animals were approved by the Ethics Committee of the National Institute of Animal Health in Japan.

RESULTS

Characterization of monoclonal antibodies. The anti-WNV MAb-secreting hybridomas SHW-2D8, SHW-6F10, SHW-7D12, SHW7G8, SHW-11A9, SHW-18C10, SHW-25C1, and SHW-32B1 were obtained. The immunoglobulin classes and specificity are summarized in Table 2.

Western blot analysis was performed to determine the reactivity of the MAbs. SHW-2D8, SHW-25C1, and SHW-32B1 reacted with NS1, SHW-7D12, SHW-6F10, SHW-7G8, and SHW-18C10 reacted with the E protein, and SHW-11A9 reacted with prM (Fig. 2).

The cross-reactivity of the established MAbs was examined against that of other JEV serocomplex flaviviruses using an indirect ELISA. SHW-2D8, SHW25C1, SHW-32B1, and SHW-11A9 were highly reactive for the WNV NY99 antigen and also reacted with the WNV group (Kunjin, g2266, and Eg-101 [data not shown]). Importantly, they showed no cross-reactivity with JEV, SLEV, and MVEV. The four other MAbs showed cross-reactivity with JEV, SLEV, and MVEV (Fig. 3).

The developed MAbs did not neutralize WNV in PRNT (data not shown).

Immunohistochemistry. SHW-32B1, SHW-7A11, and the anti-WNV rabbit polyclonal antibody strongly reacted with the E protein, and SHW-11A9 reacted with prM (Fig. 2).

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### Table 1: Immunohistochemistry of WNV-infected goose section and JEV-infected pig section

| Antibody | Antibody dilution | WNV-infected white Embden goose | JEV-infected pig brain |
|----------|-------------------|---------------------------------|-----------------------|
| SHW-32B1 | 16 50,000         | ++                              | ND                    |
| SHW-7A11 | 16 50,000         | ++                              | ND                    |
| Anti-WNV monoclonal antibody 7H2 | 125 8 | – – | – – |
| Anti-WNV polyclonal antibody | 512 4,096 | ++ | ++ |
| Anti-JEV polyclonal antibody | 256 4,096 | – – | – – |

- **ID**: Antibody identification.
- **a**: Subjective grading scheme of antibody reactivity: –, no reactivity; +, weak reactivity; ++, strong reactivity. The selection criteria of antibody reactivity are indicated in Fig. 1.
- **ND**: Not done.

The indirect ELISA was performed as described previously (7). All virus antigens were used at protein concentrations of 100 ng/well. The optical density (OD) values were corrected to facilitate comparison using the following formula: (OD value for each viral antigen)/(OD value for the WNV NY99 strain antigen) × 100.

The developed MAbs did not neutralize WNV in PRNT (data not shown).

**Immunohistochemistry.** SHW-32B1, SHW-7A11, and the anti-WNV rabbit polyclonal antibody strongly reacted with the cytoplasm of degenerated cells in the heart (Fig. 4A, D, and G) and brain (Fig. 4B, E, and H) of a WNV-infected goose. The staining
patterns of each antibody were very similar. Neither SHW-32B1 nor SHW-7A11 showed any positivity when used to stain the degenerated cells in the brain of a JEV-infected pig (Fig. 4C and F). In contrast, anti-WNV rabbit polyclonal antibody showed positive staining of the degenerated cells in the brain of a JEV-infected pig (Fig. 4I), indicating cross-reactivity. The staining pattern was similar to that seen with the anti-JEV rabbit polyclonal antibody (data not shown). These results indicate that we have successfully established MAbs that specifically recognize WNV antigens in formalin-fixed tissue sections.

**TABLE 2 Immunoglobulin class and specificity of monoclonal antibodies**

| Clone name | Immunoglobulin class | Virus specificity in an indirect ELISA | Virus component specificity in a Western blot
|------------|----------------------|----------------------------------------|----------------------------------|
| SHW-2D8    | IgM                  | WNV                                    | NS1                              |
| SHW-25C1   | IgM                  | WNV                                    | NS1                              |
| SHW-32B1   | IgG1                 | WNV                                    | NS1                              |
| SHW-6F10   | IgG1                 | JEV serogroup flavivirus E              | E                                |
| SHW-7D12   | IgG2a                | JEV serogroup flavivirus E              | E                                |
| SHW-7G8    | IgG1                 | JEV serogroup flavivirus E              | E                                |
| SHW-18C10  | IgA                  | JEV serogroup flavivirus E              | E                                |
| SHW-11A9   | IgG1                 | WNV                                    | prM                              |
| SHW-7A11   | IgG1                 | WNV                                    | NS1                              |

* NS1, nonstructural protein 1; E, envelope protein; prM, precursor membrane protein.

b SHW-7A11 was previously developed and evaluated elsewhere (7).

**DISCUSSION**

Here we have described the establishment of eight MAbs against WNV, four of which were specific to WNV and four which also reacted with other JEV serocomplex flaviviruses. Flaviviruses are constructed of three structural proteins (the E, prM/M, and capsid proteins) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (3, 14). The established MAbs re-
acted with the NS1, E, and prM proteins of WNV. Among the four WNV-specific MAbs, three reacted with NS1 and 1 reacted with prM. All four of the JEV serocomplex flavivirus-reactive MAbs reacted with the E protein. And the developed MAbs did not react with antigen in Western blot analysis under reduced conditions (data not shown); consequently, their epitopes are thought to be conformational.

NS1 is expressed on the cell surface (23) or secreted into virus-infected animal blood at high concentrations (11), and it is reported that flavivirus NS1 was found in membrane, organelle, nucleus, and cytoskeletal cell fractions (17). These observations suggest that a lot of NS1 will be present in the cells of birds that have died owing to WNV infection. Thus, it is expected that detection of NS1 would be very useful when surveying birds that have died owing to WNV infection. However, currently there is no available anti-NS1 MAb that can be used for IHC. WNV surveillance in dead birds is extremely important for public health, and IHC is one of the main diagnostic methods. IHC of WNV has been performed utilizing commercially available anti-WNV rabbit polyclonal antibody or E-specific MAbs (20). The anti-WNV rabbit polyclonal antibody strongly cross-reacts with degenerated cells in the brain of a JEV-infected pig (Fig. 4). This cross-reactivity would be a problem for WNV surveillance in areas in which JEV is endemic. On the other hand, the anti-E MAb did not react with heart sections from a WNV-infected white Embden goose. It is reported that the anti-E MAb-based IHC shows low sensitivity compared with that of IHC that utilizes polyclonal antibodies (20). For example, approximately 50% of heart sections from WNV-infected American crows were judged as negative using the anti-E MAb-based IHC (20). SHW-32B1 and SHW-7A11 showed strong staining of tissue sections from a WNV-infected bird, with the strength of staining similar to that for the polyclonal antibody, although the anti-E MAb did not show any staining. Strong staining of the cytoplasm of degenerated cells in the heart of a WNV-infected goose was observed in IHC using the anti-NS1 MAbs, SHW-32B1 and the previously reported SHW-7A11 (7), and an anti-WNV polyclonal antibody. Furthermore, the patterns of staining were very similar. Neither of the established MAbs reacted with JEV, unlike the WNV polyclonal antibody. These results strongly indicate that SHW-32B1 and SHW-7A11 are good candidates for WNV-specific IHC.

SHW-11A9 reacted with prM of WNV in Western blot analysis. The high specificity of the anti-prM SHW-11A9 MAb against WNV indicates the existence of a WNV-specific epitope on prM. This finding suggests that this MAb would be useful for WNV research.

The E protein is the major surface protein of flaviviruses, and it plays a critical role in cell attachment and membrane fusion (14). Therefore, the E protein is a principal target of neutralizing antibodies. There are a number of WNV-specific anti-E protein MAbs

![FIG 3 Monoclonal antibody cross-reactivity in an indirect ELISA. Each bar indicates the percentage of the optical density (OD) value obtained for the indicated monoclonal antibody for each JEV serocomplex virus antigen. OD values were calculated using the following formula: (OD value for each viral antigen)/(OD value for the WNV NY99 strain antigen) × 100. All virus antigens were used at protein concentrations of 100 ng/well.](http://cvi.asm.org/)
that have been previously developed (6, 10), but all of our developed anti-E protein MAbs showed cross-reactivity with the E protein from SLEV, MVEV, and JEV in an indirect ELISA.

In summary, 3 WNV-specific anti-NS1 MAbs were developed, with one of the MAbs, SHW-32B1, and the previously developed MAb, SHW-7A11, being suitable for use in IHC of formalin-fixed, paraffin-embedded sections for the specific detection of WNV. Further investigation is required to determine the applicability of SHW-32B1 and SHW-7A11 in actual WNV surveillance by IHC, but they are, however, good candidates for this application. This study is the first report showing that antibodies to NS1 can be used for WNV IHC of formalin-fixed, paraffin-embedded sections. We further showed that the SHW-11A9 MAb to prM was highly specific for WNV. These MAbs would be useful for WNV surveillance and research.

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