HLA class II polymorphism can direct the antigen-specific T-cell response, it is probable that patients with HLA-DRB1*0901 may be prevented from the shift toward Th2 dominance. Further studies are needed to clarify this point.

In conclusion, our study confirmed the previous reported HLA association between HLA-A*24 and DHF or DSS and moreover showed that A*24 with Histidine at codon 70 to be susceptible to DSS and DHF, and HLA-DRB1*0901 to be protective against the development of DSS, particularly in patients with DEN-2 infection. This study represents another attempt to improve our understanding of the risk of HLA-class I for severe outcome of DV infection in the light of peptide anchor binding site and provides a novel evidence that HLA-class II may control disease severity (DHF to DSS) in DV infection.

Supporting Information

Alternative Language Abstract S1 Translation of the Abstract into Japanese by Kenji Hirayama
Found at: doi:10.1371/journal.pntd.0000304.s001 (0.09 MB PDF)

Alternative Language Abstract S2 Translation of the Abstract into Vietnamese by Nguyen Thi Phuong Lan
Found at: doi:10.1371/journal.pntd.0000304.s002 (0.04 MB DOC)

Table S1 Phenotype frequencies of HLA-A, HLA-B and HLA-DRB1 alleles
Found at: doi:10.1371/journal.pntd.0000304.s003 (0.17 MB DOC)

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Author Contributions

Conceived and designed the experiments: NTPL MK VTQH DQH TTT VDT HMT TVD KM MY KH. Performed the experiments: NTPL MK VTQH MY KH. Analyzed the data: NTPL MK VTQH MY KH. Contributed reagents/materials/analysis tools: NTPL MK VTQH DQH TTT VDT HMT VVT CTPN TVD TO KM MY KH. Wrote the paper: NTPL MK VTQH KM MY KH.

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Characterization and application of monoclonal antibodies specific to West Nile virus envelope protein

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ABSTRACT

Recent epidemics of West Nile virus (WNV) around the world have been associated with significant rates of mortality and morbidity in humans. To develop standard WNV diagnostic tools that can differentiate WNV from Japanese encephalitis virus (JEV), four monoclonal antibodies (MAbs) specific to WNV envelope (E) protein were produced and characterized by isotyping, reactivity with denatured and native antigens, affinity assay, immunofluorescence assay (IFA), and epitope competition, as well as cross-reactivity with JEV. Two of the MAbs (6A11 and 4B3) showed stronger reactivity with E protein than the others (2F5 and 6H7) in Western blot analysis. 4B3 could bind with denatured antigen, as well as native antigens in indirect ELISA, flow cytometry analysis, and IFA; whereas 2F5 showed highest affinity with native antigen. 4B3 and 2F5 were therefore used to establish an antigen capture-ELISA (AC-ELISA) detection system. The sensitivity of this AC-ELISA was 3.95 $TCID_{50}$/0.1 ml for WNV-infected cell culture supernatant. Notably, these MAbs showed no cross-reactivity with JEV, which suggests that they are useful for further development of highly sensitive, easy handling, and less time-consuming detection kits/tools in WNV surveillance in areas where JEV is epidemic.

1. Introduction

West Nile virus (WNV) is a member of the Japanese encephalitis virus (JEV) serocomplex of the genus *Flavivirus*, family *Flaviviridae*. Recent epidemics of WNV around the world have been associated with significant rates of mortality and morbidity in humans (Lanciotti et al., 1999; Gea-Banacloche et al., 2004; Gubler, 2007; Murgue et al., 2002). However, neither a specific treatment for WNV infection nor a preventive vaccine is available at present. In nature, WNV exists in an enzootic cycle between mosquitoes and birds, with birds being the principal amplifying host (Glaser, 2004). The rapid spread of WNV is most likely caused by the migration of infected wild birds after contact with pools of *Culex* mosquitoes (Malkinson et al., 2002; Rappole et al., 2000). As the clinical symptoms of WNV infection are non-specific compared to those of other encephalitis viruses, diagnosis relies mainly on laboratory tests.

Serological testing is the primary method of diagnosing WNV infection. The plaque reduction neutralization tests for type-specific diagnosis are laborious, expensive, and require live virus, which limits their application in large-scale surveillance. ELISA-based detection for IgM, IgG or IgA has been developed, and some of these assays are commercially available (Hogrefe et al., 2004; Levett et al., 2005; Martin et al., 2000; Prince and Lape-Nixon, 2005). However, the serological cross-reactions and cross-neutralizations found in the JEV serocomplex viruses limit the specificity of serological tests (Hogrefe et al., 2004; Martin et al., 2000; Niedrig et al., 2007).

WNV viremia can serve as a clear indicator of recent infection and is suitable for early detection because it begins within a few days after infection and is short-lived. WNV-infected mosquitoes can be easily detected by various virus-detection methods (Hunt et al., 2002; Marfin and Gubler, 2001). Viral isolation depends

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heavily on the quality of samples and requires the use of cell culture and a BSL-3 laboratory, with 6-day delay. Reverse-transcriptase polymerase chain reaction (RT-PCR) is expensive and prone to contamination. Indirect immunofluorescence assay (IFA) with well-identified specific monoclonal antibodies (MAbs) can confirm virus infection. WNV antigen detection tests with specific MAbs have been used for dead birds and mosquito surveillance programs in North America (Dauphin and Zientara, 2007). An MAb-based antigen capture-ELISA (AC-ELISA) that can differentiate WNV from St Louis encephalitis virus has also been developed (Hunt et al., 2002).

As a result of the antigenic cross-reaction in the JEV serocomplex flaviviruses, it is critical to distinguish between WNV and JEV in areas such as China and Japan where JEV is endemic. Molecular diagnostic methods that simultaneously discriminate between WNV and JEV using RT-PCR analyses have previously been reported (Shirato et al., 2003, 2005). MAb is the most attractive option for the development of standardized viral diagnostic assays. In this study, four MAbs against WNV envelope protein domain III (EDIII) were characterized by isotyping, affinity assay, reactivity with denatured and native antigens, and epitope competition, as well as cross-reactivity with JEV. The results suggest the applicability of the MAbs to various analytical methods, such as immunoblotting, IFA, and AC-ELISA, for detection and pathogenic study of WNV.

2. Materials and methods

2.1. Preparation of recombinant WNV EDIII protein

The EDIII (residues 298–415) of WNV bird 5810 strain was expressed, purified and refolded as described previously (Yuan et al., 2005). Briefly, the recombinant protein was expressed in *Escherichia coli* as an inclusion body and refolded in an appropriate buffer. The refolded protein was purified by gel-filtration chromatography.

2.2. Production and purification of MAbs

Six BALB/c mice (from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), aged 8 weeks, were primed intraperitoneally with 50 μg recombinant EDIII protein, mixed with complete Freund's adjuvant (Sigma–Aldrich). Two boosts were given at days 14 and 28 with 50 μg EDIII mixed with incomplete adjuvant (Sigma–Aldrich). Three days after the last boost, the titer of polyclonal antiserum was assessed using indirect ELISA (described below) with EDIII as antigen. The mouse with the highest titer was chosen to harvest splenocytes. Separated splenocytes were fused with SP2/0 myeloma cells at a ratio of 5:1 using 50% (w/v) polyethylene glycol, according to a previously described protocol (Kohler and Milstein, 1975). The hybridoma cells were obtained and subsequently cloned by limiting dilution. The cell lines that produced specific antibodies were subcloned successively 3–5 times by limiting dilution to ensure monoclonality and stability. Positive clones that secreted high-titer EDIII-specific antibodies in indirect ELISA were further identified. The immunoglobulin subclass was determined using SBA Clonotyping System/AP kit (Southern Biotechnology Associates). Four positive cell lines (6A11, 4B3, 2F5 and 6H7) were used to generate ascites in BALB/c mice and MAbs were purified by protein A or protein G chromatography, according to manufacturer's protocols (Pharmacia). The concentration of purified MAb was determined by bicinchoninic acid protein assay (Pierce Biotechnology).

2.3. Western blot analysis

To examine whether the ascites MAbs recognized the linear epitope of EDIII protein, Western blot analysis was performed under denaturing conditions. EDIII protein was run on 12% SDS-PAGE, then electrotransferred onto a nitrocellulose membrane (Amersham Biosciences UK) and blocked with 5% non-fat dry milk in Tris-buffered saline (TBS). Membranes were incubated for 2 h at room temperature with four ascites MAbs (1:2000), respectively, and then washed 3 times with 0.05% Tween-20 in TBS (TBST), and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000 dilution; Santa Cruz) for 1 h at room temperature, and detected by SuperSignal West Pico Chemiluminescent substrate solution (Pierce Biotechnology). In the control experiment, EDIII protein was incubated with an irrelevant MAb H5, which is an anti-influenza antibody (1:2000).

The specificity of purified MAbs for WNV E protein was also evaluated by Western blot analysis. The recombinant E proteins of WNV (bird 5810 strain) and JEV (Beijing-1 strain) with a His tag were expressed on the membrane of 293T cells, by transiently transfecting pcDNA4-WNV E or pcDNA4-JEV E plasmids into 293T cells. The cell lysate and inactivated WNV (Chin01 strain) or JEV (Beijing-1 strain) were separated by 10% SDS-PAGE, and were then electrotransferred onto a nitrocellulose membrane and blocked. Membrane was incubated for 2 h with purified MAb (1 μg/ml) or anti-His MAb (0.5 μg/ml; Santa Cruz), as a positive control for the expression of JEV E protein. The membrane was washed 3 times with TBST, and incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (1:5000) for 1 h, and detected by substrate solution.

2.4. Indirect ELISA

All ELISAs were carried out in 96-well microtiter ELISA Plates (Greiner Bio-One). Titers of hybridoma-cell-secreted MAbs were detected by indirect ELISA. Briefly, the wells were coated overnight at 4 °C with 20 ng/well of purified EDIII, or an equal amount of bovine serum albumin (BSA; Sigma–Aldrich), as a negative control, and diluted in 50 mM carbonate saline (pH 9.6). After blocked for 1 h at 37 °C with PBS containing 3% BSA (PBSA), the wells were washed 4 times with PBS containing 0.05% Tween-20 (PBST). Serially diluted MAbs in PBSA (100 μl) were added to each well in triplicate and incubated for 1 h at 37 °C. After wells were washed 4 times with PBST, HRP-conjugated anti-mouse IgG (1:2000) was added to each well and incubated at 37 °C for 40 min, then washed again. Antibody binding was visualized by addition of the mixture of H_2O_2 and 3,3′,5,5′-tetramethyl-benzidene substrate (TMB; Sigma–Aldrich). After incubation for 15 min at 37 °C, the reaction was stopped by addition of 0.1 M H_2SO_4, and absorbance was read at 450 nm with a reference wavelength of 595 nm on a model Sunrise plate reader (Tecan). The endpoint titers of purified MAbs were also determined by 10-fold serial dilution with indirect ELISA. In all ELISAs, the irrelevant MAb H5 was used as an antibody control. The positive cutoff ratio was set at 2 (ratio of OD value coated with EDIII/OD value coated with BSA). This value is comparable to "positive to negative" cutoff ratios used in other WNV diagnostic assays (Davidson et al., 2005; Estrada-Franco et al., 2003).

2.5. Cell surface staining detection by flow cytometry analysis

The percentage of 293T cells expressing WNV E protein was determined by cell surface staining with MAbs. A FACSCalibur flow cytometer (BD Biosciences) was used for flow cytometry analysis. WNV E protein was expressed on the membrane of 293T cells by transfection of pcDNA4-WNV E plasmids into 293T cells and cultured for 48 h. Single-cell suspensions were prepared and incubated with ascites (1:2000) at 4 °C for 1 h in 100 μl PBSA buffer, then washed 3 times with PBS buffer. Cells were adsorbed with FITC-conjugated anti-mouse IgG (1:500; Santa Cruz) at 4 °C for 1 h, and

washed again. Fluorescent signals on the cell surface were detected and the percentage of positive cells was counted among 3×10^4 cells. Controls included cells without addition of primary MAb, cells with H5 MAb and normal mouse IgG.

2.6. Affinity analysis by surface plasmon resonance (SPR)

The affinity between MAb and purified EDIII was determined by SPR on a Biacore 3000 (Biacore, Inc). Firstly, EDIII was immobilized on the surface of a CM5 chip by amine coupling and then used to capture purified MAb. Analysis was performed at 25 °C at a constant flow rate of 30 μl/min, using HBS-EP buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20] as a running buffer. To determine the association rate, dissociation rate and affinity constant (K_D), a concentration series from 0.4 to 400 nM of purified MAb was injected (240 μl, associated for 8 min and then dissociated over 10 min). The EDIII surface was regenerated by injection of 50 mM NaOH before each EDIII injection. Binding curves and kinetic parameters were analyzed with a global fit 1:1 binding algorithm with drifting baseline by BIAevaluation software version 3.2 (Biacore). The affinity constant K_D was determined as k_{off}/k_{on}, using data from three independent experiments.

2.7. Immunofluorescence assay

Binding of mouse ascites MAbs with WNV- or JEV-infected cells was determined by IFA. Sub-confluent BHK-21 cells, which were grown in 24-well microplates with slides, were infected with WNV (Chin-01 strain) or JEV (Beijing-1 strain) at a multiplicity of infection of 0.1. After incubation for 3 days, serially diluted MAbs were added to virus-infected BHK-21 cells. After incubation at room temperature for 2 h, slides were washed 3 times with PBST, and FITC-labeled anti-mouse IgG was added at dilution of 1:1000. Slides were washed again after 1 h incubation, stained with Evans blue, and observed under fluorescence microscope at 200× magnification. Cells showing strong green fluorescence were recorded as positive. The highest dilution of mouse ascites MAb that showed a strong positive fluorescence signal was recorded as the IFA titer. The uninfected cells were used as a negative control at each dilution, and JEV-infected BHK-21 cells were used to evaluate the cross-reactivity of MAbs with JEV. The experiments that involved the use of WNV were performed in a BSL-3 laboratory.

2.8. Competitive-binding ELISA and AC-ELISA

The detector MAb was labeled with biotin using an EZ-link Sulfo-NHS-LC- Biotinylation kit (Pierce Biotechnology) according to the manufacturer's instructions. Experiments on epitope competition of the three purified MAbs (6A11, 4B3 and 2F5) were carried out using competitive-binding ELISA. The wells were coated and blocked as described in Section 2.4. After 100 μl unlabeled MAbs (5 μg/ml) were added and incubated for 1 h at 37 °C, wells were washed 3 times, followed by incubation with an equal amount of another biotin-labeled MAb for 1 h at 37 °C. Plates were washed again and incubated with HRP-conjugated streptavidin (diluted 1:2000 in PBS; Zhongshan Goldenbridge Biotechnology). After washing, the color was developed with the addition of 100 μl freshly prepared substrate solution (1:1 mixture of TMB and H_2O_2 solution) for 15 min at 37 °C. The color reaction was stopped by 100 μl 0.1 M H_2SO_4, and absorbance was read at 450 nm, with a reference wavelength of 595 nm. Wells with addition of the irrelevantly unlabeled MAb (H5) were used as a negative control, and wells with addition of unlabeled MAb (the same as the biotin-labeled MAb) were a positive control. Each pair of MAbs was assayed in triplicate. Results were expressed as a percentage of inhibition and derived by the following formula: percentage of inhibition (PI) = [(negative control OD − test MAb OD)/(negative control OD − positive control OD)] × 100%.

For the AC-ELISA, the purified MAb (1 μg/well), diluted in 50 mM carbonate saline (pH 9.6), was coated on wells overnight at 4 °C. After blocking for 3 h at 37 °C with PBS containing 5% non-fat dry milk, wells were washed 3 times with PBST. All the following washing procedures were the same as described above. Virus culture supernatant ($10^{3.5}$ $TCID_{50}$/ml) or recombinant EDIII protein, serially diluted in PBS containing 1% non-fat dry milk (PBSM) was added to the wells (100 μl/well) and incubated for 3 h. Cell culture supernatant or BSA was used as a negative control. After washing, 100 μl per well biotin-labeled detector MAb (2 μg/well, diluted in PBSM) was added and incubated for 1 h at 37 °C. After washing, the wells were incubated for 30 min at 37 °C with 100 μl per well HRP-conjugated streptavidin and detected as above. In this ELISA test, the positive cutoff was also set at 2 (ratio of positive/negative).

3. Results

3.1. Generation and purification of MAbs against WNV EDIII protein

The positive-fused cell clones were screened using indirect ELISA with recombinant EDIII as antigen. The hybridomas with higher ELISA titers were selected for screening, and four MAbs (6A11, 4B3, 2F5 and 6H7) were finally isolated and cloned. Ascites was produced in BALB/c mouse by hybridomas. The heavy chain subclasses of MAbs were determined as IgG2a (6A11) and IgG1 (4B3, 2F5 and 6H7), and the light chains of all of these were kappa isotype. 6A11 was efficiently purified by protein A chromatography, and 4B3, 2F5 and 6H7 by protein G chromatography. The concentrations of purified MAbs were determined as 10–18 mg/ml.

3.2. Western blot analysis

The binding specificity and cross-reactivity of the MAbs against denatured EDIII protein and E protein were determined by Western blot analysis. Four MAbs reacted with both EDIII and E proteins. Two of the ascites MAbs (6A11 and 4B3) showed stronger reactivity with recombinant EDIII protein (12.5 kDa) than the others (2F5 and 6H7), and the irrelevant MAb (H5) against influenza virus did not bind to EDIII (Fig. 1A). WNV E protein, which was expressed on the surface of 293T cells, as well as that from inactivated WNV, showed specific binding to 2F5, and there was no cross-reactivity with recombinant E protein or inactivated JEV (Fig. 1B). MAb 2F5

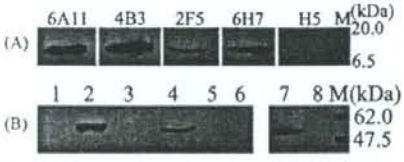

Fig. 1. Western blot analysis of anti-EDIII MAbs with denatured antigen. (A) Reactivity of four MAbs with recombinant EDIII protein, using irrelevant MAb against influenza virus (H5) as a negative control. (B) Reactivity of MAb 2F5 with E proteins from WNV and JEV. Lanes 1–3: lysates of 293T cells transfected with pcDNA4, pcDNA4-WNV E and pcDNA4-JEV E plasmids, respectively; lane 4: inactivated WNV; lane 5: inactivated JEV; lane 6: cell culture supernatant of BHK-21 cells; lanes 7 and 8: the same as lanes 3 and 1, respectively. M: protein molecular weight markers. Left panel (lanes 1–6) was detected with MAb 2F5 and right panel (lanes 7 and 8) was detected with anti-His antibody.

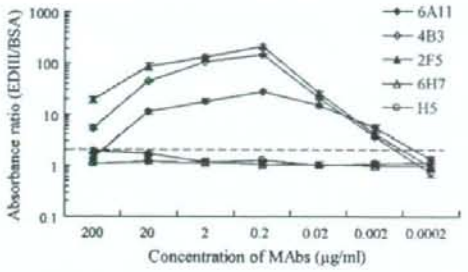

Fig. 2. Absorbance ratio of MAbs binding to EDIII and BSA. The broken line indicates the absorbance ratio cutoff value, which was set at 2.

did not show any non-specific binding to negative controls, which were 293T cells transfected with pcDNA4 vector and cultured BHK-21 cells. The binding analysis with other MAbs (6A11, 4B3 and 6H7) yielded similar results (data not shown). The JEV E protein was fused with a His tag at its C terminus, and expression of JEV E on 293T cells was confirmed by anti-His antibody (Fig. 1B). This indicated that the four MAbs recognized denatured WNV EDIII and E protein with different binding affinity and did not show cross-reactivity with E protein from JEV.

3.3. Reactivity of MAbs with WNV EDIII protein in indirect ELISA

To examine the reactivity of the MAbs with EDIII protein under non-denaturing conditions, the indirect ELISA was performed with folded EDIII protein. The titers of four unpurified ascites MAbs were higher than 10^7 in indirect ELISA (data not shown). The reactivity of purified MAbs with EDIII is shown in Fig. 2. Three MAbs (6A11, 4B3 and 2F5) showed strong positive binding with EDIII at concentrations of 0.02–20 μg/ml (absorbance ratio >10), compared with BSA-coated wells. Meanwhile, they showed obvious positive signals at the concentration of 0.002 μg/ml (absorbance ratio >2). Among these, MAb 2F5 showed highest sensitivity. The low absorbance ratios at the highest concentration tested (200 μg/ml) were due to the high non-specific binding of MAbs with BSA at this concentration. The irrelevant MAb H5 did not show specific binding to EDIII. It was surprising that MAb 6H7 showed no specific reactivity with EDIII after purification, so it was excluded from the following ELISAs.

3.4. Flow cytometry analysis of MAbs

Cell surface expression of WNV E protein was detected by MAb staining and determined by flow cytometry. When stained with different MAbs, the percentage of fluorescent cells varied greatly. Representative profiles are shown in Fig. 3. The percentage of fluorescent positive cells was 4.5 ± 2.6%, 26.9 ± 8.6%, 44.6 ± 8.0%, and 8.9 ± 1.2%, when stained with MAb 6A11, 4B3, 2F5 and 6H7, respectively. Controls, which included cells without MAb, and cells stained with the normal mouse serum or H5, were all negative. Among the four MAbs against EDIII, 2F5 showed the strongest binding to native E protein.

3.5. Binding affinity between purified MAbs and recombinant EDIII protein

The binding affinity between recombinant EDIII protein and purified MAbs (6A11, 4B3 and 2F5) was analyzed by SPR in the solid phase. MAb 6H7 did not bind to immobilized EDIII protein under experimental conditions, so the binding affinity between 6H7 and EDIII was undetectable. MAb 2F5 bound to EDIII with an affinity of 1.8 ± 0.3 nM, which was the highest among the three MAbs. The affinity of 4B3 and 6A11 was similar, with a K_D range from 407.1 ± 96.3 to 692.5 ± 112.1 nM (Fig. 4).

3.6. IFA

IFA was performed to further analyze whether the MAbs recognized the endogenously produced E protein in WNV-infected BHK-21 cells. Both normal mouse serum and three MAbs did not show non-specific binding to uninfected cells (data not shown). 6A11, 4B3 and 2F5 showed strong reactivity with WNV-infected cells, whereas normal mouse serum did not bind to infected

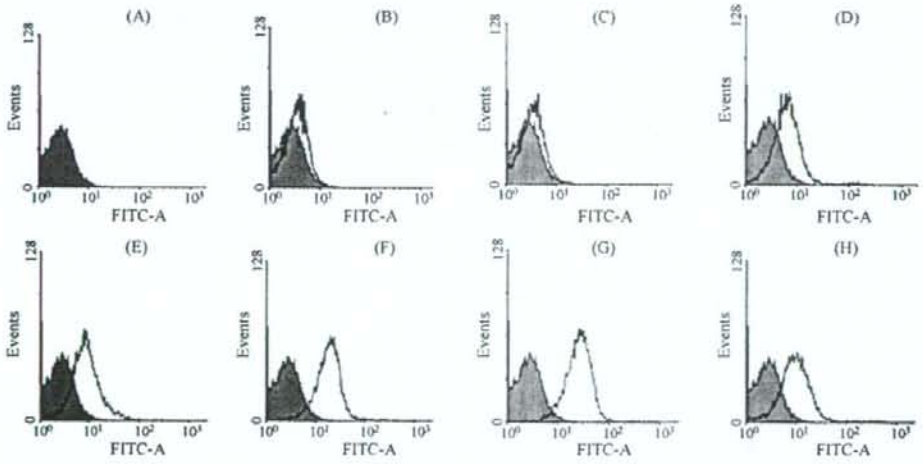

Fig. 3. Flow cytometry analysis of cells that expressed WNV E proteins. The profile of transfected 293T cells without antibody staining was used to define the background of fluorescent intensity (A–H, red). The white profiles in (B–H) were compared with reference to the red profile. (B) Stained directly with FITC-conjugated anti-mouse IgG, without addition of primary antibody; (C) stained with normal mouse serum; (D–H) stained with H5, 6A11, 4B3, 2F5 and 6H7, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

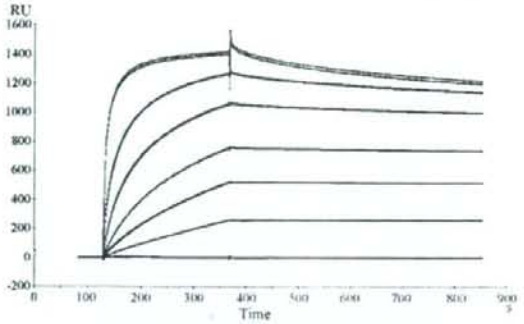

Fig. 4. Biacore binding curves of purified MAbs 4B3 with immobilized EDIII protein. A concentration series from 0.4 to 400 nM of purified 4B3 was injected (240 μl, associated for 8 min and then dissociated over 10 min). The affinity constant K_D was determined as k_{off}/k_{on}.

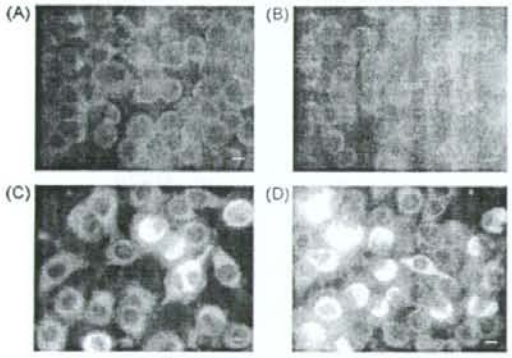

Fig. 5. Detection of WNV-infected BHK-21 cells by immunofluorescence assay. The slides were stained with Evans blue and then observed by fluorescence microscopy. Scale bar: 20 μm. (A) WNV-infected cells stained with normal mouse serum. (B) JEV-infected cells stained with MAb 2F5. (C) WNV-infected cells stained with MAb 4B3. (D) WNV-infected cells stained with MAb 2F5.

cells (Fig. 5). The IFA titer of each MAb was determined, based on the highest dilution of ascites that gave a strong signal on WNV-infected BHK-21 cells. When BHK-21 cells were infected with WNV Chin-01 strain, the IFA titers of 6A11, 4B3, 2F5 and 6H7 were 2560, 7680, 5120 and 40, respectively. Notably, these MAbs showed no cross-reactivity with cells infected with JEV (Fig. 5). Validity of

Table 1
Properties of MAbs against WNV EDIII protein in different assays.

Assay	MAbs			
	6A11	4B3	2F5	6H7
Indirect ELISA	++[a]	+++	++++	–[b]
Western blot	+++	++++	+	++
Flow cytometry	+	++	++++	+
Binding affinity	++	++	++++	–
Immunofluorescence	+	+++	++	–
Antigen capture-ELISA	+	+++	+++	–

[a] +: Weak positive; ++++: strong positive.
[b] –: Not detectable.

the MAbs used in IFA was confirmed in an independent laboratory using BHK-21 cells infected with WNV strain NY99 (data not shown).

3.7. Epitope competitions of purified MAbs and AC-ELISA

After biotinylation, the epitope competitions of three MAbs (6A11, 4B3 and 2F5) were assayed by competitive-binding indirect ELISA. The biotin-labeled 6A11 inhibited the binding of 4B3 to EDIII (57.2 ± 6.1%) and vice versa, which indicated that 6A11 and 4B3 recognized overlapping epitopes. 2F5 showed no competitive binding with 6A11 or 4B3, which meant that 2F5 recognized a different epitope from 6A11 or 4B3.

In order to establish a sensitive AC-ELISA for WNV detection, each pair of the three MAbs was evaluated. The highest sensitivity was obtained by using 2F5 as capture antibody, and biotin-conjugated 4B3 as detector antibody. To determine the detection limit of AC-ELISA, a serial dilution of EDIII protein and WNV culture supernatant ($10^{3.5}$ $TCID_{50}$/ml) were used to construct the binding curve (Fig. 6). Cell culture supernatant without WNV infection was used as a negative control. According to the cutoff threshold (2, which is the ratio of positive/negative), it was deduced that as little as 10 ng/0.1 ml of recombinant EDIII protein and 3.95 $TCID_{50}$/0.1 ml of virus culture supernatant could be detected (Fig. 6). This result revealed that MAbs 4B3 and 2F5 could be used to detect WNV in cell culture supernatant.

3.8. Properties of MAbs against WNV EDIII protein

Four MAbs against WNV EDIII protein were identified by isotyping, reactivity with denatured and native antigens, affinity assay and epitope competition ELISA, and IFA, and the results of these analyses were used to design the AC-ELISA. The properties of these MAbs are summarized in Table 1.

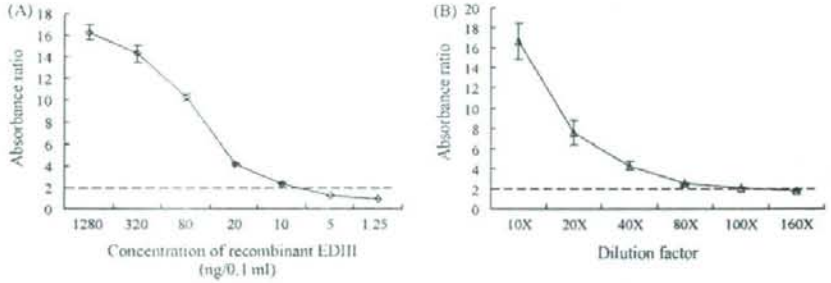

Fig. 6. Sensitivity of AC-ELISA using MAbs. (A) Quantitative analysis using recombinant EDIII protein. 10 ng/0.1 ml was the detection limit. (B) Quantitative analysis using WNV cell culture supernatant. 80-fold dilution of cell culture supernatant was the detection limit, which was 3.95 $TCID_{50}$/0.1 ml. The broken line indicates the absorbance ratio cutoff value, which was set at 2.

4. Discussion

WNV with greater epidemic potential and virulence emerged in the early 1990s and has spread rapidly across many countries (Lanciotti et al., 1999; Gea-Banacloche et al., 2004; Gubler, 2007; Murgue et al., 2002). Many methods have been developed for WNV diagnosis, in which serological testing is the primary method (Dauphin and Zientara, 2007). However, the cross-reactivity of the antibody response against flaviviruses limits the specificity of these tests (Calisher et al., 1989; Niedrig et al., 2007). As JEV is endemic in Southeast Asia, the cross-reactivity between WNV and JEV should be considered in WNV surveillance. MAbs with strong and specific reactivity to WNV antigens are the most attractive option for the development of standardized diagnostic tools. WNV EDIII-reactive MAbs have been reported by several groups (Beasley and Barrett, 2002; Oliphant et al., 2005; Sanchez et al., 2005; Throsby et al., 2006). Beasley and Barrett (2002) have described four MAbs that reacted with recombinant EDIII protein in a non-reducing Western blot assay and one of them bound to JEV. However, there is no further investigation of these MAbs expect for the neutralization activity. Furthermore, a panel of EDIII-specific MAbs has been identified by ELISA and neutralization assay, but the cross-reactivity of these MAbs with JEV has not been examined (Throsby et al., 2006).

Immunization with different forms of flavivirus antigens can produce antibodies with different properties (Aberle et al., 1999; Raviprakash et al., 2000; Sanchez et al., 2005). WNV-specific and neutralizing Abs have been mapped to EDIII (Oliphant et al., 2005; Throsby et al., 2006), and EDIII can induce specific immune responses and protection against WNV infection (Chu et al., 2007). To increase the possibility of obtaining EDIII-specific antibodies in the present study, mice were immunized with refolded EDIII protein, which was shown to be in native conformation by crystal structure analysis (PDB:2P5P).

Mouse ascites or purified MAbs were used in different assays in this study. The results of 6A11, 4B3 and 2F5 were similar in indirect ELISA for ascites or purified MAbs. The reason that 6H7 lost its specific reactivity with EDIII protein in indirect ELISA after purification may have been caused by the low pH of the elution buffer during purification, which probable destroyed the antigen binding site of this antibody. The binding affinity of MAb 2F5 (1.8 ± 0.3 nM) was comparable to that of MAb E16 (3.4 nM) (Oliphant et al., 2005) and CR4353 (6.5 nM) (Throsby et al., 2006), however, 4B3 and 6A11 showed a much lower K_D (407.1 nM for 4B3 and 692.5 nM for 6A11). One reason is that the SPR analysis was performed by using refolded EDIII protein. The binding activity of 2F5 to refolded EDIII was higher than 4B3 and 6A11 in other assays, so the lower K_D of 4B3 and 6A11 is reasonable. 4B3 and 6A11 seemed to bind with overlapping epitopes in competitive ELISA, but they showed different binding ability with the same antigen for denatured EDIII or E protein, as well as for native E protein or intact WNV particles. This may be explained by the different binding affinity constants of 4B3 and 6A11 with EDIII. It is interesting that 4B3 could bind to both denatured and native antigens. Crystal structural analysis of EDIII protein (Mukhopadhyay et al., 2003; Nybakken et al., 2005; Volk et al., 2004) has shown that EDIII is an Ig-like domain with seven β-strands, which constitute its main structure. Single β-strand is linear in native as well as in denatured protein. If 4B3 happens to recognize one of the seven β-strands, it is highly likely that 4B3 shows reactivity with both denatured and native antigens. This needs further investigation. In the present study, none of the MAbs showed protective activity in microtiter-based neutralization assays (data not shown). One possible reason is the natural scarcity of EDIII-specific neutralization antibodies. This is comparable with other study. In the 119 WNV-specific MAbs that have been cloned from three WNV-infected patients, only 10 exhibited neutralizing activity, and as few as two targeted domain III (Throsby et al., 2006).

Notably, none of the four MAbs showed cross-reactivity with JEV in any of our assays. WNV and JEV are genetically closely related, and the serological cross-reactions are usually found with these viruses (Martin et al., 2000). Considering that WNV and JEV are maintained in similar transmission cycles, and have overlapping geographic distributions in Southeast Asia, MAbs which can differentiate WNV from JEV are very useful for the surveillance system in these areas.

Of the many techniques developed for the rapid diagnosis of viral infections, the AC-ELISA is a sensitive and specific method that is capable of large-scale screening in surveillance programs. It also offers advantages over more traditional antigen detection methods, such as isolation in cell culture and plaque titration, which rely on the inoculation of samples into cells, or RT-PCR, which is expensive and prone to contamination. The availability of MAbs with strong reactivity to the target antigen is a crucial component for AC-ELISA development. Because of the possible presence of both conformational and linear antigens from live viruses or denatured samples, the recognition of the two forms of antigens by MAbs is extremely important for successful detection (He et al., 2007). To obtain the strongest signal for WNV detection by the AC-ELISA, different combination of MAbs were evaluated. It was found that a combination of MAbs 4B3 and 2F5 gave the highest signal. This is reasonable because, among the four MAbs, 4B3 showed the strongest reactivity with denatured antigen, and 2F5 had the highest affinity with native antigen. Therefore, the combination of MAbs 4B3 and 2F5 might contribute greatly to the sensitivity of the AC-ELISA. The detection limit was 3.95 $TCID_{50}$/0.1 ml for WNV-infected cell culture supernatant in the present study. This sensitivity is comparable with that for severe acute respiratory syndrome coronavirus (Che et al., 2004; Shin et al., 2006), and higher than some AC-ELISAs for other arboviruses (Hall et al., 1987; Hildreth and Beaty, 1984). Sensitivity comparisons with other previously published AC-ELISAs for flaviviruses are difficult to make, because previous studies have used plaque forming units as endpoints to assess infectivity level (Hunt et al., 2002; Tsai et al., 1987).

In conclusion, four MAbs specific to WNV EDIII protein were produced and characterized. These MAbs could be used in immunoblot assay, flow cytometry analysis, IFA, and studies in WNV pathology. As they showed no cross-reactivity with JEV, these MAbs could be used to discriminate WNV from JEV in areas where JEV is epidemic. The detection limit of the established AC-ELISA re-emphasizes the sensitivity of specific MAbs for viral antigen detection, which suggests that these MAbs will be useful for further development of highly sensitive, easy handling, and less time-consuming detection kits/tools in WNV surveillance.

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西ナイル熱

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はじめに●

西ナイル熱 West Nile fever は蚊で媒介される西ナイルウイルス West Nile virus（ウエストナイルウイルス）の感染による急性の熱性感染症である．このウイルスは 1937 年にアフリカのウガンダで発見され[1]，日本脳炎ウイルスと同じフラビウイルス科に分類されている．かつてはアフリカや中近東，西アジアの一部での散発的な流行にとどまっていたが，1999 年に初めてアメリカ大陸に侵入し毎年拡大をつづけ現在では北米大陸の大部分とカリブ海諸国，中米，南米でウイルスの活動が確認されている．特に米国では 2002 年以来，毎年数千名の感染者が報告されており，重大な公衆衛生上の問題となっている．

世界の現状●

西ナイルウイルスはもともとアフリカ，中近東，地中海地域，ヨーロッパの一部さらに西アジア，インドまで，いわゆる旧大陸にひろく分布しており，散発的な流行を繰り返してきた．さらに 1999 年には中近東から特に神経向性の強い西ナイルウイルス株が米国へ侵入して[2]，北米全域とカリブ海諸国へ伝播し，さらに中米，南米へと拡大を続けている（図 1）．自然界では，西ナイルウイルスは蚊と鳥に交互に感染する感染サイクルにより生存しており，感染した鳥とともに長距離を移動する（図 2）．さらにベクターとなる蚊は都会でも多数生息するイエカ類の蚊であるためニューヨークのような大都市，田舎の別なく患者発生がみられることが特徴といえる．特に，米国においては表 1 に示すように 2002 年以来，継続的に毎年数千名の患者が発生している．さらに感染者数の増加に伴って，輸血や移植による西ナイルウイルス感染が発生し一般医療の現場でも影響が無視できない状況である．

臨床症状●

西ナイルウイルス感染では通常，感染者の約 80% は不顕性に経過すると見積られているが発症した場合には多彩な臨床形態を示す[3]．すなわ

図 1　西ナイルウイルスの生息地域
1999 年に米国へ侵入したウイルスは現在中米，南米へと拡大をつづけている．

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- 西ナイルウイルス感染は多彩な臨床像を示す.
- わが国に侵入した場合には日本脳炎との鑑別が重要.
- 米国に侵入した西ナイルウイルスは神経向性が強い.

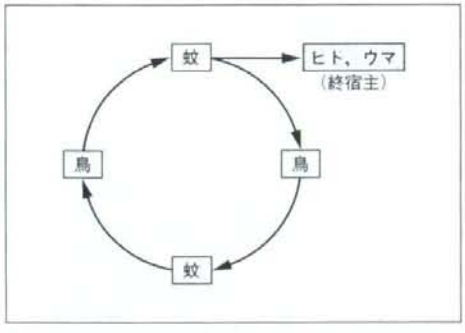

図2　自然界における西ナイルウイルスの感染サイクル
西ナイルウイルスは鳥と蚊の間で感染を繰り返すことで種を維持している.

表1　米国における西ナイルウイルス感染者数の推移

年度	流行地域	感染者数
1999	1 州	62 例
2000	3 州	21 例
2001	10 州	66 例
2002	41 州	4,156 例
2003	46 州	9,862 例
2004	41 州	2,539 例
2005	44 州	3,000 例
2006	44 州	4,269 例
2007	43 州	3,404 例（12 月 11 日現在）

2002 年以降，米国では毎年数千名の感染者が発生している．そのうち約 30% は中枢神経感染の病状を示している．
（CDC，MMWR 報告より改変）

ち，1）軽度の発熱，2）デング熱のような皮疹を伴う急性熱性疾患，3）日本脳炎に類似した中枢神経感染症状，4）黄熱病に似た黄疸を伴う肝炎症状（中央アフリカ共和国での流行で報告）などが旧大陸の西ナイルウイルス常在地域においては報告されている．しかし多くは良性の自然治癒する発熱性の急性ウイルス感染症として認識されており，「西ナイル熱」と呼ばれる所以である．

典型的な症例では 3～14 日の潜伏期間の後，発熱，頭痛，筋肉痛，発疹，リンパ節腫脹，倦怠感，食欲不振などの症状が 1 週間程度持続し後遺症なく回復する．デング熱様の皮疹は約半数の症例（米国では約 20%）にみられ発熱の中期から胸，背，上肢にみられる．ただし旧大陸の流行でも中枢神経系の感染は高齢者においてまれにみられている．

しかし現在，米国で流行している西ナイルウイルスの感染では，毎年高い頻度で髄膜炎，脳炎の患者が発生している．例えば，2006 年には 4,269 名の感染者のなかで，1,459 名（34%），2007 年（12 月 11 日時点）では 3,404 名の感染者のなかで，1,130 名（33%）が髄膜炎・脳炎などの中枢神経感染症状を示している．また，一部の患者では，四肢の筋力低下やポリオ様四肢麻痺がみられることから，米国で流行中の西ナイルウイルスはヒトの脊髄にも感染し増殖するという推測もある．さらに米国ではウイルスに感染した多数の鳥が脳炎で死んでおり，旧大陸のアフリカや中東で従来からの西ナイルウイルス生息地域ではみられない現象が報告されている．これらのことから，米国に侵入した西ナイルウイルスは神経親和性（神経毒性）の強いウイルス株であると思われる．この西ナイルウイルスは今のところ，わが国を含むアジアへ侵入した形跡なないが，将来わが国に侵入した場合には日本脳炎を含むウイルス性脳炎との鑑別が必要である．

診断上の注意点●

一般臨床検査では末梢血中の白血球減少，脳炎患者では髄液中の細胞数増多，蛋白増加，などがみられるが西ナイルウイルス感染に特徴的な所見はなく確定診断にはウイルス学的診断が必須であ

●西ナイルウイルスと日本脳炎ウイルス感染の血清診断には特異的IgM検査が必要.
●西ナイルウイルス感染を診断した場合には届出義務がある.
●特効的治療法はない.

る．特に西ナイルウイルスは日本脳炎ウイルスときわめて近縁のウイルスであり抗原性でも高い交差性があり両者の鑑別は重要である．ウイルス学的検査として急性期には血液や脊髄液からのウイルスの分離，RT-PCR法，リアルタイムRT-PCR法によるウイルス遺伝子の検出，また近年，新たな遺伝子増幅技術であるLAMP法(loop mediated isothermal amplification)を用いた西ナイルウイルス遺伝子検出法[4]などが有用である．しかし発症後7日目以降ではIgM捕捉ELISA法を用いた特異的抗体検査により西ナイルウイルス特異的IgM抗体検出により日本脳炎と鑑別可能である．日本脳炎の血清診断で一般的によく用いられる日本脳炎HI試験(赤血球凝集阻止反応試験)では両ウイルスの交差反応のため西ナイルウイルス感染者も日本脳炎陽性と判定される可能性があるので注意が必要である[5]．しかし，この交差性は西ナイルウイルスに対する予備的抗体検査として有用であるともいえる．つまり西ナイルウイルス感染が疑われる症例では発症後数日を経過していればまず日本脳炎のHIテストを実施し，陽性であった場合にさらに専門機関に特異的検査を依頼するのも一つの方法である．無論，西ナイルウイルス感染が強く疑われる場合(米国からの帰国者など)には特異的検査を直接，関係機関に依頼する必要がある．確定診断した場合には新感染症法の新四類感染症として直ちに保健所(都道府県知事)に届け出ることが義務づけられている．

おわりに●

今のところ，西ナイルウイルスはわが国に侵入した形跡はないが，西ナイルウイルスに対するヒト用の予防ワクチンや治療薬はないので米国，カリブ海諸国をはじめ西ナイルウイルス活動地域を旅行する場合には長袖のシャツや長ズボンの着用，皮膚露出部への蚊忌避剤の塗布などの蚊に刺されないような注意を喚起する必要がある．また，ロシア国内にはすでにアメリカ株が伝播した形跡があり，将来，日本にアメリカ株が伝播した場合には西ナイルウイルスの宿主となる鳥もベクターとなる蚊もわが国には沢山生息しているので米国のように甚大な健康被害が発生する可能性がある．被害を最小限にするためには何よりもまずウイルスの侵入をできるだけ早く検知することが必要であり，一般医療の場においてウイルス性脳炎と思われる症例の原因ウイルスの確定診断をすることは重要である．1999年米国において最初に西ナイルウイルス侵入を発見したのはニューヨーク市マンハッタン地区の病院に勤務する1人の内科医であった．

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ミニ解説

日本脳炎ウイルス

Japanese encephalitis virus

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病原体と伝播形式

日本脳炎ウイルスは，フラビウイルス属に分類されるエンベロープを持つRNAウイルスであり，直径約50 nmの球状構造をしている(図1)．日本脳炎ウイルスはイエカ類の蚊によって媒介されるが，日本においては特にコガタアカイエカが重要な媒介蚊である．

蚊はウイルス血症を起こしている感染ブタを吸血することで消化管からウイルスに感染し，約1週間のうちにウイルスが蚊の唾液腺に到達，増殖して感染力を持つようになる．ブタは日本脳炎ウイルスに感受性が高いので，ウイルス保有蚊に吸血されることにより効率よく感染して高いレベルのウイルス粒子を血液中に放出し，蚊へのウイルス供給源なる最も重要なウイルス増幅動物である(図2)．自然界ではこの感染サイクルにより日本脳炎ウイルスは維持されている．

疫　学

日本においては6～9月にかけてコガタアカイエカが大量発生する季節になると，日本脳炎ウイルスは蚊とブタとの間で感染を繰り返し，ウイルス汚染蚊の増加とともに患者の発生がみられる．1960年代には年間の患者発生が数千名を超える大流行が繰り返し発生したが，ワクチンの普及，蚊に刺されないような生活様式(網戸，エアコンなど)，およびコガタアカイエカの減少などの影響により患者数は減少し，1990年代以降は年間10名以下の発生数である(図3)．

しかし，いまでも関東以南の地域で8～9月にかけて日本脳炎ウイルスの活動が確認されており，コガタアカイエカからもウイルスを分離することができるので感染リスクは存在していると考えられる．

実際に小西ら(Vaccine 24：516-524，2006)は，ヒトと同じく日本脳炎ウイルスに感受性の高いウマについて，日本脳炎ワクチン接種では上昇せず日本脳炎ウイルスの自然感染でのみ上昇する日本脳炎NS1蛋白特異的抗体の測定により自然感染の状況を調査しているが，この研究によると茨城において4.2～26.7%，滋賀では0～41.7%のウマが自然感染したと判定され，依然として日本においての日本脳炎ウイルスの感染リスクは存在することが示されている．

一方，日本以外の地域では，日本脳炎ウイルスは図4に示したように，中国，韓国などの東アジアからインドネシア，タイ，ベトナム，フィリピンなどの東南アジア，インド，パキスタンなどの西アジア，南はパプアニューギニア，オーストラリア北部にまで生息しており患者発生がみられる．世界保健機関(WHO)の推計では，この地域で年間に2万～3万人日本脳炎患者が発生しているとの報告がある．また，ネパールやインドの一部地域で，近

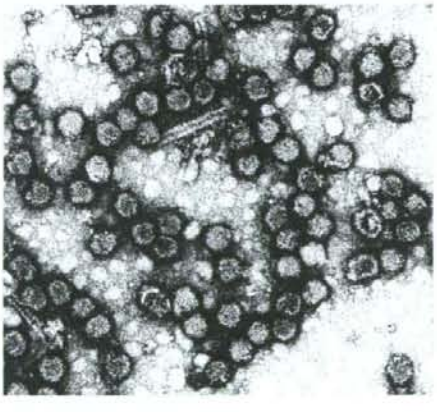

図1
日本脳炎ウイルス粒子
(電子顕微鏡写真)
直径約50 nmの球状構造をしたRNAウイルスである．

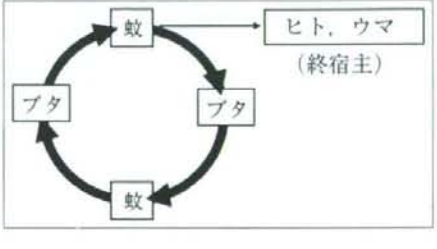

図2
日本脳炎ウイルスの
感染サイクル

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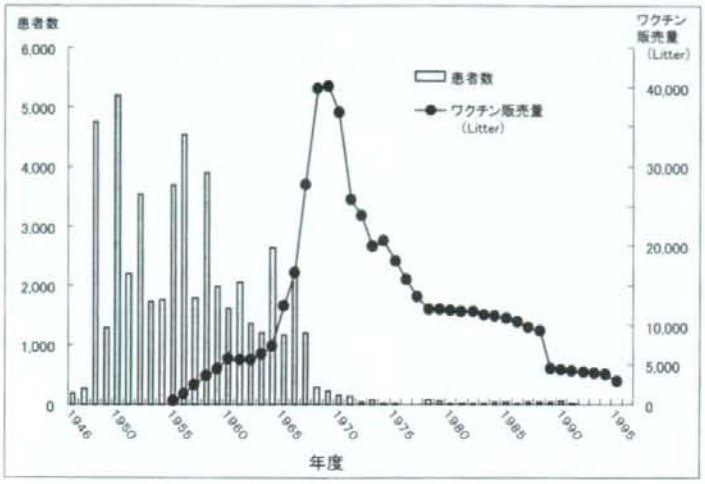

図3　日本における日本脳炎患者数の推移とワクチン販売量

図4　世界の日本脳炎ウイルスの活動地域

年，灌漑施設が整備され大規模な稲作が導入された地域では，多くの媒介蚊が発生するために患者発生が増加している．

越冬問題とウイルスの飛来

日本脳炎ウイルスは，蚊とブタとの感染サイクルにより維持されていることは先に述べたが，日本のように冬に蚊がいなくなるような地域でウイルスがどのように冬を越しているのかは謎であり，ウイルスが日本本土のなんらかの動物の体内で越冬するという“越冬説”と，毎年春に南方からウイルスが日本に持ち込まれるとする“飛来説”が長く2者択一的に議論されてきた．

しかし，Ngaと森田らは，1980～2001年の間に，東南アジアと日本で分離された日本脳炎ウイルスの遺伝子塩基配列を詳細に比較した分子疫学研究により，日本脳炎ウイルスは比較的頻繁に東南アジアから朝鮮半島や日本へ飛来していることを明らかにした．一方で，飛来したウイルスが日本国内で冬を越し，翌年の初夏に再び出現する可能性も遺伝子解析結果では示されており(飛来したウイルスの遺伝子は日本で独自の変化を遂げている事実がある)，日本脳炎ウイルスの越冬のメカニズムは現在でも解明されていない日本脳炎ウイルスに関する謎の一つである．

臨床症状と診断

日本脳炎は，日本脳炎ウイルスが中枢神経系に侵入して神経細胞で増殖することによって発症する急性のウイルス性脳炎であるが，ウイルスに感染したヒトの約300～3,000人に一人が脳炎になると見積もられており，顕性感染率は低い．

脳炎を発症するケースでは潜伏期は約1～2週間で，初発症状は発熱・頭痛である場合が多い．さらに，2～4日間の頭痛，高熱，悪寒，食欲不振，嘔気，嘔吐，傾眠の状態が持続し，進行すると項部硬直，Kernig症候，筋強剛などの髄膜刺激症状が出現し，意識障害，異常反射，四肢麻痺(特に上肢)，痙攣，昏睡がみられ，ついには死に至る．重症例の場合は死を免れても約半数に重い精神障害，運動麻痺などの後遺症がみられる．

一般的に，脳炎を発症した場合の死亡率は10～30％であり，回復した患者において重篤な後遺症は約50％の患者にみられる．また，重症例ではCTおよびMRI検査で視床，基底核(多くは両側性)に異常所見がみられると報告されている．

ほかに同様の症状を示すウイルス性脳炎は多いので，確定診断には実験室診断が必須であり，急性期には髄液からのウイルス分離やRT-PCR法またはRT-LAMP法を用いてウイルス(遺伝子)の検出を行い，回復期にあってはELISA法やHI法により特異的抗体を定量して急性期と比較して4倍以上の上昇を証明するか，ELISA法では日本脳炎特異的IgM抗体の検出によりウイルス感染を証明する必要がある．

治療と予防

日本脳炎に効果的な抗ウイルス薬はいまのところ開発されていないので，発症した場合には対症療法を行い，細菌感染などを予防することが必要とされる．日本脳炎への対策は予防が最も重要であり，日本脳炎の流行地域やウイルスの活動が確実な地域では蚊に刺されないような方策をとること，また最も確実な予防方

法としてワクチンの接種が推奨される．実際，日本や韓国，台湾においては，不活化ワクチンを学童層に集団接種したあと患者発生は激減している．このワクチンは初回接種においては1～4週間の間隔で2回の接種，1年後に1回の追加接種を行い，その後は数年おきに1回の追加免疫を行う．

現行の不活化ワクチンの副作用については，重篤な副作用（神経系の障害）の出現頻度は被接種者100万人に一人，あるいはそれ以下と見積もられており，きわめて安全なワクチンの一つと考えられるが，2005年に厚生労働省は，2004年にワクチン接種後に発生した1例の重症な急性散在性脳脊髄炎（ADEM：acute disseminated encephalomyelitis）の報告を受けて，現行の日本脳炎接種を推奨しない方針を打ち出した．これはワクチンに残留しているマウス脳成分（現行の日本脳炎ワクチンはウイルス感染マウス脳を原料としてつくられている）が誘因となって神経細胞の脱髄が発生した可能性を否定できないためとされている．ADEMは種々のウイルス感染のあと，あるいは原因不明で200万人に1～2人の割合で発生すると見積もられており，毎年数百万ドースが消費された従来型の不活化日本脳炎ワクチンとADEMの因果関係を科学的に肯定することもまた困難なことであり，頭の痛い問題である．

しかし現在，日本脳炎の第二世代ワクチン（ウイルス感染培養細胞を原料とする日本脳炎ワクチン）が認可の最終段階にあり，この第二世代日本脳炎ワクチンでは原理的にマウスの脳成分が存在する可能性はないので，厚生労働省は新型ワクチンの認可を待って日本脳炎ワクチンの推奨を再開するという方針のようである（2007年9月時点ではいまだ認可されていない）．

おわりに

日本脳炎は患者数が減るとともに社会的な関心も確実に低下しているようだ．しかし，日本脳炎の感染リスクはいまも確実に存在している．日本脳炎の流行地域，すなわちワクチンの行き届いていない東南アジアの国々では患者の多くは小児である．

現在，わが国では厚生労働省の「接種の積極的な勧奨をしない」との勧告を受け，多くの自治体で学校での集団接種を中止している．このため現在，ワクチンを打っていない子どもの数が確実に増えてきている．このままでは2009年には6歳以下の子どもの大半は日本脳炎に対して免疫を持たないことになる．

2006年9月，熊本県内で3歳児の日本脳炎の発症が確認されたが，5歳以下の発症はなんと15年ぶりのことであった．このまま新型ワクチンの認可が遅れると“日本脳炎”にかかる危険の高い子どもが増えることになり，新型ワクチンの早期の認可が望まれる．

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Seroepidemiological study on hantavirus infections in India

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Summary Hantaviruses are etiological agents of hemorrhagic fever with renal syndrome in many parts of Asia and Europe. There has been no documented case of hantavirus disease from India, although serological evidence exists. We investigated the prevalence of hantavirus in the Indian population and tried to identify potential risk groups for hantavirus infections. The presence of hantavirus-specific IgG antibodies was prospectively evaluated in 661 subjects belonging to different groups, i.e. patients with chronic renal disease, warehouse workers and tribal members engaged in rodent trapping. Healthy volunteer blood donors were included as a control group. Thirty-eight seropositive samples were found using a combination of a commercial ELISA followed by an indirect immunofluorescence assay. Western blot using recombinant Hantaan virus nucleocapsid antigen confirmed the presence of anti-hantavirus IgG in 28 (74%) of the 38 sera tested. This study confirms the presence of hantaviruses in India and warrants increasing awareness of the problems of emerging pathogens and the threats they may pose to the public health system.

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1. Introduction

Hantaviruses represent a group of emerging viruses. The genus *Hantavirus*, belonging to the family Bunyaviridae, comprises more than 20 species that can cause two diseases in humans: hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas (Lednicky, 2003). Almost all hantaviruses are maintained in rodents of the family Muridae, and are transmitted to humans via aerosolized urine, saliva and feces of infected rodents.

Although hantavirus infections were recognized in Asia for centuries, it was only during investigations initiated after the Korean conflict in the 1950s, during which thousands of UN soldiers were affected by HFRS, that the Hantaan virus (HTNV) serotype was isolated. The circulation of hantavirus serotypes, namely Seoul virus (SEOV) and Thailand virus (THAIV), has been demonstrated in several Southeast Asian countries, i.e. Thailand, Cambodia, Viet Nam and Indonesia (Plyusnina et al., 2004; Reynes et al., 2003; Truong et al., 2004). Recently a report from Thailand documented the first serological evidence of THAIV causing HFRS in humans (Pattamadilok et al., 2006). The Thottapalyam virus (TPMV), which was isolated from an insectivore, *Suncus murinus*, in 1964, is the only known hantavirus species indigenous to India (Cary et al., 1971).

The association of chronic renal disease and hantavirus seropositivity has frequently been speculated upon as studies conducted in the United States (Baltimore) suggested that hantavirus seropositives had higher rates of chronic renal disease and hypertensive renal disease than age-matched seronegative controls (Glass et al., 1990). Similar studies have been reported from Israel (George et al., 1998) and Egypt (Botros et al., 2004). An epidemiological study from Taiwan has reported detection of anti-hantavirus antibody in various risk groups such as garbage collectors and animal handlers (Chen et al., 1998).

Although the isolation of TPMV pre-dates that of HTNV, interest in hantaviruses was revived in India in 2005, with two reports on the serological evidence of hantavirus infections in patients with febrile illnesses (Chandy et al., 2005; Clement et al., 2006). However, seroepidemiological surveys have not been reported from India, and this study is the first attempt to investigate the epidemiology of hantavirus infections in India.

2. Materials and methods

2.1. Study population

The study subjects belonged to different groups and were recruited at the Christian Medical College, Vellore, south India. Healthy volunteer blood donors ($n = 360$) comprised the control group. The potential risk groups included 99 sera from asymptomatic Irulas, a tribal community living in Tamil Nadu, a state in south India (the tribe members are professional rat catchers and eat rats) and 51 sera from people working in warehouses (warehouse workers) in and around Vellore. An additional group was represented by 151 serum samples from patients with chronic renal disease with serum creatinine and urea levels of >1.4 mg/dl and >40 mg/dl, respectively.

The mean (±SD) age of the subjects in the Irula group was 32 (±11.2) years, and this group included 41 males and 58 females; in the warehouse workers it was 38 (±10.17) years and all were males; and in the renal disease patients it was 43 (±12.11) years and there were 47 females and 104 males. The subjects in the control group were aged 18–60 years.

The sample size was calculated based on a previous study of hantavirus infections in India (Chandy et al., 2005). The study period was from August 2004 to May 2007. Samples from healthy blood donors and patients with chronic renal disease were collected at the Department of Clinical Virology, Christian Medical College, Vellore and were included in the study by convenient sampling. All samples collected from asymptomatic Irulas and warehouse workers were included in the study. Written informed consent was obtained before collecting blood.

2.2. ELISA and immunofluorescence assay

Serological screening was done using a commercial (ISO certified) hantavirus IgG ELISA (Focus Technologies, Cypress, CA, USA) according to the manufacturer's instructions. This ELISA uses a cocktail of hantavirus antigens of HTNV, SEOV, Puumala virus (PUUV), Sin Nombre virus (SNV) and Dobrava-Belgrade virus (DOBV) to coat the polystyrene microwells and can detect IgG antibodies against these serotypes. The screening ELISA is an indirect test in which the optical density (OD) is directly proportional to the antigen-specific IgG antibodies present in the sample. The results were obtained by comparison of the sample OD readings with reference cut-off OD readings.

Results were reported as index values relative to the cut-off calibrator. To calculate index values, each sample OD value was divided by the mean of the cut-off calibrator OD values.

Sera positive by ELISA were re-tested by an indirect immunofluorescence assay (IFA) using HTNV-infected Vero E6 cells as antigens (Yoshimatsu et al., 1993). The secondary antibody used was fluorescein-isothiocyanate-conjugated rabbit anti-human IgG (DakoCytomation, Glostrup, Denmark). For the IFA, all spots with at least half of the infected cells showing a characteristic apple green granular cytoplasmic fluorescence with $\geq 2+$ intensity were scored positive.

Finally, a sample was considered positive if reactive by both ELISA and IFA. The screening ELISA and IFA were evaluated using a panel of positive and negative control sera kindly supplied by the European Network for Diagnostics of Imported Viral Diseases [ENIVD (Biel et al., 2003)].

2.3. Western blot analysis

Western blot analysis was performed on 38 positive sera using recombinant nucleocapsid protein (NP) of HTNV (Fojnica strain) as antigen (Razanskiene et al., 2004). Sera at dilutions of 1:1000 were applied to the membrane, and goat anti-human IgG conjugated with alkaline phosphatase (Genelabs Diagnostics Pte Ltd, Singapore Science Park, Singapore 118259, Republic of Singapore) at 1:1000

Table 1 Results of evaluation of the screening ELISA and Hantaan virus-immunofluorescence assay (IFA) using European Network for Diagnostics of Imported Viral Diseases (ENIVD) sera

ENIVD no.	Sample type	IgG level	Origin	Hantavirus strain	Serum status	ELISA result	IFA result
2	Positive serum	++	Sweden	Puumala	Convalescent	+	+
8	Positive serum	+	Kosovo	Dobrava	Convalescent	+	+
9	Positive serum	++	Sweden	Puumala	Convalescent	+	+
16	Positive serum	++	Sweden	Puumala	Convalescent	+	+
17	Positive serum	+	Finland	Puumala	Convalescent	–	–
4	Negative serum	–	Germany	–	Control	–	–
12	Negative plasma	–	Germany	–	Control	–	–
18	Negative serum	–	Germany	–	Control	–	–
19	Negative serum	–	Germany	–	Control	–	–

+, positive; ++, strong positive; –, negative.

dilutions was used as the secondary antibody. Thereafter the membrane was developed with substrate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT). In the Western blot the molecular size of the expected product was about 49 kDa.

2.4. Statistical analysis

The statistical analysis was done using EpiInfo version 6.04b (CDC, Atlanta, GA, USA) to compare two categorical variables. Percentages were calculated for categorical outcomes (positives/negatives). A P-value <0.05 was considered significant.

3. Results

ENIVD sera were used to evaluate the commercial IgG ELISA and the IFA using HTNV antigen; these assays could detect anti-Puumala virus and anti-Dobrava virus IgG-positive sera. However, one ENIVD anti-Puumala virus serum (origin Finland) could not be detected by either of these assays (Table 1).

In the initial screening, 661 serum samples were tested by a commercial IgG ELISA (Table 2). Forty-seven of the 661 sera were found to be reactive in the ELISA. The majority (38/47) of the ELISA-reactive sera were also detected by IFA using HTNV antigen. Seropositivity in the Irula tribal group (11%) was significantly higher than in the control group (4%, $P<0.05$). There was no statistically significant difference between seropositivity in the chronic renal disease patient group (7%) compared to that of the control group. The level of seropositivity in the warehouse workers (2%) was very similar to that of the control group. Sex as a demographic factor was not significantly associated with hantavirus infections in the different subject groups, suggesting that males and females are equally likely to contract hantavirus infections. The mean age of the seropositives in the renal disease patient group, the Irulas and the warehouse workers was 50 (SD ± 11.29), 29 (SD ± 8.9) and 31 years, respectively. Twenty-eight of the 38 positive sera (74%) were positive by Western blot (Table 2).

4. Discussion

Studies on hantavirus infections in India are in the early stages. The data given here represent the first attempt to characterize the epidemiology of hantavirus infections in India and strengthen previous reports on serological evidence of hantavirus infections in India (Chandy et al., 2005; Clement et al., 2006).

Forty-seven of 661 serum samples were positive by ELISA. Serology is the mainstay of diagnosis of hantavirus infections. ELISAs are highly sensitive and are the preferred diagnostic tool for serological surveys. The commercial ELISA used in this study uses a cocktail of six antigens, and can be used in areas where the circulating hantavirus species are unknown. The specificity of the hantavirus assays used in the study is acceptably good, as evaluated by the ENIVD-negative control sera. There may be problems with diagnostic sensitivity, as one ENIVD anti-Puumala IgG-positive serum was not detected by both the assays.

Table 2 Results of serological studies

Group tested	No. tested	No. positive by ELISA (%)	No. positive (ELISA and HTNV-IFA) (%)	No. positive/no. tested by Western blot (%)
Blood donors	360	19 (5)	16 (4)	11/16 (69)
Renal disease patient group	151	14 (9)	10 (7)	9/10 (90)
Irulas	99	12 (12)	11 (11)	7/11 (64)
Warehouse workers	51	2 (4)	1 (2)	1/1 (100)
Total	661	47 (7)	38 (6)	28/38 (74)

HTNV-IFA: Hantaan virus-immunofluorescence assay.

The lower seroprevalence observed in the IFA and Western blot analysis might be due to the HTNV antigen, which suggests that hantavirus species other than HTNV are circulating and causing human disease in India. Alternatively, we cannot exclude the possibility that the ELISA picked up false positives.

It has been documented that TPMV is phylogenetically and antigenically quite distinct from the other well-characterized hantaviruses (Song et al., 2007), and although antibodies against hantavirus NP are cross-reactive between different hantavirus species, we cannot speculate about the efficiency of the assays used in the study to detect antibodies against TPMV. Moreover, there may be other hantavirus species circulating in India that may be as diverse as TPMV, and in the case of an antigenic mismatch the assays used here may fail to detect seropositives. It is thus important to define the hantavirus species circulating in India and develop sensitive assays using homogeneous antigens.

In this study, the renal disease patient group appears to have a higher risk of hantavirus seropositivity compared with the control group, but the difference is not statistically significant. It is still not clear whether patients with chronic renal disease are at a higher risk of acquiring hantavirus infections or that hantavirus infections by themselves contribute to the development of chronic renal disease. These results are preliminary, and follow-up studies are needed to prove any significant association of hantavirus infections with chronic renal disease.

The tribal group in this study has a relatively high level of contact with rodents, as they are traditionally rat catchers and also eat rats. They display a high seropositivity when compared with the control group. By contrast, warehouse workers showed a low seropositivity in our study (2%). This may reflect a lower risk of contracting hantavirus infections due to the fact that many warehouses in India adopt stringent rodent-control measures.

No well-documented hantavirus case, as defined by virus isolation or molecular evidence, has been reported from India to date. However, our study confirms that one or more hantaviruses are circulating in the Indian population and indicates that the threat from emerging pathogens must be continually assessed. Studies to identify the hantaviruses that might cause problems for public health systems are important, as they will aid the development of new strategies for the prevention and control of such emerging infections.

Authors' contributions: GS and PA designed the study protocol; SC carried out the immunoassays and drafted the manuscript; KY, RGU, MM, MO and JA supplied IFA slides and Western blot strips and helped analyse results obtained; RP, GTJ, VB, JM and JM helped with the identification of subjects in the various groups and collection of samples. All authors read and approved the final manuscript. GS is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: The institutional research ethics committee of the Christian Medical College, Vellore, Tamil Nadu, India (R.C. Min. No. 5838 dated 21 February 2006) and the Indian Council for Medical Research (ICMR), New Delhi, India.

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