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Human Monoclonal Fab Antibodies Against West Nile Virus and its Neutralizing Activity Analyzed *in vitro* and *in vivo*

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Abstract

The disease progression with West Nile virus (WNV) infection in humans leads to meningitis or encephalitis and may cause death, particularly among elderly and immunocompromised individuals. Passive immunity using immunoglobulins has shown efficacy in treating some patients with WNV infection, which makes the development of human anti-WNV antibodies significant. The goal of this study was to construct a Fab-specific phage display library against WNV, and to identify and select clones with neutralizing activities. Total RNA was extracted from peripheral blood lymphocytes (PBLs) of two immunized individuals, and RT-PCR was used to amplify the Fab fragments containing the heavy (V_H) and light (V_L) chains. The amplified genes were sequentially cloned into the recombinant antibody expression vector pComb3-H, and the Fab-specific phage display library was packaged with helper phage VCS-M13. Five rounds of panning were carried out with WNV E protein domain III, and then binding antibodies were selected by ELISA. Antigen binding specificity, complementarity determining region (CDR) sequence of V_H and V_L, and neutralizing activity against WNV were analyzed *in vitro* and *in vivo*. Eight Fab monoclonal antibodies recognized E protein domain III from a library of 7×10^7 clones /ml. Of the eight, one (Fab 1), exhibited significant neutralizing activity, and completely blocked 100 pfu WNV infection in Vero cells at a concentration 160 µg/ml. In contrast, Fab 13 and Fab 25, showed weaker neutralizing activities, and modestly blocked 100 pfu WNV infections at concentrations of 320 µg/ml and 160 µg/ml, respectively. However, animal studies showed that Fab 1 failed to protect mice from death at the concentration of 160µg/ml indicating that the neutralizing potential of an antibody *in vivo* is determined by the strength of binding and the abundance of its epitope for the virion.

Keywords: West Nile virus; Phage display; Fab antibody; Neutralizing activity

Introduction

West Nile virus (WNV) is a single-stranded, positive-polarity RNA flavivirus that is related to viruses causing dengue fever, yellow fever, St. Louis, tick-borne and Japanese encephalitis. Human infections with WNV develop a febrile illness that can progress to meningitis or encephalitis and may lead to death, particularly among those elderly and immunocompromised (Granwehr et al., 2004; Marfin and Gubler, 2001). The clinical manifestations of WNV infection are well defined, but the mechanism of pathogenesis has not been elucidated completely. Previous studies have proven that WNV could infect and induce cytopathogenicity in various cell cultures of human, primate, rodent and insect origin. Both necrosis and apoptosis in WNV-infected cells and tissues were observed in patients, as well as in experimental animal models of fatal WNV infections (Xiao et al., 2001).

Currently, treatment is supportive and no approved vaccine exists for clinical use. The innate and adaptive immune responses can prevent WNV dissemination within the central nervous system (CNS) (Diamond et al., 2003), and the antiviral antibody may work directly in the CNS by preventing replication and spread in neurons (Agrawal and Petersen, 2003). Recently, various groups showed therapeutic efficacy of immune human γ-globulin and humanized monoclonal antibody in mice infected with WNV (Agrawal and Petersen, 2003; Engle and Diamond, 2003; Oliphant et al., 2005; Tesh et al., 2002; Gould et al., 2005). The passive administration of immune γ-globulin or monoclonal antibody improved survival even after virus had spread to the CNS (Engle and Diamond, 2003; Oliphant et al., 2005). These results suggest that a potent neutralizing monoclonal antibody could represent another potential direction to influence disease outcome.

Most neutralizing antibodies against flaviviruses recognize the envelope (E) glycoprotein. Monoclonal antibodies produced against the E protein have been found to protect mice from lethal infection (Oliphant et al., 2005; Gould et al., 2005; Nybakken et al., 2005; Kaufmann et al., 2006; Pereboev et al., 2008). Crys-

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tallographic analysis of the soluble ectodomain of flavivirus E proteins has shown that there are three domains. Domain I is an eight-stranded β-barrel which participates in the conformational changes associated with the acidification in the endosome. Domain II contains 12 β-strands and has roles in dimerization, trimerization and fusion (Modis et al., 2003; Rey et al., 1995; Rey, 2003; Modis et al., 2004). Domain III adopts an immunoglobulin-like fold, and contains surfaced exposed loops which putatively play a role in receptor attachment in the mature virion (Mukhopadhyay et al., 2003; Chu et al., 2005; Bhardwaj et al., 2001). Many neutralizing antibodies against flaviviruses recognize Domain III of E protein.

There is an urgent need to develop human antibodies against WNV which could be used for therapeutic purposes. Based on the importance of Domain III of E protein, we aimed to develop human antibodies against this domain.

In this study, we constructed Fab antibody phage display library generated from the PBLs of immunized donors, and obtained human Fab antibodies binding to WNV E protein domain III. We evaluated the neutralizing activities of three antibodies which have high binding activities *in vitro* and further evaluated the protection efficiency of one antibody *in vivo*.

Materials and Methods

Cells and viruses

Vero cells (ATCC CCL-81) were cultured as previously described (Mou et al., 2006). We performed neutralization experiments with the WNV strain Egypt 101 (Samoilova et al., 2003), and the titer of the WNV strain was 10^7 PFU/ml, and animal experiments with the WNV strain NY385-99 (Melnick et al., 1951).

RNA preparation and construction of expression vector

Ten milliliters of blood was drawn from two individuals with high neutralizing antibody titer against WNV. Lymphocytes were isolated by centrifugation with a lymphocyte separation medium (Pharmacia). Total RNA was isolated using Trizol reagent (Invitrogen) and was used to synthesize first-strand cDNAs using Superscript III™ first-strand synthesis system for RT-PCR (Invitrogen). DNA of the antibody Fab portion was amplified using specific primers for the antibody heavy- and light-chain genes. The primers were designed according to the previous study (Barbas and Burton, 1994) (data not shown). The V_H region of the heavy chains and the light chains were amplified. PCR was performed as follows: 35 cycles of denaturing at 95°C for 1 min, annealing at 52°C for 1 min, and extending at 72°C for 2 min followed by a final incubation at 72°C for 10 min. The amplified light chains were digested with *XbaI* and *SacI*, and purified by electrophoresis in 1.5% agarose gel. The relevant DNA bands were excised from the gels and extracted using a QIAquick gel extraction kit (Qiagen). Purified DNAs were ligated with *XbaI* / *SacI* - linearized pComb3-H vector (provided by the Scripps Research Institute). Heavy chain Fd fragments were cut with excess of the restriction enzymes *Xho I* and *Spe I* and were cloned into *Xho I* / *Spe I* – linearized pComb3-H harboring light chains. The DNA pellet was transformed into electrocompetent *E.coli XL1*-Blue cells (Invitrogen). After transformation, *XL1*-blue -DNA mix (10, 1, 0.1 µl) was plated to determine the efficiency of transformation. The insertion of target genes was detected by digestion with specific enzymes from the plasmids which were purified from several random *XL1*-blue monoclones.

Packaging of phage libraries

Following electroporation, the library was cultured in 50 ml 2×YT medium with 100 µg/ml ampicillin, 30 µg/ml tetracycline, 100 mM glucose to OD_{600} value of 0.025. The culture was incubated at 37°C for 2 h. Helper phage VCS-M13 (10^{12} pfu, Stratagene) was added at a m.o.i. of 8 at OD_{600} value of 0.1. The culture was continued at 80 rpm for 30 min and at 260 rpm for 30 min. The culture was centrifuged at 4000 rpm and the medium was changed to 40 ml 2×YT with 100 µg/ml ampicillin and 50µg/ml kanamycin. The culture was further incubated for 6 h at 30°C, then centrifuged. Supernatant was added with PEG to 4% and NaCl to 3%. The phage library was precipitated at 4°C overnight before it was centrifuged at 8000 rpm for 30 min. The phage pellet was resuspended in PBS (pH 7.4) and transferred to microcentrifuge tubes at 14000 rpm for 10 min to remove insoluble material. The Fab phage display library was then used for panning experiments.

Panning of anti-WNV fab phage display library

Several 96-well plates were coated with 200 µl highly purified recombinant WNV envelope protein containing domain III (500ng/well), which was provided by Dr. David Beasley (University of Texas Medical, Galveston, TX), and was incubated at 4°C overnight. After blocking with 3% BSA at 37°C for an hour, 50 µl phage suspensions were added to each well (total of about 10^7 pfu). The panning procedure is a modification of procedure originally described by Parmley and Smith (Barbas and Lerner, 1991). Following 5 rounds of panning, the percent yield of phage was determined as (No. of phage eluted/No. of phage applied) × 100.

ELISA analysis of fab antibodies

Fab clones were selected and cultured in 2YT medium overnight, then the cultures were induced by 1 mM isopropylbeta-D-thiogaractopyranoside (IPTG). The cells were recovered by centrifugation, and re-suspended in PBS. Fab antibodies were extracted by freezing at -70°C and thawing at 4°C for three times, then were centrifuged at 10,000 rpm and the supernatants were collected. Ninety-six well plates were coated with 100 µl highly purified recombinant WNV envelope protein containing domain III and blocked with 3% BSA as described above. After extensive washing with PBST, the supernatants of Fab antibody were added to the wells (50 µl / well) and incubated at 37°C for 2h. Following 3 washes with PBST, 50 µl of a 1:20,000 dilution of horseradish peroxidase (HRP) conjugated goat anti-human Fab (Sigma, Ronkonkoma, NY) was added and incubated at 37°C for 1 h. Finally, 50 µl of TMB (Sigma, Ronkonkoma, NY) was added and color development was monitored at 490nm. The A_{490} values of positive clones were higher than that of negative clones at least two-fold.

Sequence analysis of WNV-specific fab clones

Nucleotide sequences of the heavy and light chain variable regions of the three positive clones (Granwehr et al., 2004; Modis et al., 2003; Haard et al., 1999) were determined by the University of Texas Medical Branch Protein Chemistry Laboratory.

DNA and deduced amino acid sequences were analyzed by using Pubmed Igblast Software (http://www.ncbi.nlm.nih.gov/igblast/). Further analysis of amino acid sequences of V_H and V_L of WNV-specific human Fab antibodies was performed according to (Kim et al., 2004).

Western blotting analysis of WNV-specific fab antibodies

Maltose binding protein (MBP) WNV domain III was heated to 100°C for 10 min in equal volume of 2x SDS gel-loading buffer, and loaded up to 20 µl of each of the samples into the bottom of the wells for electrophoresis (100V for about 4 h until the bromophenol blue reached the bottom of the resolving gel). Electrophoresis was performed in duplicates. One gel was fixed with glacial acetic acid: methanol: water (10:20:70) for 10 min and washed in deionized water, then stained with Coomassie Brilliant Blue, the other was used for Western blot analysis. The proteins were transferred to nitrocellulose membrane, and nonspecific binding sites were blocked with nonfat dried milk by incubating the membrane for 2 h at room temperature with gentle agitation on a platform shaker. The membrane was washed with TBST for 3 times (10 min each). Then the membrane was cut into five strips according to the lanes, and respectively incubated with 1:2 dilution of antibodies Fab 1, Fab 13, Fab 25 and 1:200 dilution of positive serum for 2 h at room temperature with gentle agitation on a platform shaker, and after washing as above, membranes were incubated with 1:20,000 dilution of HRP-conjugated goat anti-human Fab for 1 h, and were detected by ECL™ Western blotting detection reagent (Amersham). The membranes were exposed to X-ray film for 30 sec, and the film was developed immediately.

Indirect fluorescent antibody (IFA) assay to detect WNV in vero 76 cells

Sterilized round cover slips were placed into 6-well plates, and Vero 76 cells (5× 10^5 cells/well) were seeded into the plates and cultured for 20 h. Cells were inoculated with 100 µl of virus suspension in test medium containing equal volumes of WNV (approximately 100 plaque forming units [pfu]), and incubated at 37°C for 1 h. The inocula were aspirated and fresh MEM medium containing 2% FBS were added. Sixty hours later, the cover slips were carefully removed and examined by IFA assay. Fab 1, 13, and 25, diluted by 1: 100 in PBS, human convalescent WNV serum, diluted by 1:200 in PBS, and human pooled immune globulin (without WNV antibody), diluted by 1:200 in PBS, were added to each slides and incubated at 37 °C for 1 h. These were washed with PBST and incubated at 37 °C for 50 min with 50 µl of fluorescein isothiocyanate (FITC)-conjugated goat anti-human Fab (Sigma, St. Louis, MO), diluted 1:60 in PBS and containing 1% Evan's blue dye. Evidence of specific fluorescence was monitored by fluorescence microscopy (microscopic field 20X), using an Olympus BX51 microscope.

Plaque reduction neutralization tests (PRNT) for analysis of neutralizing activity of fab antibodies

This assay was performed in triplicates. Briefly, after removal of the cell growth medium, confluent 24 h Vero 76 cell monolayer in 24-well plates (Becton Dickinson labware, NJ USA) were inoculated with 100 µl of the respective virus suspension in test medium containing equal volumes of WNV (approximately 100 pfu and serial two-fold dilutions of the test human recombinant antibodies, which had been mixed and inoculated at 37°C for 1 h. After adsorption at 37°C and 5% CO_2 for 1 h, the inocula were aspirated and each well was overlayed with 1 ml mixture of MEM medium containing 2% FBS and 1% carboxymethyl-cellulose(Sigma).Heat-inactivated anti-WNV human positive/negative serum and recombinant plasmid were used as positive, negative and mock controls at the same time. Three untreated virus controls and one uninfected cell control were included in all assays. Each compound concentration was tested in duplicate. The tests were incubated at 37°C and 5% CO_2 for 72 h until plaques appeared and fixed by 10% formalin for 48 h (changing once after 24 h) and then stained with a solution of 0.5% crystal violet in PBS. Plaques were counted over a light box after removal of the crystal violet. Neutralizing antibody activity was considered as the concentration of the antibody dilution with an 80% reduction in the number of plaques (PRNT80), as compared to the virus control and other a series of controls.

Mice passive immunization and viral challenge

Eight groups of 32 female C57BL/6 mice (Jackson Laboratories) between 4 and 6 weeks age were used in this experiment. Mice were infected with lethal dose WNV strain NY385-99 (10^3 PFU) intraperitoneally (i.p.) on day 1, and administered with the indicated doses of serum (positive control, negative control) or Fab 1 at times ranging from 1 day prior to 1 day post infection (Table 1). Survivals were recorded daily until no further deaths occurred for at least 21 days after infection.

Results

Table 1: Animal experimental design and protection results.

Groups	Virus only	Serum PC	Serum NC	Fab1	Mortality Rate
A	*				4/4
B		*			0/4
C		*			0/4
D		*			0/4
E			*		3/4
F				*	3/4
G				*	3/4
H				*	4/4

Note: Thirty-two female C57/BL/6 mice (4-6 weeks of age) were inoculated with 10^3 PFU of West Nile virus strain NY385-99, via IP. There were 4 mice per group, as shown in the table above:

A = Virus control (virus but no antibody)

B, C, and D = Received human convalescent WNV serum (T-35582), 200 uL of a 1:2 dilution given IP, one day prior to infection (B), day of infection (C) and 24 hours after infection (D).

E = Human pooled immune globulin (without WNV antibody); 200 uL of 1:2 dilution given IP given 24 hours prior to infection.

F, G and H = Received 200 uL of Fab 1, given IP one day prior to infection (F), day of infection (G) and 24 hours after infection (H).

All mice were infected on day 1. The animals were observed for 21 days post-infection. Deaths occurred between days 8-14.

Cloning of anti-WNV fab genes

A mixture of PCR-amplified κ/λ-chain products that had been digested by the appropriate restriction enzymes were ligated to the pComb3-H vector and introduced into *E. coli XL1-Blue* by electroporation. Titration ampicillin-resistant clones indicated that the light chain library contained 5×10^6 independent clones. PCR-amplified heavy chain products were ligated to DNAs extracted from the light chain library to generate a phage display Fab library with 2×10^7 clones. To examine the authenticity of the library, 30 clones were picked at random and analyzed. Light chain and heavy chain insert efficiency was approximately 53.3% and 33.3%, respectively.

ELISA analysis of anti-WNV fab antibodies

The library was panned to select clones which have binding activities to WNV domain III antigen. After 5 rounds of panning, phagemid DNAs obtained were introduced into *E. coli XL1-Blue* to develop Fab antibodies, and each clone was tested by ELISA; 8 clones (Fab 1, 6, 13, 16, 22, 23, 24, 25) which have Fab antibody proteins showed binding activities to WNV domain III protein in ELISA; three (Fab 1, 13, 25) had higher affinity than the others (Figure 1).

Sequence analysis of WNV-specific fab clones

Sequence analysis of the heavy and light chain variable regions of Fab 1, 13, 25 clones showed that their heavy chain variable region (V_H) sequences which include complementarity determining regions (CDR_S) that directly interact with the epitope of the antigen were significantly different from each other. They originated from different germline V_H segments and also had somatic hypermutations. They belonged to V_{H1} (Fab 25) and V_{H3} (Fab 1, 13) gene family, and the light chain variable region (V_L) sequences were also highly different to each other and originated from human $V_{κ1}$ gene family. The results of the sequence analyses are shown in Figure 2.

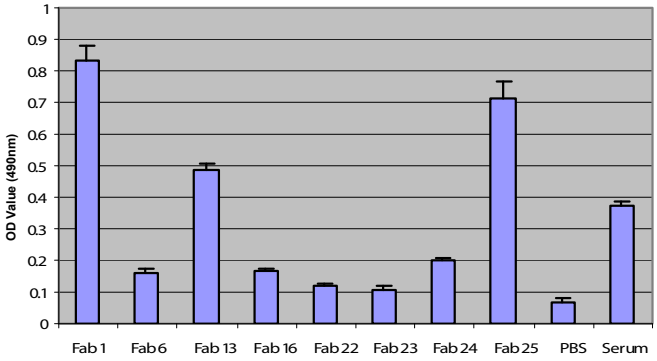

Figure 1: ELISA analysis of Fab antibodies against WNV E protein domain III.

Western blotting analysis of specificity and affinity of fab antibodies

The three positive clones (Fab 1, 13, 25) with higher activities in ELISA (Figure 1) were further identified by Western blotting and IFA assay for their specificity and affinity. The results showed each antibody binding specifically to the WNV protein domain III, and WNV proteins. Fab 1 showed the strongest activity compared to Fab 13, and Fab 25 (Figure 3 A and B).

A Variable region of light chains (V_L).

	FWR1	CDR1	FWR2	CDR2
Fab1	DIVMTQSPDSLAVSLGERATINC	KSSQSVLYSSNNKNYLA	WYQQKPGQPPKLLIY	WASTRES
Fab13	DIQMTQSPSSLSASVGDRVIISC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS
Fab25	DIVMTQSPDSLAVSGTPGQTISC	RGDSSNIGSNHFVS	WYQQLPGQAPVLVIY	DDRVRPS

	FWR3	CDR3	FWR4
Fab1	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC	QQYYSTPFT	FGPGTKVDIKR
Fab13	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYTIPYT	FGPGTKLEIKR
Fab25	GVPSRFSGSKSGTNASLTTSGLQAEDEADYYC	QVSTGDPPT	FGTGTKLTIKR

B Variable region of heavy chains (V_H)

	FWR1	CDR1	FWR2	CDR2
Fab1	EVQLLESGGGLEQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG
Fab13	EVQLVESGGGLVKPGGSLRLSCAASGFTFS	DYYMS	WIRQAPGKGLEWVS	YISSSGSTIYYADSVKG
Fab25	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPIFGTANYAQKFQG

	FWR3	CDR3	FWR4
Fab1	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCVK	DPGYGDPIDYWGQGTLVT	VSSASTKGPSV
Fab13	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR	DKGWLIDPDYWGQGTLVT	VSSASTKGPSV
Fab25	RVTITADGSTNTVYMQVRSLGAEDTATYYCAR	EESSVYASGGTYDNTTVT	VSSASTKGPSV

Figure 2: Amino acid sequences of V_L and V_H of anti-WNV-E specific human Fabs
Amino acid sequences were derived from the DNA sequences of the Fabs. Shown are the framework regions 1 to 4 (FWR1 to FWR4) and complementarity-determining regions 1 to 3 (CDR1 to CDR3) for V_H and V_L.

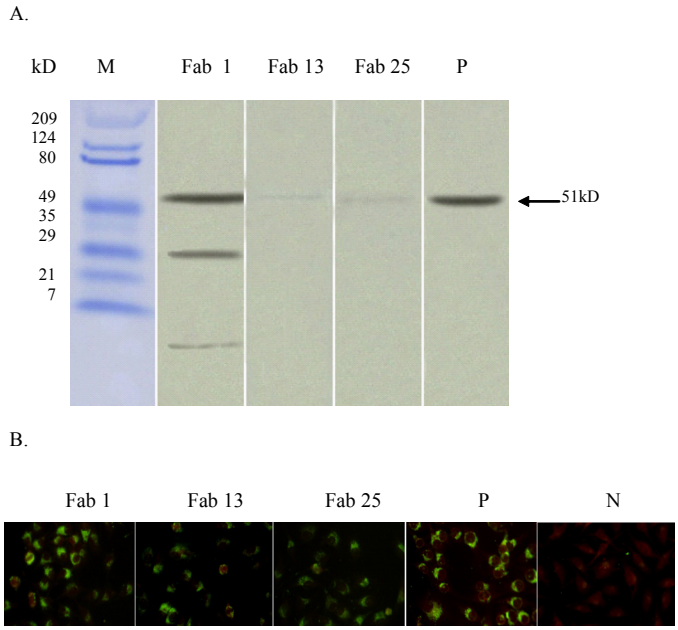

Figure 3: A. Western blotting analysis of Fab antibodies against WNV domain III, B. IFA assay to detect WNV in Vero cells. M: protein standards; P: positive control, human convalescent WNV serum (T-35582); N: human pooled immune globulin (without WNV antibody).

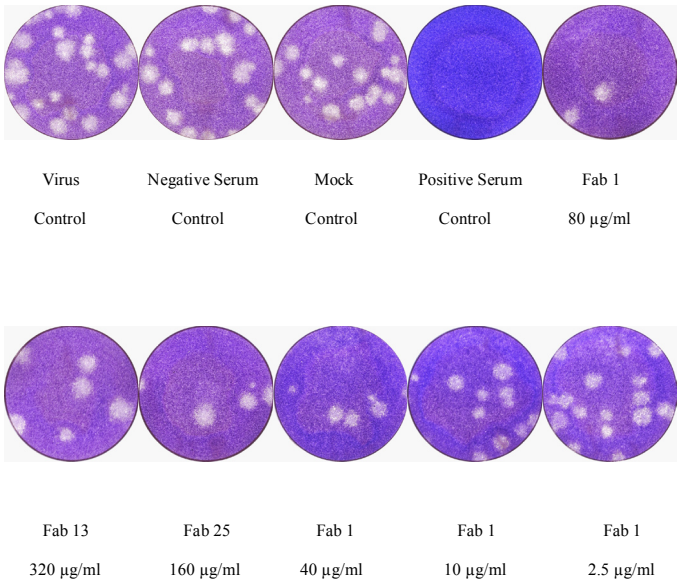

Figure 4: PRNT for virus neutralization by anti-WNV Fab antibodies. The "virus control" represents the result obtained by infection of virus only without addition of antibody. The "Negative Serum Control", "Mock Control", and "Positive Serum Control" represent the results obtained by the mixtures of virus with no anti-WNV antibody serum, supernatant of lytic XL1-blue, and anti-WNV serum, respectively. The final concentration of antibody in the virus-antibody mixture is indicated.

Neutralizing activity of fab antibodies *in vitro*

The three clones (Fab 1, 13, 25) showing specificity and affinity to WNV protein domain III were further analyzed by performing neutralization assay. The experiments were repeated three times and the results each time were consistent. The neutralizing activity associated with crude Fab antibodies was estimated by observing cytopathic effect (CPE) of Vero cells along with a series of controls. Fab 1 antibody exhibited significant neutralizing activity and blocked 100 pfu WNV infection at a concentration of 80 µg/ml (Figure 4), however, Fab 13 and Fab 25 antibodies showed weak neutralizing activity, and modestly blocked 100 pfu WNV infection at a concentration of 320 µg/ml and 160 µg/ml, respectively (Figure 4). In the PRNT, Fab 1 (PRNT80 = 80 ug/ml), Fab 13 (PRNT80 = 320 ug/ml), and Fab 25 (PRNT80 = 160 ug/ml) inhibited infection slightly than positive control (anti-WNV serum). The results demonstrated that the Fab antibodies could neutralize the Egypt 101 strain of WNV and the neutralizing activities of the antibodies were correlated with their affinities to WNV E protein.

Protection of fab antibody *in vivo*

To further examine the ability of the antibodies to neutralize WNV, Fab 1 was tested in *vivo* in a prophylactic viral infection model system using C57/BL/6 mice as described previously (Xiao et al., 2001). All mice were infected on day 1, and post infection, the animals were observed for 21 days. Deaths occurred between days 8-14, and mortality rates for each group are shown in Table 1, indicating no protection for Fab 1 in mice.

Discussion

Humoral immune response plays an important role in the control of flavivirus infection and disease. Therapeutic efficacy of immune human γ-globulin and humanized monoclonal antibody in mice infected with WNV were demonstrated by several investigators (Agrawal and Petersen, 2003; Engle and Diamond, 2003; Oliphant et al., 2005; Tesh et al., 2002; Gould et al., 2005). Among them, gene-based delivery of recombinant antibody genes seems to be a promising therapeutic strategy which has the advantages including sustained antibody levels, better safety profile and lower production costs (Kaufmann et al., 2006). Phage display system is powerful tool to generate human genetic antibodies (Haard et al., 1999). Many human genetic antibodies have already been developed with this system, though the mechanism of immune repertories generated in response to acute WNV infection or any flavivirus infection has not been well characterized in humans or primates. Antibodies against Dengue virus has been achieved from antibody phage display repertoires from Dengue virus-infected chimpanzees (Men et al., 2004). The use of partially and completely human antibodies has elicited no or minimal immune response when administered to patients (Holliger and Hoogenboom, 1998; Holliger and Hudson, 2005). Due to the absence of a WNV vaccine for humans, passive immunization represents an important alternative strategy to prevent and treat WNV infection.

In this study, we designed and constructed a phage antibody library specifically to Fab. We used a small volume of peripheral blood (20 ml) from two healthy donors with high WNV antibody titers as source to construct our Fab library. We used total RNA to synthesize the cDNA in the maximum extent. We obtained a phage library with 7×10^7 clones, which allows the rapid isolation and affinity analysis of antigen-specific human antibody fragments. Three neutralizing Fabs antibodies against WNV envelop protein domain III were developed from our li-

brary. These antibodies proved useful for generating Fab antibodies against WNV by plaque reduction neutralization test.

Fab is a construct in which the heavy chain and light chain are joined by a flexible polypeptide linker preventing dissociation. Antibody Fab fragments comprise both V_H and V_L domains and usually retain the specific, monovalent, antigen-binding affinity of the parent IgG, while showing improved pharmacokinetics for tissue penetration. Many of these products are currently in preclinical studies and clinical trials which supports our strategy in constructing Fab antibody phage display libraries, and selecting and identifying the antibodies against WNV.

The affinities of the selected antibody fragments are dependent on the antigen used for selection. Hoogenboom and colleagues reported an affinity varying between 2.7 and 38 nM for the selected Fab fragments specific for the gonadotropin (Haard et al., 1999), whereas Sheets and colleagues reported the affinity of scFv antibodies to the extracellular domain of human ErbB-2 varied between 0.22 and 4.03 nM (Sheets et al., 1998). This shows that it is very important to select appropriate antigens for the panning. Therefore, in this study, we used three different recombinant WNV envelope proteins for the panning the high-affinity Fab antibodies, and identified a panel of eight Fab antibodies that bound to the recombinant WNV envelope protein. Among those eight Fab antibodies, three of them were high-affinity antibodies to WNV. The sequences of the antibodies we obtained were blasted in Genebank, and the results showed that the sequences were unique and not previously reported. The heavy chain belong to the IgG1 subclass V_H1 and V_H3, the light chains belong to κ isotype. PRNT showed 80 μg/ml Fab 1 antibody can protect Vero cells from 100 pfu of WNV infection, which demonstrated neutralizing activity.

Passive administration of immune human γ-globulin after WNV infection improved survival in mice (Oliphant et al., 2005; Ben-Nathan et al., 2003; Engle and Diamond, 2003). In contrast our Fab antibody failed to protect mice from death partly because the neutralization potential of an antibody is determined by the strength of binding and abundance of its epitope on the virion (Burton et al., 2001; Oliphant et al., 2007). It may be limited by its own low–titer neutralizing activity, variability, and risk infectability. The therapeutic efficacy of mAbs is determined by properties in addition to neutralization. One research group found that the mAb with strongest neutralizing activity *in vitro* did not have the greatest efficacy *in vivo*, and Fab antibody was less potent in mice that lacked Fc γ receptors (Gould et al., 2005; Sheets et al., 1998); our *in vivo* data is consistent with the reported study of other research groups (Throsby et al., 2006). Although we found a neutralization potency *in vitro*, there was no association between potent *in vivo* a7ctivity and *in vitro* protection. Our experiments suggest that the highly neutralizing antibody has little significant role in primary infection and that the antibody for humans may be skewed toward the induction of cross-reactive and less-neutralization in animal studies. (Roehrig et al., 2001).

Potential conflicts of interest: All authors report no conflicts.

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