Humanized Monoclonal Antibodies Derived from Chimpanzee Fabs Protect against Japanese Encephalitis Virus In Vitro and In Vivo

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Japanese encephalitis virus (JEV)-specific Fab antibodies were recovered by repertoire cloning from chimpanzees initially immunized with inactivated JEV-VAX and then boosted with attenuated JEV SA14-14-2. From a panel of 11 Fabs recovered by different panning strategies, three highly potent neutralizing antibodies, termed Fabs A3, B2, and E3, which recognized spatially separated regions on the virion, were identified. These antibodies reacted with epitopes in different domains: the major determinant for Fab A3 was Lys172 (domain I), that for Fab B2 was Ile126 (domain II), and that for Fab E3 was Gly302 (domain III) in the envelope protein, suggesting that these antibodies neutralize the virus by different mechanisms. Potent neutralizing antibodies reacted with a low number of binding sites available on the virion. These three Fabs and derived humanized monoclonal antibodies (MAbs) exhibited high neutralizing activities against a broad spectrum of JEV genotype strains. Demonstration of antibody-mediated protection of JEV infection in vivo is provided using the mouse encephalitis model. MAb B2 was most potent, with a 50% protective dose (ED50) of 0.84 μg, followed by MAb A3 (ED50 of 5.8 μg) and then MAb E3 (ED50 of 24.7 μg) for a 4-week-old mouse. Administration of 200 μg/mouse of MAB B2 1 day after otherwise lethal JEV infection protected 50% of mice and significantly prolonged the average survival time compared to that of mice in the unprotected group, suggesting a therapeutic potential for use of MAB B2 in humans.

Japanese encephalitis virus (JEV) is the prototype virus of the Japanese encephalitis (JE) group belonging to the Flaviviridae family. Other members of the group include Kunjin virus, St. Louis encephalitis virus, and West Nile encephalitis virus (WNV). JEV is widely distributed in South Asia, Southeast Asia, and the Asian Pacific Rim. In recent years, JE epidemics have spread to previously unaffected areas, such as northern Australia (14, 47), Pakistan (17), and India and Indonesia (27). The JE outbreak in India during July to November of 2005 was the longest and most severe in recent years, affecting >5,000 persons and causing >1,000 deaths (42). It is estimated that JEV causes 35,000 to 50,000 cases of encephalitis, including 10,000 deaths and as many neurologic sequelae, each year (61). Although only one JEV serotype is known to exist, cross-neutralization experiments have demonstrated antigenic differences among JEV strains (1). Phylogenetic studies have identified five JEV genotypes, four of which are presently recognized (5, 55, 62). The wide geographical distribution and the existence of multiple strains, coupled with the high rate of mortality and residual neurological complications in survivors, make JEV infection an important public health problem.

The JE-VAX vaccine currently available in most countries is an inactivated whole-virus vaccine prepared from virus grown in mouse brain, and a three-dose regimen is required for young children (34). The requirements of multiple doses and the high vaccine manufacturing cost have prevented many countries from adapting an effective JEV vaccination campaign. A live-attenuated vaccine, JEV strain SA14-14-2, has been developed and extensively used in China and appears to be efficacious after one dose in a recent case-controlled study (59). A potentially promising, chimeric JEV vaccine constructed from the attenuated yellow fever 17D strain is in a late experimental stage (35). Until a JEV vaccine becomes generally available, passive immunization with potently neutralizing anti-JEV antibodies remains an attractive strategy for short-term prevention of and therapeutic intervention in encephalitic JEV infections.

Like other flaviviruses, JEV contains a single-stranded RNA genome that codes for the three virion proteins, i.e., the capsid (C), premembrane/membrane (prM/M), and envelope (E) proteins, and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The E protein is the major protective antigen, eliciting neutralizing antibodies that play an important role in protective immune responses. In the replication cycle, the E protein mediates virus attachment to a putative cell receptor(s) and viral fusion with the endosomal membranes. Three-dimensional structures of several flavivirus
E proteins have been determined by X-ray crystallography (20, 32, 33, 49). The head-to-tail dimers of E are tightly organized in the virion surface. The monomeric E is folded into three structurally distinct domains (domains I to III). Domain III adopts an immunoglobulin-like structure consisting of seven antiparallel β-strands. This domain is linked by a flexible region to domain I, which folds into an eight-stranded antiparallel β-barrel. Domain I contains approximately 120 amino acids in three segments disrupted by two inserts in the form of looped sequences, which together form the dimerization domain (domain II). At the distal end of one of these domain II inserts is a flavivirus-conserved peptide shown to be involved in membrane fusion (2, 23, 49).

Studies of mouse monoclonal antibodies (MAbs) from flavivirus infections have provided much information about E functional specificities and antigenic structures. A majority of cross-reactive, weakly to nonneutralizing antibodies react with epitope determinants involving the fusion peptide in domain II (56). Antibodies that recognize domain III epitopes are type-specific and efficient neutralizers of viral infection (39, 50). Domain III-reactive antibodies can neutralize the virus at an early infection step presumably by blocking viral attachment to cell receptors or by interfering with conformational changes to E, thereby preventing membrane fusion (6, 37). Mouse MAbs that neutralize flaviviruses, such as St. Louis encephalitis virus, yellow fever virus, and dengue virus (DENV), at high titers in vitro have also been shown to mediate protection of infection in vivo (4, 28). In the case of JEV, studies have shown that passive transfer of mouse MAbs can protect against prior and subsequent infection in mice, goats, and monkeys (21, 65). However, the possible immunogenicity of these antibodies limits their clinical utility in humans. Only a relatively few MAbs that efficiently neutralize flaviviruses and map to domain I or II have been characterized (7, 16, 24, 29, 36, 51). Consequently, the antigenic structures of these domains and their involvement in the protective immune response remain poorly understood.

Until recently, there has been a lack of primate-derived antibodies for characterization of flavivirus antigenic epitopes discovered with mouse antibodies. However, DENV type-specific and cross-reactive antibodies recently have been recovered from infected chimpanzees by repertoire cloning. A DENV-4-specific, highly neutralizing MAb (5H2) has been shown to react with epitope determinants in domain I, and a DENV-cross-reactive antibody (1A5) was shown to react with the fusion peptide in domain II (11, 24). It was also demonstrated that passively transferred MAb 1A5, which shares characteristics with a major subset of flavivirus cross-reactive antibodies, upregulates DENV replication by a mechanism of antibody-dependent enhancement (9). We have also shown that passive transfer with the highly neutralizing antibody MAB 5H2 protects mice and monkeys against DENV-4 challenge (24). As an extension of these studies of antibodies against DENV, the present study describes repertoire cloning, epitope mapping, and functional characterization of JEV-neutralizing MAbs from immunized chimpanzees. Several planning strategies were applied to recover Fabs that bind to epitopes in different antigenic domains. Representative MAbs that neutralized JEV efficiently and mapped to each of the three domains in E were selected for analysis of binding activities for JEV and evaluation of their in vitro neutralizing titers against strains belonging to the four JEV genotypes. The protective capacities of these humanized antibodies were analyzed in a mouse model of JE.

MATERIALS AND METHODS

Viruses and cultured cells. Simian Vero cells and mosquito C6/36 cells were grown in minimum essential medium (MEM). Schneider's Drosophila melanogaster line 2 (S2) cells were cultured in Schneider's Drosophila medium, and human embryonic kidney 293 T cells were cultured in Dulbecco's modified essential medium. All media were supplemented with 10% fetal bovine serum, 0.05 mg/ml gentamicin, and 2.5 units/ml amphotericin B (Fungizone). Media were purchased from Invitrogen (Carlsbad, CA), and cells were from the American Type Culture Collection (Manassas, VA). The inactivated JEV vaccine, JE-VAX, was obtained from Sanofi Pasteur Inc. (Swiftwater, PA). The attenuated JEV SA14-14-2 strain was kindly provided by K. Eckerl and R. Putnak. The JEV stock used for infection of chimpanzees, phage library panning, and plaque reduction neutralization tests (PRNT) was prepared from infected C6/36 cells grown in Voges-Proskauer-serum-free medium (Invitrogen). The virus titer was approximately 10^7 focus-forming units (FFU)/ml as determined on Vero cell monolayers. PRNT using the four genotype strains of wild-type JEV was performed at the Center for Vaccine Development, Mahidol University (Nakhonpathom, Thailand). These strains were JE 1991 (genotype I), JE B1034/8 (genotype II), Beijing (genotype III), and JKT 9092 (genotype IV). The JEV prototype strain Nakayama, belonging to genotype III, was used for mouse challenge experiments performed at Adimmune Corporation (Taiichung, Taiwan). Experiments to detect antibody-binding specificities were performed by enzyme-linked immunosorbent assay (ELISA) with DENV-1 (Hawaii), DENV-2 (New Guinea B), DENV-3 (H87), DENV-4 (814669), Langat virus (LTV) strain TP 21, and a WNV/DENV-4 chimera as described previously (10).

Antibodies. Humanized MAbs 1A5 and 5H2 derived from chimpanzee Fabs were prepared by transient transfection of 293 T cells (10, 30) (Kemp Biotechnology, Gaithersburg, MD). Hyperimmune mouse ascites fluid (HMAF) raised against JEV was purchased from the American Type Culture Collection (Manassas, VA). Mouse JEV complex-reactive MAb 8743 (MAb 6B4A-10) was purchased from Chemicon (Temecula, CA). JEV E domain III-specific mouse MAb E3.3 was kindly provided by S-C. Wu (26).

JEV E antigen preparations. Three different E antigen preparations from JEV SA14-14-2 were used: (i) JEV virions, (ii) domain III-specific E, and (iii) N-terminal 80% E. To prepare JEV virions, mosquito C6/36 cells grown in MEM plus supplements were infected with the virus at a multiplicity of infection of 0.1 in Voges-Proskauer-serum-free medium (Invitrogen, Carlsbad, CA) and incubated at 32°C. The culture medium was harvested 8 days after infection and kept frozen at ~80°C. The virus preparation was used for panning, ELISA, and neutralization assays, as well as for selection of neutralization-escape variants. The recombinant domain III-specific E was constructed for use as panning antigen. The protein was expressed in bacteria with a histidine tag, essentially as described elsewhere (18, 64). The DNA sequence corresponding to amino acids 296 to 398 (DIII) near the C terminus of E was amplified by PCR from the viral cDNA of JEV SA 14-14-2. The DNA product was then purified and digested with pET21 plasmid containing the insert. The histidine-tagged, domain III E protein was affinity purified through a column of Talon metal affinity resin (Chontech, Mountain View, CA). Western blot analysis and ELISA were performed using JEV HMAF and MAB E3.3 to confirm the identity and proper folding of the recombinant domain III E protein.

Recombinant 80% E was generated in Drosophila S2 cells essentially as described elsewhere (25, 31, 46). The DNA encoding amino acids 131 to 692 of the prM/N-terminal 80% E fusion protein was amplified by PCR from JEV cDNA using the primers GGAGCCATGAAGAGATCTAATTTCCAGGGG and GC CCAGCGTGCTCCCGGTTGTGGCCAAATGGTG. The DNA product was digested with BglII and SacII and inserted into the pMTBiP/V5-HisB expression vector (Invitrogen, Carlsbad, CA). The recombinant plasmid and a bacteriophage 

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affinity purified with Talon metal affinity resin. Western blot analysis and ELISA were performed with HMAF, MAb E3,3, and MAB 8743 to verify the identity of recombinant E. Variants of recombinant 80% E containing single amino acid substitutions were constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**Immunization of chimpanzees with JEV vaccines and construction of yMAb library.** Two chimpanzees (96A07 and 1620) were administered subcutaneously three doses of JE-VAX of 1 ml each at days 0, 7, and 30, according to the indicated regimen. One year later the chimpanzees were infected with a mixture of attenuated JEV strain SA14-14-2 and WNV/DENV-4 chimera each at 10^6 FFU, diluted in MEM plus 0.25% human serum albumin to boost the antibody response. Eight weeks after infection, bone marrow was aspirated from each chimpanzee and the lymphocytes were prepared by centrifugation on a Ficoll-Paque gradient. Repertoire cloning of chimpanzee Fab fragments was described earlier (30). Approximately 1 × 10^10 bone marrow lymphocytes from chimpanzee 96A07, which developed a higher JEV-neutralizing antibody titer than did chimpanzee 1620, were used for phage library construction. A library with a diversity of 2 × 10^10 to 1 × 10^11 was obtained at each cloning step.

**Panning of phage library and selection of JEV-specific Fabs.** The pComb 3H DNA library that contained the V_L-CL and VH-CH1 inserts was used for phage production as described earlier (30). To increase the possibility of recovering antibodies against different epitopes on the JEV E, three different panning strategies were used. The phage library was first panned using JEV virions captured by chimpanzee convalescent-phase sera applied as a coating to the wells of an ELISA plate. Panning of the phage library by epitope masking was also conducted as described previously (8). Briefly, wells of a microtiter plate coated with JEV virions were incubated with purified Fab A3 (isolated in the panning described earlier) at a concentration of 50 μg/ml for 1 h at 37°C. One-fourth of the volume was removed before addition of 50 μl of the phage library. The third strategy of antibody selection was performed using domain III-specific E as panpping antigen. Briefly, wells of a 96-well ELISA plate were coated with 5 μg/well of purified domain III E in 0.1 M carbonate buffer, pH 9.0. After being washed with phosphate-buffered saline (PBS), antigen-coated wells were blocked with 3% bovine serum albumin. The phage library was then added as described. Following three rounds of panning in each case, the selected phage population was used for infection of E. coli XL-1 to produce phagemid DNA. Phagemid DNA was cleaved with SpeI and NheI to remove the phage gene III segment and was used for infection of E. coli XL-1. Transformed E. coli colonies were screened by ELISA to identify clones producing soluble Fab fragments reactive with JEV. Individual Fabs were prepared and screened for binding specificity to JEV virions or domain III E. Plasmids were sequenced to identify Fab clones with distinct V_L and V_H DNA inserts.

**Production of Fabs and humanized MAb s.** The histidine-tagged Fab produced in E. coli was affinity purified using Talon metal affinity resin. The Fab purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the concentration was determined using the bichromatic acid protein assay kit (Pierce, Rockford, IL). Construction of plasmids for expression of the humanized MAb was performed according to the V5 and CH1 designations (thereafter) from cloned Fab DNA was carried out as described previously (30). MAb expression was verified by transfection of 293 T cells (purchased from ATCC) in the presence of Lipofectamine (Invitrogen, Carlsbad, CA) and grown in Optimem medium. One day after transfection, cells were washed and Dulbecco’s modified essential medium was added. Cells were incubated for 5 to 7 days, and the culture medium was harvested. The medium was concentrated, and the MAb product was purified on a protein A column (Pierce, Rockford, IL). Scale-up MAb production was performed by Kemb Biotechnology (Gaithersburg, MD).

**Neutralization of neutralizing titers of Fab and MAb s.** The neutralizing titer of Fab or MAb was determined by PRNT against the representative JEV strains essentially as described elsewhere (30, 38). Virus foci that formed on the cell monolayer were immunostained, and the antibody 50% plaque reduction neutralization test (PRNT<sub>50</sub>) titer in μg/ml was calculated. The neutralization tests using the attenuated and wild-type JEV strains were performed in appropriate biologic containment laboratories at NIH and the University of Mahidol, Thailand, respectively.

**Biotinylation of purified Fab and competition ELISA.** Purified Fabs were biotinylated with EZ-Link N-hydrosuccinimide–LC-biotin (Pierce, Rockford, IL) and used in competition ELISA. Briefly, biotin-labeled Fab at a fixed concentration (0.05 μg/ml) was added to JEV virion-coated wells and incubated at 37°C. After washing, streptavidin–alkaline phosphatase (Pierce, Rockford, IL) was added to detect the amount of biotinylated Fab attached to the virus.

**Measurement of binding affinity.** Affinity binding analysis by ELISA or with a surface plasmon resonance (SPR) biosensor was performed to determine the Fab or MAb binding activity for JEV virions. ELISA was performed as described previously with minor modifications, i.e., in the absence of detergent at all steps (11, 48). JEV HMAF was used to coat the microtiter plate. Following blocking with 3% bovine serum albumin, JEV at a predetermined concentration was added and incubated at 37°C for 1 h. Dilutions of affinity-purified Fab were added and incubated at 37°C for 1 h. The Fab bound to JEV on the microtiter plate was detected using a goat anti-human IgG–alkaline phosphatase conjugate (Sigma, St. Louis, MO). The steady-state equilibrium affinity constant (K<sub>D</sub>) was calculated as the Fab concentration that produced 50% maximum binding.

The SPR biosensor experiments were conducted using a Biacore 3000 instrument (Biacore Inc., Piscataway, NJ) with short carboxymethylated dextran sensor surfaces (CM3; GE Healthcare, Piscataway, NJ) and standard amine coupling as described elsewhere (54). Since the recombinant E protein showed self-binding in preliminary experiments, the E protein was immobilized on the chip surface and the kinetics of Fab binding and dissociation were recorded for 40 to 50 min and 2 h to 10 h, respectively, at various Fab concentrations (53). Analysis of antibodies was conducted at a flow rate of 2 μl/min for Fab B2 and 5 μl/min for Fabs A3 and E3, using PBS-P buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>PO<sub>4</sub>, 2.3 mM KH<sub>2</sub>PO<sub>4</sub>, 0.005% surfactant P20, pH 7.4) at 25°C. The chip surface was regenerated with 0.05% Triton X-100–2 M NaCl in the case of Fab B2. No regeneration conditions were applied with Fabs A3 and E3. The kinetic traces were globally fitted with a model for continuous ligand distributions combined with a two-compartment approximation of mass transport (58).

**Immunoprecipitation and Western blot analysis.** Immunoprecipitation was performed with lysates of JEV-infected mosquito cells or parental chimpanzee E. C6/36 cells were infected with the virus at a multiplicity of infection of 1 and incubated for 5 days at 32°C. Infected cells were rinsed with PBS, and lysis buffer containing 1% NP-40, 0.15 M NaCl, and 0.1% Triton X, pH 7.5, was added. The cell lysate or recombinant E was incubated with the test antibody for 2 h at 4°C. A 10-μl suspension of protein A Sepharose beads (Calbiochem, La Jolla, CA) was added, and the mixture was incubated overnight at 4°C. The beads containing immunocomplexes were collected by centrifugation and washed three times with the lysis buffer. The immunocomplexes were supplemented with 4× loading buffer (Invitrogen, Carlsbad, CA) and separated by SDS-PAGE. After being transferred onto a nitrocellulose membrane, the E protein was detected by a mouse or humanized anti-JEV antibody followed by anti-mouse or anti-human IgG–horseradish peroxidase (HRP, Pierce, Rockford, IL) or by a mouse anti-V5 epitope MAb-HRP conjugate (Invitrogen, Carlsbad, CA) for chemiluminescence development (Pierce, Rockford, IL).

**Selection of JEV antigenic variants.** Affinity-purified Fabs A3, B2, and E3 were used for selection of neutralization-escape mutants (11). Briefly, approximately 1 × 10^6 FFU of parental JEV SA14-14-2 was mixed with 25 μg/ml of Fab in MEM and incubated at 37°C for 1 h. The mixture was added to the Vero cell monolayer and incubated at 37°C for 1 h. Following removal of the inoculum, the plate was rinsed once with PBS, refed with 3 ml of MEM containing 2% fetal bovine serum and 5 μg/ml of the selecting Fab, and incubated at 37°C for 5 days. Antibody-resistant variants were isolated by plaque-to-plaque purification on Vero cells, and the individual isolates were amplified in infected C6/36 cells in the presence of the selecting Fab. Sequence analysis of JEV antigenic variants was conducted as described previously (11). The JEV E structure modeling was performed with the crystal coordinates of WNV, accession code 1T69, as a template (20) and SwissModel (13, 43). Graphical development was performed using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics (University of California, San Francisco) (44).

**Mouse model for JEV challenge.** Experiments involving passive antibody transfer in mice were performed at Adimmune Corporation (Taihing, Taiwan). The laboratory is approved for good manufacturing practices. For analysis of efficacy, groups of 4-week-old inbred ddY mice (either sex, n = 12) were infected with 0.5 ml of MAb at doses of 200, 40, 8, 1.6, and 0.32 μg per mouse by the intraperitoneal (i.p.) route and the control group received PBS diluent only. One day later, mice in all groups were challenged by the intracerebral (i.c.) route with 40% 50% lethal doses (LD<sub>50</sub> (1.5 FFU) of JEV strain Nakayama in 30 μl. The animals were monitored daily for clinical signs of infection, including ruffled hair, hunched back, paralysis, and death, for 2 weeks. When signs of encephalitis paralysis developed, mice were euthanized as the experiment end point. In the infection-intervention experiment by passive antibody transfer, a single dose of test MAB at 200 μg was administrated by the i.p. route at day 1, 3, or 5 following i.c. inoculation of 40 LD<sub>50</sub> of JEV strain Nakayama. Mice were monitored daily for symptoms of encephalitis for 3 weeks. Student’s t test was used to compare the average survival times between the mouse groups that received MAb and those that received PBS.

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RESULTS

Chimpanzee antibody response to JEV vaccines and isolation of Fabs. Two chimpanzees were initially immunized with three doses of inactivated vaccine JE-VAX. After 2 months chimpanzees 96A007 and 1620 developed only moderate PRNT50 titers against JEV SA14-14-2 (1/100 and 1/71, respectively). After inoculation with a mixture of JEV SA14-14-2 and WNV/DENV-4 chimera, high JEV-neutralizing antibody titers, 1/10,633 and 1/3,114, were detected in the serum of chimpanzees 96A007 and 1620, respectively. Chimpanzee bone marrow was aspirated 8 weeks after infection, and the cells of chimpanzee 96A007 were used for a phage library construction.

Selection of Fabs from a combinatorial library with a single panning antigen often yields only a dominant antibody subset that may or may not be neutralizing. Highly neutralizing antibodies may be present as a minor subset. Therefore, three different panning strategies were performed in order to assemble a collection of JEV-neutralizing Fab antibodies for further functional characterizations.

(i) Fabs recovered from panning with JEV virions (group 1 Fabs). The phage library was first panned with JEV virions captured by chimpanzee polyclonal sera. A total of 200 E. coli clones were screened for Fabs reactive to the virus. Sequence alignment of 48 positive Fab clones identified four V\text{H} sequences, three of which, i.e., Fabs A3, G9, and B3, were similar but not identical (Fig. 1). These Fabs appeared to represent a dominant subset of antibodies in the library. The V\text{L} sequences of these four Fab clones showed three distinct patterns. Binding assay by ELISA showed that, with the exception of Fab A3, which was weakly reactive to WNV (detected only at 1/10 dilution), the other three Fabs reacted with JEV but not with DENV-1 to -4, WNV, or LGTV. These Fabs neutralized JEV efficiently at PRNT50 titers ranging from 2.55 to 7.91 nM (0.12 to 0.36 μg/ml) (Table 1).

(ii) Fabs recovered from panning by epitope masking (group 2 Fabs). To increase the possibility of recovering a different subset of antibodies binding to minor epitopes on E, Fab A3 (described above) was used for epitope masking in a new

<table>
<thead>
<tr>
<th>Group (panning antigen)</th>
<th>Fab</th>
<th>PRNT50 titer (nM)</th>
<th>ELISA titer (1/\text{log}_{10} \text{dilution})</th>
<th>binding of ( a )</th>
<th>binding of ( b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (JEV SA14-14-2)</td>
<td>A3</td>
<td>2.55 ± 0.42</td>
<td>4.7</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B3</td>
<td>4.14 ± 0.65</td>
<td>4.5</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G9</td>
<td>4.38 ± 1.10</td>
<td>4.4</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C8</td>
<td>7.91 ± 3.29</td>
<td>4.3</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>2 (masking with Fab A3)</td>
<td>B2</td>
<td>0.25 ± 0.09</td>
<td>4.2</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>0.41 ± 0.21</td>
<td>4.1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>0.45 ± 0.22</td>
<td>4.2</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A8</td>
<td>&gt;1,100</td>
<td>4.2</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>&gt;1,040</td>
<td>3.0</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>3 (recombinant DIII E)</td>
<td>B12</td>
<td>&gt;1,070</td>
<td>4.0</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

\begin{itemize}
\item \( a \) 1 nM = ~0.046 μg/ml.
\item \( b \) Microtiter plates were coated with virions of the indicated flavivirus (10^6 FFU/ml). The ELISA titer was the \text{log}_{10} reciprocal dilution of Fab that gave an optical density reading twofold or greater than the background. The initial Fab concentration was ~100 μg/ml.
\item DENV-1, DENV-2, DENV-3, DENV-4, WNV, and LGTV were tested. Nonneutralizing Fabs A8, G1, and B12 were included for comparison.
\end{itemize}
Evidence indicates that flavivirus infections elicit a major class of cross-reactive but weakly neutralizing antibodies that react with epitopes involving the fusion peptide in domain II E (41, 56). Studies of cloning of DENV-neutralizing antibodies from chimpanzees (10) and of cloning of WNV-neutralizing antibodies indicate that domain III E is an antigenic target in the murine model (39). The third strategy to recover chimpanzee antibodies against JEV used domain III-specific E as the panning antigen. Twenty-three Fabs were identified, and sequence analysis revealed two distinct VH segments, as present in Fabs E3 and B12, with Fab E3 representing 78% of the clones. Both Fabs were JEV specific. Fab E3 neutralized the virus at a relatively low titer (84.9 nM) compared to the neutralizing titers of Fabs selected with the previous panning strategies (Table 1). Fab B12 did not neutralize JEV (>1,070 nM).

(iv) Human homologs of chimpanzee antibodies. A search for sequence homology in the database showed the most closely related human IgG gene homologs of the panel of chimpanzee Fabs (Table 2). The γ1 heavy chain sequences of these Fabs demonstrated similarity to the human VH1, VH3, VH4, or VH7 gene families with sequence homologies ranging from 67 to 83%, excluding the CDR-3 regions. The λ light chain sequences exhibited the highest identity with human VK1, VK2, or VK3 gene families with sequence homologies of 80 to 95%, excluding the CDR-3 regions. The four Fabs in group 1 were most closely related to VH1 and VK1 germ line genes. The γ1 heavy chain sequences of the most highly neutralizing Fabs in group 2 had the highest identity with the human VH3 gene family.

Fab binding sites on JEV shown by competition ELISA. Six Fabs that were distinct in their CDR-3H sequences were selected for analysis of the relatedness of their binding sites on JEV by competition ELISA (Fig. 1). Fabs A3, B2, and E3 were representatives of the three Fab groups that neutralized JEV most efficiently. Additionally, Fabs A8 and G1 (group 2) and Fab B12 (group 3) were selected for analysis of their binding sites on JEV. Binding competition was not detected among these Fabs with each other nor with DENV-4-specific Fab 5H2 as a negative control (30) (Fig. 2). Further, binding competition was not observed with Fab 1A5, a flavivirus-broadly reactive antibody that binds to determinants in the flavivirus E fusion loop. JEV Fab clones were grouped according to the panning procedure: group 1, orange; group 2, green; and group 3, blue.

**TABLE 2. Sequence similarities between chimpanzee Fabs and their most closely related human immunoglobulin homologs**

<table>
<thead>
<tr>
<th>Chimpanzee Fab</th>
<th>( V_H ) Family (gene)</th>
<th>Identity(^a)</th>
<th>( V_L ) Family (gene)</th>
<th>Identity(^a)</th>
</tr>
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<tbody>
<tr>
<td></td>
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<tr>
<td><strong>Group 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>VH1 (VH1-69)</td>
<td>70</td>
<td>VK1</td>
<td>95</td>
</tr>
<tr>
<td>G9</td>
<td>VH1 (VH1-69)</td>
<td>70</td>
<td>VK1</td>
<td>80</td>
</tr>
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<td>B3</td>
<td>VH1 (VH1-69)</td>
<td>70</td>
<td>VK1 (VK1D-16)</td>
<td>91</td>
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<td>C8</td>
<td>VH1 (VH1-69)</td>
<td>67</td>
<td>VK1 (VK1D-27)</td>
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<tr>
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<td>VK2 (VK2-28)</td>
<td>87</td>
</tr>
<tr>
<td>A8</td>
<td>VH3 (VH3-74)</td>
<td>82</td>
<td>VK1 (VK1-9)</td>
<td>95</td>
</tr>
<tr>
<td>B2</td>
<td>VH7 (VH7-4-1)</td>
<td>83</td>
<td>VK1 (VK1D-16)</td>
<td>86</td>
</tr>
<tr>
<td>F1</td>
<td>VH7 (VH7-4-1)</td>
<td>79</td>
<td>VK1 (VK1D-39)</td>
<td>85</td>
</tr>
<tr>
<td>F3</td>
<td>VH7 (VH7-4-1)</td>
<td>82</td>
<td>VK3</td>
<td>83</td>
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<tr>
<td><strong>Group 3</strong></td>
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<td></td>
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<tr>
<td>B12</td>
<td>VH3 (VH3-74)</td>
<td>77</td>
<td>VK1 (KV1-9)</td>
<td>86</td>
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<tr>
<td>E3</td>
<td>VH4</td>
<td>74</td>
<td>VK3 (KV3D-7)</td>
<td>90</td>
</tr>
</tbody>
</table>

\(^a\) The DNAPLOT program was used to search for the most closely related homologs of human germ line IgG genes in the database.

\(^b\) The percent amino acid identity in the \( V_H \) or \( V_L \) segment, excluding the CDR-3 region, was determined with the MEGA program.

**FIG. 2. Analysis of Fab binding to JEV SA14-14-2.** Fabs A3 (A), B2 (B), and E3 (C) were affinity purified, biotinylated, and used for analysis of binding activity to JEV SA14-14-2 by competition ELISA in the presence of competing, unlabeled Fabs. Fab 5H2, which did not react with JEV, was used as a negative control. Fab 1A5 is a flavivirus cross-reactive antibody that binds to determinants in the flavivirus E fusion loop. JEV Fab clones were grouped according to the panning procedure: group 1, orange; group 2, green; and group 3, blue.
The binding activities of Fabs and derived MAbs for JEV. The binding activities of Fabs and their derived MAbs for JEV virions in the absence of detergents were determined by ELISA. The concentration of each antibody required to attain 50% maximum binding was calculated by nonlinear regression (Fig. 4A). The concentration provides an estimate of the binding affinity $K_D$. Accordingly, the $K_D$ for Fab A3, $0.45 \pm 0.06$ nM for Fab A3, $0.28 \pm 0.11$ nM for Fab B2, and $0.98 \pm 0.07$ nM for Fab E3 (Table 3). Conversion from the monovalent Fab to the bivalent MAb form increased the antibody avidity three- to fourfold. A consistent correlation was observed for each antibody when the $K_D$ value was compared with the neutralization titer ($r = 0.97$).

The question arises whether there is a similar correlation between Fab binding affinity for recombinant E and the neutralization potency. SPR measurements allow a precise, real-time determination of Fab-E association and dissociation rates. Representative tracings for Fabs A3 and B2 are shown in Fig. 4B. Using this analysis, the affinity constant $K_d$ measured for Fab A3 was $0.72$ nM and that for Fab E3 was $0.35$ nM, comparable to the $K_D$ values measured by ELISA (Table 3). The $K_d$ of Fab B2 for binding to recombinant E was $\approx 150$-fold weaker than that measured for Fab A3 or E. The $K_d$ of Fab B2 was also significantly weaker than the $K_D$ measured with virions by ELISA ($110$ nM versus $0.15$ nM, respectively). Remarkably, the off-rate of Fab B2 was $\approx 120$-fold higher than that of Fab A3 or E.
Localization of epitope determinants on E. Fabs A3, B2, and E3 were each used to isolate neutralization-escape mutants of JEV SA14-14-2. Antigenic variant JEV-v1 was isolated from Fab A3, JEV-v2 was isolated from Fab B2, and JEV-v3 was isolated from Fab E3. When the selecting Fab was used in the neutralization assay, variants JEV-v1 and JEV-v2 showed approximately 340- and 132-fold more resistance than the parental virus, respectively (Fig. 5A and B). JEV-v3 was completely resistant to neutralization by 1,080 nM Fab E3 (Fig. 5C).

To map the epitope determinants of JEV-neutralizing antibodies, the C-prM-E sequences of variants v1, v2, and v3 and the parental virus were determined. Figure 6A shows the sequence alignment in the regions surrounding the amino acid substitutions in the variants. Variant v1 contained two substitutions in E, Lys136-Asn (β-strand E0) and Lys179-Glu (β-strand G0), both located in domain I. These two amino acids are conserved, but the surrounding amino acids vary among members of the JE group, which possibly accounts for the lack of reactivity of Fab A3 with WNV by ELISA (Table 1). These amino acids were 20.7 Å apart and exposed on the surface of E, according to the three-dimensional JEV E protein model based on the WNV E crystal coordinates (Fig. 6B). Variant v2 also contained two substitutions, Ile126-Thr (β-strand e) and Tyr219-His (α-A), at a distance of 16.2 Å in domain II. Binding of Fab B2 to WNV was not observed, despite the conservation of Ile126 and surrounding amino acids (Fig. 6A). Variant v3 also contained two substitutions, Gly302-Asp in domain III and Ile126-Thr in domain II. The two positions were approximately 64.6 Å apart, and Fab E3 reacted with domain III sequences, suggesting that Ile126-Thr was probably not responsible for resistance to neutralization by this antibody. Based on the comparison of the escape variant and the wild-type virus PRNT50 titers, it is possible that there might be other major epitope determinants, especially for Fabs A3 and B2.

JEV recombinant E proteins containing single amino acid substitutions. Since there were two mutations in E of each JEV variant, the effect of each mutation on antibody binding was analyzed. JEV E proteins containing the single substitution Ile126-Thr, Lys136-Asn, Lys179-Asp, His219-Tyr, or Gly302-Asp were generated. Immunoblots showed that Lys136-Asn substitution had no effect, whereas the Ile126-Thr substitution lost the reactivity for MAb B2, whereas His219-Tyr did not affect binding. As a positive control, mouse MAb 6B4A-10 reacted with all of these mutant E constructs in both assays. These results support the conclusion that Lys136 in domain I,
Ile_126 in domain II, and Gly_302 in domain III are the major epitope determinants of MAbs A3, B2, and E3, respectively.

Neutralization of the attenuated and wild-type JEV strains by Fabs and humanized MAbs. Earlier we determined the neutralizing titers of Fabs using attenuated JEV strain SA14-14-2 (genotype III). Three highly neutralizing Fabs were further evaluated for neutralization of wild-type JEV strains representing each of the four genotypes. Each of these Fabs neutralized wild-type members of genotypes I to IV as efficiently as the attenuated strain, with the exception of Fab B2, which neutralized strain JKT 9092 (genotype IV) at a PRNT_{50} titer reduced by greater than 10^{3}-fold (Table 4). Fab B2 was the most efficient neutralizer of other strains, and Fab E3 was the least efficient. Humanized MAbs derived from these Fabs were also used for neutralization of the attenuated strain and the wild-type strains representing each genotype. MAbs A3 and B2 showed a PRNT_{50} titer 3- to 100-fold higher than that of the Fab counterpart. MAb E3 had a PRNT_{50} titer 40- to >1,000-fold higher than that measured for Fab E3 against all genotype strains. JEV JKT 9092, like other strains, was efficiently neutralized by MAbs A3 and E3, although it was only moderately neutralized by the highly neutralizing MAb B2.

Protective capacity of humanized MAbs against JEV infection in mice. The mouse JEV encephalitis model used for validation of the inactivated JEV vaccine in commercial production was employed to evaluate the protective capacity of
humanized MAbs. Four-week-old, inbred ddy mice were each inoculated by the i.p. route with a single dose of MAb ranging from 0.32 to 200 μg/9262 g. Mice were challenged with 40 LD50 of JEV strain Nakayama i.c. 24 h later. At a dose of 200 μg/per mouse, MAbs A3 and B2 protected 100% and MAb E3 protected 75% of mice in the groups, compared to no survivals in the unprotected group following virus challenge (Fig. 8). Titration of MAbs against virus infection showed a dose-dependent response in terms of the survival rate and average survival time. The 50% protective dose per mouse calculated by non-linear regression analysis was 0.84 μg/9262 g for MAB B2, 5.8 μg/9262 g for MAb A3, and 24.7 μg/9262 g for MAb E3.

The possibility of using these MAbs for therapy of JEV encephalitis was also investigated. MAb B2 administered at a single dose of 200 μg/9262 g 1 day after JEV infection resulted in a 50% survival rate (Table 5). Although fewer survivals were found after similar transfer with the less-protective MAb A3 or E3 1 day after JEV infection, the average survival time increased significantly with MAb B2 (8.0 ± 1.4 days; P = 0.015, t test) or MAb A3 (7.4 ± 0.7 days; P = 0.0001, t test), compared with 5.9 ± 0.8 days for unprotected animals. Thus, passive transfer with either of these MAbs improved the outcome of JEV infection when administered 1 day prior to infection (Table 5). However, the average survival time was in the range of 6.2 ± 1.1 to 6.3 ± 0.5 days for MAb A3 and 5.2 ± 0.4 to 6.3 ± 0.5 days for MAb B2, not significantly different from the 5.8 ±

FIG. 7. Binding analysis of mutant E proteins containing a single substitution at position 126, 136, 179, 219, or 302. (A) Binding of humanized MAbs A3 and E3 (top) and the control mouse MAb anti-V5 epitope (bottom) to various mutant proteins as analyzed by Western blotting. The wild-type (WT) and mutant E proteins reacted with the indicated antibody and were developed with an HRP-conjugated secondary antibody. (B) Binding of MAbs A3, B2, and E3 to WT and mutant E proteins analyzed by immunoprecipitation in the absence of detergents. The immunoprecipitates were developed by Western blotting using MAb anti-V5 epitope-HRP conjugate.

FIG. 8. Protective activity of humanized JEV IgG1 antibodies (MAbs) using a mouse JEV challenge model. Inbred ddy mice (n = 12) were injected i.p. with MAb A3 (A), MAb B2 (B), or MAb E3 (C) at various doses indicated. Unprotected control mice were administered PBS diluent. Twenty-four hours later mice were infected i.c. with JEV strain Nakayama. The animals were monitored daily and euthanized when clinical signs of infection appeared. Kaplan-Meier survival curves are shown.

### TABLE 4. Neutralization of JEV strains representing genotypes I to IV by Fab and humanized MAbs

<table>
<thead>
<tr>
<th>Genotype (strain)</th>
<th>PRNT50 titer (nM) of antibody:</th>
<th>A3</th>
<th>B2</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fab</td>
<td>MAb</td>
<td>Fab</td>
<td>MAb</td>
</tr>
<tr>
<td>I (JE1991)</td>
<td>1.30</td>
<td>0.04</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>II (JE B1034/8)</td>
<td>3.47</td>
<td>0.04</td>
<td>1.85</td>
<td>0.02</td>
</tr>
<tr>
<td>III (Beijing)</td>
<td>2.82</td>
<td>0.20</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>III (SA14-14-2)</td>
<td>2.55</td>
<td>0.20</td>
<td>0.25</td>
<td>0.03</td>
</tr>
<tr>
<td>IV (9092)</td>
<td>1.74</td>
<td>0.07</td>
<td>&gt;2.170</td>
<td>2.00</td>
</tr>
</tbody>
</table>

* 1 nM = 0.15 μg/ml.
Recent studies with DENV, WNV, and tick-borne encephalitis virus suggest that a major subset of broadly cross-reactive antibodies are directed against immunodominant epitopes that include the fusion peptide in the E protein (11, 56, 60). The most plausible explanation for the lack of such antibodies in the current study is that the SA14-14-2 virus used for panning binds weakly to the cross-reactive antibodies, thereby preventing their isolation. Previously we have shown that the Phe107 to Leu substitution in the E fusion loop alone was responsible for the reduced binding affinity of SA14-14-2 virions to the broadly cross-reactive chimpanzee MAb 1A5 (11).

ELISA provided useful insights into Fab binding activities for JEV virions. Highly neutralizing Fabs B2 and A3 reached 100 nM (Fig. 4A). The concentra-
tion for half-maximum binding, together with the PRNT<sub>50</sub> titers, allowed measurement of antibody neutralization potency, based on the calculation of the threshold occupancy of accessible antibody sites on the virion (45). According to the multiple-hit theory and stoichiometric analysis of epitope occupancy for neutralization (22), the most potent antibodies neutralize the virus at concentrations with low occupancy of the epitopes available for binding on the virion. The occupancy for the most potent JEV-neutralizing MAb B2 was approximately 28% of available sites, whereas the occupancies for MAbs A3 and E3 were calculated at 45% and 66% of the accessible sites, respectively (data not shown) (12, 45). It should be noted that in the current study the three JEV-neutralizing antibodies bind to specific epitopes in three separate E domains and most probably neutralize the virus by different mechanisms. Other contributing factors for assessment and interpretation of the antibody binding stoichiometry may also include the epitope presentation of antigen preparations. To that effect, the binding affinity of Fab B2 for the recombinant E protein measured by SPR was very different from that determined for the virion by ELISA, i.e., K<sub>d</sub> of 110 nM versus K<sub>d</sub> of 0.28 nM (Table 3). One possible explanation is the conformational dependency of the B2 epitope, as shown by the loss of MAb B2 binding to the recombinant E or the virion in a Western blot assay. Accordingly, the number of accessible sites for B2 binding differed between the recombinant E and the virion on a molar basis. Taken together, one could speculate that the high neutralization potency of MAb B2 is partially determined by a higher affinity for a limited subset of E protein conformations that most closely mimic E on the viral surface.

The presently recognized four JEV genotypes show a 7% or greater nucleotide sequence divergence based on limited sequences (5, 55). Strains of genotype IV are the least similar and probably represent the ancestral lineage with up to 20% nucleotide and 6.5% amino acid divergence compared to other genotype strains. Genotypes I to III are most widespread and responsible for epidemic disease. Our analysis demonstrates that each of the three Fabs and derived humanized MAbs exhibits a high neutralizing activity against a broad spectrum of JEV genotype strains. One single exception is that the neutralizing activity of MAb B2 against JEV strain 9092 (genotype IV) was reduced by approximately 100-fold compared to that against other genotype strains. A sequence search of strain 9092 (accession no. U70409) in the database revealed that the substitution Ile<sub>126</sub>-Thr identified earlier in the B2 escape mutant was not present. This observation suggests that other mutations in E of the JEV strain affecting MAb B2 binding and neutralization are present. It would be of interest to determine the mutation(s) involved in the antibody-resistant phenotype of strain 9092 in order to map additional determinants of the MAB B2 epitope. On the other hand, a sequence analysis of other genotype IV strains revealed the presence of the Ile<sub>126</sub>-Thr substitution in strains JKT 6468 (accession no. AY184212) and JKT 7003 (accession no. 70408) in E, indicating that both JEV strains may exhibit resistance to neutralization by MAB B2. Strains of genotype IV were all isolated in 1980 and 1981 from mosquitoes and are believed to have remained in the Indonesia-Malaysia region (5). The significance of their involvement in epidemic viral encephalitis is not clear.

Unlike JEV genotype IV strains, strains of genotypes I to III have spread widely in Asia in recent years. Immunization using the inactivated or live SA14-14-2 JEV vaccine, each prepared from genotype III strains, has effectively controlled JE epidemics in most countries. However, JEV outbreaks remain a public health problem for residents in the regions where JEV vaccination is inadequate and a concern for travelers to these regions as well. Antibody-mediated prevention of JEV infection represents an attractive short-term alternative to vaccines. Demonstration of passive protection with humanized chimpanzee MAbs against JEV infection in vivo is provided herein. The 50% protective dose was measured for MAbs B2 (0.84 µg), A3 (5.8 µg), and E3 (24.7 µg) for 21-g mice. These experiments confirm the feasibility of MAbs for prevention of JEV encephalitis by passive transfer as described earlier (21). Administration of 200 µg/mouse of MAB B2 1 day after lethal i.c. JEV infection protected 50% of mice, whereas all mice in the control group died. A significant improvement of JEV infection survival time after administration of MAbs B2 and A3 was also evident. These results suggest a therapeutic potential for use of these MAbs.

In contrast, the average survival time was not prolonged when mice were inoculated with any of the antibodies 3 or 5 days after JEV challenge. Virus titers can reach ~1 × 10<sup>7</sup> to 1 × 10<sup>9</sup> FFU/g in the brain of 3-week-old mice 3 to 5 days after i.c. inoculation of JEV (35a). Other mouse JE models employing less-severe ip. inoculation have also been described (19, 21). Studies have shown that inoculation of 200 µg of mouse MAB 503 on day 5 after i.p. challenge protected 82% of the animals (21). Passive protection at 3 or 5 days after infection by our humanized antibodies would probably have been possible if the virus were introduced into the animals ip. However, we chose a more stringent test of protection by challenging mice ic. Conceivably, early precise diagnosis of JEV infection and timely administration of effective, neutralizing antibody would help improve the infection outcome. Additionally, infection intervention may be further improved by the combined use of two or more MAbs, such as B2 and A3, that react to separate domains and possibly neutralize the virus by different mechanisms.

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