Dengue virus (DEN), the pathogen behind dengue hemorrhagic fever, remains a public health problem in Asia and South America. In this study, monoclonal antibodies (MAbs) against DEN serotype 1 (DEN-1) were generated by fusing NSI/1-Ag4-1 mouse myeloma cells with lymphocytes from BALB/c mice immunized with DEN-1. Twelve MAbs were found to react specifically to the DENs by enzyme-linked immunosorbent assay, immunofluorescence analysis, and immunoblotting analysis. Five MAbs, namely, DA4-7, DA6-7, DA9-5, DA10-2, and DA11-13, were found to react with envelope proteins of DEN-1. Two serotype-specific MAbs of DEN-1, DA6-7 and DA11-13, were further shown to neutralize DEN-1 infection by a plaque reduction neutralization test. The neutralizing epitopes of these MAbs were further identified from a random peptide library displayed on phage. Immunopositive phage clones reacted specifically with these MAbs and did not react with normal mouse serum. Epitope-based peptide antigens were proved able to detect antibodies in serum samples collected from DEN-1-infected patients but not in those taken from DEN-2-infected patients or healthy controls. We believe that these MAbs and neutralizing epitopes will provide information that will lead to the development of DEN-1 serotype-specific diagnostic reagents and vaccines.

Dengue virus (DEN) causes a variety of illnesses that range in severity from mild, in such syndromes as dengue fever (DF), to severe, in the syndromes dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (18, 19). DF is manifested as a typical biphasic fever, headache, body pain, and rash. DHF, however, is characterized by abnormalities of hemostasis and vascular permeability and is often fatal. Two-fifths of the world’s population is at risk of infection, and it is estimated that 50 million people a year are infected with this virus. One percent of people infected with this virus will develop DHF (59).

Until now, no effective method has been reported to be capable of preventing the development of DHF/DSS because the pathogenic mechanisms of this disease are unclear (5, 6, 41). However, several relevant hypotheses exist, including antibody-dependent enhancement of infection and virus variation (20, 35, 37). DENs are divided into four serotypes, DEN-1, -2, -3, and -4, which have very similar genome sequences and antigenic properties. A secondary infection with a different DEN serotype may increase the risk of DHF (20). One possibility is that monocytes/macrophages take up the virus complexes by binding to nonneutralizing antibodies or subneutralizing cross-reactive antibodies (6, 19, 20). Antibody-dependent enhancement, in conjunction with

activation of memory T-cell responses, is believed to contribute to the immunopathogenic disease process (50).

Virus variation may also account for differences in severity of dengue-related diseases (32, 47, 49). Moreover, since DEN infection is often accompanied by the production of cytokines or chemokines and the activation of complement or immune cells, these may also contribute to the pathogenesis of DHF/DSS (15, 16, 24, 28). The severity of disease also depends on the serotype of the infecting DEN, the degree of viremia, and the genetic background (51, 55). In summary, several complicated mechanisms have been hypothesized to be involved in the pathogenesis of DEN infection, though their relative roles need further investigation.

Because DEN is a major cause of pediatric morbidity and mortality in tropical regions (19), a safe vaccine and a simple reliable test for the serodiagnosis of DEN infection could significantly reduce morbidity and mortality. The ideal vaccine would protect against all four DEN serotypes and provide long-lasting immunity against DEN infection. Importantly, vaccination should not predispose patients to the development of DHF/DSS. Prerequisites to the development of such a vaccine are epitope mapping and the discovery of serotype-specific and neutralizing epitopes of DENs.

In addition to vaccine development, the identification of neutralizing epitopes is useful in the study of virus-host cell interactions and the pathogenesis of DHF. Measuring the ability of a monoclonal antibody (MAB) to bind to fragments of the E protein expressed in bacteria can give us an understanding of the antigenic map of the DEN-2 E protein (40, 48). Polyclonal sera from dengue patients and dengue-immune rabbits were also used to identify the linear serological epitopes
(six to eight amino acids) in the DEN-2 E protein by overlap-
ing synthetic peptides (PEPSCAN) (1, 23). However, oli-
gopeptide antigens cannot be used to identify epitopes that are con-
formationally or discontinuously recognized by neutralizing 
antibodies. Only two epitopes have been found to be involved in 
neutralization in DEN-2, at E307 (34) and at E383 to -385 (22). However, there is no evidence yet that either epitope is recognized by serum samples from dengue patients.

Alternatively, through a selection process called biopanning, the phage display technique makes possible the rapid identi-
cation of linear epitopes (36, 60) or conformational epitopes (13, 61, 62). Phage-displayed random peptide libraries provide opportunities to map B-cell epitopes (11, 14, 52, 60, 61) and organ-specific (2, 12, 45) peptides.

In the present study, two neutralizing MAbs against DEN-1 
were generated. The neutralizing epitopes of both antibodies 
were further identified with a phage-displayed random peptide 
library. Nine serotype-specific antibodies (DA4-7, DA6-6, 
DA6-7, DA10-2, DA11-9, DA11-13, DA15-2, DA31-6, and 
DA32-9) and epitope-based peptide antigens can be used to 
develop a convenient, efficient serologic test and to address the 
role of antibodies in the pathogenesis of primary and second-
ary DEN-1 infections. The neutralizing MAbs and epitopes of 
DEN-1 may be useful for studying the mechanism of viral entry 
and may provide information for the development of vaccines.

MATERIALS AND METHODS

Cells and viruses. DEN-1 strain 760733 is a local Taiwanese strain isolated from patients with DF. Four prototype dengue viruses—DEN-1 (HI), DEN-2 (New Guinea C), DEN-3 (HST), and DEN-4 (H241)—were provided by Duane J. Gubler from the Centers for Disease Control and Prevention, Fort Collins, CO. All viral strains were used to infect mosquito C6/36 cells with growth medium containing 50% Mitsuami and Maramorsch insect medium (Sigma) plus 50% Dulbecco’s modified Eagle’s minimal essential medium (DMEM; GIBCO). The DEN-infected C6/36 cells were incubated at 28°C for 7 to 9 days. The viruses were harvested from the supernatants and titrated in BHK cells by plaque assay inoculated with 1% volume of the sample. The titer of the unamplified third-round 
MA). Phage display biopanning procedures were performed according to our 
previously published method (61). The titer of the unamplified third-round 
MAbs were diluted with serum-
free MEM and mixed with virus at a multiplicity of infection of 0.1 and incubated for 1 h at 4°C. The antibody-virus mixture was incubated in duplicate with BHK-21 cells in 12-well plates. After adsorption of viruses for 2 h, 2 ml of medium (MEM containing 2% FBS, antibiotic, and 1% carboxymethyl cellulose) was added to each well. Plates were incubated in 5% CO2 at 37°C for 5 to 7 days. Cells were stained with 0.5% crystal violet added directly to the medium and left for 60 min. After being washed three times with tap water, the plaques were counted.

Inhibition of DEN infection in BHK-21 cells. Cells were seeded in monolayers on sterile glass slides. MAbs were diluted with serum-free MEM and mixed with virus at a multiplicity of infection of 0.1 and incubated for 1 h at 4°C. The inhibition of DEN infection in BHK-21 cells by neutralizing 
MAbs was observed with a fluorescence microscope.

Identification of neutralizing epitopes by phage display. An ELISA plate was coated with 100 μg/ml of neutralizing MAbs in 0.1 M sodium bicarbonate buffer (pH 8.6). The plate was then incubated with blocking buffer (1% bovine serum albumin and 0.05% Tween 20 at 4°C overnight) and washed with PBS/100. A phage-displayed 12-mer peptide library was purchased from New England Biolabs, Inc. (Beverly, MA). Phage display biopanning procedures were performed according to our previously published method (61). The titer of the unamplified third-round phage particles was determined on Luria-Bertani medium—IPTG (isopropyl-
β-D-thiogalactopyranoside)–X-Gal (5-bromo-4-chloro-3-indolyl-
β-D-galactopyranoside) plates, and immunopositive phage clones were screened by ELISA. An ELISA plate was coated with 100 μg/ml of antibody in 0.1 M sodium bicarbonate buffer (pH 8.6) and blocked with blocking buffer. Serially diluted phage was added to the anti-
body-coated plate and incubated at room temperature for 1 h. The plate was washed with PBS/Ts, and HRP-conjugated anti-M13 antibody (Pharmacia) di-
luted in blocking buffer was added. The plate was incubated and washed with PBS/Ts, and then the same procedures were followed as those described in “Screening of MAbs against DEN by ELISA.”

DNA sequencing and computer analysis. Immunopositive phage clones were further characterized by DNA sequencing. DNA sequences of purified phages were determined by the dideoxynucleotide chain termination method with an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer, CA). The primer used for phage DNA sequencing was 5′-CCCTCATAGTTAGGCTAA-3′. The phage-displayed peptide sequences were translated and aligned with the Genet-
ics Computer Group program.

Phage competitive inhibition assay. Antigens were mixed with an equal volume 
of native sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions, and transferred to a nitrocel-
 lulose membrane. Nonspecific antibody-binding sites were blocked with 5% skim
TABLE 1. Generation and characterization of MAbs against DEN-1 by Western blotting, ELISA, and PRNT assay

<table>
<thead>
<tr>
<th>MAb</th>
<th>Western blot result</th>
<th>ELISA result</th>
<th>DEN-1 PRNT&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</th>
<th>Specificity&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> E, envelope proteins; NS1, nonstructural protein 1.

RESULTS

Generation and identification of MAbs against DEN-1. In this study, we generated serotype-specific and neutralizing MAbs against DEN-1 to aid in our exploration of the immunopathogenesis of DHF and development of serologic diagnostic reagents and therapeutic antibodies. Immunoblotting and ELISA were used to test the specificities of MAbs for four serotypes of DEN (Table 1). We generated five MAbs against E proteins of DENs. DA4-7, DA6-7, DA10-2, and DA11-13 reacted only with E proteins of DEN-1. DA9-5 reacted with E proteins of both DEN-1 and DEN-2 (Fig. 1A). We also generated seven MAbs against nonstructural protein 1 (NS1 protein) specific to DENs. They were DA6-6, DA11-9, DA15-2, DA31-6, and DA32-9, which reacted specifically with NS1 protein of DEN-1, and DA8-4 and DA11-1, which reacted with NS1 proteins of DEN-1 and DEN-3 (Fig. 1B). These MAbs recognized only the viral proteins of DEN-infected cells, not mock-infected cells, by immunoblotting analysis (data not shown). The specificities of these MAbs for viral proteins were further confirmed with MAbs 4G2 (ATCC HB197; against E proteins of four serotypes of DENs) (21) and 15F3 (ATCC HB47; against NS1 proteins of DEN-1) (21, 60) by an immunoblotting assay (data not shown). ELISA revealed C6/36 cells to be infected with DEN-1, -2, -3, and -4. Infected cells were fixed and incubated with MAbs against DEN, normal mouse IgG (NM-IgG), and normal mouse serum (NMS). All MAbs recognized DEN-1-infected C6/36 cells (Table 1).

Inhibition of DEN-1 entry into BHK-21 cells by neutralizing antibodies. In screening for neutralizing MAbs, we tested the neutralizing activity of all MAbs against DEN-1 by PRNT assay (see Materials and Methods for details). We identified two neutralizing MAbs, DA6-7 and DA11-13. Both had a 50% reduction in plaque formation at 1.25 µg/ml. NM-IgG (10 µg/ml) did not inhibit the formation of plaques (Fig. 2A).

To further confirm the neutralizing ability of DA6-7 and DA11-13, the antibodies were incubated with DEN-1 before being used to infect BHK-21 cells. E proteins of DEN-1 in BHK-21 cells were detected by an indirect immunofluorescence assay. E proteins were found on BHK-21 cells infected with nonneutralized virions (Fig. 2B, no antibody and NM-IgG). Fluorescence staining for DEN E proteins was detected most intensely in the cytoplasm of the cell. In contrast, cells

FIG. 1. Identification of MAbs against E and NS1 proteins of DENs by immunoblot analysis. Four serotypes of DEN antigens (D1 to D4) from DEN-infected C6/36 cell lysates were size fractionated in polyacrylamide gels. The gels were incubated with MAbs. (A) MAbs DA4-7, DA6-7, DA9-5, DA10-2, and DA11-13, recognizing E proteins (55 kDa) of DENs, were identified by immunoblot analysis using a nonreducing gel. (B) MAbs DA6-6, DA8-4, DA11-1, DA11-9, DA15-2, DA31-6, and DA32-9 against the dimeric form of NS1 proteins (75 kDa) were identified by immunoblot analysis using a nonreducing gel.
infected with DEN pretreated with DA6-7 and DA11-13 MAbs showed consistently negative staining (Fig. 2B). DEN entry into BHK-21 cells was inhibited by these neutralizing MAbs, and the viruses stopped replicating.

Screening of phage-displayed peptide library with neutralizing antibodies against DEN-1. Neutralizing epitopes of DA6-7 and DA11-13 were identified by the phage display technique. To select immunopositive phage clones binding to neutralizing MAbs, DA6-7 and DA11-13 ascitic fluids were purified using a protein G affinity column. Purified antibodies were immobilized on ELISA plates, and bound phage clones were selected after biopanning three times. Further screening of immunopositive phage clones was performed by single-phage clone isolation and amplification for MAb screening by ELISA.

Of the 30 phage clones selected by DA6-7, 15 (DA6-7-C2, -C3, -C4, -C10, -C12, -C14, -C16, -C17, -C18, -C25, -C26, -C27, -C28, -C29, and -C30) were highly reactive with antibody DA6-7 and did not bind to NMS (Fig. 3A). Of the 36 phage clones selected by DA11-13, 32 (DA11-13-C1, -C2, -C3, -C4, -C5, -C6, -C7, -C8, -C9, -C10, -C11, -C12, -C13, -C14, -C15, -C16, -C17, -C19, -C20, -C21, -C22, -C24, -C25, -C27, -C28, -C29, -C30, -C31, -C32, -C33, -C34, and -C36) were highly reactive with antibody DA11-13 and did not bind to NMS (Fig. 3B).

Characterization of neutralizing B-cell epitopes. Fifteen immunopositive phage clones (DA6-7-C2, -C3, -C4, -C10, -C12, -C14, -C16, -C17, -C18, -C25, -C26, -C27, -C28, -C29, and -C30) were highly reactive with antibody DA6-7 and did not bind to NMS (Fig. 3A). Of the 36 phage clones selected by DA11-13, 32 (DA11-13-C1, -C2, -C3, -C4, -C5, -C6, -C7, -C8, -C9, -C10, -C11, -C12, -C13, -C14, -C15, -C16, -C17, -C19, -C20, -C21, -C22, -C24, -C25, -C27, -C28, -C29, -C30, -C31, -C32, -C33, -C34, and -C36) were highly reactive with antibody DA11-13 and did not bind to NMS (Fig. 3B).

Characterization of neutralizing B-cell epitopes. Fifteen immunopositive phage clones (DA6-7-C2, -C3, -C4, -C10, -C12, -C14, -C16, -C17, -C18, -C25, -C26, -C27, -C28, -C29, and -C30) that were highly reactive with DA6-7 were amplified, and phage DNAs were isolated for DNA sequencing. All of the phage clones displayed the 12 amino acid residues NTYFTAFLDGPK.

Similarly, 17 immunopositive phage clones (DA11-13-C1, -C2, -C3, -C4, -C5, -C6, -C8, -C11, -C12, -C13, -C14, -C20, -C24, -C28, -C30, -C33, and -C36) that were highly reactive with DA11-13 were amplified, and phage DNAs were isolated for DNA sequencing. These immunopositive phage clones displayed four different peptide sequences. Phage clones DA11-13-C2, -C3, -C8, -C11, -C13, -C20, -C28, and -C30 displayed the same amino acid sequence, QVPSSLLLQSR, clones DA11-13-C5 and -C36 displayed the sequence HKYSSLQQR, and clone DA11-13-C12 displayed the sequence TAPSSIHLAR. Three amino acid residues, i.e., serine (S)/threonine (T)-S-leucine (L)/isoleucine (I) (shown in bold), were highly conserved in all of these immunopositive phage clones.

To confirm that the peptides displayed on immunopositive phage clones bound DA6-7 and DA11-13 specifically, a serial-dilution ELISA binding assay was performed. The DA6-7-selected phage clone (DA6-7-C4) reacted with DA6-7 specifically and dose dependently but not with DA11-13 or NM-IgG (Fig. 4). DA11-13-selected phage clones (DA11-13-C1, -C3, -C12, and -C36) reacted with DA11-13 specifically and dose dependently but not with DA6-7 or NM-IgG (Fig. 4).

To further confirm that the phage-displayed peptide sequences were the B-cell epitopes of neutralizing MAbs, phage competitive inhibition assays were performed to determine whether the immunopositive phage competed with E proteins for reactivity with DA6-7 and DA11-13. The reactivity of DA6-7 with E proteins was inhibited markedly by DA6-7-C4 at 10^{11} and 10^{10} PFU/ml of phage (Fig. 5A). Similarly, the reactivity of DA11-13 with E proteins was inhibited completely by DA11-13-C2 and -C4 at 10^{10} and 10^{9} PFU/ml of phage (Fig. 5B and C). The binding activities of these neutralizing MAbs to E proteins were inhibited by immunopositive phage clones. These findings strongly suggest that the phage-displayed peptide sequences are indeed the B-cell epitopes of DA6-7 and DA11-13.
Detection of serum samples from DEN-infected patients by using immunopositive phage clones. We tried to evaluate whether the phage-displayed epitopes could be used as a diagnostic tool in the detection of antibodies in serum samples from dengue patients. Six of eight serum samples from DEN-1-infected patients had positive ELISA antibody reactivity with DA6-7-C4 (Fig. 6C). Five of eight serum samples from DEN-1-infected patients had positive ELISA antibody reactivity with DA11-13-C1 (Fig. 6D). All serum samples obtained from DEN-2-infected patients were seronegative, and none of the control NHS samples had positive responses with these two phage clones (Fig. 6C and D).

DISCUSSION

The identification of viral B-cell epitopes is important for the development of subunit vaccines and virus-specific serological diagnostic reagents as well as for studying virus-antibody interactions at the molecular level. In this study, we generated 12 MAbs, including two neutralizing antibodies against DEN-1. We also identified and characterized two neutralizing epitopes of DEN-1 by using a phage-displayed random peptide library. We found that the neutralizing MAb DA11-13-selected phage clones displayed 12-mer peptide sequences that had a consensus motif, S/TSL/I. The reactivities of the neutralizing MAbs with E proteins were inhibited markedly and dose dependently by the epitopes displayed by immunopositive phage clones (Fig. 5). Our data strongly suggest that the phage-displayed peptides are mimic epitopes of DA11-13 and DA6-7. We also used phage-displayed epitopes to detect antibodies in serum samples from DEN-infected patients (Fig. 6).

The similarities of amino acid sequences for the four serotypes of DEN range from 63.2 to 78.7% (58). The high similarity of amino acid sequences within the four serotypes of DEN makes them difficult to distinguish when using antigens obtained from overlapping synthetic peptides (1, 23, 48) or from recombinant or enzyme cleavage antigen fragments (38, 40, 54). Using epitope-based peptide antigens displayed on phage makes it easier to identify the antibodies in serum samples from DEN-1-infected patients. Furthermore, the serotype-specific epitopes are useful for differentiating between serum samples from DEN-1- and DEN-2-infected patients (Fig. 6). The sensitivities of ELISA for detecting serum samples from DEN-1-infected patients by using phage-displayed serotype-specific epitopes of DEN-1, namely, DA6-7-C4 and DA11-13-C1, were 75% (6/8) and 62.5% (5/8), respectively (Fig. 6C and D). Using the same serologic test, we found that serum samples from all eight healthy adults were seronegative, yielding a specificity of 100% for healthy donors for these two serotype-specific epitopes (Fig. 6C and D). The identification of more serotype-specific epitopes and combination of these epitope-based peptide antigens for serological diagnosis will
increase the sensitivity of serotype-specific detection of DEN-infected patients.

Severe and sometimes fatal DHF and DSS often occur in regions where more than one DEN serotype is circulating (17,

FIG. 4. Specific reactivities of selected phage clones to neutralizing MAbs. An ELISA plate was coated with DA6-7 (A), DA11-13 (B), or NM-IgG (C). The antibodies were then incubated with 10-fold serially diluted phage clones (10⁶, 10⁵, 10⁴, 10³, and 0 PFU). The DA6-7-selected phage clone (DA6-7-C4) bound to DA6-7 specifically but did not react with DA11-13 and NM-IgG. DA11-13-selected phage clones (DA11-13-C1, -C3, -C12, and -C36) bound to DA11-13 specifically but did not react with DA6-7 and NM-IgG.

FIG. 5. Phage competitive inhibition assay by immunoblot analysis. (A) The reactivity of DA6-7 with E protein was inhibited by phage clone DA6-7-C4. The reactivity of DA11-13 with E protein was inhibited by phage clones DA11-13-C1 and DA11-13-C4 (C).

FIG. 6. (A and B) Capture ELISA for serum samples from patients with DEN-1 infection. Serum samples (200-fold dilution) from DEN-infected patients were analyzed. Representative data are shown to illustrate the MAb responses. All of the serum samples from DEN-1- and DEN-2-infected patients could be detected by DA6-7-captured DEN-1 (A) and DA11-13-captured DEN-1 (B). (C and D) ELISA reactivities of phage clones with serum samples (200-fold dilution) from DEN-infected patients. (C) Six of eight serum samples from DEN-1-infected patients could be identified by DA6-7-C4, but all serum samples from DEN-2-infected patients and NHS from healthy control subjects did not reveal such reactivity. (D) Five of eight serum samples from DEN-1-infected patients could be identified by DA11-13-C1, but eight serum samples from DEN-2-infected patients and NHS revealed no such reactivity. Cutoff values are represented by solid lines.
19). It has been hypothesized that nonneutralizing cross-reactive antibodies acquired during the first DEN infection enhance a second infection by a different DEN serotype (19, 20). Two new dengue diagnostic tests, the MRL Diagnostics dengue fever virus IgM capture ELISA and the PanBio rapid immunochromatographic test, have been used to detect DEN antibodies. However, they cannot distinguish between the four serotypes of DEN and have 45 to 50% cross-reactivity with Japanese encephalitis virus-infected samples (29, 56, 57). In the present study, we generated serotype-specific MAbs recognizing DEN-1. We also identified the serotype-specific epitopes of DEN-1 and used epitope-based peptides as antigens to detect DEN-1 antibodies in serum samples from DEN-1-infected patients. Our serotype-specific MAbs and epitope-based peptide antigens may be useful in the development of diagnostic laboratory tests for DEN-1 infection.

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