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activation of the host's humoral immunity. Identification of these epitopes on viral proteins is important for understanding the pathogenesis of viral infectious diseases as well as for developing vaccines and diagnostic reagents. Serotype-specific monoclonal antibody, Ab4, against dengue virus type 2 (DEN-2) was generated and identified by ELISA, immunofluorescence and immunoblotting analysis. The B-cell epitope of Ab4 was further identified from a random peptide library displayed on phage. The selected phage clones were specifically reacted with Ab4 and did not reacted with other monoclonal antibodies. These immunopositive phage clones displayed a consensus motif, His-Arg-Leu/Ile. Synthetic peptide corresponding to the phage-borne peptide can bind the antibody specifically. The epitope-based synthetic peptide could detect serum samples from DEN-2 immunized mice and rabbits by ELISA assay. The peptide was also able to differential very clearly between serum samples from DEN-2 and Japanese encephalitis virus (JEV) immunized mice. The results show that this monoclonal antibody and its epitope-based peptide will be useful for serologic diagnosis of DEN-2. Furthermore, the identification of the epitope of DEN-2 will permit us to functionally dissect the antibody response and to address the role of antibody in the pathogenesis of primary or secondary DEN-2 infection.

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ELISA, immunofluorescence immunoblotting

(Ab4)

peptide　　　　His-Arg/Lys-Leu/Ile motif
synthetic peptide
peptide

epitope-based peptide
epitope-based peptide

epitope-based peptide antigen

ABSTRACT

Viral infection usually results in the production of antibodies directed against the epitopes of viral proteins through the

KEY WORDS: monoclonal antibody; B-cell epitope; synthetic peptide; serologic diagnosis

INTRODUCTION

Dengue virus (DEN) causes serious febrile illness in humans, including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Henchal, et al. 1990; Halstead, 1988). Primary dengue virus infection often results in a painful, debilitating, but nonfatal dengue fever and appears to against reinfection with the same serotype. However, the more severe and sometimes fatal forms DHF and DSS have been frequently seen in region where more than one serotype of dengue virus is circulating (Halstead, 1988; Gubler, 1998). Secondary infection with a different serotype is associated with an increased risk for DHF and maybe caused by the uptake by monocytes/macrophages of virus complexes to nonneutralizing antibodies, sub-neutralizing cross-reactive antibodies or low-titer neutralizing antibodies (Halstead, 1988; Bielefeldt-Ohmann, 1997; Halstead, et al. 1984). Viral infection usually results in the production of antibodies directed against the epitopes of the viral proteins through the activation of the host's humoral immunity. The epitopes have been divided into linear or continuous epitopes and conformational or discontinuous epitopes (Sela, 1969; Barlow et al., 1986). Linear epitopes are short stretches of the primary structure of the protein and are made up of some continuous amino acid residues of the primary sequence. Conformational epitopes consist of several amino acid residues which are discrete in the primary sequence but assemble to form an antigenic determinant on the tertiary structure of the native protein. Identification of these epitopes on viral proteins is important in understanding the pathogenesis of viral infectious diseases as well as in developing effective vaccines and diagnostic reagent.

Recent advance in peptide technology has allowed the development of combinatorial peptide libraries expressed either on a solid phase support or displayed on bacteriophages. The high molecular diversity displayed by these libraries provides the possibility to study B-cell epitope mapping (Scott and Smith 1990; Young et al., 1997; Wu et al., 2001)

Recently, we have identified serotype-specific B-cell epitope of DEN-1 (Wu et al., 2001). In this study, we used a phage-displayed peptide library to identify the serotype-specific B-cell epitope for DEN-2. Up to now, it is still not clear whether DHF/DSS is due to a primary or secondary infection of DEN or other immunopathologic mechanisms (Halstead, 1988; Gubler, 1998). Therefore, the identification of B-cell epitopes for DEN can provide important information for the development of a safe and effective dengue vaccine and contribute to the understanding of the pathogenesis and immunological responses in DEN infection.

RESULTS

Identification of serotype-specific MAb against DEN-2

To show that the MAb Ab4 was specific for DEN-2 and did not cross-react with other serotype of DENs, the immunoblotting and ELISA assay was perform. The AB4 reacted only to the NS-1 of DEN-2 and did not cross-react with DEN-1, 3, and 4 (Fig 1).
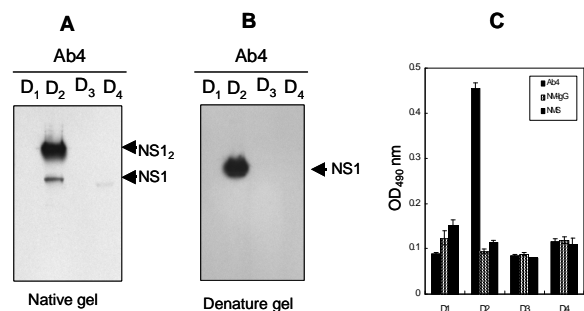

Fig. 1. Identification the specificity of DEN-2 monoclonal antibody (Ab4) by ELISA and immunoblotting assay.

Screening of phage displayed peptide library with DEN-2 serotype-specific antibody

To select the phage clones by DEN-2 specific monoclonal antibody (Ab4), the ascites were purified by protein G affinity column. The purified antibodies were immobilized on ELISA plate and the bound phage clones were selected after three times

of biopanning. For further screen of these selected phage clones, the single phage clones were isolated and amplified for Ab4 screening by ELISA assay. Selected phage clones showed significant enhancement of reactivity to antibody Ab4 and these phage clones did not bind to normal mouse serum or IgG (Fig. 2).
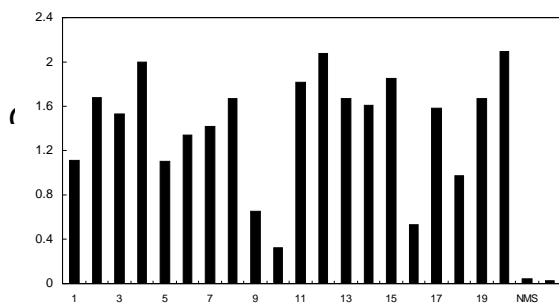

Fig. 2. Identification of DEN-2 specific monoclonal antibody (Ab4) selected phage clones by ELISA assay. Phage displayed random peptide library was screened by Ab4. After three rounds of screening, 17 phage clones from 20 selected phage clones showed were significant highly reactivity to antibody Ab4 but not to normal mouse serum (NMS) or PBS.

Characterization of the B cell epitope

Ten phage clones selected by Ab4 were amplified and the phage DNAs were isolated for DNA sequencing. The primer used for phage DNA sequencing is 5'-CCCTCATAGTTAGCGTAA-3'. This primer locates in antisense strand of gene III of the M13 phage and has 96 nucleotides separated from the inserted DNA. The inserted nucleotides of selected phage clones were sequenced and all of them contain 36 inserted nucleotides. The inserted oligonucleotide sequences of phage DNAs were selected and translated to peptide sequences. The peptide sequences were aligned using a MacDNASIS software to analyze the epitopes of the antibody Ab4 and its binding motif. The binding motif for the Ab4 is H-R/K-L/I and all of the selected phage clones contain this motif.

Binding assay of synthetic peptide

In order to verify that the peptide sequences identified by phage display were indeed recognized by antibody Ab4, synthetic peptide binding assays were carried out. The phage-displayed peptides were synthesized in multiple antigen peptide (MAP) form because the ELISA sensitivity of this eight-chain MAP were more than single-chain peptides (Tam and Zavala, 1989). As shown in Fig. 3, the synthetic peptide (P7M) XXXXXXTMPSES, selected with MAb Ab4 from phage displayed RPLs, binds the antibody in a concentration-dependent manner. An unrelated control peptide KGTFDPLQEPRT (P4M) and EHKYSWKS (P14M) showed no reactivity.
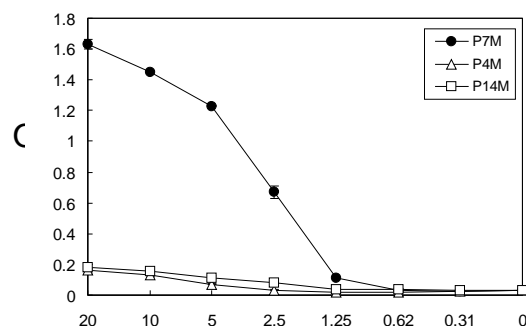

Fig. 3. ELISA reactivity of synthetic peptide corresponding with selected phage clone. The phage-displayed peptide was synthesized and immobilized on a 96-well plate as an antigen for detection of Ab4. The synthetic peptide (P7M), corresponding with the selected phage clone, reacted with Ab4 specifically, but not the control peptide (P4M and P14M).

Detection of DEN-2-infected animal sera using synthetic peptide P7M

To test whether the antibodies produced from outbred rabbit sera that had been intravenously infected with the DEN-2 would react with synthetic peptide (P7M), we collected hyperimmune serum samples after four doses of DEN-2 for ELISA assay. The results revealed that all serum samples from DEN-2-hyperimmune rabbits demonstrated a higher ELISA antibody reactivity with P7M peptide than the preimmune serum samples (Fig. 4B). Furthermore, our additional results revealed that serum samples obtained from five JEV-hyperimmune and eight normal ICR

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mice showed no ELISA reactivity with P7M peptide, but eight DEN-2-hyperimmue ICR mice showed significant ELISA reactivity with the peptide antigen (Fig. 4A), which suggested its differentiation capability between JEV and DEN-2 in mouse serum sa
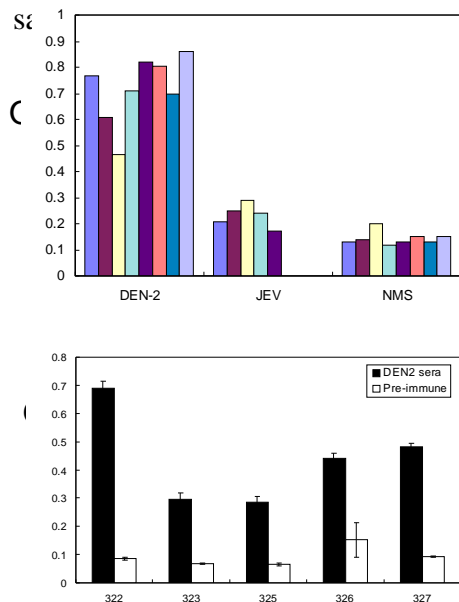

Fig. 4. (A). ELISA reactivities of DEN-2 synthetic peptide P7M with DEN-2 and JEV immunized mice sera (100-fold dilution). Eight DEN-2 immunized mice sera could be identified by the peptide P7M. (B). ELISA reactivities of DEN-2 synthetic peptide P7M with pre-immunized and DEN-2 immunized rabbit sera (100-fold dilution). Five DEN-2 immunized rabbit sera could be identified by the peptide P7M.

DISCUSSION

Identification of viral B-cell epitopes is of importance in the selection of peptides for inclusion into subunit vaccines, the development of virus-specific serological diagnosis and understanding the interaction of antibodies with viruses at a molecular level. This is the first study to characterize serotype-specific B-cell epitope of DEN-2 MAb (Ab4) and detect antibodies of DEN-2 from serum samples of DEN-2-immunized animal using the epitope-based peptide antigen. Our results found that the selected phage displayed 12mer peptide sequence revealed a consensus motif, His-Arg/Lys-Leu/Ile. It is feasible that our phage displayed epitope and epitope-based

peptide antigen can be used to develop laboratory diagnosis serologic tests for dengue viruses in the future.

Using our epitope-based peptide antigen to detect anti-DEN antibody is relatively simple and specific. Therefore, it will be promising to become routine procedures for viral diagnosis in clinical and public health laboratories. On the other hand, conventional IgM- and IgG-capture ELISA tests, which require the preparation of DEN antigen and antibody, have been primarily used in specialized virology centers. Several reliable dengue serologic diagnosis tests are now available, they still display difficulty distinguishing among the four serotypes of DEN and still show a 45-50% cross-reaction with antibody of the Japanese encephalitis virus (JEV) (Lam et al., 1998., Vaughn et al., 1998; Vaughn et al., 1999). Otherwise, in our results suggested the epitope-based synthetic peptide P7M differentiation capability between JEV and DEN-2 in mouse serum samples.

Moreover, our developed method can also be applied to detect future DF and DHF patients who had secondary infection with a heterologous serotype of DEN, which would minimize possible morbidity and mortality. Finally, our test will be very valuable for further development of a serotype-specific diagnostic reagent that can be used to serological distinguish four serotypes dengue patients and thus help combat dengue diseases.

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