行政院國家科學委員會專題研究計畫　成果報告

研究登革病毒血清型專一性抗體的B細胞抗原決定位及評估其為偵檢試劑之可行性

計畫類別：個別型計畫
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執行單位：國立臺灣大學醫學院口腔生物科學研究所

計畫主持人：吳漢忠

計畫參與人員：張博欽

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行政院國家科學委員會補助專題研究計畫成果報告

研究第二型登革血清型專一性抗體的 B 細胞抗原決定位之及評估其為偵檢試劑之可行性

計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC 91-2314-B-002-257

執行期間：91 年 8 月 1 日至 92 年 7 月 31 日

計畫主持人：吳 漢 忠

共同主持人：

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□國際合作研究計畫國外研究報告書一份

執行單位：臺大醫學院口腔生物研究所

中　華　民　國　92 年　10 月　27 日

行政院國家科學委員會專題研究計畫成果報告

計畫編號：NSC 91-2314-B-002-257
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主持人：吳 漢 忠　臺大醫學院口腔生物研究所
共同主持人：xxxxxx　執行機構及單位名稱
計畫參與人員：張博欽

一、中文摘要

本計畫主要是要研究登革病毒的 B 細胞抗原決定位。經由 ELISA, immunofluorescence 和 immunoblotting 方法，我們找出具專一性的第二型登革病毒單株抗體(Ab4)。此抗體之抗原決定位的確認，有助於病毒感染機制的瞭解，疫苗及偵檢試劑的研發。在此研究中，我們以噬菌體顯現法來研究第二型登革病毒的抗原決定位。我們也已經篩選出多株的噬菌體可以專一性的與第二型登革病毒抗體結合。這些專一性噬菌體所攜帶的外來 peptide，都含有 His-Arg/Lys-Leu/Ile motif。以 synthetic peptide 代替噬菌體所攜帶的外來 peptide，可與第二型登革病毒抗體明顯反應。第二型登革病毒感染之老鼠及兔子血清，可以用此 epitope-based peptide 來確認。另外，此 epitope-based peptide 可有效的分辨登革病毒及日本腦炎病毒感染之老鼠血清，這些抗原決定位的確認與研究，將有助於 epitope-based peptide antigen 之研發，及了解出血性登革之病理機制。

關鍵詞：第二型登革病毒，噬菌體顯現法，抗原決定位，登革偵檢試劑

ABSTRACT

Serotype-specific monoclonal antibody (MAb), D_2 16-1 (Ab4), against dengue virus type 2 (DEN-2) was generated. This MAb recognized non-structural protein 1 (NS1) of DEN-2; its specificity was determined by ELISA, immunofluorescence and immunoblotting analysis. Serotype-specific B-cell epitope of Ab4 was further identified from a random phage-displayed peptide library. Selected phage clones specifically reacted with Ab4 and did not react with other MAbs. Immunopositive phage clones displayed a consensus motif, His-Arg/Lys-Leu/Ile. Synthetic peptide corresponding to phage-borne peptide specifically bound to antibody. His and Arg in this epitope were crucial for peptide binding to Ab4: binding activity dramatically decreased when His or Arg was changed to Leu. Epitope-based synthetic peptide identified serum samples from DEN-2-immunized mice and rabbits by ELISA assay. The peptide clearly differentiated between serum samples from DEN-2- and Japanese encephalitis virus (JEV)-immunized mice. This monoclonal antibody and its epitope-based peptide antigen will be useful for serologic diagnosis of DEN-2 infection. Furthermore, DEN-2 epitope identification makes it feasible to dissect the antibody response and to address the role of antibodies in the pathogenesis of primary and secondary DEN-2 infections.

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

Immunoblotting and ELISA assays determined reactivity of MAb D_2 16-1 (Ab4) with DEN-2 and other DEN serotypes. Ab4 reacted only to NS-1 of DEN-2 and did not cross-react with DEN-1, -3 or -4 (Fig. 1).

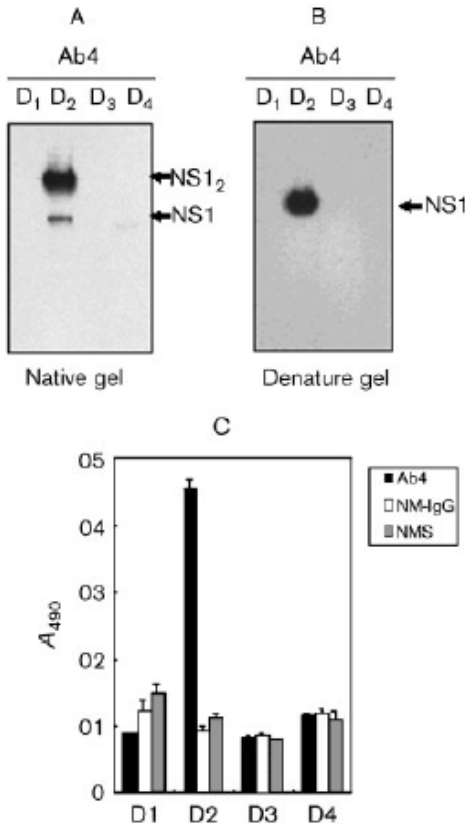

Fig. 1. Identification the specificity of DEN-2 monoclonal antibody (Ab4) by ELISA and immunoblotting assay.

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).

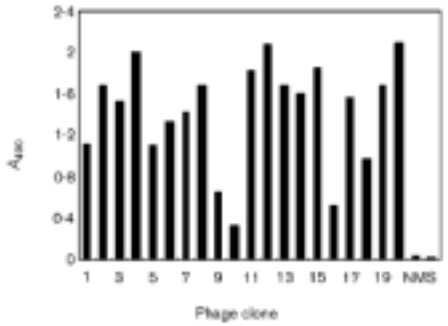

Fig. 2. Identification of DEN-2 specific monoclonal antibody (Ab4) selected phage clones by ELISA assay. Phage displayed random peptide library was screened by Ab4. After three rounds of screening, 17 phage clones from 20 selected phage clones showed were significant highly reactivity to antibody Ab4 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. Inserted oligonucleotide sequences of phage DNAs were selected and translated to peptide sequences. Peptide sequences were aligned using MacDNASIS software to analyze epitopes and binding motif of Ab4. Interestingly, three amino acid residues, histidine (His, H)-arginine (Arg, R)/lysine (Lys, K)-leucine (Leu, L)/isoleucine (Ile, I) were highly conserved in these immunopositive phage clones; the other nine amino acid residues were randomized (Table 1). We concluded that the binding motif for Ab4 is H-R/K-L/I and that all selected phage clones contained this motif (Table 1).

Table 1. Alignment of phage-displayed peptide sequences selected by DEN-2 serotype-specific mAb Ab4

Phage-displayed consensus amino acids are shown in bold.

Clone	Insert										
Ab4-C2	S	**H**	**R**	**L**	H	A	T	Stop	I	L	M	P
Ab4-C3	S	**H**	**R**	I	L	S	S	A	L	V	Stop	V
Ab4-C4	S	**H**	**R**	**L**	H	N	T	M	P	S	E	S
Ab4-C8	T	**H**	**R**	**L**	Y	L	A	S	T	L	P	G
Ab4-C11	S	**H**	**R**	**L**	P	H	P	N	L	I	R	L
Ab4-C12	Q	**H**	**R**	I	H	N	P	A	P	V	S	L
Ab4-C13	H	**H**	**R**	I	L	I	E	P	P	S	M	H
Ab4-C14	S	**H**	**R**	**L**	P	P	V	S	N	P	S	L
Ab4-C15	H	**H**	**R**	**L**	N	N	Q	D	D	L	L	K
Ab4-C17	S	**H**	**K**	**L**	F	Y	Q	M	G	T	Q	L
Ab4-C19	Q	**H**	**R**	M	T	W	M	Q	G	L	S	S
Ab4-C20	Q	**H**	**K**	**L**	Y	P	V	I	P	L	P	S

Binding assay of synthetic peptide

To verify that peptide sequences identified by phage display were recognized by antibody Ab4, synthetic peptide binding assays were carried out. Phage-displayed peptides were synthesized in multiple antigen peptide (MAP) form because ELISA sensitivity for an eight-chain MAP is greater than for a single-chain peptide (Tam and Zavala, 1989; Wu et al., 2001a). As shown in Fig. 3, synthetic peptide P7M (SHRLHNTMPSES), corresponding to immunopositive phage clones Ab4-C4 (Table 1) that had been selected with Ab4 from phage-displayed peptide library, bound antibody in a concentration-dependent manner. Unrelated control MAP peptides, KGTFDPLQEPRT (P4M) and EHKYSWKS (P14M), were not reactive.

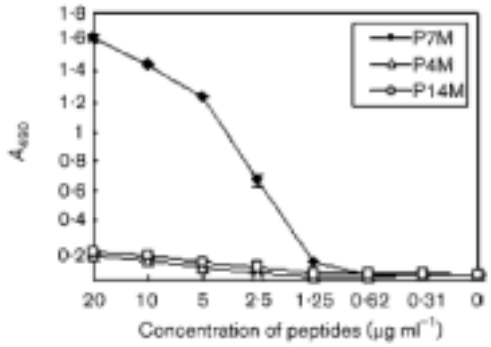

Fig. 3. ELISA reactivity of synthetic peptide corresponding with selected phage clone. The phage-displayed peptide was synthesized and immobilized on a 96-well plate as an antigen for detection of Ab4. The synthetic peptide (P7M), corresponding with the selected phage clone, reacted with Ab4 specifically,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

Using the phage display method, we determined the binding motif for Ab4 was His-Arg-Leu/Ile. His and Arg were found in most immunopositive phage clones (Table 1). We proposed that these two amino acid residues play a crucial role in binding to the antibody. To prove this hypothesis, we synthesized two peptides: P7M-m1 (SLRLHNTMPSES) and P7M-m2 (SHLLHNTMPSES). There is only one amino acid residue difference between these peptides and P7M (SHRLHNTMPSES). ELISA assay indicated that binding activity was dramatically decreased when positively charged His or Arg in P7M was changed to aliphatic side-chain Leu in P7M-m1 or P7M-m2, respectively (Fig. 4A).

To further confirm that the phage-displayed peptide was the epitope of Ab4, peptide-competitive inhibition assay was performed to determine whether the synthetic peptide P7M competed with NS1 proteins for reactivity with Ab4. The reaction activity of Ab4 with NS1 proteins was markedly inhibited by P7M. The mutated peptide P7M-m1 had no effect on Ab4 reaction with NS1 proteins (Fig. 4B). Our observations strongly suggest that P7M is the B-cell epitope of Ab4 and that His and Arg are an important for binding to MAb.

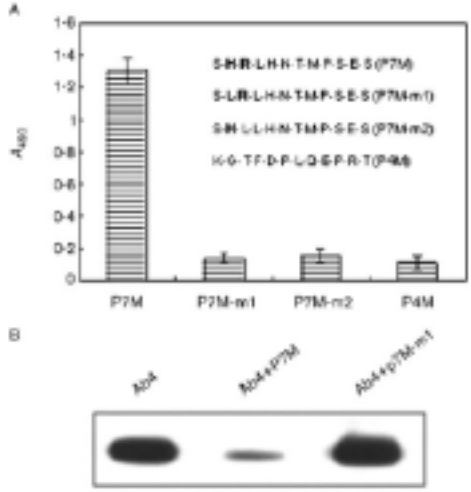

Fig. 4

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

We evaluated whether the epitope-based peptide antigen P7M could be used as a diagnostic reagent to detect immunized animal serum samples. Two different species of animals were immunized with DEN-2. Serum samples were collected to test detection efficacy. To test whether antibodies produced from DEN-2-immunized rabbit sera would react with synthetic peptide P7M, we collected hyperimmune serum samples after four inoculations with DEN-2 for ELISA assay. All serum samples from DEN-2-hyperimmune rabbits had higher ELISA antibody reactivity with P7M peptide than with preimmune serum samples (Fig. 5B). Serum samples obtained from five JEV-hyperimmune and eight normal BALB/c mice had no significant ELISA reactivity with P7M; however, serum samples from all eight DEN-2-hyperimmune BALB/c mice had significant ELISA reactivity with peptide antigen (Fig. 5A). This suggests that P7M can differentiate between JEV and DEN-2 in immunized mouse serum samples. Using different animals and different immunization techniques (i.p. inoculation in mice and i.v. inoculation in rabbits), we determined that P7M could identify DEN-2-immunized animal serum samples. To further test the specificity of P7M, we used this peptide antigen to detect DEN-1, -2, and -3

immunized mouse serum samples. P7M detected all eight DEN-2-hyperimmune BALB/c mice by ELISA (Fig. 5C). In contrast, all serum samples obtained from DEN-1 and -3-hyperimmune mice were seronegative. These results indicated that vertebrates like rabbits and mice immunized with DEN-2 can produce antibodies recognized by this epitope-based synthetic peptide antigen.

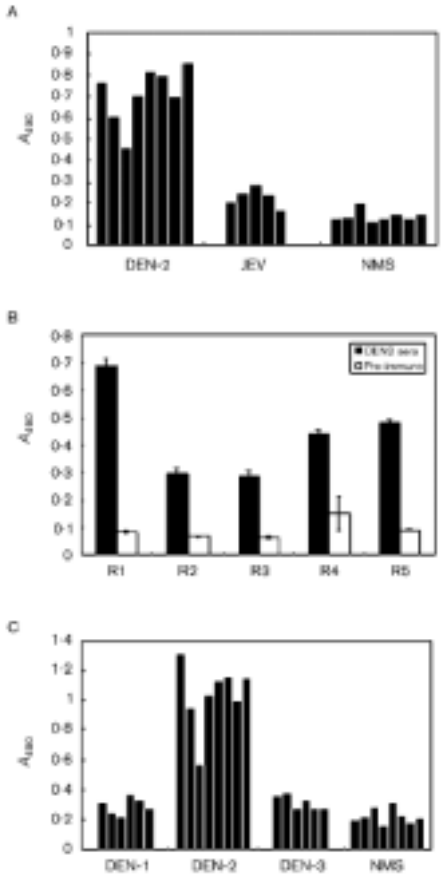

Fig. 5

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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