行政院國家科學委員會補助專題研究計畫成果報告

登革病毒中和抗原決定位之研究及其疫苗研發

計畫類別：■個別型計畫　　□整合型計畫
計畫編號：NSC 89－2320－B－002－207
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計畫主持人：吳 漢 忠
共同主持人：

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共同主持人：xxxxxx　執行機構及單位名稱
計畫參與人員：李國豪

一、中文摘要

本計畫主要是要研究登革病毒中和性的 B 細胞抗原決定位。經由 ELISA, 和 immunoblotting 方法, 我們確認具專一性的第二型登革病毒單株抗體(3H5)。3H5 經由中和力測試實驗, 証明具有很高中和登革病毒感染的能力。動物實驗測試, 也發現此抗體在 100 倍 LD_{50} 的登革病毒的劑量下, 仍可以保護 80％乳鼠的存活率。此中和性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中和性抗原決定位。篩選出的噬菌體可以專一性的與第二型登革病毒抗體 3H5 結合。這些專一性噬菌體所攜帶的外來 peptide, 都含有 **XXXXXX**SELEXXW。此位置恰好位於第二型登革病毒套膜蛋白的第 51－56 amino acid。我們也更進一步証明篩選出的噬菌體與 3H5 具有很高的專一性。當我們用 10 倍稀釋的噬菌體與 3H5 反應, 發現此噬菌體與 3H5 有專一性結合, 且呈現 dose-dependent 現象, 而 control phage 則完全不反應。另外篩選出的噬菌體也証明只與 3H5 抗體結合, 而不與其它四種單株抗體、正常小白鼠血清及純化 IgG 結合。中和性抗原決定位的確認與研究, 將有助於 epitope-based peptide vaccine 之研發, 及了解出血性登革之病理機制。

關鍵詞：登革病毒, 中和性單株抗體, 噬菌體顯現法, 抗原決定位, 登革疫苗

ABSTRACT

In this report, serotype-specific monoclonal antibody (MAb) against dengue virus type 2 (DEN-2), 3H5 (ATCC HB46), was identified by ELISA and immunoblotting analysis. 3H5 possess highly neutralizing activity against DEN-2 infection using plaque reduction neutralization test. This MAb also has 80% protection activity against 100-fold LD_{50} DEN-2 challenge. We also identified the neutralizing epitopes of this antibody using phage display method. Eighteen immunopositive phage clones that bound specifically to MAb 3H5 were selected. All of these phage-borne peptides had displayed a peptide sequence of **XXXXXX**SELEXXW that mimicked the sequence, which corresponded to the amino acid residues 51-56 of the envelope protein (E protein) of DEN-2. For further conformation of the selected phage clone bound 3H5 specifically, the antibodies were incubated with ten-fold serial dilution of phage clone. The result shown that only the selected phage clone bound the antibodies specifically and dose dependently. The selected phage clone only specific interacted with 3H5 and did not react with other four MAbs, normal mouse serum, and IgG. Furthermore, serotype-specific MAb of DEN-2, 3H5, bound its selected phage clone specifically, but had no effect on other five MAbs-selected phage clones. The identification of the epitopes of envelope protein of DEN will permit us to functionally dissect the antibody response and to address the role of anti-E protein antibodies either in the protection from or in the immunopathogenesis of DEN infection. Discovery this neutralizing epitope of DEN-2 may be useful for vaccine development in the future.

KEY WORDS: Dengue, Neutralizing monoclonal antibodies, Phage display, B-cell epitope, dengue vaccine

INTRODUCTION

Dengue viruses (DEN) are mosquito-borne pathogens of the family *Flaviviridae* and are transmitted to humans by *Aedes aegypti* and *Aedes albopictus* mosquitoes. DEN causes serious febrile illness in humans, including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Henchal and Putnak 1990). The number of annual dengue fever (DF) cases worldwide is estimated to be 50 - 100 million, and 250,000 to 500,000 cases of dengue haemorrhagic fever (DHF) (Gubler 1997; Gubler and Clark 1995). Dengue is the most important human viral disease transmitted by arthropod vectors and over half of the world's population lives in areas at risk of infection (Rigau-Perez et al., 1998). The occurrence of DHF/DDS have been strongly associated with sequential infections with two different serotypes of DEN virus. Infection with the second serotype of DEN can be enhanced by the presence of non-neutralizing cross-reactive antibody acquired during the previous infection with a different serotype (Halstead, 1988; Halstead, 1989). DHF and DSS are believed to be the result of an ADE of the viral infection, caused by the uptake by monocytes/macrophages of virus complexes to nonneutralizing, cross-react or to low-titer neutralizing antibodies (Halstead, 1988; Bielefeldt-Ohmann, 1997; Halstead, et al. 1984). Because some patients develop DHF during primary dengue virus infection, viral virulence may also contribute to the pathogenesis of DHF and DSS (Rosen 1977; Gubler et al., 1978). Furthermore, some reports also mention that antibodies against dengue virus E proteins cross-reactive to human plasminogen during the immune response to dengue virus infection (Markoff et al., 1991; Chungue et al., 1994; Huang et al., 1997). The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells and may potential implications in dengue hemorrhagic fever (Falconar, 1997).

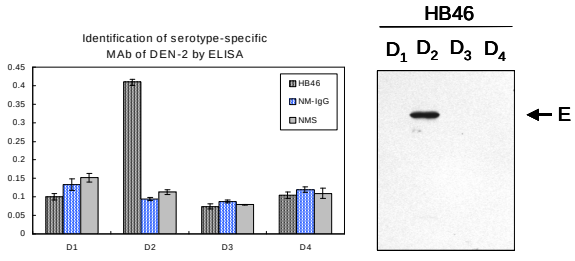
Recent advance in peptide technology has allowed the development of combinatorial peptide libraries expressed either on a solid phase support or displayed on bacteriophages. The high molecular diversity displayed by these libraries provides the possibility to study B-cell epitope mapping (Scott and Smith 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 neutralizing 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 neutralizing 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.

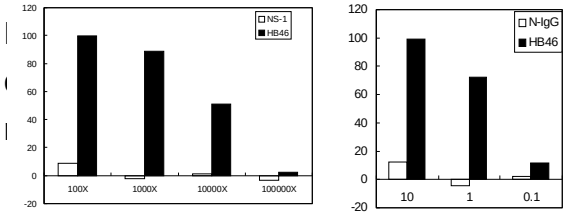
RESULTS

Functional characteristics of neutralizing monoclonal antibodies

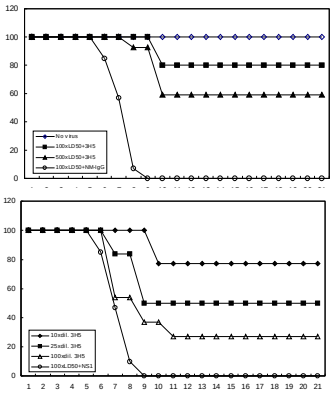
3H5 MAbs that exhibited highly neutralizing and protective activities against DEN-2 was proved in this study. 3H5 recognized E protein of DEN-2 and is a DEN-2 serotype-specific MAbs. (Fig. 1B). Using the ELISA assays, we also identified the 3H5 was specific for DEN-2 and did not cross-react with DEN-1, 3, and 4 (Fig 1A).



The neutralizing activities of the MAbs were measured in BHK-21 cells by using the 50% plaque reduction end point ($PRNT_{50}$; Russell, et al. 1967). 3H5 (ATCC HB46) is a DEN-2 specific MAbs and shown to have a significantly high neutralization titer ($PRNT_{50}$: 10,000-fold dilutions) in this study (Fig. 2A) and previously reports (Gentry et al., 1982; Henchal et al., 1985; Kaufman et al., 1987). Control normal ascites (NS-1) has no neutralizing activity (Fig. 2A). The neutralizing activities of purified IgG of 3H5 also showed in Fig. 2B, 10 and 1.0 μg/ml of purified 3H5 inhibited 99.2% and 72.5% of DEN-2 infection, respectively (Fig. 2B).



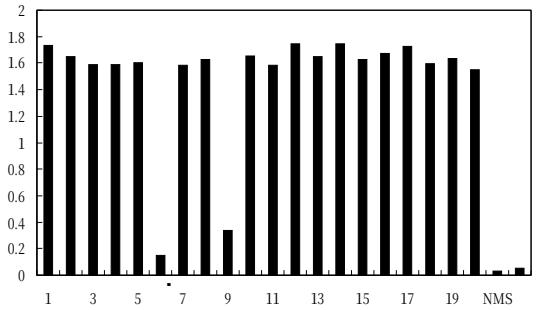
The protective activities against DEN-2 were demonstrated by challenging ICR suckling mice with lethal doses of DEN-2. A 50% lethal dose (LD_{50}) of DEN-2 was determined by i.c. injection to ICR mice as described in Methods. The protective ability of the MAb was determined by using them against 10^2-folds LD_{50} of DEN-2 and comparing their activities. Our results revealed that 3H5 protected 80% of the mice from 10^2 times the LD_{50} of DEN-2 challenge (Fig. 3A). This MAb protected or prolonged the life of the mice against 10^2 times the LD_{50} of DEN-2 challenge revealed dose-dependent manner (Fig. 3B). Otherwise, all of the mice died when 10^2-folds LD_{50} of DEN-2 incubated with control ascites were used (Fig. 3).



3H5 against DEN-2 showed good neutralizing activity and protective ability. These results indicate that neutralizing MAb recognized epitopes on DEN-2 could protect animals against lethal DEN-2 challenge. Identification of the specific protein sequences of these protective epitopes is valuable for investigation the immunopathogenesis of DHF and as a step toward the design of safe and efficacious DEN vaccines. Therefore, we further identify the B-cell epitopes of these protective MAbs by phage display method.

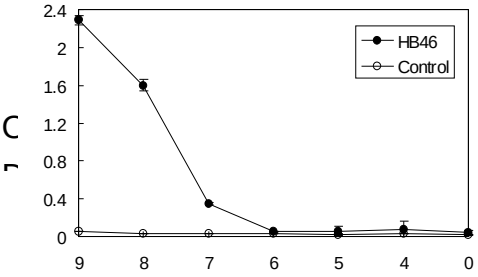
Screening of phage-displayed peptide libraries with neutralizing antibody

To study the B-cell epitope of neutralizing MAb against DEN-2, the phage display method was performed. The affinity-purified antibodies were immobilized on the ELISA plate and the bound phage clones were selected after three biopanning cycles. The selected phage clones produced by 3H5 showed significant increases in reactivity to its antibody. Eighteen of 20 selected phage clones (HB46-1, 2, 3, 4, 5, 7, 8, 10, 11, 12, 13, 14, 16, 17, 18, 19 and 20), which showed significant enhancement of reactivity to antibody 3H5 (Fig. 4), did not bind to normal mouse serum (NMS) and normal mouse IgG (Fig. 4).



To further confirm that the immunopositive phage clone bound 3H5 specifically, the antibody was incubated with a ten-fold serial dilution of selected (HB46-1) and control phage clones (control, selected by other MAb). The results showed that only the 3H5 selected phage clone (HB46-1) bound its antibody specifically in a dose-responsive

manner, whereas the control phage clone did not react with the antibody (Fig. 5).



Identification of neutralizing epitopes

Eighteen (HB46-1, 2, 3, 4, 5, 7, 8, 10, 11, 12, 13, 14, 16, 17, 18, 19 and 20) immunopositive phage clones highly reactive with neutralizing antibodies 3H5 were further sequenced. The phage-displayed 12 residues peptide sequences were aligned using the MacDNASIS software to analyze the epitopes of the neutralizing antibodies. All of these phage-borne peptides had displayed a peptide sequence of **XXXXXX**SELEXXW that mimicked the sequence, which corresponded to the amino acid residues 51-56 of the envelope protein (E protein) of DEN-2.

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. Viral surface proteins are generally major determinants of host range, cell tropism and virulence or attenuation. Investigation of neutralizing epitopes on the E proteins may provide the framework for a detailed understanding of specific mechanisms of the viral infection; domain participates in attachment to a cellular receptor and vaccine design. In this report, we identified 3H5 MAb that exhibited highly neutralizing and protective activities against DEN-2.

The problems of developing new vaccines include limited material for vaccine production; biohazard considerations; genetic variations of the pathogen; and poor immunogenicity in young infants due to the presence of maternal antibodies. There is an urgent need for development of improved vaccines, which will be effective in children even in the presence of maternal antibodies. New approaches for vaccine design are clearly required and the development of epitope-based vaccines represents one important approach.

3H5 (ATCC HB46) was a DEN-2 specific monoclonal antibodies and shown to have a significantly high neutralization titer (Gentry et al., 1982; Henchal et al., 1985) as well as in this study. The binding site of 3H5 was linear and may be mapped to residues 386-397 of E protein (Trirawatanapong et al., 1992), but a synthetic peptide containing this sequence has failed to bind to the 3H5 (Yao et al., 1995). Therefore, B-cell epitope of this antibody is not well document. In this study, we have identified the B-cell epitope of 3H5 using phage display method. Identification the neutralizing epitope of DEN-2 may be useful to develop epitope-based vaccine against dengue viral infection.

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