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Selection and identification of B-cell epitope on NS1 protein of dengue virus type 2

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ABSTRACT

NS1 of dengue virus (DENV) is an important non-structural protein, which plays an important role in DENV replication and dengue infection. In this study, using the phage-displayed peptide library screening method and purified anti-DENV2-NS1 polyclonal antibody immunoglobulin G (IgG) as target, which was generated from the purified recombinant expressed DENV2-NS1 protein immunization on rabbit, seven B-cell epitopes of DENV2-NS1 protein were screened. Considering the results of comprehensive bioinformatic analysis on NS1 B-cell epitopes, possible dominant B-cell epitopes are located in amino acids residues 36–45, 80–89, 103–112, 121–130, 187–196, 295–304, and 315–324 of the NS1, and two epitope-based NS1 protein dodecapeptides corresponding to the predominant epitopes (PA10: ^{36}PESPSKLASA^{45} and AA10: ^{187}AIKDNRAVHA^{196}) were chosen for synthesis. Results of binding assay and competitive-inhibition assays indicated the two peptides were the specific epitopes of DENV2-NS1 protein. These epitopes could be useful in understanding the pathogenesis of DENV and as dengue vaccine constituents in further study.

1. Introduction

Dengue viruses (DENV1, 2, 3, and 4), members of the family *Flaviviridae*, have a single-stranded, positive-sense RNA genome of approximately 11 kb. Dengue infections may be flu-like self-limited dengue fever or life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which are a serious cause of morbidity and mortality in most tropical and subtropical areas of the world (Gubler, 2002; Rigau-Perez et al., 1998). However, presently the pathogenesis of dengue is poorly defined, and the protective versus the pathogenic nature of the immune response to DENV infection is as yet unclear. DHF/DSS have an immunological basis including antibody-dependent enhancement and other antibody-mediated events, T-cell activation, cytokine cascades, and complement and other mediates (Pang et al., 2007). There are lack of available effective vaccines and specificity anti-DENV drugs.

The dengue virus non-structural NS1 protein is the only glycoprotein among its 7 NS proteins, which is an obbligato component in the DENV replication complex. All non-structural proteins are intracellular proteins with the exception of dengue NS1 protein, which exists as secreted as well as a membrane-associated protein. Both forms are demonstrated to be immunogenic (Flamand et al., 1999; Mackenzie et al., 1996). The molecular weight of NS1 is about 42–50 kDa and it approximately consists of 352 amino acids, which possesses both group specific and type specific determinants and has been postulated to contribute to the pathogenesis of dengue. First, elevated NS1 plasma concentrations early in illness are associated with more severe disease, possibly reflecting higher viral burdens in these patients (Avirutnan et al., 2006; Garcia et al., 1997; Libraty et al., 2002). The potential for early NS1 concentrations to predict clinical outcome has been postulated but not assessed. Next, it has been suggested that high NS1 levels may activate complement in solution and/or by directly binding endothelial cells, and may establish foci for immune complex formation leading to complement activation, endothelial damage and capillary leakage (Falconar, 1997; Lin et al., 2002, 2003; Mackenzie et al., 1998).

B-cell epitopes are defined as regions on the surface of the native antigen that are recognized and bind to B-cell receptors or specific antibodies. These epitopes are the focus of pathogenesis and immunological research as well as the targets of development of vaccine and diagnostic reagent (Vanniasinkam et al., 2001; Viudes et al., 2001). Therefore, the identification of B-cell epitopes for DENV-NS1 can provide important information for the development of dengue vaccine and contribute to the understanding of the pathogenesis of dengue and immunological responses in DENV infection. In the present study, we screened and identified B-cell epitopes of DENV2-NS1 using phage-displayed peptide library, anti-DENV2-NS1 polyclonal antibody immunoglobulin G (IgG) as target (Jiang et al., 2009), and an improved biopanning procedure, as well as bioinformatics comprehensive analysis.

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2. Materials and methods

2.1. Expression of recombinant NS1

The recombinant plasmid pPICZαB-NS1 containing the full-length gene of NS1 from DENV-2 virus *New Guinea C* strain (NGC) was constructed in our laboratory and the transformant *Pichia Pastoris*-NS1 was induced by methanol for expression as described before (Zhou et al., 2006).

2.2. Generation of polyclonal antibody (PAb) against NS1

For preparation of immunogen, the area of the SDS-PAGE gel corresponding to the bands representing NS1 protein were cut and comminuted with a proper volume of PBS. Adult male New Zealand rabbits were immunized subcutaneously with SDS-PAGE purified NS1 protein emulsified with an equal volume of Freund's complete adjuvant (CFA, Sigma, St. Louis, MO, USA). After 2 weeks, three booster injections were given at 1-week intervals with the same immunogen and equal volume of Freund's incomplete adjuvant. The polyclonal antiserum was analyzed by Western blot and enzyme-linked immunosorbent assay (ELISA). Anti-NS1 polyclonal antibody was purified by caprylic acid–ammonium sulfate precipitation and protein A resin (GenScript, USA), then, the specificity, sensitivity, and valence of the antibody were detected by Western blot and ELISA (Jiang et al., 2009).

2.3. Phage-display biopanning procedures

Four cycles of biopanning with affinity bead capture were performed. A microfuge tube was transferred with 50 μl 50% aqueous suspension of protein A agarose and added with 1 ml TBS + 0.1% Tween (TBST) for washing. The resin was suspended in 1 ml of blocking buffer and incubated for 60 min at 4 °C. The Ph.D.™ phage display 12-mer peptide library (New England Biolabs, Inc., Beverly, Mass.), which is a random dodecapeptide libraries, was diluted to 1.5×10^{11} phage particles, added with 300 ng of the antibody (10 nM) to a final volume of 200 μl with TBST and incubated for 20 min at room temperature. The phage–antibody mixture was washed 4 times with 1 ml of TBST after the blocking reaction and then transferred to the tube containing the washed resin and mixed gently for 15 min at room temperature. The tube was then washed 10 times with 1 ml of TBST. Bound phage was eluted by suspending the resin in 1 ml elution buffer (0.2 M glycine–HCl, pH 2.2, 1 mg/ml BSA) and incubated for 10 min at room temperature. The elution mixture was centrifuged for 1 min in a low-speed benchtop microcentrifuge and then the supernatant was transferred carefully to a new microfuge tube. The eluate was neutralized immediately with 150 μl 1 M Tris–HCl (pH 9.1). The eluted phage was amplified in 20 ml ER2738 culture at 37 °C with vigorous shaking for 4.5 h and amplified phage was titrated onto LB medium plate containing IPTG and X-gal. The protocol for second-round panning was almost identical to the first, with the addition of $1–2 \times 10^{11}$ pfu from first-round panning phage to each microfuge tube. In addition, a negative selection procedure was performed in which the amplified phage was pre-incubated with the bead in the absence of the antibody. The supernatant was then reacted with the antibody in a positive selection. Third- and fourth-round panning were identical to the second.

2.4. Identification of phage clones by ELISA

Individual target-combined phage clones were prepared after fourth-round biopanning. The ELISA plates were coated with anti-NS1 PAbs at room temperature for 2 h and blocked at 4 °C overnight. Phage clones which had been diluted to 10^{12} pfu phage particles were added to the antibody-coated plates and incubated at room temperature for 1 h. The plates were then washed with washing buffer, and 1:5000-diluted horseradish-peroxidase (HRP)-conjugated anti-M13 antibody (Amersham Biosciences, Pharmacia) in blocking buffer was added. The plates were incubated at room temperature for 1 h with agitation, washed with washing buffer, and incubated with the peroxidase substrate azinobis(3-ethylbenzothiazole sulfonic acid) diammonium salt (ABTS, Pharmacia). The reaction was stopped with 3 N HCl and the plates were read using a microplate reader (Multiskan MK3, Thermo Labsystems, Shanghai, China) at 405 nm.

2.5. Phage DNA sequencing and computer analysis

The amplified immunopositive phage clones were precipitated by 2/7 volume of PEG/NaCl (20% [w/v] polyethylene glycol–8000, 2.5 M NaCl). The phage pellet was resuspended in 100 μl of iodide buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA, 4 M NaI) with 250 μl of ethanol, and incubated at room temperature for 10 min. Phage DNA was isolated from the pellet after centrifugation at 14,000 rpm for 10 min at 4 °C, washed in 70% ethanol, dried, and resuspended in 30 μl TE (10 mM Tris–HCl [pH 8.0)], 1 mM EDTA). DNA sequences of purified phages were determined according to the dideoxynucleotide chain termination method using an automated DNA sequencer (ABI PRISM 377; PerkinElmer) by Invitrogen Company. Sequences of DNA inserted into target phage clones were translated into amino acid sequences and compared with that of NS1 protein of DENV2 which were retrieved from the GenBank site (http://www.ncbi.nlm.nih.gov/) and Iubio Archive site (ftp://ftp.bio.indiana.edu/) using Standard protein–protein BLAST [blast] and Clustal W Multiple Sequence Alignment [clustal] public software.

2.6. Bioinformatics aggregate analysis of DENV2-NS1 B-cell epitopes

Using multiple bioinformation software online (http://www.expasy.org/tools/protscale.html), we performed general evaluation of DENV2-NS1 B-cell epitopes including Hopp & Woods hydrophilicity, Welling antigenicity, polarity, accessibility, flexibility, and secondary structure. In addition, Wu Yuzhang antigenicity index was calculated (Wu and Zhu, 1995). Considering the results of phage biopanning together, we chose two predominant epitopes to synthesize for further study. The epitope-based dodecapeptides were synthesized in the form of an eight-branch multiple antigen peptide (MAP) construct (purity >90%, Jetway Biotech company, Guangzhou, China).

2.7. Properties analysis of synthetic peptides

2.7.1. Antibody-binding assay

ELISA plates were coated with 50 μl per well of individual synthetic peptides at a concentration of 10 μg/ml and blocked with 2% BSA-PBS. For the sensitivity binding assay, 2-fold serial peptide antigens (concentrations ranging from 2 to 0.0625 μg/ml) were coated to the plates. Anti-DENV2-NS1 PAb diluted in 1:500 was added to each well and incubated at room temperature for 1 h. After being washed, the wells were incubated with corresponding HRP-conjugated second antibodies for 1 h, then tetramethyl benzidine (TMB) to develop the color, and finally, optical density (OD) values at 405 nm were measured. Normal rabbit and mouse sera were used as negative control. PBS was used as blank control.

2.7.2. Competitive-inhibition assay

In competitive-inhibition experiments, coating with anti-DENV2-NS1 PAb, blocking, and washing were performed as