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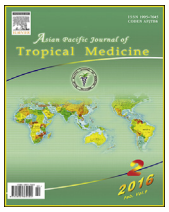

Original research http://dx.doi.org/10.1016/j.apjtm.2016.01.019

Recognition of a multiple antigen peptide containing sequence from mimotope of the dengue type 3 virus NS4B protein by human antibodies

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ARTICLE INFO

Article history:
Received 15 Nov 2015
Received in revised form 20 Dec 2015
Accepted 30 Dec 2015
Available online 11 Jan 2016

Keywords:
MAP
Antibodies
NS4B
Dengue virus

ABSTRACT

Objective: To evaluate the recognition of NS4B mimotope, as multiple antigen peptide (MAP), by dengue antibodies presents in serum samples from patients with different serotype infections.

Methods: A MAP containing mimotope sequence was synthesized and used to evaluate the recognition of NS4B mimotope as MAP by a panel of 66 human sera from dengue cases by an indirect ELISA assay.

Results: The MAP differentiated between sera from dengue viruses infected patients and sera from healthy individuals and the best reactivity was shown by serum from dengue type 3 virus patients. The recognition was more intense with serum from patients with secondary infection.

Conclusions: The findings suggest the potential use of NS4B mimotope on the development of a multi-epitope diagnostic tool. These results are important for further immunogenicity studies.

1. Introduction

Dengue viruses (DENV) belong to the family *Flaviviridae*, and the genome encodes 3 structural proteins: the capsid (C), precursor membrane (prM), envelope (E) and 7 non-structural proteins (NS1–NS5). Robust antibody responses are generated to 3 proteins: E, which contains 3 distinct domains, I, II, and III (potent neutralizing activity); prM, which augments infectivity of poorly infectious immature virions; and NS1, which directs complement-mediated lysis of infected cells [1]. Serological studies in humans have suggested, that after a DENV infection, people develop serum antibodies against some of the non-structural proteins (NS1, NS3, NS5) [2]. However, few studies have been carried out to characterize the immune response to others non-structural proteins [3].

The advent of the phage-displayed peptide technology, in which large, complex libraries of filamentous bacteriophage bearing random peptide sequences on their coat proteins can be generated and screened with antibodies, has provided a new approach to identify previously unknown antigens and/or new epitopes on already known antigens [4]. In this approach, phage-displayed peptide libraries are screened with sera from patients who have suffered a particular disease or pathological condition to discover peptide epitopes that are specifically recognized by patient's sera [5–7].

The present study extends previous results obtained with the mimotope of the non-structural NS4B DENV protein. The NS4 protein is composed of two distinct domains: NS4A and NS4B which may play a role in viral replication [8], however, the role in humoral immunity of NS4B has been poorly studied. Nevertheless, detection of antibody specific to purified recombinant GST-NS4B antigen was reported in serum samples from dengue patients [9]. Here we evaluate the recognition of NS4B mimotope as MAP by dengue antibodies in serum samples from patients with different dengue serotype infections.

2. Materials and methods

2.1. Serum samples

All the serum specimens used in the study came from a human sera collection stored at −20 °C. To obtain these sera, all procedures followed were in accordance with the ethical

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Peer review under responsibility of Hainan Medical College.

standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 [Cuadernos de Bioética (Bs As) 2010; 15/16:289–348].

Sera collected from 66 dengue confirmed adult patients and stored at the Dengue Sera Bank of the Virology Department at the 'Pedro Kouri' Tropical Medicine Institute of Havana in Cuba were analyzed in this study. Dengue diagnostics was confirmed by virus isolation and identification, RT-PCR and IgM-IgG determination. Cases were classified in primary or secondary infection according to anti-dengue IgG level [10,11]. Serum samples were collected five to seven days or two months after the onset of fever. Sera from healthy donors were obtained from the Cuban National Blood Bank and used as a negative control.

2.2. Synthetic peptides

Peptides consisting of the mimotope from NS4B DENV protein, mimotope from E DENV protein (as positive control of antigen) [12] and mimotopes from Hepatitis A virus (HAV) (as negative control of antigen) [13] were synthesized at the Synthetic Peptide Laboratory at the Center for Genetic Engineering and Biotechnology (Havana City) (Table 1). The peptides were named NS4B MAP, E peptide and MAP 46–56, respectively.

2.3. Indirect ELISA-NS4 MAP and controls

Multi-well plates (Nunc Maxisorp F8, Life Technologies Limited, Paisley, UK) were coated with 100 µL/well of NS4B MAP: 5 µg/mL, E peptide: 20 µg/mL or MAP 46–56: 5 µg/mL diluted in 0.05 M sodium carbonate buffer pH 9.6 (coating buffer), and incubated overnight at 4 °C. Plates were washed 3 times with phosphate buffered saline in 0.05% Tween 20 (PBS/T) and blocked with skimmed milk (3% w/v) in PBS/T (blocking solution) for 1 h at 37 °C. Human serum samples diluted in blocking solution (1:20, 100 µL/well) were added to the plates and incubated 1 h at 37 °C. After repeating the washes, bound antibodies were detected with HRP-conjugated anti-human IgG antibodies (Sigma–Aldrich, UK), H_2O_2 and *o*-phenylenediamine dihydrochloride (Sigma). The reaction was stopped with 0.1 M H_2SO_4. The absorbance at 492 nm was measured by an automated ELISA reader. All samples were run in duplicate and results were expressed as mean values of optical density (OD) ratios.

Control ELISAs were run in parallel, in which the E peptide and HAV MAP were used. Each ELISA was performed twice.

Positivity criteria: The formula ratio = P/N where P is the OD obtained in the sample and N is the OD mean values obtained with sera of healthy donors for the screening of the serum sample with NS4B peptide was employed. A sample with a ratio $\geq$ 2 was considered positive.

Table 1

Sequence of mimotopes contained in synthetic antigens.

Peptide constructs	Sequence
NS4B MAP (four arms)	FERVPEGV
E peptide linear	LGQSVGQDS
HAV MAP/46–56 (four arms)	46 SHSQLGPPVGPP 56 SHSVTKSLRVFGGPP

2.4. Statistical analysis

The GraphPad Prism statistical software (San Diego, CA, USA) was used. The Mann Whitney test was used to compare the results of OD ratios obtained in each serotype and values of $P < 0.05$ were considered significant.

3. Results

To analyze the potential of NS4B mimotope as MAP to behave as antigen, their ability to detect anti-DENV antibodies was assessed. ELISA screening was performed with human sera collected from dengue patients with NS4B MAP. The reactivity of 30 serum samples from secondary cases to DENV-1 (n = 10), DENV-3 (n = 10) and DENV-4 (n = 10) is shown in Figure 1. Serum samples of DENV-2 infection were not available for the study. The OD ratio values for DENV-3 were statistically significant when compared with DENV-1 (P = 0.03) and DENV-4 (P = 0.04).

Dengue antibodies from the studied samples were not detected with MAP 46–56 used as control, demonstrating a specific recognition of NS4B MAP by antibodies produced in response to the natural dengue infection. Figure 1 shows the results obtained with dengue 3 sera against MAP 46–56.

The unpaired sera of 48 patients from DENV-3 positive cases (20 serum samples collected between five to seven days, and 28 collected after two months of fever onset) were used to compare the response to NS4B antibodies by ELISA-NS4B MAP (Figure 2). These patients were classified in primary or secondary type of infection. NS4B MAP was recognized strongly by samples collected at five to seven days in secondary infection (70%) and only 2 were positive in primary cases. Sera collected $\geq$ two months showed the highest percentage of recognition in both primary (93%) and secondary cases (100%); however, the OD ratios were lowest in relation with secondary cases (samples five to seven days). A greater reactivity to E peptide, used as positive control, was observed in sera collected at the convalescent phase of illness (data not shown).

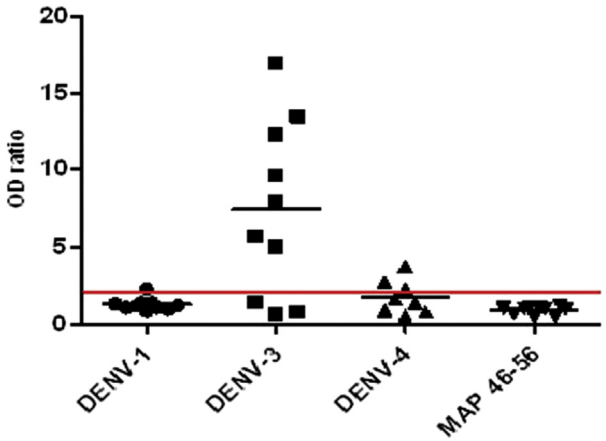

Figure 1. ELISA reactivity of human sera to NS4B mimotope as MAP. ELISA was performed using serum samples collected from confirmed DENV-1, DENV-3, DENV-4 patients. Results are expressed as OD ratio, and are presented for individual samples (10 samples to each serotype). MAP 46–56 was used as not related peptide (negative control). Values were considered as positive when OD ratio $\geq$ 2.0 and is represented by the dotted horizontal line.

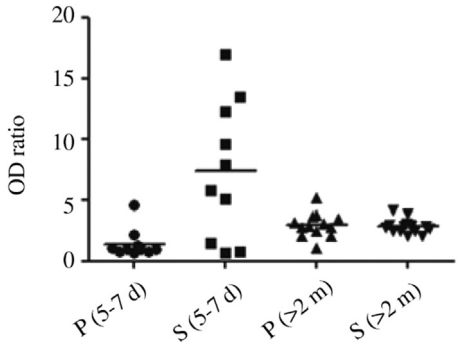

Figure 2. Recognition of NS4B MAP of serum samples from patients with primary (P 5–7 = 10 and $P \geq 2$ m = 13) or secondary (S 5–7 = 10 and S $\geq$ 2 m = 15) dengue 3 infections by an Indirect ELISA. Results are expressed as OD ratio and are presented for individual samples.

4. Discussion

One of the advantages of the phage display technology is to reduce complex protein antigens to small structured peptides that retain immune recognition. Mimotopes can potentially serve as lead compounds to develop low molecular weight substitutes of the template protein for the development of diagnostic assays [6,14]. This approach has been widely used in epitope mapping of flavivirus [15–18]. Recently, a mimotope of NS4B protein of DENV-3 was obtained by screening a solid-phase 9mer random peptide library using human immune sera. The selected sequences mimic the binding properties of natural antigen epitopes [19].

In this study, we detected antibodies against DENV from serum samples of dengue patients using the NS4B mimotope by indirect ELISA. The mimotope was synthesized in the MAP form, because the binding efficiency of an MAP is greater than that of a single-chain peptide. Short synthetic peptides are usually ineffective antigens for solid phase immunoassays owing to their poor ability to attach to solid surfaces. The multimeric nature of MAP constructs have been found to overcome these deficiencies and provide consistently reproducible results in increased surface-binding properties and sensitivity of detection [20]. The high reactivity with DENV-3 can be explained by the fact that the NS4B mimotope was selected with an anti-dengue 3 sera [19]. These data correlate well with the results reported by several authors who identified serotype specific epitopes using the phage-displayed peptide library screening method [15–18,21].

The serum antibody responses are different following primary and secondary DENV infections. In secondary infections, the stimulation of B-cell memory leads to a rapid rise in DENV-specific IgG that is measurable even on the first day of symptoms. Moreover DENV specific serum IgG titers are much higher in secondary compared to primary infections [1]. Valdes *et al.* [22] have demonstrated that the antibody response to NS5 and NS3 proteins in acute-phase samples from secondary cases is greater than in primary cases, including the intensity of the reaction, due to the high levels of IgG antibodies in secondary cases. The present study confirms these results, with the presence of anti-NS4B antibodies in acute-phase samples from secondary cases, using NS4B mimotope as MAP. Previously, NS4B MAP was shown to induce robust humoral response in mice and these antibodies were able to recognize NS4B protein, which is produced in the first step of viral replication [12]. On the other hand, 'in silico' analysis predicted potential B-cell epitopes on the NS4B protein including the epitope mimic of NS4B [23]. These preliminary data support the usefulness of NS4B MAP to detect antibodies to DENV in human sera suggesting that the NS4B protein of dengue virus could be implicated in the humoral response. Our findings suggest the potential use of NS4B mimotope for the development of a multi-epitope diagnostic tool. These results are important for further immunogenicity studies towards the evaluation of the mimotope as experimental vaccine.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors would like to thank Dr. Marta Romano for the critical reading of the manuscript.

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