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ORIGINAL ARTICLE

Identification of Dengue-specific B-Cell Epitopes by Phage-display Random Peptide Library

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Abstract

Background: **Dengue is the most important human viral disease transmitted by arthropod vectors. The availability of random peptide libraries (RPL) displayed on phage has provided a powerful tool for selecting sequences that mimic epitopes from microorganisms that are useful for diagnostic and vaccine development purposes. In this paper, we describe peptides that resemble the antigenic structure of B-cell epitopes of dengue virus identified from a phage-peptide library using human sera containing polyclonal antibodies against dengue virus.**

Materials and Methods: **Eighteen phage clones were isolated from the phage-display peptide library, J404, by affinity selection using human antisera against dengue virus type 3. These clones were tested for reactivity by ELISA with a panel of hyperimmune ascitic fluids (HAFs) containing antibodies either against all four dengue serotypes, West Nile virus (WNV) or Eastern equine encephalitis virus (EEEV) with control ascitic fluid (NAF) used as a negative control.**

Results: **Eight clones were recognized by HAFs against the four dengue serotypes, of which four significantly inhibited binding of anti-dengue antibodies to the virus. Two peptides with similar sequences to regions of NS3 and NS4B non-structural dengue virus proteins were identified.**

Conclusion: **Our results suggest that these peptides could be used for the development of diagnostic tools for the detection of dengue virus infection and for a potential vaccine against this pathogen.**

Keywords: *medical sciences, virology, dengue, epitopes*

Introduction

Dengue is the most important human viral disease transmitted by arthropod vectors. Annually, it is estimated that 100 million cases of dengue fever (DF) occur in tropical and subtropical regions, of which 500 000 result in dengue haemorrhagic fever (DHF) and 25 000 cases result in death. DF and DHF are caused by the four dengue viruses, DEN 1, 2, 3, and 4, which are closely related antigenically. Dengue virus belongs to the Flaviviridae family whose members are enveloped, positive-sense, single-stranded RNA viruses, such as those that cause Yellow fever, Japanese encephalitis, West Nile fever and hepatitis C (1). The flaviviral genome is translated as a single polypeptide that is post-translationally processed by cleavage into three structural proteins (C, M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (2). The E protein is considered to be the immunodominant protein (3). C-prM and prM proteins are able to induce an immune response and long lasting antibodies (4). The presence of antibodies against some non-structural proteins has also been demonstrated (5–9).

Prevention and control of DF and DHF has become more urgent with their expanding geographic distribution and increased disease incidence (10). Active laboratory-based surveillance and effective use of vaccines should be

components of disease prevention programs (11). Dengue diagnosis based on antibody identification has emerged as the most practical approach (1). Most methods of antibody detection rely on the use of whole dengue virus antigens produced in tissue culture or in suckling mouse brain. The use of such material is expensive and production costs associated with virus cultivation are generally high (12). Commercial kits are available for serological dengue diagnosis, but they still need careful evaluation. Although dengue diagnosis has improved, better tools are still needed for early, rapid, specific, sensitive and inexpensive diagnosis (13). One of the major difficulties associated with the development of a dengue virus vaccine is attributed to observations that most cases of DHF occur in individuals experiencing a secondary viral infection by a different dengue virus serotype, which therefore requires a safe and effective tetravalent vaccine (14). The absence of a suitable animal model, poor understanding of the pathogenesis of the disease, poor financial support and several other problems need to be solved before effective and safe dengue vaccines become available (13).

The availability of RPL displayed on bacteriophage has provided a powerful tool for selecting peptide sequences that mimic epitopes of infectious agents (15). Peptides mimicking epitopes of dengue virus proteins present in an RPL could be an alternative source of antigens for the development of diagnostic assays, and selection of peptides mimicking immunologically relevant B- and T-cell epitopes of dengue virus could be useful for disease prevention. B-cell epitopes of dengue proteins have been previously identified using mouse monoclonal antibodies (16–19). In the present work using human polyclonal antibodies against dengue virus, we report the identification of peptides capable of mimicking antigenic determinants of dengue virus non-structural proteins that could be useful in the development of a diagnostic kit or a potential antigen for vaccine production.

Materials and Methods

Human sera

The serum samples used in the study were obtained from the collection of the Arbovirus Laboratory, Department of Virology, "Pedro Kourí" Tropical Medicine Institute, Havana City, Cuba. All of the sera were tested for dengue virus-specific IgM and/or IgG antibodies (20) and by plaque reduction neutralization test (PRNT) (21). A panel of 21 sera was used, including 8 negative sera for IgM and IgG antibodies to dengue virus and 13 positive sera of dengue infection with DEN 1 (n=1), DEN 3 (n=11), and DEN 4 (n=1). All of the serum samples were classified as primary infection and showed IgG antibodies

Affinity selection: Reactivity of phage clones with hyperimmune ascitic fluids (HAFs) and a dengue anti-complex monoclonal antibody (H3/6).

The methodology used to identify dengue virus epitopes using polyclonal antisera is essentially similar to that described by Larralde et al. (22). The J404 bacteriophage-display peptide library (PDPL) (kindly donated by Dr. Jim Burritt, Montana State University, USA) (23), human serum samples positive for dengue virus type 3 antibodies and sera collected from healthy donors (negative controls) were used.

Clones derived from the affinity selection were immune-screened by slot blot with three positive sera against dengue virus type 3 and three negative sera as follows: serial dilutions of the phage sample were performed in LB broth and 100 µL of each dilution was mixed with melted soft agar, followed by the addition of TG1 Escherichia coli cells in stationary phase. This mixture was poured onto a solid LB agar plate followed by an overnight incubation at 37 °C. Isolated clones were picked using a Pasteur pipette, transferred to tubes containing 1 mL of LB broth and incubated overnight at 37 °C with gentle mixing. The supernatant was then transferred into a new tube and centrifuged at 6000 g for 30 min at 4 °C. Supernatants from phage clones and M13K07 (negative control) were titred and their concentrations were adjusted to $7.5x10^7$ pfu/mL. Fifty microlitres of each sample was applied onto a nitrocellulose membrane in a slot blot apparatus. The membranes were blocked with PBS containing non-fat dry milk (PBSNM) for 2 h at room temperature (RT) with four buffer changes. Three positive sera against dengue virus type 3 and three negative sera were pre-adsorbed with TG1 Escherichia coli extract and UV-inactivated M13K07 phage for 2 h at RT. The pre-adsorbed sera were added to the nitrocellulose discs

and incubated overnight at 4 °C with gentle mixing, followed by 10 washes with PBS/0.1% NP40. The washed membranes were incubated with alkaline phosphatase-conjugated goat anti-human IgG (Sigma, 1:5000 in PBSNM) for 4 h at 4 °C, washed and developed in NBT/BCIP chromogen for 2–5 min.

To confirm their specific reactivity with dengue virus antibodies, the selected phage clones were evaluated with HAF either against the four dengue serotypes, West Nile virus (WNV) or Eastern equine encephalitis virus (EEEV), and a control ascitic fluid (NAF) using an indirect ELISA. Briefly, multi-well plates (Nunc Maxisorp F8, Life Technologies Limited, Paisley, UK) were coated with 100 µL of anti-M13 monoclonal antibody (Amersham Pharmacia Biotech, UK) (10 µg/mL in 50 mM $NaHCO_3$, pH 9.6). Plates were incubated overnight at 4 °C, washed three times with PBS/0.05 % Tween 20 (v/v) (PBS-T) and blocked with PBS-T/5 % non-fat dry milk (w/v) for 1 h at 37 °C. Phage clones and wild-type phage (as controls) were added (100 µL/well) and incubated for 4 h at RT. Plates were washed three times with PBS-T and test serum was added (1/100, pre-adsorbed with TG1 E. coli extract and UV-inactivated M13K07 phage, for 4 h at RT). Plates were washed four times with PBS-T, incubated for 4 h at 37 °C with 100 µL/well of goat anti-human IgG/alkaline phosphatase conjugate (Sigma-Aldrich, UK) diluted 1:5000. They were then washed and developed with p-nitrophenyl phosphate substrate. The absorbance at 405 nm was recorded by an automated ELISA reader (Dynex Technologies, UK). For each serum sample, the average results from two independent experiments were evaluated. Values were considered positive when the ratio of absorbance of phage clones over absorbance of phage M13K07 (wild-type control) (P/N) was >2 and was more than twice the NAF. Phage clones with a P/N ratio of >2 in relation to NAF were discarded due to nonspecific reactions. Data were further analysed statistically by principal component analysis, cluster analysis and exploratory data analysis.

The ability of phage clones to be recognized by H3/6, a dengue anti-complex monoclonal antibody (25), was evaluated by the following ELISA: multi-well plates (Nunc Maxisorp F8, Life Technologies Limited, Paisley, UK) were coated with 10 µg/mL of H3/6 monoclonal antibody. Plates were incubated overnight at 4 °C, washed three times with PBS-T and blocked with PBS-T/5 % non-fat dry milk (w/v) for 1 h at 37 °C. Phage clones and wild-type phage (as controls) were added (100 µL/well) and incubated for 4 h at RT. Plates were washed four times with PBS-T, incubated for 4 h at 37 °C with 100 µL/well of goat anti-human IgG/alkaline phosphatase conjugate (Sigma Aldrich, UK) diluted 1:5000. The plates were then washed and developed with p-nitrophenyl phosphate substrate. The absorbance at 405 nm was recorded by an automated ELISA reader (Dynex Technologies, UK). Phage clones generated with the dengue anti-complex monoclonal antibody H3/6 (unpublished data) were used as positive control. Values were considered positive when the ratio of absorbance of phage clones over absorbance of phage M13K07 (wild-type control) (P/N) was >2.

Competitive inhibition assay

The ability of peptides displayed in the phage clones to compete with dengue virus for binding to antibodies present in the sera from dengue patients was evaluated by an inhibition ELISA (20). Sera positive for antibodies against DEN 1, 3 and 4 and negative sera were tested with and without pre-incubation with phage clones and M13K07 (109 phage particles). The percent inhibition of anti-dengue virus antibodies by the phage clones was estimated:

$$\%\ inhibition = 1 - \frac{\text{O.D serum sample without absorption by phages}}{\text{O.D serum sample with absorption by phages}} \times 100$$

DNA sequencing and similarity search

Selected phages were used to infect exponentially growing TG1 Escherichia coli cells. Infected cells were grown overnight in LB agar plates containing kanamycin (Sigma Aldrich, UK) at 75 µg/mL. Single colonies were picked and grown in LB broth containing kanamycin at the same concentration as above, and phage DNA was purified (QIAprep Spin Miniprep Kit, Qiagen, USA). The phage DNA was sequenced using a geneIII-specific primer, which anneals to 50 nucleotides from the 27-mer insert, as described Burrit et al. (26). Amino acid sequences were deduced using the GENERUNNER program. The phage-displayed peptide sequences were ran against the proteomes of the four dengue serotypes using the stand-alone BLAST program (27).

Results

Affinity selection: Reactivity of phage clones with hyperimmune ascitic fluids (HAFs) and a dengue anti-complex monoclonal antibody

Eighty-four phage clones were obtained after affinity selection of the RPL with a human serum sample containing a high titre of anti-dengue virus antibodies. Supernatants of the 84 isolated phages were tested by immunodot assay and ELISA, against three positive sera and also against three sera from non-infected individuals, resulting in the selection of 18 phage clones. These clones did not react with the negative sera. The reactivities of these 18 phage clones with different HAFs, as measured by ELISA, are shown in Table 1. Clones Ph2, Ph15, Ph24, Ph34, Ph35, Ph37, Ph79, and Ph84 showed the strongest antibody binding against serotype 2 and serotype 3. Clones Ph8, Ph26 and Ph64 were excluded because they reacted with NAF, suggesting a nonspecific reaction. The statistical analysis allowed the segregation of the phage clones into two clusters. Cluster 1 is comprised of the phage clones Ph2, Ph24, Ph34, Ph37, Ph79, and Ph84 and Cluster 2 is comprised of clones Ph15, Ph16, Ph26, Ph27, Ph27, Ph35, and Ph64 (Fig. 1A). The best recognition was obtained with HAF against dengue virus 3. Differences in the recognition of phage clones included in both clusters were not found by the HAF against WNV, EEEV, and the NAF negative control (Fig. 1B). None of the phage clones were recognized by the H3/6 monoclonal antibody (data not shown).

Competitive inhibition assay

The reaction of the eight previously selected phage clones with anti-dengue virus type 3 antibodies is shown in Table 2 as the percent inhibition of binding of anti-dengue 3 to dengue virus compared with the unabsorbed sera. Clones Ph2, Ph15, Ph35, and Ph37 inhibited the binding of anti-dengue 3 antibodies to the virus. The same clones were also evaluated with antisera against

Table 1: ELISA reactivity of HAFs to phage clones

Phage clone	HAF						
	D1	**D2**	**D3**	**D4**	**WN**	**EEE**	**NAF**
Ph2	1.23	3.00	3.36	0.88	0.61	1.20	1.28
Ph4	0.61	1.37	1.53	0.43	0.94	1.14	1.09
Ph5	0.59	1.15	1.32	0.50	1.15	1.30	1.11
Ph6	0.72	1.54	1.58	0.49	0.67	1.14	1.16
Ph8 [a]	1.70	5.80	6.56	1.95	2.05	1.31	2.08
Ph15	1.18	2.32	2.76	0.90	1.03	1.04	1.00
Ph16	1.15	1.43	1.28	0.47	0.97	1.29	1.03
Ph24	1.42	2.78	3.92	0.93	1.42	1.37	1.73
Ph26 [a]	1.09	1.23	2.07	0.80	1.89	1.40	2.08
Ph27	1.14	1.62	1.76	0.57	1.49	1.39	1.24
Ph34	1.31	4.10	5.75	1.37	1.42	1.09	1.55
Ph35	1.31	2.42	3.21	0.78	1.34	1.31	1.48
Ph37	1.24	2.82	3.91	0.84	1.21	1.36	1.85
Ph64 [a]	1.22	2.29	2.99	0.78	1.32	1.35	2.23
Ph78	1.22	2.26	2.69	0.70	1.16	1.63	1.68
Ph79	2.69	3.10	5.17	2.56	1.21	1.15	1.70
Ph84	1.57	3.46	4.30	1.48	1.81	1.38	1.71

Results are expressed as the ratio of absorbance of phage clones over absorbance of wild-type phage (P/N). Values were considered positive when (P/N) >2 and differed by more than twice the NAF value. Shaded cells indicate positive results.

[a] Discarded by reactivity with NAF

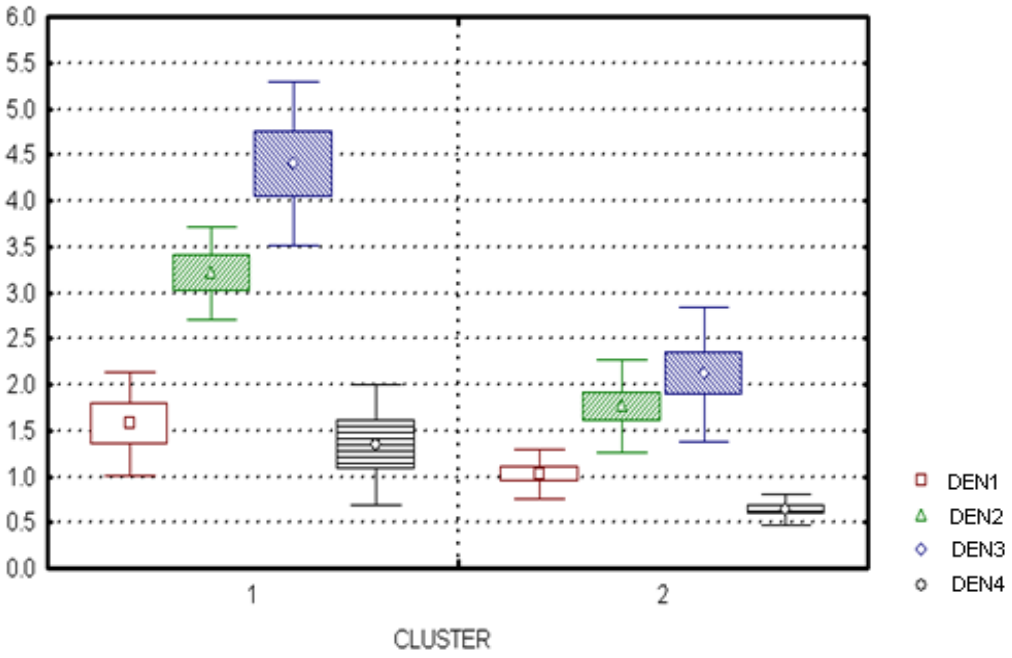

Figure 1A: Reactivity of phage clones by HAFs against DEN serotypes 1 to 4. Cluster 1 (Ph2, Ph24, Ph34, Ph37, Ph79, and Ph84) and Cluster 2 (Ph4, Ph5, Ph6, Ph15, Ph16, Ph26, Ph27, Ph35, and Ph64)

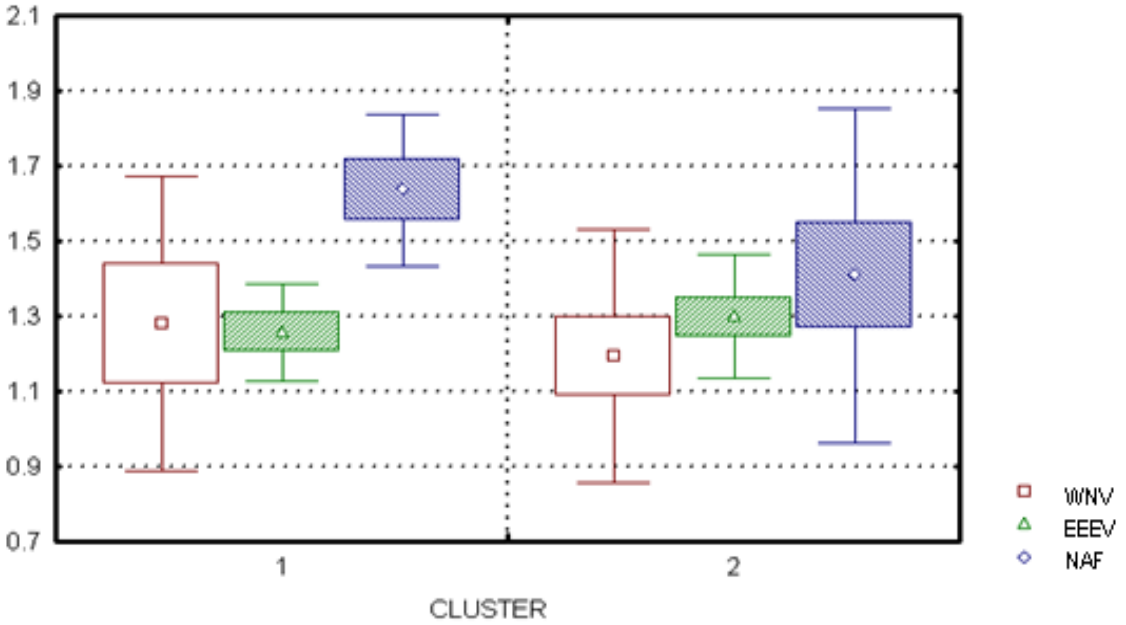

Figure 1B: Reactivity of phage clones by HAFs against WNV, EEEV and NAF.

Table 2: Inhibition of binding of anti-dengue 3 antibodies to dengue virus by phage clones

Phage clones	Inhibition (%)[a]		
	Positive sera DEN 3		
	Serum1	Serum 2	Serum 3
Ph2	21.73	4.55	9.86
Ph15	6.37	4.08	7.98
Ph24	1.34	1.01	0
Ph34	1.17	0.67	3.2
Ph35	4.24	8.99	3.58
Ph37	16.61	13.80	9.36
Ph79	-8.90	-13.79	0.81
Ph84	-5.57	-9.92	-2.54
M13	0.84	-14.02	-8.52

[a]Inhibition of binding was calculated as: % inhibition = 1–(O.D. without serum sample absorption by phages/O.D. with serum sample absorption by phages) x 100. M13 = wild-type phage M13K07. Phage clones with the highest percent inhibition of binding of antibodies to the virus were selected for the next experiment.

DEN 1, 3, and 4 (Table 3). Each of the clones inhibited each anti-serotype by approximately 13 to 46 %. Negative percent of the inhibition of binding of anti-dengue antibodies to dengue virus by phage clones were considered negative.

DNA sequencing and similarity search

The deduced amino acid sequences of clones Ph2, Ph15, Ph35, and Ph37 have a range of similarity from 50% to 70% with regions of the NS3 and NS4B dengue proteins of the four dengue serotypes. Figures 2A and 2B show the comparison of these peptides with NS3 and NS4B proteins of DEN serotype 3. The BLAST results were similar for all dengue serotypes. The peptide sequence, FERVPGEVT, was found in Ph2, Ph15, and Ph35 and exhibited several amino acids at the same position as the NS4B protein in residues 164-172 (dengue 1, 2 and 3), and 161-168 (dengue 4). Peptide RRALPPVSS from Ph37 showed a high similarity with two regions of the NS3 protein of the four dengue serotypes in regions corresponding to residues 425-432 and 537-544.

Discussion

Recent studies have shown that phage-displayed peptides selected using antibodies raised against pathological antigens can be important tools for both diagnosis and disease prevention (28-36). This approach has previously been used to identify serotype-specific epitopes of dengue virus using mouse monoclonal antibodies (16-19). In this work, peptides that resemble the antigenic structure of B-cell epitopes of dengue virus were identified from a phage-peptide library using human polyclonal antisera from patients who had recovered from dengue virus infection. Eighteen phage clones were isolated by the following procedure: affinity selection of the random peptide library with a positive serum containing a high titre of anti-dengue antibodies; screening by slot blot with a panel of antisera (three positive and three negative sera); ELISA using three positive and three negative sera. Assessing the reactivities of these 18 phage clones with different HAFs by ELISA facilitated the selection of eight dengue virus-specific phagotopes. The fact that they reacted only with HAFs against dengue and did not react with HAFs against WNV, EEEV, or NAF suggests that they do not share epitopes with these arboviruses. Clones Ph8, Ph26, and Ph64 were rejected based on their reactivity with other HAFs, so as to minimise the possibility of enriching for "false positive" clones which may display unrelated target peptides.

Table 3: Inhibition of binding of anti-dengue antibodies to dengue virus by phage clones

Phage clones	Inhibition (%)[a]				
	Positive sera			Negative sera	
	DEN 1	DEN 3	DEN 4	M30	M37
Ph2	39.0	27.08	13.3	1.033	1.069
Ph15	36.9	33.12	13.8	-7.20	-9.0
Ph35	46.01	35.5	35.9	2.04	8.81
Ph37	44.06	32.9	25	0.13	0.757
M13	1.05	1.80	1.08	0.59	0.23

[a]The inhibition of binding of anti-dengue antibodies to dengue virus is expressed as the percent inhibition compared to M13 (wild-type phage M13K07)

(A)

```
>YP_001531175|NS4B
          Length = 248

 Score = 12.7 bits (21), Expect = 4.9
 Identities = 4/8 (50%), Positives = 6/8 (75%), Gaps = 0/7 (0%)

Query: 1   FERVPGEV 8
           FE+  G+V
Sbjct: 164 FEKQLGQV 171
```

(B)

```
>YP_001531172|NS3
          Length = 619

 Score = 14.2 bits (26),  Expect = 1.6
 Identities = 5/7 (71%), Positives = 5/7 (71%), Gaps = 0/7 (0%)

Query  1     RRALPPV  7
             RR L PV
Sbjct  426   RRCLKPV  432


 Score = 10.0 bits (16),  Expect = 30
 Identities = 5/8 (62%), Positives = 5/8 (62%), Gaps = 2/8 (25%)

Query  1     RRA-LPPV  7
             RR  LP V
Sbjct  538   RRGDLP-V  544
```

Figure 2: Deduced amino acid sequences of mimotopes by BLAST search against the dengue virus serotype 3 proteome. (A) Pairwise alignment of peptide FERVPGEVT displayed on Ph2, 15 and 35 with NS4B protein. (B) Pairwise alignment of peptide RRALPPVSS displayed on Ph37 with NS3 protein. The BLAST results were similar for all dengue serotypes

Phage panning is a very dynamic process that is influenced by affinity, avidity, the nature of target and the combined impact of multiple experimental parameters (29). The serological diagnosis of dengue virus infection is complicated by the existence of cross-reactive antigenic determinants shared by all four dengue serotypes and some other flaviviruses (38). The absence of cross-reactivity between HAFs against WNV and the selected phage clones makes these phage-peptides very attractive for diagnostic purposes. The use of anti-dengue 3 sera in the selection process could explain the optimal recognition of phage clones included in both clusters by HAFs against dengue 3. H3/6 has been characterized as a dengue anti-complex monoclonal antibody specific to the E protein that is non-reactive with other flaviviruses (25). The fact that none of the phage clones was recognized by this monoclonal antibody suggests that they do not display peptides mimicking the epitope of the E protein recognized by this monoclonal antibody.

Competition ELISAs with the original antigen are necessary to ensure that the phage clones are specific for the antigen binding site of the antibody (29). Inhibition of the reaction of the human sera positive for dengue virus antibodies after absorption with the phage clones supports the hypothesis that the peptides mimic dengue virus epitopes and block the reaction of serum antibodies with the virus. Differences in absorption of the antibodies by the different phage clones can be explained by differences in the concentrations of antibodies against the corresponding epitope and the affinity of the antibodies for the mimotope. Only four phage clones were able to compete with the virus for binding to dengue virus antibodies. Peptides exposed on these clones could mimic specific dengue mimotopes. Competitive assays are particularly useful in cases in which a large panel of sera containing antibodies of the studied entity is not available or when it is not possible to sequence all of the selected clones.

Two peptides mimicking B-cell epitopes of the NS3 and NS4B non-structural proteins of the four dengue virus serotypes were identified. Three phage clones (Ph2, Ph15, and Ph35) displayed peptides with the same amino acid sequence (FERVPGEVT). This peptide shares partial homology with the NS4B dengue protein. Although NS4B has not been previously reported as one of the principal proteins involved in dengue virus antibody responses, consistent antibody responses to NS4B were recently found in a study of 100 sera samples from dengue patients that were tested against recombinant NS4B by ELISA (9). Phage clone Ph37 displays a peptide that is similar to two regions of the NS3 protein of the four serotypes. This peptide shares five residues with the amino acid region 537–547. The NS3 amino acid region 537–547 is highly conserved with at least 80% identity between a total of 44 strains from the four serotypes (39). In the same study, this sequence was not shared with 64 other flaviviruses, suggesting that this sequence is dengue virus-specific, which corroborated the results obtained for reactivity with HAFs. It has been proposed that this peptide could possibly function in cell attachment (39). The peptide expressed on clone Ph37 also exhibited similarity with five residues in the NS3 region (amino acids 421–481). This sequence has been reported as a strong inducer of T-cell responses in dengue virus-infected patients (40). Although the NS3 protein induces a strong T-cell response, and a preponderance of T-cell epitopes have been identified (41), the functional significance of antibody responses against this protein remains to be elucidated (5–9). Further studies should be performed to determine the participation of the selected peptides in T-cell responses. There have been several reports of the identification of B-cell epitopes of dengue virus using serotype-specific monoclonal antibodies of dengue virus (16–19). In our work, two peptides were selected using serum samples of confirmed dengue patients, suggesting this method could be used to develop a reagent for the diagnosis of dengue patients.

During a primary infection, individuals develop IgM after 5–6 days and IgG antibodies after 7–10 days. During a secondary infection, high levels of IgG are detectable even during the acute phase and they rise considerably over the next two weeks. IgM levels are lower and, in some cases, absent during secondary infection. The presence of IgM antibodies suggests a recent infection, although they are still present after 2–3 months. High titres of IgG are a criterion of secondary infection (13). The ability of the phage-displayed peptides to bind to IgG antibodies from dengue patients could be potentially useful to discriminate between primary and secondary infection.

Several studies have been conducted on antibody responses to non-structural proteins (9). A comparison of the amino acid sequences of the selected clones showed similarity with NS3 and NS4B proteins of dengue virus. Further investigations are needed to evaluate the immunogenicity of these peptides as experimental anti-dengue subunit vaccines. Synthetic peptide vaccines are relatively cheap, safe to produce, and heat stable. The antibody dependent enhancement (ADE) hypothesis emphasizes the importance of the immune response in the development of DHF/

Dengue Shock Syndrome (DSS). Therefore it is necessary to determine if antibodies against these peptides can enhance dengue virus infection and study its possible role in the immune-amplification phenomenon.

In the present study, two B-cell epitopes of dengue virus were identified using a phage-display peptide library and polyclonal anti-dengue virus antibodies. Our results suggest that these two peptides are immunologically important and could be used for the development of diagnostic systems and a potential vaccine against this pathogen.

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Author's contributions

Conception and design: NA
Data analysis and interpretation: NA, AA, FC, JCR, EMP
Data collection and assembly , drafting of the article: NA, AA
Critical revision of the article: FC, MP, DIS, EMP, A.Acosta
Provision of study materials or patients: FC, YV, MP
Statistical expertise: LI
Obtaining of funding: FD, DIS
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