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Serotype specificity of recombinant fusion protein containing domain III and capsid protein of dengue virus 2

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ABSTRACT

Recombinant fusion protein containing domain III of the dengue envelope protein fused to capsid protein from dengue 2 virus was immunogenic and conferred protection in mice against lethal challenge in previously report. Here, the antigenic specificity of this recombinant protein using anti-dengue antibodies from mice and humans and the cross-reactive humoral and cellular response induced in immunized mice were evaluated. The homologous anti-dengue antibodies showed a higher reactivity to the recombinant protein compared to the wide cross-reactivity observed for viral antigen as determined by ELISA. The IgG anti-dengue and functional antibodies, induced by the recombinant proteins in mice, were highly serotype specific by ELISA, hemaglutination inhibition and plaque reduction neutralizing tests. Accordingly, the cellular immune response determined by the IFNγ and TNFα secretion, was serotype specific. The specificity of serotype associated to this recombinant protein in addition to its high antigenicity, immunogenicity and protecting capacity suggest its advantage as a possible functional and safe vaccine candidate against dengue in a future tetravalent formulation.

1. Introduction

Dengue virus (DEN) belongs to the genus flavivirus of the family Flaviviridae. The four DEN serotypes (DEN-1, DEN-2, DEN-3, and DEN-4) are the leading cause of arboviral diseases in the tropical and subtropical areas (Guzman and Kouri, 2002). While most DEN infections are asymptomatic or result in a self-limited illness, dengue fever (DF), some people may present with the severe and potentially life-threatening disease dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Gubler, 2002; Guzman and Kouri, 2002).

The infection with one DEN serotype presumably results in life-long immunity to that serotype, but does not confer immunity to the other serotypes (Gubler, 1998). In fact, DHF/DSS is most often observed in individuals experiencing a secondary infection with a heterologous serotype (Burke et al., 1988; Guzman et al., 2010b; Sangkawibha et al., 1984). It has been postulated that cross-reactive antibodies and T cells are involved in the immunopathogenesis of this disease. Because of the lack of a good mouse model, the most of these studies were done using patient samples and were therefore descriptive in nature (Green and Rothman, 2006; Kurane, 2007; Rothman, 2009). *In vitro* studies have demonstrated that non-neutralizing concentrations or cross-reactive antibodies enhance the viral replication, suggesting that antibodies produced during previous infection or passively acquired contributes to DHF/DSS via antibody dependent enhancement (ADE) (Goncalvez et al., 2007; Kliks et al., 1989; Morens and Halstead, 1990).

In addition to antibodies, cross-reactive T cells may also contribute to the immunopathogenesis of secondary infections. It is hypothesized that low-affinity T cells raised against the original infecting serotype dominate during a secondary heterologous infection in a phenomenon termed 'original antigenic sin' (Mongkolsapaya et al., 2003). Human studies have found that serotype cross-reactive T cells are preferentially activated during secondary infection, and these cross-reactive T cells exhibit suboptimal degranulation and altered cytokine production (Imrie et al., 2007; Mangada and Rothman, 2005; Mongkolsapaya et al., 2003, 2006). Activation of cross-reactive memory T cells likely contributes to severe disease via the activation of innate immune cells and enhanced cytokine production (Rothman, 2010). Cytokines, such as tumor necrosis factor α (TNFα), may play a pathogenic role in DHF/DSS by causing endothelial cell dysfunction and subsequent plasma leakage, which is a hallmark of DHF/DSS (Green et al., 1999; Mangada et al., 2002).

There is currently no licenced vaccine for dengue. Nevertheless, there are several candidates at different stages of development live

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Glossary

DEN	dengue virus	HI	hemaglutination inhibition
DF	dengue fever	Mock	negative control
DHF	dengue hemorrhagic fever	HMAFs	hyperimmune mouse ascitic fluids
DSS	dengue shock syndrome	PHS	primary human sera
ADE	antibody dependent enhancement	LD50	median lethal doses
TNFα	tumor necrosis factor α	BSA	bovine serum albumin
E	envelope	GMT	geometric mean titer
CMI	cell-mediated immunity	PRNT	plaque reduction neutralization test
DIII-C-2	domain III-capsid protein of DEN-2	FBS	fetal bovine serum
ELISA	enzyme- linked immunosorbent assay	ConA	concanavalin A

attenuated virus, inactivated virus, subunit, vectored, and DNA vaccines (Guzman et al., 2010a; Swaminathan et al., 2010). Specifically, protein subunits as vaccine candidates for dengue constitutes the most potentially safe (Babu et al., 2008; Clements et al., 2010; Hermida et al., 2006; Robert Putnak et al., 2005; Valdés et al., 2009). Many of them use fragments of the domain III of the Envelope (E) glycoprotein, which is the major structural protein on the surface of the mature virion and contains neutralizing epitopes (Rey et al., 1995).

We have previously described the functionality of fusion proteins containing domain III of the E protein from DEN in terms of their induction of neutralizing antibodies and protection in mice and monkeys (Bernardo et al., 2008b; Hermida et al., 2006, 2004a,b; Valdés et al., 2009, 2009).

Moreover, recently, it has been recognized the cell-mediated immunity (CMI) as an important factor in mice protection against DEN (Gil et al., 2009; van der Most et al., 2003, 2000; Yauch et al., 2010, 2009).

Recently, a chimeric domain III-capsid protein, containing antigen fragments potentially inducer of both neutralizing antibodies and CMI, respectively, as a unique molecule has been obtained. This protein was able to induce humoral immune response, CMI, and confers protection in mice (Valdés et al., 2009).

Here we evaluate the antigenic specificity of the recombinant domain III-capsid protein of DEN-2 (DIII-C-2) using a panel of murine and human antibodies to the four DEN serotypes. In addition, the humoral and cellular response cross-reactivity in immunized mice was also assessed. As a result, a significant serotype specificity of the induced immune response was detected. This result suggests the possible use of this protein in a vaccine formulation with minimal risk to induce the immunopathological mechanisms upon vaccination.

2. Materials and methods

2.1. Recombinant protein

The recombinant protein DIII-C-2 contains the region corresponding to the domain III of the E protein of DEN-2 virus (Jamaica strain) fused at the N-terminus region of the capsid protein of the same dengue virus. The genetic construction, expression and immunological evaluation of the recombinant protein has been previously published (Valdés et al., 2009). The recombinant protein was purified to about 90% (Marcos et al., in press).

2.2. Virus strains

The standard strains, DEN-1 Hawaii, DEN-2 New Guinea C (NGC), DEN-3 H87, and DEN-4 H241 (kindly provided by Dr. Robert Shope) were grown in suckling mouse brain and extracted by the sucrose-acetone method. The obtained extract was employed as antigen for evaluating the humoral immune response by enzyme-linked immunosorbent assay (ELISA) and hemaglutination inhibition (HI) test. A similar preparation from non-infected mice brains was used as negative control (Mock) (Clarke and Casals, 1958). For animal immunization and virus challenge, infective preparations of previous DEN strains were employed.

Neutralization assay was performed using cell-culture supernatant from African green monkey kidney (Vero) cells (ATCC) infected with DEN-1 WP74, DEN-2 SP 16803, DEN-3 CH 53489 and DEN-4 TVP360 provided by the Department of Immunization, Vaccines and Biologicals, WHO (Roehrig et al., 2008).

A concentrated preparation of virus was used for the *in vitro* stimulation of mouse splenocytes. Supernatant from infected Vero cells (100 ml), with 10^6 pfu/ml of DEN-1 Angola, DEN-2 SB8553, DEN-3 CS81.1 and DEN-4 Dominica, was concentrated by centrifugation at 80,000*g* for 4 h at 4 °C. The pellet containing the virus was resuspended in 1 ml of PBS (Gibco, Paisley, UK). A mock preparation was similarly prepared from the supernatant of non infected Vero cells.

2.3. Antibodies and cells

Pools of polyclonal hyperimmune mouse ascitic fluids (HMAFs) corresponding to the four dengue virus serotypes were employed for the antigenic characterization of the recombinant protein. Also, serum samples collected after 15 days of onset of fever from 10 individuals with a primary infection to DEN-1, DEN-2, DEN-3 and DEN-4 were employed.

Sera collected from 14 week-old female Balb/c (Bc, H-2d) mice (CENPALAB, Cuba) immunized by intraperitoneal route with 23 μg of DIII-C-2 protein on schedule 0, 15, and 30 were used to determining the serotype specificity of the humoral immune response induced by the recombinant protein. The formulation containing the recombinant protein was prepared employing aluminum hydroxide (alum) (Alhydrogel) (Brenntag Biosector, Denmark) as adjuvant at a final concentration of 1.44 mg/mL. Spleen cells of immunized mice with the recombinant protein and DEN virus were employed for the serotype specificity study of the induced cellular immune response.

2.4. Animal protection assay

One month after the last dose, non-bled mice ($n = 10$) were injected intracranially (i.c.) with 20 μL of a suspension of DEN-1, DEN-2 and DEN-4 virus-infected suckling mouse brain containing 50% median lethal doses (50 LD50). Mice were observed daily for 21 days and deaths were recorded.

2.5. Antigenic characterization with HMAFs and human sera by ELISA

An indirect ELISA was used to determine the recognition of the recombinant protein DIII-C-2 with anti-DEN HMAFs and human

sera of dengue primary infection. Polystyrene plates of 96 wells MICROLON (Greiner bio-one, Germany) were coated for 2 h at 37 °C with 5 μg/mL of the protein and 100 μL of dengue 2 antigens diluted in coating buffer (0.16% Na_2CO_3, 0.29% $NaHCO_3$, pH 9.5). As negative control the plates were coated with 5 μg/mL of negative process of purification, corresponding to cells transformed with the pQE30 plasmid and 100 μL of Mock respectively. Subsequently, plates were blocked with 2% bovine serum albumin (BSA) for 1 h at 37 °C in the same buffer solution. Three washes with PBS (0.8% NaCl, 0.02% KCl, 0.014% KH_2PO_4, and 0.009% Na_2HPO_4, pH 7.4) containing 0.05% Tween 20 (Merck, Germany) (PBS-T) were completed after each step of the ELISA. After washing, HMAFs and human sera of each viral serotype were serially diluted and incubated for 1 h at 37 °C with the protein. Anti-mouse and anti-human IgG-peroxidase conjugate (Amersham-Pharmacia) were added. Plates were incubated for 1 h at 37 °C. After washing, 500 μg/mL *ortho*-phenylenediamine and 0.015% H_2O_2 in 0.1 mol/L citrate buffer (2% Na_2HPO_4, 1% citric acid, pH 5.0) was added. Plates were kept for 30 min at 25 °C and the reaction was stopped with 12.5% H_2SO_4. The microplate reader was adjusted at 492 nm (Biorad, USA). The positive cut-off value was taken as twice the absorbance value of the negative serum control.

2.6. Antiviral antibodies by ELISA

An amplified sandwich ELISA was used to detect anti-DEN virus antibodies. Polystyrene plates of 96 wells MICROLON (Greiner bio-one, Germany) were coated for 2 h at 37 °C with 100 μL per well of a mixture of anti-DEN human immunoglobulins (IgG) (5 μg/mL) in coating buffer and then blocked with 2% BSA in coating buffer for 1 h at 37 °C. Three washes with PBS-T were performed after each reaction step. The plates were incubated overnight at 4 °C with a saturating concentration of the viral antigen (suckling mice brain infected with DEN-1, DEN-2, DEN-3 or DEN-4 viral strains) and the negative control antigen (non-infected suckling mice brain) diluted in PBS-T. After a washing step, immune sera (serially diluted in PBS-T) were added and incubated for 1 h at 37 °C. Later, plates were incubated for 1 h at 37 °C with anti-mouse IgG-peroxidase conjugate (Amersham-Pharmacia). After washing, the substrate solution was added. Plates were kept for 30 min at 25 °C and the reaction was stopped with 12.5% H_2SO_4. Absorbance was read at 492 nm in a microplate reader (Biorad, USA). The positive cut-off value was set as twice the mean absorbance value of the negative control sera.

2.7. Hemaglutination inhibition assay (HI)

The presence of HI antibodies was determined as previously described by Clarke and Casals (1958). The HI antibody titers were estimated as the higher serum dilution resulted in the complete inhibition of the hemaglutination pattern produced by eight hemaglutinating units. The results were shown as the HI geometric mean titer (GMT) per group of sera.

2.8. Plaque-reduction neutralization test

Neutralizing antibody titers against to four DEN virus serotypes were evaluated by plaque reduction neutralization test (PRNT) in baby hamster kidney cells (BHK-21) as described by Morens et al. (1985) with the modification by Alvarez et al. (2005). The serum dilution that resulted in a 50% reduction of plaque count, as determined by probit analysis, was considered the end point titer. The MAb 4G2 was used as positive control, which recognizes the flavivirus E protein (Kaufman et al., 1987).

2.9. Cell culture and viral stimulation

Spleen cells were obtained in aseptic conditions. Erythrocytes were lysated by adding NH_4Cl 0.83% solution. Cells were washed twice with PBS-2% Fetal Bovine Serum (FBS) (PAA Laboratories, Ontario, Canada) and resuspended at 2×10^6 cells/mL in RPMI-1640 medium (Sigma Aldrich) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, UK), 2 mM glutamine (Gibco, UK), 5 × 10–5 M 2-mercaptoethanol (Sigma St. Louis, MO) and 5% FBS. Finally, 2×10^5 cells/well were cultured in 96-well round bottom plates with the antigens (10^3 pfu of dengue antigen or mock preparation). Concanavalin A (ConA) (Sigma St. Louis, MO) was used as a positive control. In all the experiments three wells were plated for each antigen. After 4 days of culture, culture supernatants were collected and stored at −20 °C.

2.10. Cytokine detection

The culture supernatants of splenocytes previously stimulated with each antigen were analyzed in duplicate to determine the IFNγ and TNFα concentration by ELISA using MAbs pairs (Mabtech; Nacía, Sweden). ELISA protocol recommended by manufacturers was used with minor modifications. The lower limit of detection of cytokine was 4 pg/mL.

2.11. Statistical analysis

Data were processed by the Graph Pad Prism program (version 4, 2003) using the Kruskal–Wallis non-parametric test with Dunn's corrections or ANOVA parametric test following by Newman-Keuls for multiple comparisons in relation to the analysis of normality and variance homogeneity. Data from the protection assay were analyzed by the log-rank test.

3. Results

3.1. Antigenic specificity of recombinant protein DIII-C-2 against murine and human sera

The purified protein was used to react with HMAFs against the four serotypes by indirect ELISA. As result, the main recognitions were obtained with antisera against DEN-1 and DEN-2 (1:64 000 and 1:256 000 respectively), although the recognition against DEN-2 was three fold higher than the recognition against DEN-1. The recognition of the protein with antisera against DEN-3 and DEN-4 was 64 and 480 fold lower, respectively than homologous recognition (Fig. 1). In contrast, we observed a wide reactivity to DEN-2 virus (1:256 000 with DEN-1 and DEN-4, 1:1 024 000 with DEN-2 and 1:512 000 with DEN-3).

When human serum samples from primary infection to DEN virus were used, a similar reactivity pattern was observed with both antigens (recombinant protein and DEN-2 virus). Although the ELISA titers are very low the highest levels were detected with DEN-1 and DEN-2 immune sera, being statistically significant differences for the recombinant protein recognition ($p < 0.01$) (Fig. 2).

3.2. Evaluation in mice of the protection capacity of DIII-C-2 against DEN-1, DEN-2 and DEN-4

The protective capacity of the protein DIII-C-2 was assessed in Balb/c mice. As controls, one group of animals received the placebo formulation (negative control) and other one was inoculated with the infective DEN-2 (positive control). Thirty days after the third dose, animals were challenged by intracerebral route with a lethal

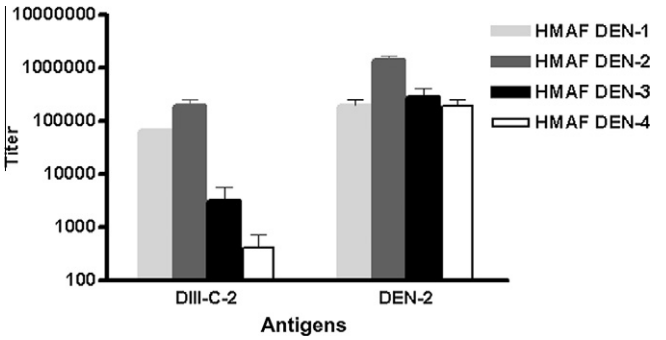

Fig. 1. Antigenic specificity by ELISA with mice antibodies. Serotype antigen specificity of the recombinant protein DIII-C-2 and DEN-2 with HMAFs corresponding to the four DEN serotypes by ELISA. The *Y* axis shows the GMT + standard deviation.

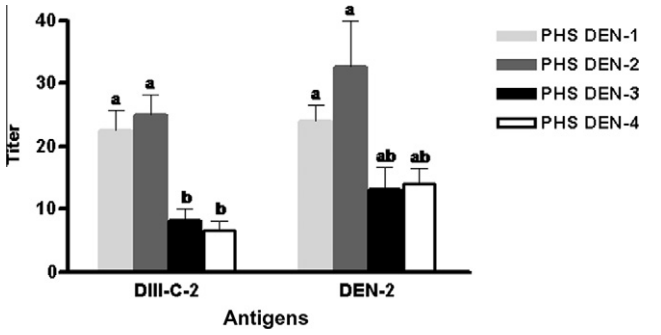

Fig. 2. Antigenic specificity by ELISA with human antibodies. Serotype antigen specificity of the recombinant protein DIII-C-2 and DEN-2 with sera collected from individuals with primary infection with DEN-1, DEN-2, DEN-3 and DEN-4 by ELISA. Different letters indicate significant differences ($p < 0.01$). The *Y* axis shows the GMT + standard deviation. PHS: primary human sera.

dose of DEN-1, DEN-2 and DEN-4. Challenge with DEN-3 was not performed because of the lack of a suitable neurovirulent strain.

As shown in Fig. 3A, after the observation period, 86.6% of animals that received DIII-C-2 survived upon viral DEN-2 challenge, whereas all the animals of the negative control group died ($p < 0.001$). As expected, the positive control group (DEN-2 immune animals) exhibited the highest level of protection: 100%, being statistically similar to the group receiving DIII-C-2 ($p > 0.05$).

Most of the DIII-C-2 immune animals died upon DEN-1 or DEN-4 challenge reaching only 6.6% of protection to each viral serotype, without significant differences respect to the negative control

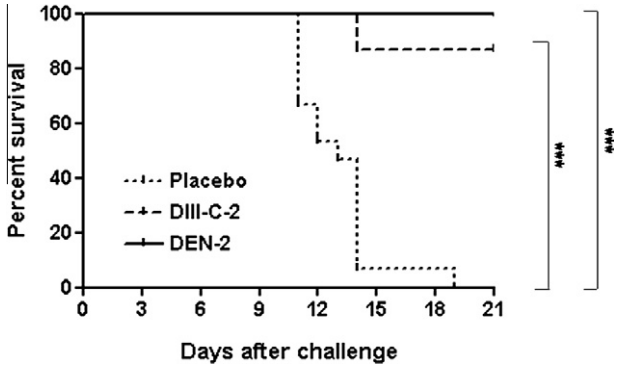

Fig. 3a. Protection assay. Survival curves of immunized mice with DIII-C-2 and DEN-2 and those challenged with the homologous lethal virus (DEN-2). Statistical analysis was performed by the log-rank test ($^{***}p < 0.001$).

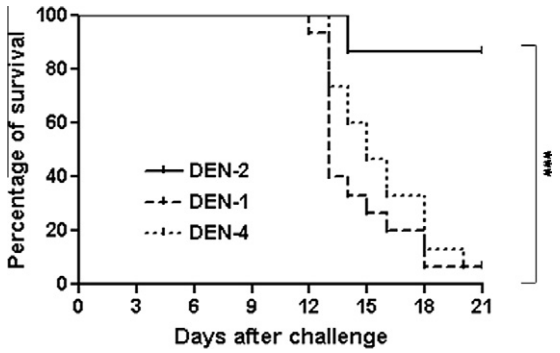

Fig. 3b. Protection assay. Survival curves of immunized mice with DIII-C-2 and those challenged with the homologous (DEN-2) and the heterologous lethal viruses (DEN-1 and DEN-4). Statistical analysis was performed by the log-rank test ($^{***}p < 0.001$).

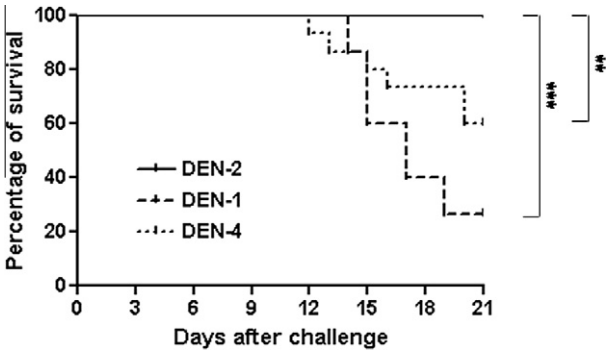

Fig. 3c. Protection assay. Survival curves of immunized mice with DEN-2 and those challenged with the homologous (DEN-2) and the heterologous lethal viruses (DEN-1 and DEN-4). Statistical analysis was performed by the log-rank test ($^{**}p < 0.01$, $^{***}p < 0.001$).

group ($p > 0.05$), (Fig. 3B). In contrast, the immunization with DEN-2 induced partial protection (26.6% and 60% of survival) against heterologous viral challenge with DEN-1 and DEN-4, respectively (Fig. 3C) ($p < 0.01$).

3.3. Dengue virus specific antibody response in immunized mice

Sera from immunized Balb/c mice were used to assess the serotype specificity of the humoral induced immune response. As

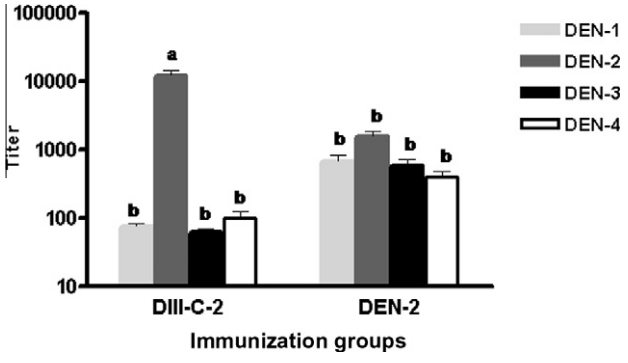

Fig. 4a. Serotype specificity of the IgG antibody response induced in Balb/c mice. Specificity of antibodies induced in Balb/c mice after immunization with the recombinant DIII-C-2 through an ELISA against antigens of the four DEN serotypes compared with the induced antibody response after infection with DEN-2. Different letters indicate significant differences ($p < 0.001$). The *Y* axis shows the GMT + standard deviation.

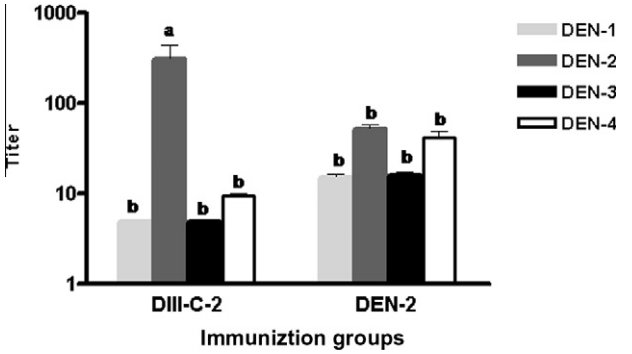

Fig. 4b. Serotype specificity of the HI antibody response induced in Balb/c mice. Specificity of hemagglutination inhibition antibodies induced in Balb/c mice after immunization with the recombinant DIII-C-2 and DEN-2. The murine sera were tested by HI against each serotype. Different letters indicate significant differences ($p < 0.001$). The *Y* axis shows the GMT + standard deviation.

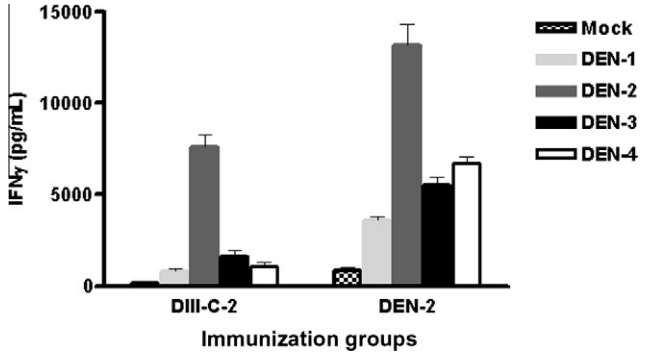

Fig. 5a. Serotype specificity of the IFNγ response induced in Balb/c mice. IFNγ levels secreted after viral stimulation with homologous and heterologous serotypes of mice splenocytes previously immunized with the recombinant DIII-C-2 and DEN-2.

shown in Fig. 4A, the group immunized with DIII-C-2 developed high levels of anti-DEN-2 antibodies (1:16 000) with significant differences compared to those raised against DEN-1 (<1:100, $p < 0.001$), DEN-3 (<1:100, $p < 0.001$) and DEN-4 (1:100, $p < 0.001$). Conversely, a wide cross-reactivity was observed in sera collected from DEN-2 immunized mice: 1:1000, 1:2000; 1:500 and 1:250 for DEN-1, DEN-2, DEN-3 and DEN-4, respectively without statistically differences among them ($p > 0.05$) (Fig. 4A).

The specificity of the functional antibody response, in terms of HI antibodies, was also measured. As shown in Fig. 4B, the response induced DIII-C-2 was highly specific to the homologous serotype (1:640, $p < 0.001$) whereas antibodies elicited in immunized mice with DEN-2 virus were broadly cross-reactive to all DEN serotypes without significant differences among them ($p > 0.05$).

Finally the PRNT titers were determined. As expected, both DIII-C-2 and DEN-2 induced neutralizing antibodies only against the homologous virus ($p < 0.001$) (Table 1).

3.4. Dengue virus specific cellular response in immunized mice

The splenocytes obtained from DIII-C-2 immunized mice were *in vitro* stimulated with the four viral antigens to evaluate the serotype specificity of the cellular immune response, by IFNγ and TNFα secretions. As shown in Fig. 5A, the highest level of IFNγ was secreted in the culture supernatants from splenocytes stimulated with the homologous serotype (7 558.1 ± 1241). The cytokine levels upon stimulation with DEN-1, DEN-3 or DEN-4 (771.7 ± 296.7; 1619.9 ± 554.4 and 1076.9 ± 405.5 respectively) was 10, 5 and 7-fold lower respectively, than levels upon homologous stimulation. In contrast, high IFNγ levels were detected in the supernatant from DEN-2 immunized mice after stimulation with the four dengue serotypes. Specifically, the ratio homotypic/heterotypic secretion was 4, 2 and 2 after stimulation with DEN-1 (3570.4 ± 376), DEN-3 (5461.6 ± 717.3) and DEN-4 (6724.7 ± 473.7), respectively (Fig. 5A).

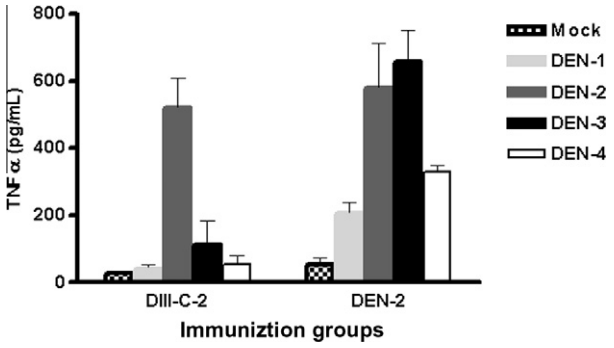

Fig. 5b. Serotype specificity of the TNFα response induced in Balb/c mice. TNFα levels secreted after viral stimulation with homologous and heterologous serotypes of mice splenocytes previously immunized with the recombinant DIII-C-2 and DEN-2.

Consistent with the IFNγ pattern, the TNFα levels induced by homologous stimulation (520.9 ± 152.1) were higher than those induced by heterologous stimulation (DEN-1 42.6 ± 19.7, DEN-3 112.3 ± 124.8 and DEN-4 53.7 ± 46.5) of the group immunized with DIII-C-2. The homologous secretion was 6, 5 and 10-fold higher

Table 1
Neutralizing antibody titers induced after immunization of Balb/c mice with the recombinant protein DIII-C-2 and DEN-2 against all four DEN serotypes. The neutralization titer was defined as the maximal dilution yielding a 50% reduction in the number of plaques. Different letters indicate significant differences ($p < 0.001$).

No. mice	DIII-C-2				DEN-2			
	DEN-1 (a)	DEN-2 (b)	DEN-3 (a)	DEN-4 (a)	DEN-1 (a)	DEN-2 (b)	DEN-3 (a)	DEN-4 (a)
1	<10	23	<10	<10	<10	46	<10	<10
2	<10	16	<10	<10	<10	32	<10	<10
3	<10	25	<10	<10	<10	48	<10	<10
4	<10	50	<10	<10	<10	35	<10	<10
5	<10	65	<10	<10	<10	71	<10	<10
6	<10	26	<10	<10	<10	33	<10	<10
7	<10	18	<10	<10	<10	42	<10	<10
8	<10	30	<10	<10	<10	46	<10	<10
9	<10	45	<10	<10	<10	50	<10	<10
10	<10	63	<10	<10	<10	33	<10	<10
GMT	<10	36.1	<10	<10	<10	43.6	<10	<10

than secretion upon stimulation with heterologous antigens DEN-1, DEN-3 and DEN-4, respectively. Conversely, upon homologous and heterologous stimulation of the splenocytes from mice inoculated with dengue virus, high levels of TNFα were detected (Fig. 5B). The highest values were detected with DEN-2 and DEN-3 stimulations (580.28 ± 228 and 658.80 ± 160 respectively), with a reversal in the ratio homotypic/heterotypic secretion favoring the DEN-3 heterologous secretion.

4. Discussion

A vaccine candidate against dengue should ideally induce high serotype-specific immune response against the four serotypes to guarantee that it does not enhance subsequent infections from natural exposure to heterologous dengue viruses. In this sense, the antigenic characterization of the recombinant protein DIII-C-2 and the analysis of the cross-reactivity of the humoral and cellular immune response induced are the main purpose of this study. We use in this paper multiple sets of dengue virus in order to standardize our results with previous studies. Also, is a tough test for the candidate vaccine to be assayed against heterologous strains.

The antigenic characterization using mice antibodies showed a major reactivity of DEN-1 and DEN-2 HMAFs against the recombinant protein while the recognition of DEN-2 virus was highly cross-reactive. Similar results were previously obtained with the fusion proteins P64k-domain III by dot blot assay (Izquierdo et al., 2008). The cross reactive recognition with DEN-1 HMAFs has also been earlier reported for MBP-domain III recombinant protein of serotype 2, suggesting the presence of some subcomplex-reactive epitopes (Simmons et al., 1998).

The cross reactive recognition by HMAFs of the DEN-2 virus was expected. In turn, the results of the recognition of DEN-2 virus with human sera showed similar levels of reactivity with homologous and heterologous primary sera, as it has been previously demonstrated (Hapugoda et al., 2007; Holbrook et al., 2004). This can be explained due to the majority of anti-E antibodies after primary infection are cross-reactive, and only a minor proportion are type specific against the homologous serotype (Lai et al., 2008).

We also observed a preferential cross-reactive recognition of DIII-C-2 protein with DEN-1 human sera. A similar result was earlier reported by Kochel et al. (2002, 2005). They found even that serum from humans immune to dengue serotype 1 neutralized the infectivity of some strains of dengue serotype 2 (Kochel et al., 2005, 2002). On the other hand, it has been reported that the DEN-2-specific E-binding activity relative to DEN-1 was 0.6% and the E-binding activity cross-reactive to DEN-1 was 99.4%, suggesting that the majority of anti-DEN-2 E antibodies generated during primary DEN-2 infection cross-reacted with DEN-1 E protein and that only a minor proportion was DEN-2 specific (Lai et al., 2008).

As a next step, the protective capacity of DIII-C-2 against homologous and heterologous serotypes was assessed. We use for this purpose the mouse model of i.c. challenge. This model does not mimic the infection route; neither produces the same symptoms as dengue virus infection in humans and also are necessary the use of neuroadapted strains, pathogenic in mice but not in humans (Yauch and Shresta, 2008). However, the model of the intracerebral infection of mice with mouse-brain-adapted DEN has been predominately used to test the efficacy of DEN vaccines (Clements et al., 2010; Falgout et al., 1990; van Der Most et al., 2000).

It was demonstrated that protection was only directed against the homologous serotype, with similar levels to those described in a previous work (Valdés et al., 2009) whereas, the immunization with DEN-2, induced partial protection against heterologous serotypes. These results corroborate that a future vaccine against dengue, based on this chimeric construct, should contain the proteins for the four serotypes. The partial protection induced by DEN-2 immunization in mice is similar to that described for the pioneering studies in humans. Experimental inoculations, conducted by Sabin et al., demonstrated that cross-protection against disease is possible with a second DEN and lasted, at least, 2 months. Nevertheless, when the second virus was administered 9 months after the first infection, signs of the disease were detected indicating a lack of protection (Sabin, 1952).

The specificity of the humoral immune response induced by the recombinant protein in Balb/c mice was also examined. In agreement with previous reports using different recombinant proteins based on domain III, significant levels of IgG antibodies against homologous serotype were found being higher than those elicited against heterologous serotypes (Izquierdo et al., 2008; Khanam et al., 2006, 2007; Simmons et al., 2001). In general the pattern of IgG antibodies was serotype-specific.

It is postulated that the broad cross-reactivity between flaviviruses is revealed by HI assays (Stiasny et al., 2006) and that these antibodies inhibit the conformational change of the E protein at slightly acidic pH which is crucial in the virus penetration to the host cell (Simantini and Banerjee, 1995). The DIII-C-2 recombinant protein also induced highly serotype specific HI antibody response according to our previous studies with the domain III-P64k recombinant proteins in mice and monkeys (Izquierdo et al., 2008). In fact, monkeys immunized with DEN-2 domain III-P64k recombinant proteins showed full protection with earliest HI antibody response upon challenge (Bernardo et al., 2008a).

The neutralizing capacity of the antibodies elicited was another parameter measured in this work. As expected, both immunogens, DIII-C and DEN-2 virus, induced neutralizing antibodies only against DEN-2, the homologous serotype. In fact, the measurement of PRNTs is the most widely used test for determining serotype-specific antibodies against dengue virus (Roehrig et al., 2008).

In general, considering the characterization of the humoral immune elicited, we can conclude that DIII-C protein is able to induce a serotype-specific antibody response and consequently, the risk of ADE after vaccination is lower. Nevertheless, experiments of ADE using the sera of animals immunized with the recombinant protein are required to define the potential role of such cross-reactive immune in enhancement phenomenon.

However, heterologous antibodies are not the unique mechanism underlying the pathogenesis of DHF/DSS. Also, the increased viral load, through activation of preexisting cross-reactive T-cell populations (with greater avidity for the primary, but not the secondary serotype), triggers a vigorous cytokine storm that damages the vascular endothelium with concomitant capillary leakage and severe disease (Swaminathan et al., 2010). IFNγ and TNFα are pro-inflammatory cytokines with high implication in the immune response to dengue virus (Bozza et al., 2008; Wang et al., 2007; Yang et al., 2001). The levels of cross-reactivity in the secretion of these cytokines are important to define the protective or pathogenic role of the cellular immune response induced by any vaccine candidate.

In the present work we demonstrated that CMI induced in mice by DIII-C-2 protein is basically serotype-specific in contrast to the cross-reactive profile detected for the CMI induced by the DEN-2 virus immunization. The TNFα has been correlated with a pathological response and the development of DHF in patients with a secondary DEN infection (Dejnirattisai et al., 2008; Friberg et al., 2011; Mongkolsapaya et al., 2006). In turn, the ratio of TNFα-producing to IFNγ-producing T cells constitutes a correlate to characterize a pathological response in DEN infection, in human and animals models (Beaumier et al., 2008; Friberg et al., 2011; Mangada and Rothman, 2005; Mongkolsapaya et al., 2006). In our experiments, the heterologous viral stimulation of spleen cells from

DEN-2 immune animals, showed a cross-reactive TNFα secretion, with significant TNFα/IFNγ ratio for heterologous serotypes. This pattern is similar to that observed in peripheral blood mononuclear cells from dengue-vaccine or primary dengue-infected recipients after *in vitro* stimulation with heterologous DEN serotypes (Bashyam et al., 2006; Friberg et al., 2011; Mongkolsapaya et al., 2006; Zivny et al., 1999). It means, that such vaccine candidates are able to induce, besides of functional immune response, a potential pathogenic one. The balance of the immune response induced in each individual as well as the duration of the functional one will define the final effect: protection or pathogenesis.

In contrast, the high specificity of the IFNγ and TNFα secretion observed in the splenocytes from mice immunized with the recombinant protein DIII-C-2 upon stimulation with the four dengue viruses suggest the induction of protective cellular response with minimal induction of pathogenic heterotypic response.

5. Conclusions

Taken together we can assert that DIII-C protein constitutes an attractive vaccine candidate against dengue. It induces a specific immune response in mice in terms of neutralizing antibodies and CMI. In turn, it is possible that ADE as well as the phenomena of the original antigenic sin of T cells should not be favored upon vaccination. However more studies are needed to corroborate this assertion.

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