Recombinant nucleocapsid-like particles from dengue-2 induce functional serotype-specific cell-mediated immunity in mice

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The interplay of different inflammatory cytokines induced during dengue virus infection plays a role in either protection or increased disease severity. In this sense, vaccine strategies incorporating whole virus are able to elicit both functional and pathological responses. Therefore, an ideal tetravalent vaccine candidate against dengue should be focused on serotype-specific sequences. In the present work, a new formulation of nucleocapsid-like particles (NLPs) obtained from the recombinant dengue-2 capsid protein was evaluated in mice to determine the level of protection against homologous and heterologous viral challenge and to measure the cytotoxicity and cytokine-secretion profiles induced upon heterologous viral stimulation. As a result, a significant protection rate was achieved after challenge with lethal dengue-2 virus, which was dependent on CD4+ and CD8+ cells. In turn, no protection was observed after heterologous challenge. In accordance, in vitro-stimulated spleen cells from mice immunized with NLPs from the four dengue serotypes showed a serotype-specific response of gamma interferon- and tumour necrosis factor alpha-secreting cells. A similar pattern was detected when spleen cells from dengue-immunized animals were stimulated with the capsid protein. Taking these data together, we can assert that NLPs constitute an attractive vaccine candidate against dengue. They induce a functional immune response mediated by CD4+ and CD8+ cells in mice, which is protective against viral challenge. In turn, they are potentially safe due to two important facts: induction of serotype specific cell-mediated immunity and lack of induction of antiviral antibodies. Further studies in non-human primates or humanized mice should be carried out to elucidate the usefulness of the NLPs as a potential vaccine candidate against dengue disease.

INTRODUCTION

Dengue virus (DENV) is a mosquito-borne flavivirus and the causative agent of dengue fever (DF) and dengue haemorrhagic fever (DHF). It is estimated that nearly half of the world’s population is at risk of infection and up to 50 million people are infected each year with frequent epidemic activity in South-East Asia, South America and Western Pacific regions. There are four immunologically and antigenically distinct serotypes of DENV, referred to as DENV-1 to -4. These four serotypes differ in amino acid sequence by 25–30 % and, crucially, infection with one serotype does not confer immunity to infection by one of the others. Viral and host factors have been proposed to play a role in the development of DHF, but epidemiological evidence led to the realization that the majority of DHF cases occur in individuals who suffer secondary or sequential DENV infections (Sangkawibha et al., 1984; Guzmán et al., 1999). This implies that immunity to a previously encountered virus is not only ineffective at preventing secondary infection, but may also enhance the disease.

Antibody-dependent enhancement (ADE) of DENV infections of monocytic cells has been proposed as one explanation for this observation (Halstead, 1988, 1989; Morens, 1994). Several recent studies indicate that it is possible to induce ADE in vitro and in vivo with any anti-DENV antibody (Balsitis et al., 2010; Dejnirattisai et al., 2010; Zellweger et al., 2010). Other authors have also proposed that serotype-cross-reactive DENV-specific T-cells play a role in the development of the plasma-leakage
syndrome, characteristic of DHF (Mongkolsapaya et al., 2003; Rothman, 2003; Beaumier et al., 2008; Dejnirattisai et al., 2008). It has been reported that serotype-cross-reactive T-cells are preferentially activated in the heterologous infection, a phenomenon termed ‘original antigenic sin’ (Mongkolsapaya et al., 2003). These cross-reactive T-cells exhibit sub-optimal degranulation (Mongkolsapaya et al., 2006) and altered cytokine production (Mangada & Rothman, 2005; Imrie et al., 2007), contributing to severe disease. These immunopathological mechanisms have obvious implications for vaccine design: any effective and safe dengue vaccine should induce neither ADE nor serotype-cross-reactive T-cells.

Recently, our group developed a purification procedure and in vitro assembly process for the recombinant DENV-2 capsid (C) protein (López et al., 2009), which allowed us to obtain nucleocapsid-like particles (NLPs). We also demonstrated that NLPs induce protective CD4+ and CD8+ cells in the viral encephalitis murine model, without the induction of antiviral antibodies (Gil et al., 2009).

In the present work, a new formulation of NLPs was evaluated in mice to determine the protective capacity against homologous and heterologous serotype and to measure the cytotoxicity and cytokine-secretion profiles induced upon heterologous viral stimulation. Spleen cells from DENV-1-, -2-, -3- and -4-immune animals were also stimulated with the C protein.

RESULTS

A new formulation of NLPs induces cell-mediated immunity and protective response, dependent on CD4+ and CD8+ cells, without the contribution of antiviral antibodies

According to the process described by López et al. (2009), a new formulation of NLPs was obtained but, in this case, using a protein : nucleic acid molecular ratio of 3 : 1. As a result of the in vitro assembly procedure, spherical NLPs with a diameter between 25 and 30 nm were visualized by electron microscopy (Fig. 1).

With the aim to evaluate the functionality of a low dose of NLPs with respect to that published previously (Gil et al., 2009), the immunogenicity and protective capacity of 10 µg NLPs (3 : 1) adjuvanted with alum were assessed in mice. As a control, one group of animals received 10 µg non-particulate antigen (C protein) with the same adjuvant. The placebo- and DENV-2-immunized animals acted as negative and positive controls, respectively.

Thirty days after the third dose, the antigen-induced humoral and cellular immune responses were evaluated. As shown in Fig. 2(a), the sera from the animals immunized with the capsid protein or NLPs (3 : 1) did not recognize any of the four serotypes of DENV. Consistently, none of these sera neutralized the in vitro DENV-2 infection, as measured by plaque-reduction neutralization test (PRNT) (geometric mean titre <10).

Cell-mediated immunity (CMI) was measured by gamma interferon (IFN-γ) secretion upon viral stimulation of the splenocytes. High levels of IFN-γ were secreted by the splenocytes of mice immunized with NLPs (3 : 1), similar to those of the DENV-2-immunized animals (Fig. 2b). In turn, the concentration of the cytokine decreased in both CD4+ and CD8+ cell-depleted splenocytes, exhibiting significant differences with respect to the non-depleted ones (P<0.001). A similar behaviour was observed for splenocytes from DENV-2-immune animals (Fig. 2b).

The cytotoxic capacity of the antigen-induced memory T-cells was also determined. As a result, a significant in vitro cytotoxicity was detected in the splenocytes from NLPs (3 : 1)-immune mice (57.3±5.4%) and in the splenocytes from DENV-2-immunized animals (77.6±5.7%). This response was not detected in the placebo group (13.6±1.3%) (Fig. 2c).

The animals that were not used for analysis of the immune response were challenged by intracranial route with a lethal dose of DENV-2. In agreement with the results of the CMI, 80% of the animals that received NLPs (3 : 1) survived upon viral challenge, whereas only 10% of the mice from the negative-control group survived, with statistically significant differences between them (P=0.0006). On the other hand, only 20% of animals immunized with the non-particulate antigen controlled the encephalitis and survived, showing no statistically significant differences with respect to the placebo group (P=0.5925). As expected, the positive-control group (DENV-2-immune animals) exhibited the highest level of protection (100%), which was

Fig. 1. Characterization of nucleocapsid-like particles. Morphology and size of the particles were analysed by electron microscopy. Samples, previously centrifuged at 10 000 r.p.m. for 20 min, were negatively stained using uranyl acetate and analysed by transmission electron microscopy. Bar, 200 nm.
Fig. 2. Immunogenicity and protective response induced by NLPs (3:1). Thirty days after the third dose, the antigen-induced humoral and cellular immune response was evaluated and the remaining animals were challenged by the intracranial route with 50 LD_{50} DENV-2. (a) Antiviral antibody response against the four DENV serotypes, generated after inoculation with different formulations of the recombinant DENV-2 capsid protein. Data represent the geometric mean with the 95% confidence interval of 10 mice per group. The number above the bar represents the geometric mean titre of neutralizing antibodies against DENV-2 determined by PRNT. The statistical analysis was performed by one-way analysis of variance, using the Kruskal–Wallis and Dunn multiple comparison test. (b) Concentration of IFN-γ in culture supernatants measured by ELISA. Non-depleted, CD4-depleted and CD8-depleted splenocytes from immunized mice were stimulated with 10^3 p.f.u. DENV-2 antigen. Data represent means ± SD of six mice per group. Statistical analysis was performed using two-way ANOVA with the Bonferroni post-test. (c) Cytotoxicity, as measured by lactate dehydrogenase assay in splenocytes from immunized mice co-cultured with 10^3 p.f.u. DENV-2 antigen. Data represent means ± SD of five mice per group. Statistical analysis was performed using the Mann–Whitney test. (d) Mortality rates from mice immunized with 10 mg C protein (non-particulate antigen), 10 μg NLPs (3:1), placebo and DENV-2 (n=10). Statistical analysis was performed by the log-rank test. (e) Mortality rates from non-depleted, CD4-depleted and CD8-depleted mice immunized with 10 μg NLPs (3:1) (n=10). Statistical analysis was performed by the log-rank test. *P<0.05; **P<0.01; ***P<0.001.
The contribution of CD4$^+$ and CD8$^+$ cells to the protection conferred by the new formulation of NLPs was also evaluated. Only 10 or 40% of CD4-depleted or CD8-depleted animals survived the viral encephalitis, with statistically significant differences with respect to the non-depleted animals ($P=0.0001$ and 0.0355, respectively) (Fig. 2e). Also, the survival rate (80%) in the group of non-depleted mice was not affected by the inoculation of mAb 2C4, an isotype-matched control antibody (data not shown). On the other hand, all mice from the placebo group died due to the encephalitis after CD4$^+$ or CD8$^+$ cell depletion, with a similar behaviour to that observed in non-depleted animals ($P=0.5894$ and 0.4802, respectively) (data not shown).

**Immunization with NLPs (3:1) does not protect against heterologous viral challenge**

Once the protective capacity of the formulation of NLPs (3:1) against the homologous serotype had been demonstrated, a second experiment was performed in mice to assess its protective capacity against heterologous viral challenge. A similar schedule was performed. One group of mice was inoculated with 10$^5$ NLPs (3:1) adjuvanted with aluminium hydroxide and as controls; two other groups of animals received a placebo preparation or DENV-2. One month after the third dose, the immunized animals were challenged with a lethal dose of DENV-1 or -4. Challenge with DENV-3 was not performed because of the lack of a suitable neurovirulent strain.

Upon viral challenge, NLPs (3:1)-immune animals succumbed to the ensuing DENV-1 or -4 encephalitis and, finally, none of the animals survived against DENV-1 and only 10% of the animals inoculated with DENV-4 controlled the disease (Fig. 3). A similar behaviour was observed in the placebo group, without statistically significant differences in both cases ($P=0.203$ for DENV-1 challenge and $P=0.6543$ for DENV-4 challenge). In contrast, immunization with DENV-2 induced partial protection (40 and 60% survival) against heterologous viral challenge with DENV-1 and -4, respectively ($P=0.0451$ and 0.0023) (Fig. 3).

**NLPs (3:1) induces serotype-specific cell-mediated immunity**

Spleen cells from mice immunized with 10 μg NLPs (3:1), placebo or DENV-2 were cultured with the four DENV serotypes. After the incubation period, culture supernatants were tested by ELISA to measure IFN-γ and tumour necrosis factor alpha (TNF-α) concentrations. In addition, cytotoxic activity was determined by a lactate dehydrogenase assay. As a result, significant IFN-γ secretion was detected in splenocytes from NLPs (3:1)-immune animals upon homologous viral stimulation (1747.1 ± 170.3 pg ml$^{-1}$). However, low levels of this cytokine (170.4 ± 84.7, 388.9 ± 93.7 and 249.4 ± 84 pg ml$^{-1}$) were secreted upon stimulation with DENV-1, -3 and -4, respectively (Fig. 4a). In contrast, a different profile was obtained in DENV-2-immunized animals. Splenocytes from these mice secreted significant IFN-γ levels upon stimulation with the four dengue serotypes (610.8 ± 121.8, 833.1 ± 143.9 and 1317.2 ± 175.1 pg ml$^{-1}$ for DENV-1, -3 and -4, respectively). It is important to highlight that levels of IFN-γ measured in DENV-2-immune animals (2126.7 ± 288.2 pg ml$^{-1}$) were statistically similar to those secreted in spleen cells from mice immunized with 10 μg NLPs (3:1) after stimulation with homologous virus ($P=0.0621$). Splenocytes from the negative-control group did not secrete significant levels of the cytokine after stimulation with each DENV.

According to previous results, immunization with NLPs (3:1) induced a memory-cell response with cytolytic

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**Fig. 3.** Mortality rates of immunized mice after challenge with 50 LD$_{50}$ each of heterologous DENVs. (a) Survival curves from mice immunized with 10 μg NLPs (3:1), placebo and DENV-2 challenged with DENV-1. (b) Survival curves from mice immunized with 10 μg NLPs (3:1), placebo and DENV-2 challenged with DENV-4 ($n=10$). Statistical analysis was performed by the log-rank test; *$P<0.05$; **$P<0.01$; ***$P<0.001$.
activity only against DENV-2-infected cells (28.2 ± 3.3 %) (Fig. 4b). Very low cytotoxicity was detected against heterologous DENV-infected cells (6.9 ± 1.9 % for DENV-1, 7.7 ± 1.4 % for DENV-3 and 8 ± 1.6 % for DENV-4), similar to that detected for the placebo group (6 ± 1.4 % for DENV-1, 5.7 ± 1.2 % for DENV-2, 5.7 ± 1.3 % for DENV-3 and 6.5 ± 1.3 % for DENV-4). As expected, cross-cytotoxicity was detected in splenocytes of DENV-2-immune mice against DENV-infected cells (Fig. 4b). However, cytotoxic activity was significantly lower (P, 0.001) against the heterologous serotypes than against the homologous serotype (19.5 ± 1.1 % for DENV-1, 49 ± 4.9 % for DENV-2, 27.2 ± 2.7 % for DENV-3 and 40.5 ± 2.3 % for DENV-4).

Additionally, TNF-α secretion by splenocytes from NLPs-immune animals was serotype-specific (Fig. 4c). The levels of TNF-α were 57.1 ± 22.5, 784.3 ± 107.7, 72.6 ± 30.8 and 35.4 ± 11.1 pg ml−1 after stimulation with DENV-1, -2, -3 and -4, respectively. In turn, the ratio of TNF-α:IFN-γ obtained upon stimulation with the homologous serotype was similar to that calculated for stimulations with heterologous DENV serotypes (0.33 ± 0.11 for 626.3 ± 115.7 and 536.1 ± 130.4 pg ml−1). Consequently, the ratios of TNF-α:IFN-γ were higher upon stimulation with heterologous DENV serotypes (0.33 ± 0.11 for

Fig. 4. Cell-mediated immune response of immunized mice against DENV serotypes. Culture supernatants from mock-treated or DENV antigen (10^3 p.f.u.)-infected splenocytes from individual animals of all groups were tested 30 days after the last dose. (a) Concentration of IFN-γ in culture supernatants measured by ELISA. (b) Cytotoxicity measured by lactate dehydrogenase assay. (c) Concentration of TNF-α in culture supernatants measured by ELISA. Data represent means ± SD of six mice per group. Statistical analysis was performed using two-way ANOVA with the Bonferroni post-test; **P<0.01; ***P<0.001.

Fig. 5. Ratios of TNF-α:IFN-γ for each animal per group. Culture supernatants from DENV antigen (10^3 p.f.u.)-infected splenocytes from individual animals of all groups were tested 30 days after the last dose and the concentration of IFN-γ and TNF-α was determined by ELISA. ■, DENV-1; ▲, DENV-2; ●, DENV-3; ◆, DENV-4. Data represent mean±SD of six mice per group. Statistical analysis was performed using two-way ANOVA with the Bonferroni post-test; *P<0.05; ***P<0.001.
DENV-1, 0.78 ± 0.22 for DENV-3 and 0.41 ± 0.11 for DENV-4) than that calculated for the stimulation with the homologous serotype (0.24 ± 0.07) (Fig. 5).

**The C protein is able to stimulate only splenocytes from DENV-2-immunized mice**

To determine the ability of the C protein to stimulate splenocytes from mice immunized with the four DENV serotypes, in vitro stimulation experiments were subsequently carried out to measure the IFN-γ and TNF-α concentrations in the culture supernatants. As shown in Fig. 6(a), the IFN-γ levels secreted upon C stimulation were 155.7 ± 48.4, 1373.7 ± 71.3, 147 ± 44.2 and 272.3 ± 28.4 pg ml⁻¹, for DENV-1, -2, -3 and -4-immune animals, respectively. In turn, the levels of TNF-α were 31.7 ± 29.7, 42.3 ± 22.5, 16.3 ± 19.6 and 20 ± 20.2 pg ml⁻¹, respectively (Fig. 6b). On the other hand, when spleen cells were stimulated with DENV-2, they secreted 1943.7 ± 889.1, 6035.3 ± 236.7, 3100 ± 326.3 and 3927.7 ± 544.6 pg IFN-γ ml⁻¹ and 1002.3 ± 131.5, 462 ± 156.4, 581.7 ± 115.4 and 881.7 ± 132.8 pg TNF-α ml⁻¹ for DENV-1, -2, -3 and -4-immune animals, respectively (Fig. 6a, b).

**DISCUSSION**

One of the problems in developing a tetravalent DENV vaccine is the viral diversity observed among the four serotypes of this pathogen (Rothman, 2004), which results in type-specific and type cross-reactive T-cell determinants (Livingston et al., 1995). T-cells mediate not only protective immunity but also substantial immunopathology. Dengue is an example of a general immunopathological phenomenon in which prior immune experience affects the immune response against the infecting virus, because of the significant amino acid homology (approx. 70%) among the four DENV serotypes (Irie et al., 1989). In this sense, vaccine strategies incorporating whole DENV immunogens (live-attenuated virus, inactivated virus or chimeric viruses) are able to elicit both functional and pathological responses. Therefore, an ideal tetravalent vaccine candidate against dengue should be focused either on sequences that are strictly conserved in all four DENVs or on serotype-specific ones (Mongkolsapaya et al., 2003; Mangada & Rothman, 2005).

Our group has developed a new vaccine candidate based on the C protein as a recombinant construct in Escherichia coli (Lazo et al., 2007). We selected this protein because it is a target for the T-cell response (Gagnon et al., 1996, 1999) and the protective capacity of two of its epitopes was recently confirmed in mice experiments (Yauch et al., 2009). Also, unlike the non-structural proteins (Bashyam et al., 2006; Dong et al., 2007; Beaumier et al., 2008), no altered peptide ligand has been described for the C protein. In fact, several bioinformatic studies have demonstrated its serotype specificity (Khromykh & Westaway, 1996; Khan et al., 2006).

On the other hand, C protein as NLPs is able to induce CMI and protection in mice against homologous virus, without the induction of antiviral and neutralizing antibodies (Gil et al., 2009). In the present work, we developed a new formulation of these NLPs, containing half of the quantity previously assayed for the recombinant C protein, basically following the particulation process described by López et al. (2009). In the present formulation, an additional quantity of single-stranded DNA oligonucleotides (ODNs) in the assembly reactions was added at a protein:ODNs molecular ratio of 3:1. The morphology and the diameter of the particles were similar to those reported by López et al. (2009), as well as to those for the native viral nucleocapsid (Kuhn et al., 2002).

The results of this and previous studies (Gil et al., 2009) show that NLPs (3:1) do not induce antiviral antibodies and, consequently, cannot induce ADE of infection by any of the four dengue serotypes. It is well-known that none of

![Fig. 6. Concentration of IFN-γ and TNF-α in culture supernatants of splenocytes from DENV-immune animals stimulated with C protein or DENV-2. Culture supernatants from splenocytes treated with mock (empty bars), 10 μg C protein ml⁻¹ (grey-shaded bars) or 10⁸ p.f.u. DENV-2 antigen (filled bars) from individual animals were tested 30 days after one dose of infective DENV-1, -2, -3 or -4. (a) Concentration of IFN-γ; (b) concentration of TNF-α. Data represent means ± SD of six mice per group. Statistical analysis was performed using two-way ANOVA with the Bonferroni post-test; **P < 0.01; ***P < 0.001.](http://vir.sgmjournals.org)
the regions of the viral nucleocapsid are exposed on the virion surface (Kuhn et al., 2002; Mukhopadhyay et al., 2005) and therefore they are not likely to interact with B-cells during infection. However, NLPs (3:1) conferred protection against DENV-2 in the mouse encephalitis model, and the CD4+ and CD8+ cells contributed to the percentage survival reached.

The main caveat attributed to the murine encephalitis model is the inoculation of very high doses of mouse-adapted viral strains by the intracranial route. This provokes disease manifestations that are irrelevant to the human dengue disease, as nervous-system involvement in DENV infections is rare (Yauch & Shresta, 2008). However, the infection of immunocompetent mice provides a useful immunological model to study DENV-specific responses. In fact, the induction of viral encephalitis in immunocompetent mice has been the most extensively used model for the evaluation of the protective capacity of vaccine candidates against dengue (Simmons et al., 1998; van der Most et al., 2000; Clements et al., 2010).

CD4+ and CD8+ T-cells could be involved directly in protecting against the disease, as subsets of these cell populations have been found to lyse cells infected with different flaviviruses, including DENV (Green et al., 1993; Gagnon et al., 1996; van der Most et al., 2003; Yauch et al., 2009, 2010). Our results showed a high cytotoxic activity against DENV-2-infected cells in splenocytes from NLPs (3:1)-immune mice. Additionally, both subsets of cells secreted IFN-γ upon in vitro viral stimulation. This cytokine has been described as a mediator of the cellular immune response and plays a role in the antiviral activity against DENV (Shresta et al., 2004; Dejnirattisai et al., 2008; Gunther et al., 2011; Hatch et al., 2011). Based on the results of this work and on previously described evidence, we propose a theoretical model to explain the mechanism by which the effector immune response protects NLPs-immunized mice against intracranial challenge with the virus (Fig. 7).

The functionality of this new formulation of NLPs, containing 10 μg antigen, could be related to the presence of oligonucleotides within the particles, but this issue remains unanswered. There are several studies showing that oligonucleotides have adjuvant capacity for the induction of humoral and CMI to target antigens (Vollmer, 2006). It has been reported that the dose range of oligonucleotides required for the optimal adjuvant effect in mice is 10–50 μg (Riedl et al., 2002). In the present study, only 4.2 μg oligonucleotides was added, but we propose that the oligonucleotides encapsulated into NLPs protect them from nucleolytic degradation. Therefore, once NLPs enter the antigen-presenting cell, due to its aggregated nature, the oligonucleotides interact with intracellular receptors and trigger the stimulation process (Takeshita et al., 2001).

The serotype specificity of the protection and the immune response induced by NLPs (3:1) were studied thoroughly in the present work. Firstly, it was demonstrated that protection was only directed against the homologous serotype, whereas immunization with DENV-2 induced a partial protection against heterologous serotypes. These results corroborate that a future vaccine against dengue, based on these NLPs, should contain the C proteins for the four serotypes. The partial protection induced by DENV-2 immunization in mice is similar to that described for

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**Fig. 7.** Proposed model of the mechanism of protection by the effector immune response after intracranial challenge in NLP-immunized mice. Upon challenge, infection provokes an inflammatory response in the brain. As a result, the endothelial cells upregulate the expression of selectin and adhesion molecules, which attract the memory antigen-specific T-cells. In addition, the antigen-presenting cells (microglia) express MHC class I and II, as well as co-stimulatory molecules to activate the memory T-cells induced after vaccination. The infected neurons express viral peptides in the context of MHC class I molecules, which are recognized by antigen-specific T-cells. In this inflammatory environment, memory CD4+ T-cells are activated and secrete IFN-γ, activate memory CD8+ T-cells and exhibit cytolytic activity. Additionally, memory cytotoxic CD8+ T-cells can also control the infection by secreting IFN-γ and killing the infected cells. DV, Dengue virus; ICAM-1 and VCAM-1, adhesion molecules; OD, oligodendrocite.
pioneering studies in humans. Experimental inoculations conducted by Sabin (1952) demonstrated that cross-protection against disease is possible with a second DENV and can last for 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). In this study, we also detected in vitro cytotoxicity against heterologous DENV-infected cells 1 month after the viral dose, which could be related to the cross-protective capacity induced by DENV-2.

Consistent with the results from the protection assay, significant IFN-γ secretion was detected only upon homologous viral stimulation of spleen cells from NLPs-immune mice. Accordingly, when the C protein was used as antigen for the stimulation of DENV-immune splenocytes, a significant secretion of IFN-γ and TNF-α was detected only in cells from DENV-2-immunized animals. This means that the CMI induced by NLPs is basically serotype-specific, as opposed to the cross-reactive profile of CMI detected after DENV-2 virus immunization. Moreover, the cytotoxic activity was detected only against DENV-2-infected cells. No region of ≥9 aa that is conserved in ≥80% of the sequence of each DENV serotype has been found in the C protein (Khan et al., 2008). Therefore, this protein is unlikely to induce a pathological response upon DENV infection in NLP-based vaccine recipients.

The TNF-α has been correlated with a pathological response and the development of DHF in patients with secondary DENV infection (Mongkolsapaya et al., 2006; Dejinrattisai et al., 2008; Friberg et al., 2011). In turn, the ratio of TNF-α-producing:IFN-γ-producing T-cells constitutes a correlate to characterize a pathological response in DENV infections in humans and animal models (Mangada & Rothman, 2005; Beaumier et al., 2008; Friberg et al., 2011). In our experiments, heterologous viral stimulation of spleen cells from DENV-2-immune animals induced a cross-reactive TNF-α secretion, with a significant TNF-α:IFN-γ ratio for DENV-3 and -4. 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 DENV serotypes (Zivny et al., 1999; Bashyam et al., 2006; Friberg et al., 2011). Therefore, these in vitro determinations could simulate natural sequential infections in humans. In addition, a lower cytotoxicity was detected against heterologous DENV-infected cells than against DENV-2-infected cells. However, whether this impaired response is related to an immunopathological effect in mice remains unanswered. It has been previously reported that, upon secondary heterologous DENV infections, altered peptide ligands change the functionality of the memory T-cell populations, which may then have a potential immunopathogenic effect, such as an increased production of inflammatory cytokines and low cytotoxic function (Mongkolsapaya et al., 2006; Friberg et al., 2011). This altered immune response resulting from cross-reactive memory cells causes the increase of vascular permeability and plasma leakage, characteristic of severe dengue disease (Mathew et al., 1996; Libraty et al., 2002).

Taking the data together, we can assert that NLPs (3 : 1) constitute an attractive vaccine candidate against dengue. They induce a functional immune response mediated by CD4+ and CD8+ cells in mice, which is protective against viral challenge. In turn, they are potentially safe (a crucial attribute particularly for dengue vaccines) due to two important facts: induction of serotype-specific CMI and lack of induction of antiviral antibodies. It means that dengue infection in people vaccinated with the NLP tetravalent formulation will recall neither cross-reactive T-cells (they are the source of the altered cytokine profile and do not properly clear the infected cells) nor antiviral antibodies (able to provoke ADE). The next step of our approach is to demonstrate the protective capacity of the NLP tetravalent formulation in non-human primates to define whether the CMI is sufficient to clear or reduce viral load upon challenge.

**METHODS**

**Viruses.** For animal immunization, antibody detection and virus challenge, strains DENV-1 (Hawaii strain), DENV-2 (A15 strain), DENV-3 (H-87 strain) and DENV-4 (H241 strain) were used. They were obtained by homogenization of suckling mouse brain infected with each virus, in RPMI 1640 medium (Sigma-Aldrich). A similar preparation obtained from brains of non-inoculated mice was used as negative control.

A concentrated preparation of each virus (DENV-1 to -4 antigens) was used for the in vitro stimulation of mouse splenocytes. Supernatants (100 ml) from Vero cells infected with approximately 10^7 p.f.u. DENV-1 (Jamaica strain) ml^−1, 10^6 p.f.u. DENV-2 (SB8553 strain) ml^−1, 10^5 p.f.u. DENV-3 (CS81.1 strain) ml^−1 and 10^4 p.f.u. DENV-4 (Dominica strain) ml^−1 were concentrated by centrifugation at 80,000 g for 4 h at 4 °C. The pellet containing the virus was resuspended in 1 ml PBS (Gibco). A mock preparation was prepared similarly from the supernatant of uninected Vero cells. Virus titres were determined by plaque assay in BHK-21 cells (Morens et al., 1985).

**In vitro assembly of the recombinant NLPs.** The highly purified C protein was subjected to an in vitro assembly procedure as described previously (López et al., 2009). Briefly, 20 μg protein was incubated with 3.4 or 0.25 μg ODNs (random sequences) for a protein : nucleic acid molecular ratio of 3 : 1 in assembly buffer (25 mM HEPES, 100 mM potassium acetate, 1.7 mM magnesium acetate, pH 7.4). The reaction mixture was incubated for 30 min at 30 °C and finally stored at 4 °C.

**Mice.** Female BALB/c (Bc, H-2d) mice (aged 6–8 weeks) were purchased from CENPALAB (Havana City, Cuba) and housed in appropriate animal-care facilities during the experimental period. The maintenance and care of experimental animals used in this research complied with the Cuban Institute of Health guidelines for the humane use of laboratory animals.

**Mouse immunizations and protection assay**

**Immunization schedule no. 1.** Groups of 58 mice were injected by the intraperitoneal (i.p.) route with different formulations of the
recombinant C protein. Three doses of each immunogen were administered on days 0, 15 and 30. All formulations had a volume of 100 μl and were prepared using aluminium hydroxide (Alhydrogel; BRENNTAG Biosector) as adjuvant at a final concentration of 1.44 mg ml⁻¹.

Group 1: 10 μg C protein (non-particulate form)
Group 2: 10 μg NLPs (3:1)
Group 3: placebo (3.4 μg oligonucleotides per mouse in assembly buffer)

Group 4 (positive control): received one dose (0.5 ml) of infective DENV-2 (A15 strain) without adjuvant

One month after the last dose, 10 mice per group were bled and splenecotomized for further immunological analysis. The remaining animals of each group were divided into four subgroups (A, B, C and D). Subgroups A and B were inoculated by the i.p. route with a single dose of 100 μg highly purified anti-CD8 (clone YTS 169.4) and the anti-CD4-depleting (clone YTS 191.1) mAbs, respectively (kindly provided by Dr J. V. Gavilondo, Pharmaceutical Department, CIGB). Subgroup C received PBS. As an isotype-matched control antibody administration, subgroup D was inoculated with mAb 2C4, specific for the V3 region of the gp120 protein of the human immunodeficiency virus type 1 MN isolate (kindly provided by Dr C. Duarte, Vaccine Division, CIGB). Three days after administration of the depleting antibodies, animals were injected intracerebrally with 20 μl of a preparation of infective DENV-2 (A15 strain) containing 50 LD₅₀ (20 p.f.u.). Mice were observed daily over 21 days for mortality. On the same day, two animals of each subgroup were splenectomized and cell-specific depletion was confirmed by flow cytometry. More than 94 % of CD4⁺ or CD8⁺ cells were depleted in animals inoculated with the specific mAb (data not shown).

Immunization schedule no. 2. Groups of 50 mice were injected by the i.p. route with different formulations of the recombinant C protein. Three doses of each immunogen were administered on days 0, 15 and 30. All formulations had a volume of 100 μl and were prepared using aluminium hydroxide (Alhydrogel; BRENNTAG Biosector) as adjuvant at a final concentration of 1.44 mg ml⁻¹.

Group 1: 10 μg NLPs (3:1)
Group 2: placebo (3.4 μg oligonucleotides per mouse in assembly buffer)

Group 3 (positive control): received one dose (0.5 ml) of infective DENV-2 (A15 strain) without adjuvant

One month after the last dose, 10 mice were splenecotomized for further immunological analysis. On the same day, the remaining animals were divided in four groups of 10 mice and injected intracerebrally with 20 μl of a preparation of infective DENV-1 (Hawaii strain), DENV-2 (A15 strain), DENV-3 (H-87 strain) or DENV-4 (H241 strain), containing 50 LD₅₀ (20 p.f.u.). Mice were observed daily over 21 days for mortality.

Humoral immune response. The anti-DENV IgG antibodies stimulated by immunization were monitored by an amplified sandwich ELISA system (Lazo et al., 2007). Briefly, polystyrene plates of 96-wells (Costar) were coated with a mixture of anti-dengue human immunoglobulins (5 μg ml⁻¹) in coating buffer (0.16 % Na₂CO₃, 0.29 % NaHCO₃, pH 9.5), were blocked in coating buffer containing 1 % BSA and later incubated with the viral antigen and the negative-control antigen. Later, sera from each group were tested by serial dilutions starting at 1:50. The anti-dengue antibodies were detected using anti-mouse IgG–peroxidase conjugate (Amersham Biosciences). Finally, 0.04 % substrate (O-phenylenediamine in buffer: 2 % Na₂HPO₄, 1 % citric acid, pH 5.0) was added. The reaction was stopped with 2.5 M H₂SO₄. A₄₉₂ was read in a microplate reader (Sensidet Scan; Merck). Titres were defined as the dilution of serum giving twice the A₄₉₂ value of the negative-control serum.

Neutralizing-antibody titres were measured by PRNT in BHK-21 cells as described previously (Morens et al., 1985). DENV-2 strain SB8553 was used in this test. The neutralizing-antibody titre was identified as the highest serum dilution that reduced the number of virus plaques by 50%. mAb 4G2 was used as positive control (Kaufman et al., 1987).

Cell culture and in vitro stimulation. Spleen cells were obtained under aseptic conditions and erythrocytes were lysed by adding 0.83 % NH₄Cl solution. Cells from each animal were washed twice with PBS/2 % FBS (PAA Laboratories) and resuspended at 2 x 10⁶ cells ml⁻¹ in RPMI 1640 medium supplemented with 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ (Gibco), 2 mM glutamine (Gibco), 5 x 10⁻⁵ M 2-mercaptoethanol (Sigma) and 5 % FBS. Finally, 2 x 10⁵ cells per well were cultured in 96-well round-bottomed plates with the antigens (10³ p.f.u. DENV-1 to -4 antigens or mock preparation). Recombinant C protein was used as an antigen at 10 μg ml⁻¹. Concanavalin A (Sigma) was used as a positive control. In all experiments, three wells were plated for each antigen. After 4 days culture, culture supernatants were collected and stored at −20 °C.

In vitro cell depletion. For CD4⁺ or CD8⁺ cell depletion, splenocytes at 5 x 10⁶ cells ml⁻¹ in PBS were incubated for 30 min at 37 °C with 100 μg highly purified anti-CD8-depleting or anti-CD4-depleting mAbs, respectively, and rabbit complement (Cedarlane). Later, the cells were washed twice with PBS/2 % FBS and cell-specific depletion was confirmed by flow cytometry (data not shown).

Flow-cytometry analysis. For cell-specific depletion analysis, 1 x 10⁶ splenocytes in PBS were incubated for 30 min at 4 °C with anti-CD8–FITC antibody (Serotec) and anti-CD4–allophycocyanin antibody (Serotec). Samples were analysed in duplicate in a PassIII flow cytometer (Partec GmbH). Dead cells were excluded by propidium iodide incorporation. The gates used in the sample acquisition were saved and analysed by the WinMDI software version 2.8 (Purdue University, WL, USA). The percentage of CD4⁺ or CD8⁺ cells was determined by gating on the positive lineages of the CD4⁺ or CD8⁺ subsets.

In vitro cytotoxicity assay. Cytotoxic activity was determined in a 48 h in vitro lactate dehydrogenase assay using 2 x 10⁵ spleen cells, from the immunized animals, co-cultured with 10³ p.f.u. DENV antigen. Percentage of specific lysis was calculated as [(experiment release – spontaneous release)/(maximum release – spontaneous release)] x 100. Maximum release was obtained by adding 1 % Triton X-100 to cells, and spontaneous release was determined by incubating the cells with medium alone.

Cytokine detection. Culture supernatants of splenocytes previously stimulated with each viral antigen were analysed in duplicate to determine the IFN-γ concentration by ELISA using mAb pairs (INF-γ, Mabtech) and the TNF-α concentration using a Mouse TNF Alpha ELISA Ready-Set-Go! kit (ebiScience). The ELISA protocol recommended by the manufacturers was used with slight modifications. The lowest limit of detection of cytokines was 4 pg ml⁻¹.

Statistical analysis. Data from the humoral immune response were assessed using the Kruskal–Wallis and Dunn Multiple Comparison tests. Data from cytokine secretion were analysed by two-way ANOVA with the Bonferroni post-test. Cytotoxicity data were
an analysed by the non-parametric Mann–Whitney test and data from the protection assay were analysed by the log-rank test. In all cases, GraphPad Prism version 5.00 for Windows (GraphPad Software; http://www.graphpad.com) was employed.

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REFERENCES


