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DNA vaccines against dengue virus based on the *ns1* gene: The influence of different signal sequences on the protein expression and its correlation to the immune response elicited in mice

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Abstract

We analyzed four DNA vaccines based on DENV-2 NS1: pcENS1, encoding the C-terminal from E protein plus the NS1 region; pcENS1ANC, similar to pcENS1 plus the N-terminal sequence from NS2a (ANC); pcTPANS1, coding the t-PA signal sequence fused to NS1; and pcTPANS1ANC, similar to pcTPANS1 plus the ANC sequence. The NS1 was detected in lysates and culture supernatants from pcTPANS1-, pcENS1- and pcENS1ANC-transfected cells and not in cells with pcTPANS1ANC. Only the pcENS1ANC leads the expression of NS1 in plasma membrane, confirming the importance of ANC sequence for targeting NS1 to cell surface. High levels of antibodies recognizing conformational epitopes of NS1 were induced in mice immunized with pcTPANS1 and pcENS1, while only few pcENS1ANC-inoculated animals presented detectable anti-NS1 IgG. Protection against DENV-2 was verified in pcTPANS1- and pcENS1-immunized mice, although the plasmid pcTPANS1 induced slight higher protective immunity. These plasmids seem to activate distinct patterns of the immune system.

Keywords: DNA vaccine; Dengue virus; NS1; Mouse; Challenge

Introduction

Dengue virus (DENV) is a mosquito-borne virus in the genus *Flavivirus*, family *Flaviviridae*, consisting of four antigenically related serotypes: DENV-1, DENV-2, DENV-3, DENV-4 (Lindenbach and Rice, 2001). Infection with these viruses can result in a broad spectrum of effects, including a self-limiting acute febrile illness, dengue fever (DF), which may evolve to severe disease forms, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), with homeostatic and vascular permeability abnormalities (Rothman, 2004). Annually, it is estimated that 50–100 million cases of dengue occur in tropical and subtropical regions, in which 500,000 result in the DHF/DSS, with more than 20,000 deaths (Guzmán and Kourí, 2002). Because of the importance of such disease, concerning morbidity and mortality, the development of an effective vaccine against DENVs has been considered a high priority by the World Health Organization. One of the major difficulties associated with DENV vaccine is attributed to observations that most of DHF occur in individuals experiencing a secondary virus infection, which lead to the need of a safe and efficient tetravalent vaccine (Rothman, 2004). Since traditional methodologies were not successful, several new approaches have been proposed, including DNA vaccines (Edelman, 2005; Kinney and Huang, 2001).

The DENV are enveloped, single-stranded, positive-sense RNA virus of approximately 11 kb long, which contains a single open reading frame, encoding a polyprotein precursor that is processed by viral and host cell proteases to produce three structural proteins, capsid (C), premembrane/membrane (prM/M) and envelope (E), and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lindenbach and Rice, 2001). Most of experimental vaccines against DENV

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are based on the glycoproteins E and NS1, in a lesser extent. The E protein contains major epitopes responsible for eliciting neutralizing antibodies (Brinton et al., 1998; Chambers et al., 1990). However, such protein may also induce non-neutralizing antibodies involved in the phenomenon of antibody dependent enhancement (ADE) of the infection, which might be one of the major factors for the increase of DHF in secondary infections (Halstead and O'Rourke, 1977; Halstead and Deen, 2002).

The NS1 protein, in its turn, is highly immunogenic and can also induce protection in experimental animals against different flaviviruses (Brinton et al., 1998; Schlesinger et al., 1987). Several reports have shown that this protein may generate antibodies with complement fixing activity, which seems to kill infected target cells (Schlesinger et al., 1987, 1993). Since the NS1 is not present on the virion, antibodies against such protein may be elicited without the risk of the ADE. The NS1 is found in mammalian infected cells associated with plasma membrane and also secreted into the circulation as a soluble multimer (Flamand et al., 1999; Jacobs et al., 2000; Young et al., 2000). Although its function is not fully elucidated, evidences suggest that NS1 is involved in viral RNA replication (Mackenzie et al., 1996). The mature protein is essentially hydrophilic and lacks a membrane-spanning domain. However, during virus infection, such protein is translocated into the endoplasmic reticulum, using a hydrophobic signal sequence present at the C-terminal of the E protein, and associates in homodimer forms (Falgout et al., 1989; Pryor and Wright, 1993; Winkler et al., 1989). Dimeric NS1 is then anchored to cell membranes, probably via a hydrophobic sequence at N-terminal of NS2a, which appears to act as a signal sequence for glycosyl–phosphatidylinositol (GPI) linkage of NS1 (Jacobs et al., 2000; Pryor and Wright, 1993).

One advantage of applying DNA vaccines when compared to other approaches is the possibility of targeting the *in vivo* expressed recombinant antigen to different cell compartments. Thus, addition of signal sequences can retain the protein in cytosol or some sub-cellular compartments or direct it to cell membrane or secretion into extracellular milieu (Alves et al., 1998a; Donnelly et al., 2005; Lu et al., 2003). Therefore, the effectiveness of these vaccines can be distinct from each other since the different DNA construction strategy adopted may affect antigen presentation to the host immune system and consequently influence the elicited immune response (Alves et al., 1999; Donnelly et al., 2005).

Based on these evidences, in the present work, we constructed four different DNA vaccines encoding the DENV-2 NS1 in frame with its natural signal sequence, present at the C-terminal of the E protein, or with the secretory signal peptide derived from human tissue plasminogen activator (t-PA). Two of these constructs contained the hydrophobic stretch derived from the NS2a protein in order to target the recombinant NS1 to host cell surface, while the other plasmids were designed for protein secretion. Results demonstrated that these constructions differed on their abilities to drive the expression of NS1 in mammalian cells, to elicit NS1-specific antibodies in mice and to confer protection against DENV-2 challenge in these animals.

Results

Construction of different recombinant plasmids encoding the ns1 gene

Four different recombinant plasmids, pcTPANS1, pcTPANS1ANC, pcENS1 and pcENS1ANC, were constructed as described in Materials and methods. In the pcTPANS1 and pcTPANS1ANC constructs, the NS1 region from DENV-2 was cloned in frame with the t-PA signal sequence. Both plasmids are similar, except that the coding sequence of the pcTPANS1ANC was extended 138 nucleotides into the NS2A region (Fig. 1). The two other plasmids, pcENS1 and pcENS1ANC, contain the sequence of 63 nucleotides, which codes the 21 amino acid signal peptide derived from the C-terminal of the E protein, in addiction to the NS1 region (Fig. 1). The pcENS1ANC also encodes the 138 nucleotides from the NS2A region, similar to pcTPANS1ANC plasmid (Fig. 1). The t-PA signal peptide and the C-terminal hydrophobic region of the E protein were both used to target the translocation of the NS1 into endoplasmic reticulum and its secretion to extracellular space.

In vitro expression of the NS1 protein

The expression of recombinant NS1 proteins was evaluated in BHK-21 cells transiently transfected with each plasmid. Cells transfected with pcTPANS1, pcENS1 and pcENS1ANC plasmids were positively stained by immunofluorescence assays using a DENV-2 hyperimmune ascitic fluid (Fig. 2), which indicates that these constructs promote the expression of the NS1 in eukaryotic systems. On the other hand, no reaction could be detected in pcTPANS1ANC-transfected cell culture (Fig. 2f), although sequencing analysis revealed that the *ns1* gene was correctly cloned in this construct. As expected, cells transfected with control vectors (pcTPA and pcDNA3) did not react with DENV-2 antibodies (data not shown).

The expression of NS1 promoted by pcTPANS1, pcENS1 and pcENS1ANC was confirmed by Western blot (Fig. 3). A protein of approximately 48 kDa, corresponding to the NS1, was detected in pcTPANS1- and pcENS1-transfected cell lysates (Fig. 3a). In pcENS1ANC-transfected cell extract, one slight larger NS1 form was detected (Fig. 3a), probably due to the presence of the C-terminal hydrophobic region of 46 amino acids from the NS2a protein. Similar to results observed in the immunofluorescence assays, the pcTPANS1ANC-transfected cell lysate did not present any corresponding NS1 protein, confirming that this construct was not able to promote the expression of such protein. As expected, no related band was observed in cells transfected with control vectors (Fig. 3a). The presence of the NS1 protein was also detected in culture supernatants of pcTPANS1-, pcENS1- and pcENS1ANC-transfected cells (Fig. 3b), which demonstrated that the signal sequences cloned upstream the *ns1* gene (the t-PA sequence and C-terminal hydrophobic region from the E protein) indeed targeted the recombinant protein to secretion. In order to evaluate whether secreted NS1 was in monomeric or dimeric forms, samples were suspended in non-reducing conditions, submitted or not to heat

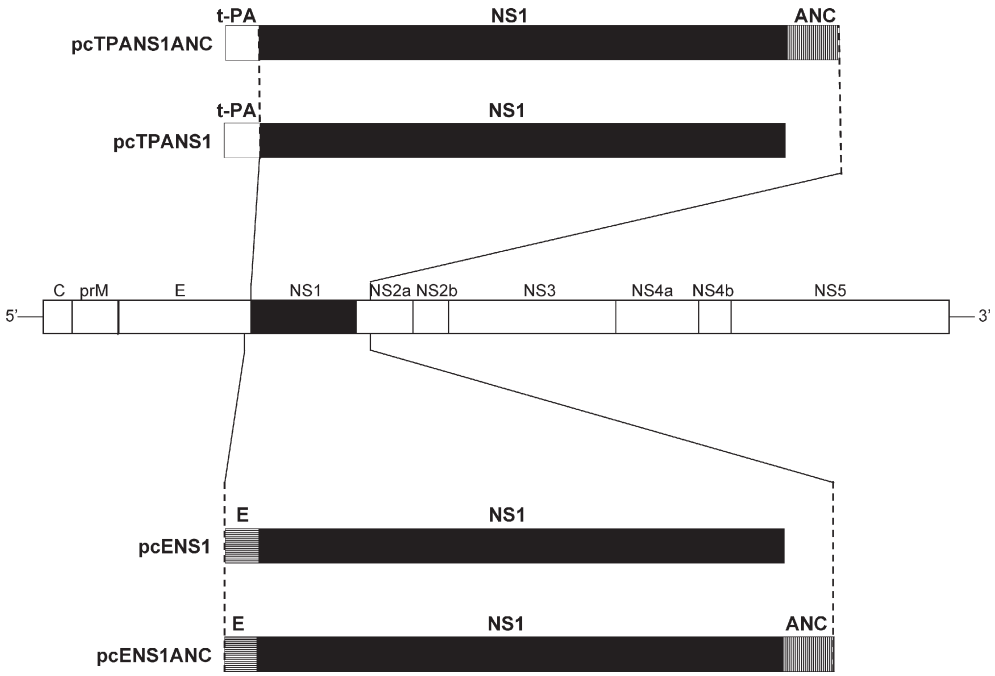

Fig. 1. Schematic diagram of DENV-2 genome map and plasmid constructs. Black area represents the NS1 region; hatched boxes represent signal sequences from the C-terminal of E protein (E) and the N-terminal of NS2a protein (ANC); open box represents the signal peptide derived from the human tissue plasminogen activator (t-PA).

treatment. Results showed that NS1 proteins were secreted mainly in a dimeric form of approximately 96 kDa, which became monomers of approximately 48 kDa after heat denaturation (Fig. 3b). A recombinant NS1 protein expressed in insect cells was used as a positive control. Such protein presented a slight size difference when compared to the NS1 expressed in BHK cells (Fig. 3b), which may be due to distinct glycosylation patterns from these two expression systems.

The NS1 localization in transfected BHK cells by electron microscopy

Cells transfected with pcTPANS1, pcENS1 and pcENS1ANC were immunostained with DENV-2 hyperimmune ascitic fluid and analyzed by electron microscopy in order to localize the NS1 protein. As expected, the protein was detected mainly in vesicles in all transfected cells, showing its targeting to

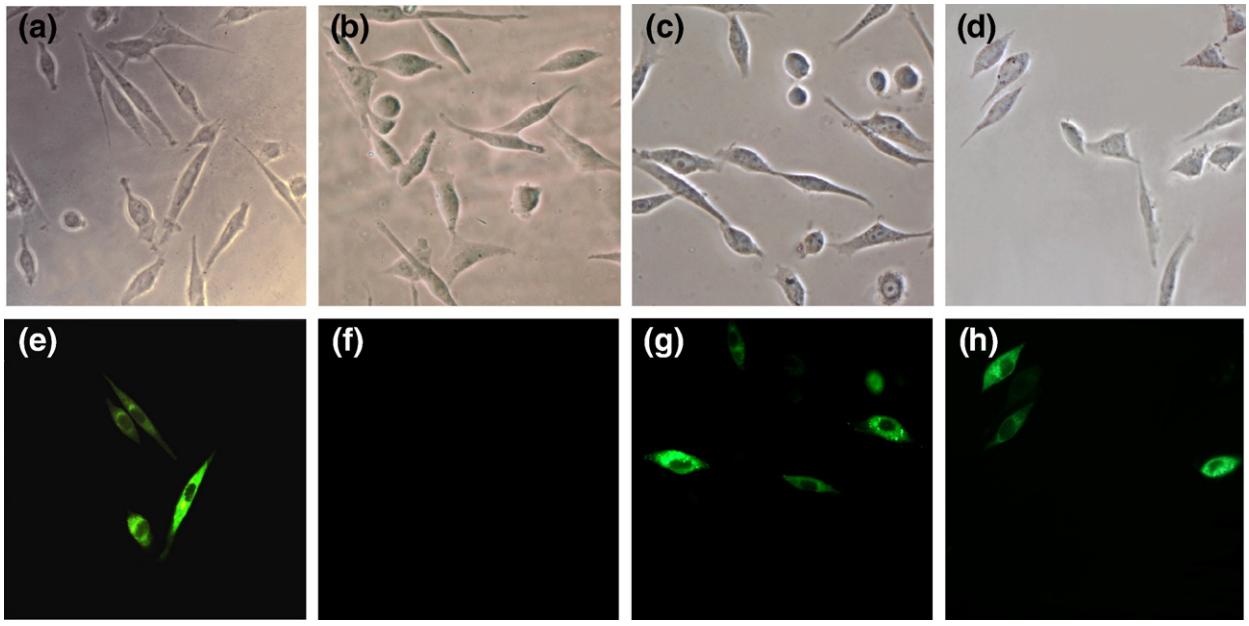

Fig. 2. BHK-21 cells transfected with the different recombinant plasmids: pcTPANS1 (a, e), pcTPANS1ANC (b, f), pcENS1 (c, g) and pcENS1ANC (d, h). Cells were permeabilized, fixed and treated with DENV-2 hyperimmune mouse ascitic fluid and anti-mouse fluorescein-conjugated goat IgG. Phase contrast (a–d); immunofluorescence (e–h). Magnification, ×400.

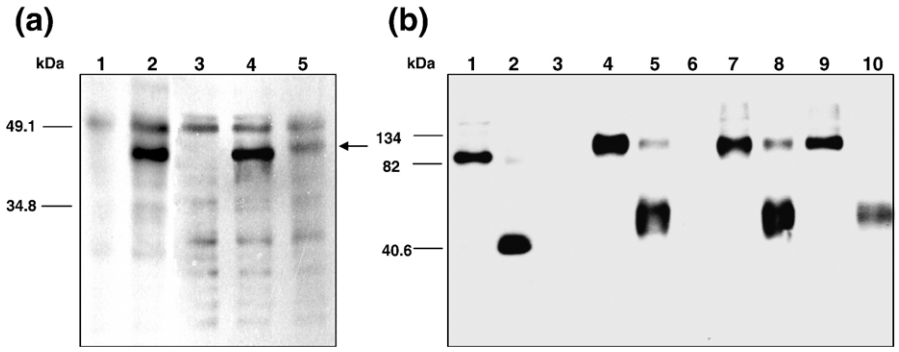

Fig. 3. NS1 protein expressed from recombinant plasmids in transfected BHK cells and detected in Western blots of SDS-PAGE, using DENV-2 hyperimmune mouse ascitic fluid. Samples were suspended in SDS-PAGE buffer with heat treatment (a) or in phosphate buffer with protease inhibitors without 2-mercaptoethanol, submitted or not to heat treatment (b). (a) Whole-cell extracts harvested from transfections with pcDNA3 (lane 1), pcTPANS1 (lane 2), pcTPANS1ANC (lane 3), pcENS1 (lane 4) and pcENS1ANC (lane 5). Arrow indicates bands corresponding to the NS1 protein. (b) Purified NS1 protein expressed in insect cells (lanes 1 and 2) and culture supernatants harvested from cells transfected with pcTPA (lane 3), pcTPANS1 (lanes 4 and 5), pcDNA3 (lane 6), pcENS1 (lanes 7 and 8) and pcENS1ANC (lanes 9 and 10), not submitted to heat treatment (lanes 1, 4, 7 and 9) or boiled for 5 min (lanes 2, 3, 5, 6, 8 and 10).

the secretory pathway (Fig. 4). In cells transfected with the pcENS1ANC, vesicles containing NS1 were seen close to and fused with the cytoplasmic membrane, which in some parts lead to its anchoring to the membrane on the surface of the cell (Figs. 4c and d). Such results confirm that the recombinant protein is predominantly secreted, due to the signal peptide sequences cloned upstream of the gene, and that the hydrophobic sequence from the NS2a protein is essential for the NS1 association with the plasma membrane.

Antibody response elicited in mice immunized with different plasmids

Serum samples from mice immunized twice with one of the recombinant plasmids were tested individually by ELISA, 4 weeks after the first DNA injection. All pcTPANS1- and pcENS1-inoculated animals presented a significant NS1-specific antibody response, with titers ranging from 20,000 to 30,000 in both cases, except for one animal in each group, which presented higher antibody levels (41,000 and 61,000 for pcTPANS1- and pcENS1-immunized mice, respectively) (Figs. 5a and b). On the other hand, only half of pcENS1ANC-inoculated animals (5 in 10) presented NS1-specific IgG response (two mice with antibody titers of approximately 1000 and three with titers ranging from 4700 to 5600) (Fig. 5c). As expected, pcTPANS1ANC-inoculated mice did not present any NS1-specific antibody response, as well as animals injected with control vectors, pcTPA or pcDNA3 (data not shown).

Time course of serum NS1-specific IgG responses were then analyzed in pooled samples from pcTPANS1- and pcENS1-immunized groups and from the five NS1-positive mice inoculated with the pcENS1ANC. Levels of NS1-specific antibodies were very similar in animals immunized with pcTPANS1 and pcENS1, which attained maximum values 4 weeks after the first DNA dose and remained stable until 10 weeks after the beginning of experiments (Fig. 6). Animals inoculated with pcENS1ANC presented considerable lower NS1-specific antibody levels in all time points tested (Fig. 6). However, humoral response patterns were similar in the three immunization groups, with a significant increase of antibody levels after the second DNA inoculation. Epitope specificities of antibodies generated by immunization with the three plasmids were analyzed, using intact or heat-denatured forms of the NS1 protein as solid-phase bound antigen. Antibodies raised in all immunizations recognized mainly the intact form of NS1 since reactivity of antibodies was drastically reduced when the heat-denatured protein was used (approximately 30-fold lower when compared to the non-denatured NS1 during peak antibody level) (Fig. 6). These results suggest that antibodies induced by the three plasmids recognized mainly conformational surface-exposed epitopes, which are practically absent in the heat-denatured NS1 protein.

Protective immunity in pcTPANS1- and pcENS1-vaccinated mice

Since the pcTPANS1 and pcENS1 elicited high and homogenous NS1-specific antibody responses, they were selected for further challenge experiments. The protective efficacy of these plasmids was then evaluated in animals vaccinated and i.c. challenged with a mouse brain adapted DENV-2, 2 weeks after the second DNA dose, when peak antibody levels were observed. As controls, mice inoculated with pcTPA or pcDNA3 vectors or non-immunized animals were also challenged with DENV-2. Mice were monitored the following 21 days for mortality and morbidity, regarding mainly the development of hind leg paralysis. Three independent challenge experiments were performed for each vaccine at the same conditions, and data are summarized in Fig. 7.

Paralysis was detected 7 days after challenge in control groups (pcTPA, pcDNA3 and non-immunized mice) and, in the end of experiments, 90% of these animals presented remarkable signs of dengue infection (Figs. 7a and c). In fact, most of these mice died 21 days after challenge, with survival rates of 33.3% in non-immunized or pcDNA3-inoculated groups and of 50% in pcTPA-injected animals (differences not significant, $p = 0.19043$) (Figs. 7b and d). In contrast, pcTPANS1- and pcENS1-vaccinated animals showed a considerable reduction in clinical signs, with morbidity rates of 10% (3/

30) and 27% (8/30), respectively (significance with $p<0.00001$ in both cases, when compared to control groups) (Figs. 7a and c). A strong correlation was observed between morbidity and survival rates. Indeed, both tested vaccines were highly protective against DENV-2, in which 96.7% (29/30) and 86.7% (26/30) of pcTPANS1- and pcENS1-immunized mice, respectively, survived to challenge (significance with $p=0.00004$ for pcTPANS1 and $p=0.00003$ for pcENS1, when compared to control groups) (Figs. 7b and d). Results revealed that the pcTPANS1 plasmid was slightly more protective than the pcENS1, although not statistically significant, regarding either survival (96.7% for pcTPANS1 and 86.7% for pcENS1, $p=0.16113$) or morbidity rates (10% for pcTPANS1 and 27% for pcENS1, $p=0.09527$). Besides, in pcENS1-immunized group, paralysis was noted on the 7th day after challenge, while in animals vaccinated with the pcTPANS1, clinical infection signs appeared only on the 9th day.

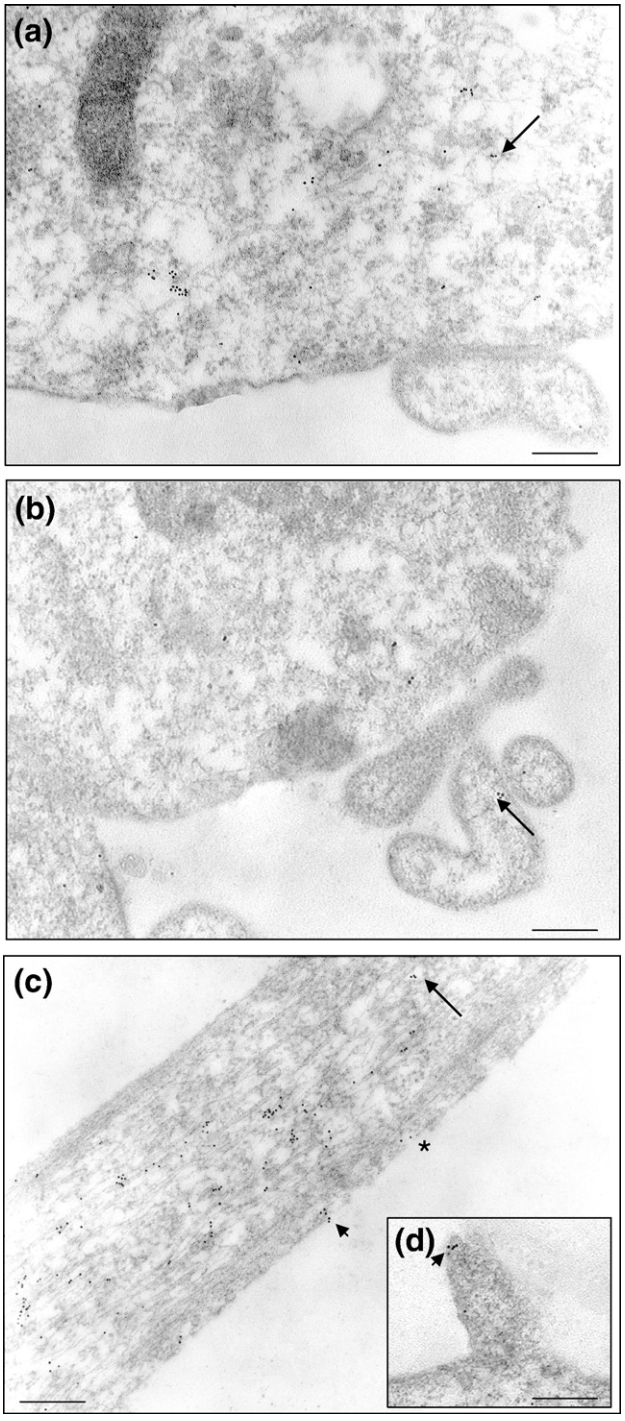

Fig. 4. Electron microscopy of transfected BHK cells, immunolabeled with DENV-2 hyperimmune mouse ascitic fluid and anti-mouse colloidal gold conjugated IgG. Cells transfected with: (a) pcTPANS1, scale bar=0.45 μm; (b) pcENS1, scale bar=0.22 μm; (c) pcENS1ANC, scale bar=0.21 μm; (d) pcENS1ANC, scale bar=0.14 μm. Note the NS1 protein present in vesicles (arrow), anchored to plasma membrane (arrowhead) and NS1 containing vesicles fused to cytoplasmic membrane (asterisk).

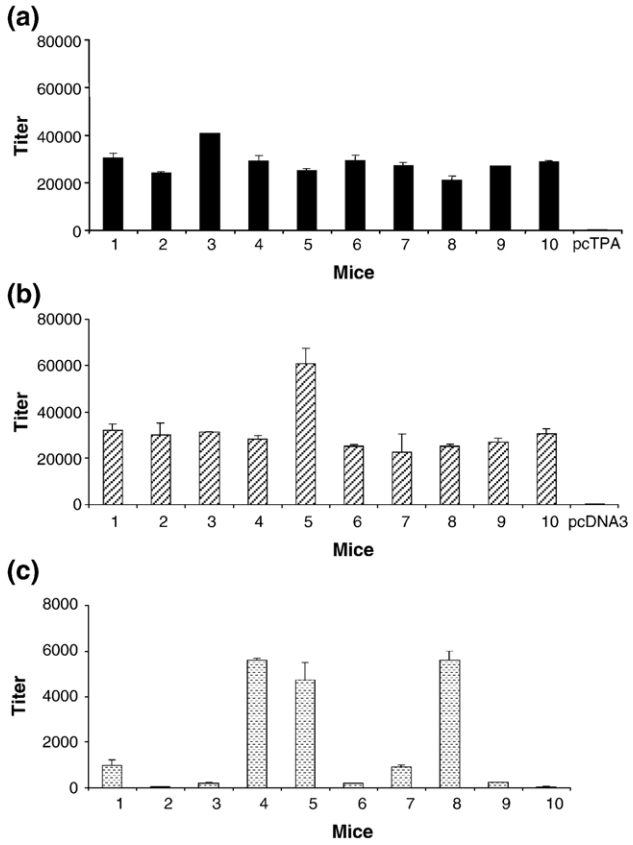

Fig. 5. Individual NS1-specific IgG response elicited in mice immunized with (a) pcTPANS1, (b) pcENS1 and (c) pcENS1ANC. Animals were i.m. inoculated with two DNA doses, blood samples were taken 4 weeks after the first immunization and tested by ELISA. Serum samples harvested from mice inoculated with control vectors pcTPA or pcDNA3 were pooled. Titers were established as the reciprocal serum dilutions which gave an absorbance above that of preimmune sera. Data represent the mean of duplicate values for each sample, and bars are standard deviation of the mean.

Antibody response in vaccinated animals after challenge

Levels of NS1-specific antibody increased more than 5 times after challenge with DENV-2 in both vaccinated animals (titers ranging from approximately 32,000 or 25,000 before challenge to 254,000 or 140,000 after virus inoculation in pcTPANS1- and pcENS1-immunized mice, respectively) (Fig. 8). In contrast, control animals presented considerable low levels of NS1-

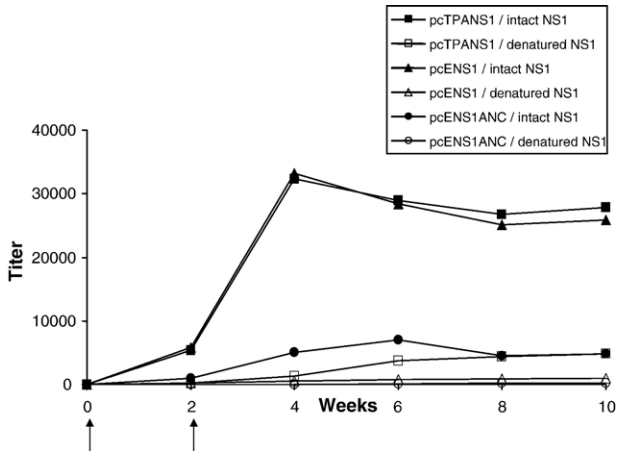

Fig. 6. Time course of serum IgG response to NS1 protein from Balb/c mice i.m. immunized with two doses of pcTPANS1, pcENS1 or pcENS1ANC. Titers were determined in ELISA using purified intact or heat-denatured NS1 protein expressed in insect cells, as solid-phase bound antigen. Data represent the mean of duplicate values of pooled serum samples harvested from immunized mice. Arrows indicate time point of DNA inoculations. Standard deviations were always under 10% of the mean value.

specific IgG after challenge (titers ranging from 30,000 to 50,000) (data not shown).

The percentage of NS1-specific IgG1 and IgG2a subclasses was then analyzed in the two vaccinated mouse groups and controls. Mice immunized with the pcTPANS1 plasmid presented mainly IgG1 antibodies, either before or after virus challenge (Fig. 9). On the other hand, immunization with the pcENS1 elicited similar levels of both IgG subclasses, and such pattern remained similar after challenge (Fig. 9). In control animals (non-immunized or pcTPA-injected mice), inoculation of DENV-2 by the i.c. route induced predominantly NS1-specific IgG2a.

Discussion

In an effort to develop an optimal DNA vaccine for dengue virus based on the NS1 protein, in the present report, we expanded our previous work with the pcTPANS1 plasmid, which contains the DENV-2 NS1 region fused to t-PA signal sequence (Costa et al., 2006a,b). Such construction was compared to three other plasmids encoding the DENV-2 *ns1* gene. In one construct (pcENS1), this gene was cloned in frame with its natural leader sequence, present at the 3′ end of the envelope gene. The same region present in the pcENS1 was cloned in frame with the sequence coding the N-terminal hydrophobic stretch of the NS2a protein in order to generate the pcENS1ANC. Another plasmid was constructed with the addition of such NS2a sequence downstream the *ns1* gene in the pcTPANS1, engendering the pcTPANS1ANC.

Expression of the recombinant NS1 protein in transfected mammalian cells was significantly different, depending on the plasmid used. The pcTPANS1, pcENS1 and pcENS1ANC

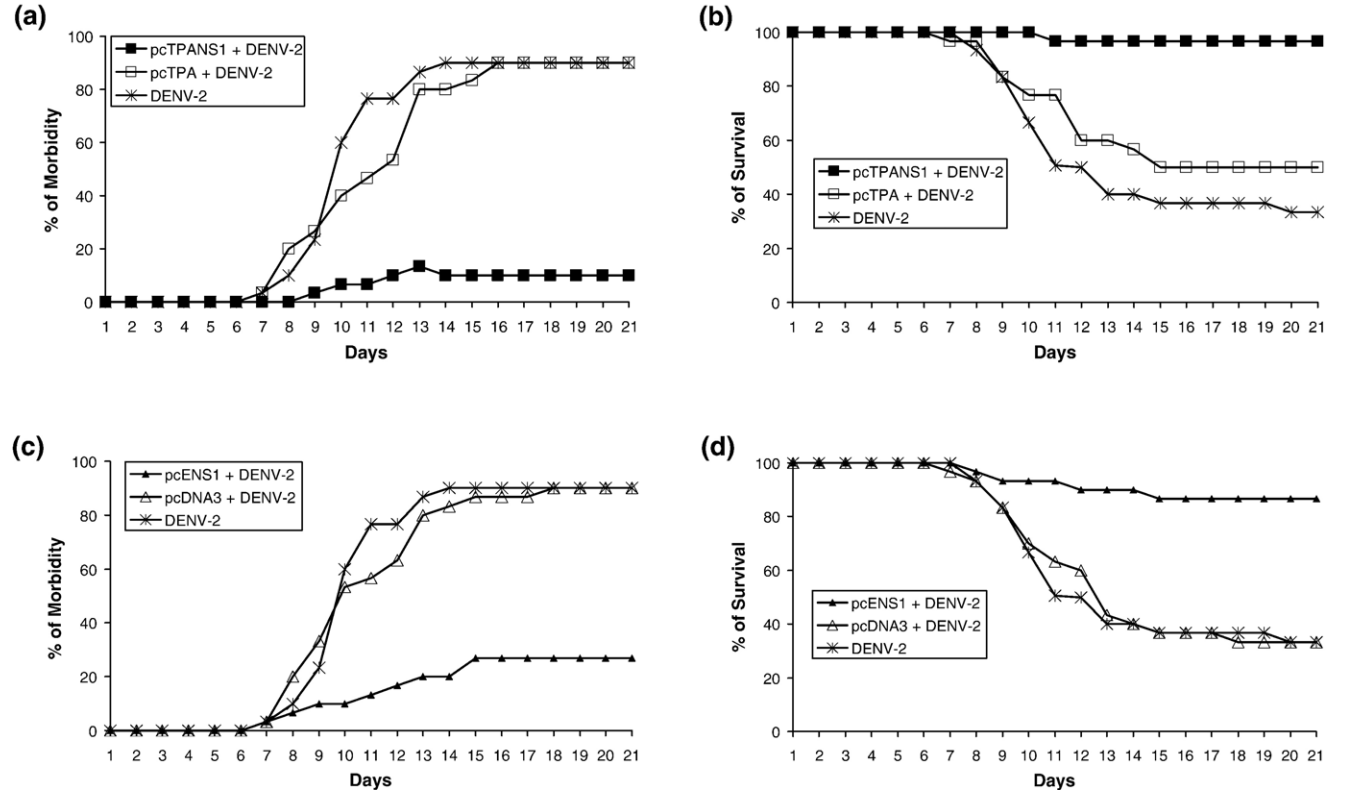

Fig. 7. Percentage of morbidity (a, c) and survival (b, d) of Balb/c mice immunized with pcTPANS1 (a, b) and pcENS1 (c, d) and i.c. challenged with NGC DENV-2. Mice were i.m. immunized with two DNA doses and challenged 4 weeks after the first plasmid inoculation. Non-immunized and pcTPA or pcDNA3-injected mice followed the same virus infection procedure. Mice were daily monitored, and pathological symptoms, mainly hind leg paralysis, were recorded. Data represent compilation of three independent experiments, with groups of 10 animals in each test ($n=30$).

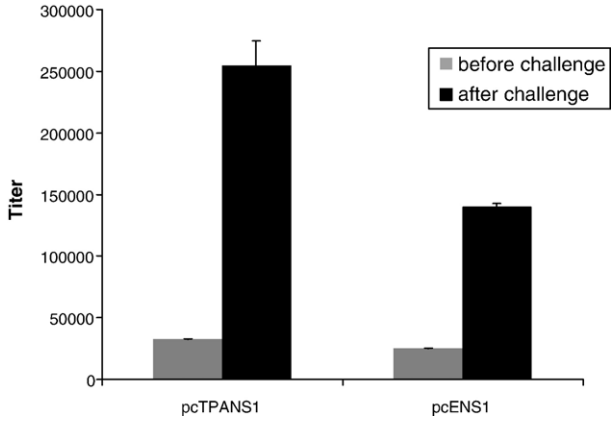

Fig. 8. NS1-specific antibody response in pcTPANS1- and pcENS1-immunized mice, before and after challenges with DENV-2. Mice were i.m. injected with two DNA doses and challenged 4 weeks after the first plasmid inoculation. Serum samples were collected 4 weeks after the first DNA immunization (before challenge) and 21 days after virus inoculation (after challenge). Titers of NS1-specific antibodies were determined in ELISA with purified NS1 protein expressed in insect cells as solid-phase bound antigen and calculated as described in Fig. 5. Data represent the mean of duplicate values for pooled blood samples, and bars are standard deviation of the mean.

plasmids were able to promote expression of NS1, while cells transfected with the pcTPANS1ANC did not present any recombinant protein, as revealed by immunofluorescence analyses. Apparently, the two signal hydrophobic sequences (t-PA and ANC) are incompatible. One possible reason for such results is that the presence of these two sequences may change the NS1 conformation, which prevented the correct protein processing. The incorrect folding of the protein could then lead to its accumulation inside the cell, which might be toxic and consequently no viable pcTPANS1ANC-transfected cells could be detected. In addition to the intracellular NS1 detected in cells transfected with the three other plasmids, this protein was also identified in Western blot of culture supernatant as dimeric forms. These results indicate that both homologous and heterologous secretory signal sequences were efficient for targeting translocation of protein into the endoplasmic reticulum. Furthermore, the presence of secreted dimeric forms of NS1 suggests that this recombinant protein was expressed similarly to the protein observed in DENV infected mammalian cells, in which dimerization is essential for its export along the secretory pathway to the plasma membrane (Pryor and Wright, 1993). Moreover, the NS1 contains two conserved N-glycosylation sites and it was demonstrated that the addition of sugar groups to this protein contributes to dimer stability, increasing its hydrophobicity (Flamand et al., 1999; Pryor and Wright, 1994). Thus, secretion of the NS1 by cells transfected with the pcTPANS1, pcENS1 and pcENS1ANC plasmids as dimers suggests that these proteins are glycosylated.

Electron microscopy analyses of pcTPANS1-, pcENS1- and pcENS1ANC-transfected cells showed that the NS1 was found mainly in cytoplasmic vesicles in the three cases. Such results were consistent with the detection of NS1 in culture supernatants, indicating that most protein is in fact target to the secretory pathway. Furthermore, in pcENS1ANC-transfected cells, the NS1 was also found anchored to plasma membrane or reaching the cell surface via membrane-bound vesicles. These data confirm previous evidences that the NH_2 terminus of the NS2a protein is in fact required for association of the NS1 to cell membrane, probably via GPI linkage (Jacobs et al., 2000).

All Balb/c mice inoculated with pcTPANS1 or pcENS1 developed a homogeneous humoral immune response with high NS1-specific antibody levels. In contrast, only few pcENS1ANC-immunized animals presented NS1-specific antibodies, with heterogeneous levels, in which highest titers were approximately six-fold lower than those found in mice inoculated with the two other plasmids. Such results might be due to differences in the NS1 expression patterns since part of the recombinant protein should be retained in plasma membrane of *in vivo* pcENS1ANC-transfected cells, similar to data observed in the *in vitro* transfections. Consequently, the amount of soluble NS1 available for the immune system to elicit antibodies might be diminished in these animals. These findings are partially in agreement with other studies using recombinant vaccinia virus expressing the DENV-4 NS1 protein and 15% of the NS2a (Falgout et al., 1990). Animals immunized with such virus presented low titers of NS1-specific antibodies, which correlated to a partial protection against DENV-4. However, in this case, authors observed that the expressed recombinant NS1 was not correctly cleaved, leading to aberrant forms of the protein (Falgout et al., 1990). On the other hand, our expression analysis results showed that the NS1 was secreted as dimeric forms by cells transfected with the three plasmids (pcTPANS1, pcENS1 and pcENS1ANC), similarly confirming the correct process of the recombinant protein. Consistent with these findings, antibodies induced by such plasmids recognized mainly intact NS1 expressed in insect cells, while the reaction was significant weakly detected when the protein was heat-denatured, with titers more than 30-fold lower even for pcENS1ANC-elicited antibodies, during peak IgG level. Such observations indicate that these antibodies were predominantly directed to conformational surface-exposed epitopes and confirmed that all the three recombinant NS1 expressed *in vivo* presented a tri-dimensional

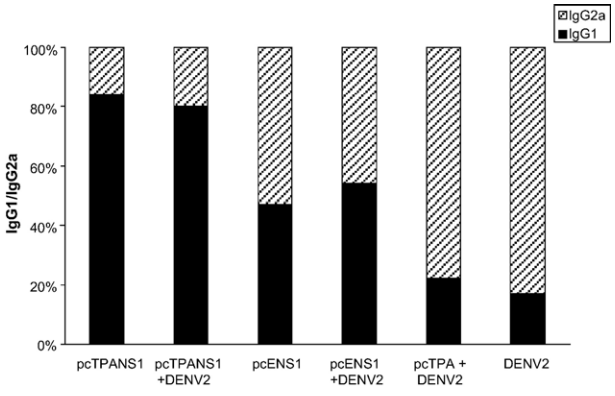

Fig. 9. NS1-specific IgG subclass responses in mice immunized with pcTPANS1 and pcENS1, before and after challenge with DENV-2. Animals were inoculated with recombinant plasmids or control vector (pcTPA), and serum samples were harvested and pooled as depicted in Fig. 8. Each column represents the relative percentage of IgG1 and IgG2a subclasses in each analyzed sample.

structure similar to the native viral protein, as previously suggested (Costa et al., 2006b).

The protective ability of pcTPANS1 and pcENS1 immunizations was investigated in challenge experiments with Balb/c i.c. injected with a mouse brain adapted NGC DENV-2. The pcENS1ANC plasmid was not used in these challenge assays due to the heterogeneous and low NS1-specific antibody response it elicited. Our results revealed that the pcTPANS1 and pcENS1 plasmids conferred high level of protection. These data are consistent with previous studies supporting that the flavivirus NS1 by itself is able to elicit protective immunity (Costa et al., 2006a,b; Falgout et al., 1990; Lin et al., 1998; Schlesinger et al., 1987; Wu et al., 2003). Although not statistically significant, the pcTPANS1 plasmid was slightly more protective than the pcENS1, regarding survival and mainly morbidity rates. Clinical infection signs appeared 2 days early, on the 7th day after challenge, in pcENS1-immunized mice (similar to control groups) when compared to what happened in pcTPANS1-inoculated animals, which presented such signs on the 9th day. In addition, morbidity rates 21 days post virus inoculation were almost 3 times higher in pcENS1- than in pcTPANS1-vaccinated animals (27% and 10% in pcENS1 and pcTPANS1 groups, respectively). Furthermore, morbidity and mortality rates varied among the three challenge experiments in pcENS1-immunized mice, while vaccination with the pcTPANS1 induced a considerable homogeneous protection in all performed tests (data not shown). These results confirmed our previous report with the pcTPANS1 plasmid (Costa et al., 2006b) and other studies with a DNA vaccine encoding the NS1 and the signal peptide derived from C-terminus of the E protein (Wu et al., 2003). The DNA vaccine constructed in Taiwan (Wu et al., 2003) is very similar to the pcENS1, although the signal peptide contains 19 amino acids, while in the case of the pcENS1, this signal sequence is composed of 21 amino acids. Besides, the *ns1* gene cloned in the pcENS1 was amplified from the NGC DENV-2 and in the other plasmid this gene came from a Taiwanese DENV-2 strain. Immunization with the plasmid DNA constructed in Taiwan also induces protection against DENV-2, but in lower level (28% of mortality and 30% of morbidity) when compared to the pcTPANS1 (3.3% of mortality and 10% of morbidity), and similar to the pcENS1 (13.3% of mortality and 27% of morbidity). Such observations are in agreement with other findings, which demonstrate that the t-PA signal sequence is a highly efficient signal peptide for induction of strong immune responses by DNA vaccines against several pathogens (Alves et al., 2001; Ashok and Rangarajan, 2002; Li et al., 1999).

Animals vaccinated with the pcTPANS1 or pcENS1 exhibited a significant increase of NS1-specific antibody levels after virus challenge, indicating the activation of an immunological memory with a rapid and strong secondary immune response. These data also demonstrated that the protective immunity induced by the NS1-based DNA vaccines were not sterilizing. Such results were not surprising since the NS1 is not present in the virion, and therefore the immune response against this protein can only be protective after initial virus replication in host cells. Supporting this fact, antibodies raised against NS1 by immunizations with our DNA vaccines presented no detectable neutralizing activity (data not shown).

The analyses of NS1-specific IgG subclasses elicited by both DNA vaccines pcTPANS1 and pcENS1 revealed distinct activation patterns of the immune system. Mice vaccinated with pcTPANS1 developed a predominant anti-NS1 IgG1 subclass response (more than 80%), while pcENS1 immunization elicited a mix of IgG1 and IgG2a (approximately 50%), before and after virus challenge. In contrast, after the DENV-2 inoculation, animals in control groups (non-immunized or pcTPA-injected) presented mainly NS1-specific IgG2a antibodies (approximately 80%). Previous reports suggested that the NS1 can be protective against flavivirus infections due to the induction of antibodies that kill infected target cells in a complement-dependent manner (Henchal et al., 1988; Lin et al., 1998). However, our results, concerning IgG subclass differences, cannot specify whether the complement activation is in fact the major mechanism involved in the protection induced by the pcTPANS1 and pcENS1 plasmids. The IgG2a subclass is generally associated with substantial complement fixing activity (Leatherbarrow and Dwek, 1984), although some studies have demonstrated that IgG1 is also efficient in complement activating. In humans, the IgG1 subclass was shown to be a good activator of the classical pathway while IgG2 was the best subclass for the alternative pathway complement activation (Lucisano and Lachmann, 1991). On the other hand, Chung et al. (2006) demonstrated that different monoclonal antibodies against the NS1 protein from West Nile virus can be protective through different mechanisms, in a complement independent pathway. Furthermore, the protective effect of these antibodies was not dependent on the IgG subclass. Thus, it is probable that mechanisms other than complement fixing might also play account for the protection induced by anti-NS1 antibodies. Besides, our results also suggest that the pcTPANS1 activated a major Th2-type immune response and the pcENS1 immunization led to an equilibrium of the two population cells (Th1 and Th2), while survived animals in control groups, which presented high paralysis signs, generated a drastically Th1 biased immune response. Therefore, we cannot discard a possible contribution of these cellular immune responses, as well as related cytokines, to the induced protection. Further studies will be necessary in order to evaluate such questions.

Materials and methods

Virus and cell lines

The dengue 2 virus (DENV-2) strain New Guinea C (NGC DENV-2) was used for cloning the NS1 sequence and challenge assays. NGC DENV-2 propagation was carried out in Vero cells cultivated in medium 199 with Earle salts (E199) buffered with sodium bicarbonate (Sigma, USA), supplemented with 10% fetal bovine serum (FBS). For *in vitro* transfection and recombinant NS1 protein expression analyses, baby hamster kidney cells (BHK-21) were propagated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA), supplemented with 5% FBS.

Construction of recombinant plasmids

Four pcDNA3-based plasmids were constructed encoding the NS1 sequence from DENV-2. Total RNA from cells infected with the virus was extracted with Trizol (Invitrogen), according to the manufacturer's protocol. The RNA was used as template for the synthesis of a cDNA performed by reverse transcriptase M-MLV (Invitrogen) with the antisense primer 5′-CAT AAG CTT ACA GAG GTT CCC CCA TG-3′, which hybridizes to nucleotides 5943 to 5958 in the NGC DENV-2 genome. Different segments containing the NS1 sequence were then amplified by PCR using primers shown in Table 1. All sense and antisense primers contained *Eco*RV and *Xho*I restriction sites, respectively. The different PCR products were electrophoresed on a 1% agarose gel, recovered with glass beads, geneclean (Stratagene, USA), restricted with *Eco*RV and *Xho*I and ligated to the vectors previously digested with the same enzymes. The pcDNA3 vector (Invitrogen) was used for construction of pcENS1 and pcENS1ANC, while the plasmids pcTPANS1 and pcTPANS1ANC were constructed based on the pcTPA, a modified pcDNA3 vector that contains the human tissue plasminogen activator (t-PA) signal sequence (Costa et al., 2006b). All recombinant plasmids were screened by restriction mapping and confirmed by sequencing, using ABI PRISM dye terminator cycle sequencing core kit (Applied Biosystems, USA). Plasmids were isolated from transformed *Escherichia coli*, DH5α strain, and purified by Qiagen Plasmid Giga Kit (Qiagen, Germany), following manufacturer's instruction. DNA concentrations were determined by measuring optical density at 260 nm, and integrity of plasmids was checked by agarose gel electrophoresis. Plasmids were suspended in sterile water and stored at −20 °C until use.

Transfection of BHK cells with different plasmids

BHK cells were transiently transfected with each recombinant plasmid or with control vectors (pDNA3 or pcTPA) and fixed or harvested 24 h after transfections. For detection of the NS1 protein by immunofluorescence, 2×10^4 cells/well were plated in chamber slides (Nunc, Denmark) with Optimen medium (Invitrogen) and transfected with 0.2 μg of each DNA, using lipofectamine (Invitrogen) under conditions suggested by the manufacturer. For Western blot and electron microscopy analyses, 5×10^5 cells were plated in 25 cm^2 bottle and transfected with 2 μg of DNA.

Immunofluorescence

Monolayers of transfected cells were washed in 0.1 M phosphate buffer pH 7.4 (PB), fixed in 4% paraformaldehyde in PB for 10 min, washed again in PB, permeabilized with 0.6% saponin in PB for 10 min and blocked with 1% bovine serum albumin (BSA) and 0.2% saponin in PB for 15 min. All such steps were conducted at room temperature. Cells were then incubated with DENV-2 hyperimmune mouse ascitic fluid (ATCC, USA) at a dilution of 1:1500 in PB for 1 h at 37 °C, washed tree times with PB and another incubation of 1 h at 37 °C with fluorescein-conjugated goat anti-mouse IgG (Southern Biotechnology, USA) diluted 1:100 in PB. Slides were mounted with Vectashield medium (Vector Laboratories Inc., USA), and cells were visualized in a fluorescence microscope.

Western blotting

Western blotting of transfected whole-cell extracts and culture supernatants was performed as previously described (Costa et al., 2006b). Briefly, cell extracts were suspended in SDS-PAGE sample buffer (Sambrook et al., 1989) and boiled for 5 min. Culture supernatants were clarified by low speed centrifugation, concentrated with Centricon YM-3 (Millipore Corporation, USA), mixed with equal volume of SDS-PAGE sample buffer without 2-mercaptoethanol and submitted or not to heat treatment (boiled for 5 min). Proteins were sorted in SDS-PAGE and transferred onto nitrocellulose membranes. Dimeric and monomeric forms of NS1 were detected with DENV-2 hyperimmune mouse ascitic fluid at a dilution of 1:1000 followed by incubation with rabbit anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Southern Biotechnology) diluted 1:4000. Membranes were developed with the ECL kit (Amersham Biosciences, UK) and exposed to Kodak X Omat films.

Table 1
Primers used for amplifications of different DENV-2 NS1 sequences and construction of recombinant plasmids

Vectors [a]	Primers	Amplified regions [b]	Recombinant plasmids
pcTPA	5′-GGGGGATATCGATAGTGGTTGCGTTG-3′ [c] 5′-GGGGCTCGAGTTAGGCTGTGACCAAG-3′ [d]	NS1 (2422–3477)	pcTPANS1
pcTPA	5′-GGGGGATATCGATAGTGGTTGCGTTG-3′ [c] 5′-GGGGCTCGAGTTACCCTGTGATCAATG-3′ [d]	NS1 (2422–3477)+NS2a (3478–3615)	pcTPANS1ANC
pcDNA3	5′-GGGGGATATCATGCTGTCTGTGTCACTAG-3′ [c] 5′-GGGGCTCGAGTTAGGCTGTGACCAAG-3′ [d]	E (2359–2421)+NS1 (2422–3477)	pcENS1
pcDNA3	5′-GGGGGATATCATGCTGTCTGTGTCACTAG-3′ [c] 5′-GGGGCTCGAGTTACCCTGTGATCAATG-3′ [d]	E (2359–2421)+NS1 (2422–3477)+NS2a (3478–3615)	pcENS1ANC

[a] Vectors used for cloning.
[b] Genome coordinates (Irie et al., 1989).
[c] Sense primer.
[d] Antisense primer.

Electron microscopy

Monolayers of transfected cells were fixed overnight with 1% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2. Cells were then scraped, washed with the cacodylate buffer, dehydrated with ethanol and embedded in LR Gold resin (London Resin Company Ltda, England). Ultrathin sections (60–70 nm) were collected on gold grids (400 mesh) and incubated for 1 h at room temperature with DENV-2 hyperimmune mouse ascitic fluid, diluted 1:1500 in 0.1 M Tris–HCl buffer, pH 7.4, with 0.1% gelatin, 1% Tween-20 and 1% BSA. Grids were washed in water and incubated for 1 h at room temperature with goat anti-mouse colloidal gold conjugated IgG, 10 nm, (Sigma) diluted 1:40 in the same buffer. Sections were washed in water, double stained with uranyl acetate and lead citrate, washed again in water and observed in a transmission electron microscope (EM 10, Zeiss, Germany).

Immunization procedure

Experiments with mice were conducted in compliance with Ethical Principles in Animal Experimentation stated in the Brazilian College of Animal Experimentation and approved by the Institute's Animal Use Ethical Committee. Balb/c mice, 4 to 6 weeks old, were inoculated by the intramuscular (i.m.) route with 50 μg of plasmid dissolved in 50 μL of phosphate buffer saline (PBS) in each tibialis posterior muscles (100 μg/mice) using 27-gauge needles. Each animal group ($n=10$) received two doses of one recombinant plasmid or control vector, given 2 weeks apart. For time course of NS1-specific antibody response evaluation, animals were bled by retro-orbital puncture, before inoculation (preimmune sera) and at several time intervals after immunization. Initially, serum samples were individually tested for reactivity against NS1 protein and then pooled and stored at −20 °C for subsequent analyses. For challenge experiments, mice were bled 4 weeks after the first DNA dose and at the end of experiments (21 days after challenge), when animals were sacrificed.

Evaluation of NS1-specific antibody response

Mouse sera were tested for the presence of NS1-specific antibodies by enzyme-linked immunosorbent assay (ELISA). Wells of MaxiSorp plates (Nunc) were coated for 1 h at 37 °C with 0.1 μg (in 100 μL PBS) of a recombinant DENV-2 NS1 protein expressed in insect cells (Hawaii Biotechnology Group Inc., USA) and blocked overnight at 4 °C with 2% skim milk in 0.05% Tween-20–PBS (PBST). Assays were performed either with intact or heat-denatured (boiled for 5 min) recombinant NS1 protein. Serum samples were serially diluted and added to wells previously washed with PBST. After 1 h at 37 °C, plates were washed with PBST and incubated with goat anti-mouse immunoglobulins IgG, IgG1 or IgG2a conjugated with horseradish peroxidase (Southern Biotechnology) for 1 h at 37 °C. The secondary antibodies used to determine IgG subclasses were highly specific and had quantitatively similar reactivity in ELISA, thus allowing direct comparison between the levels of each subclass (Alves et al., 1998b). Reactions were measured at $A_{450\ nm}$ with *ortho*-phenylenediamine dihydrochloride (Sigma) and H_2O_2 as substrate and with a 9 N H_2SO_4 stopping solution. Titers were established as the reciprocal of serum dilution, which gave an absorbance above that of the respective preimmune serum.

DENV-2 challenge in mice

Two weeks after the second DNA dose, mice i.m. immunized with recombinant or control plasmids were challenged with the NGC DENV-2, a mouse brain adapted virus. Animals were anesthetized with a mixture of ketamine-xylazine (Erhardt et al., 1984) and intracerebrally (i.c.) inoculated with 30 μL of 4.32 $\log_{10}$ PFU of DENV-2, which corresponds to 3.8 LD_{50}, diluted in E199 medium supplemented with 5% FCS. Immediately after the challenge procedure, inoculum was back-titered in Vero cells as described previously (Caufour et al., 2001). Mice were separated in five groups ($n=10$) for each test: pcTPANS1-, pcTPA-, pcENS1- and pcDNA3-inoculated mice and non-immunized animals, all challenged with the same virus sample. Animals were monitored for 21 days. Morbidity, mainly the appearance of hind leg paralysis, and mortality were recorded. After 21 days, survived animals were sacrificed and blood samples were collected. Three independent challenge tests were performed in the same experimental conditions.

Statistical analysis

Experimental results on challenge tests were analyzed for their statistical significance by chi-square test.

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