Ministério da Saúde
Fundação Oswaldo Cruz
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Pós-Graduação em Biologia Celular e Molecular

VACINAS DE DNA CONTRA O VÍRUS DA DENGUE UTILIZANDO COMO ANTÍGENOS AS PROTEÍNAS NS1 E NS3

Simone Morais da Costa

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Tese apresentada ao curso de Pós-graduação em Biologia Celular e Molecular, como requisito parcial para obtenção de grau de Doutor em Ciências na área de Biologia Celular e Molecular

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1 – Dengue 2 - Vacina de DNA

3 – NS1 4 – NS3

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Tese intitulada: **Vacinas de DNA contra o vírus da dengue utilizando como antígenos as proteínas NS1 e NS3**

Simone Morais da Costa

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“Quanto mais aumenta nosso conhecimento, mais evidente fica nossa ignorância.”

Jonh Kennedy

Às vezes estamos tão imersos nas peculiaridades do nosso trabalho que não nos damos conta da sua relação com nosso dia a dia. Durante a escrita desta tese, eu, mais uma vez, fui infectada pelo vírus da dengue. Vivenciei a maioria dos sintomas sobre os quais escrevia e a preocupação por ser uma infecção secundária. Percebi minha impotência frente àquele “personagem com quem dividia meus dias”. Eu, minha mãe e minha vizinha de 1 ano, todas casos desta epidemia de 2008 e a única coisa que eu realmente podia oferecer era meu tênue conhecimento sobre esse vírus.

Dedico esta tese aos meus pais Lourdes e Luis. E junto deles aos meus irmãos Patrícia e Luizinho, e a minha linda Carolzinha

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ABREVIATURAS E SIGLAS

ADE	aumento da replicação viral dependente de anticorpos (do inglês *antibody-dependent enhancement*)
APC	célula apresentadora de antígeno
ANC	seqüência N-terminal da proteína NS2A
BHK	célula de rim de hamster neonato (do inglês *baby hamster kidney*)
BSS	solução salina balanceada
BSS-CMF	solução BSS livre de cálcio e magnésio
cDNA	DNA complementar
C-terminal	carboxi-terminal
DENV	vírus da dengue
DF	febre do dengue (do inglês *dengue fever*)
DHF	febre hemorrágica da dengue (do inglês *dengue hemorrhagic fever*)
DMEM	meio mínimo essencial de Eagle, modificado por Dubelco
DNA	ácido desoxirribonucléico
dNTP	desoxinucleotídeo trifosfato
DSS	síndrome do choque da dengue (do inglês *dengue shock syndrome*)
EDTA	ácido etilenodiaminotetracético
ELISA	ensaio imunoenzimático (do inglês *Enzime-linked immunosorbent assay*)
FcR	receptor para porção Fc de imunoglobulinas (do inglês *Fc receptor*)
GPI	Glicosil-Fosfatidil-Inositol
i.c.	intracerebral
i.d.	intradérmica
IgG	imunoglobulina G
IgG1	imunoglobulina G1
IgG2a	imunoglobulina G2A
i.p.	intraperitoneal
i.m.	intramuscular
INF	interferon
LB	meio de cultura Luria-Bertani
LD_{50}	dose letal para 50% da população em teste (do inglês *lethal dosis 50%*)
LDH	desidrogenase lática (do inglês *Lactate dehydrogenase*)
MOI	multiplicidade de infecção
N-terminal	amino-terminal

NTPase	atividade Nucleosídeo 5' trifosfatase
OMS	Organização Mundial da Saúde
OPTI-MEM	meio mínimo de Eagle para transfecção de células
pb	pares de bases
pcENS1	plasmídeo recombinante que codifica o peptídeo sinal E (região C-terminal da proteína E) fusionado à proteína NS1
pcENS1ANC	plasmídeo recombinante que codifica o peptídeo sinal E (região C-terminal da proteína E) fusionado à proteína NS1 e a seqüência ANC
PCR	reação em cadeia da polimerase (do inglês *Polymerase Chain Reaction*)
pcTPANS1	plasmídeo recombinante que codifica o peptídeo sinal t-PA fusionado à proteína NS1 e a região N-terminal da proteína NS2A
pcTPANS1ANC	plasmídeo recombinante que codifica o peptídeo sinal t-PA fusionado à proteína NS1 e a região N-terminal da proteína NS2A
pcTPANS3	plasmídeo recombinante que codifica o peptídeo sinal t-PA fusionado à proteína NS3
pcTPANS3C	plasmídeo recombinante que codifica o peptídeo sinal t-PA fusionado à porçãoC-terminal obtida após a clivagem da NS3
pcTPANS3H	plasmídeo recombinante que codifica o peptídeo sinal t-PA fusionado ao domínio da proteína NS3 com as atividades helicase/NTPase/RTPase
pcTPANS3N	plasmídeo recombinante que codifica o peptídeo sinal t-PA fusionado à porção N-terminal gerada pela clivagem da proteína NS3
pcTPANS3P	plasmídeo recombinante que codifica o peptídeo sinal t-PA fusionado ao domínio protease da proteína NS3
PFU	unidade formadora de placa (do inglês *plaque forming unit*)
Proteína C	proteína do capsídeo
Proteína E	proteína do envelope
Proteína prM	precursor da proteína de Membrana
Proteína M	proteína de membrana
Proteína NS1	proteína não estrutural 1
Proteína NS3	proteína não estrutural 3
RPMI	meio RPMI-1640 para cultura de células
RNA	ácido ribonucléico
RTPase	atividade RNA trifosfatase 5' terminal
SFB	soro fetal bovino

TAE	tampão Tris-acetato-EDTA
TB	meio de cultura *Terrific Broth*
TBE	vírus da encefalite do carrapato (do inglês *tick-borne encephalitis*)
Th1	célula T auxiliar do tipo 1
Th2	célula T auxiliar do tipo 2
TNF	fator de necrose tumoral (do inglês *Tumor Necrosis Factor*)
t-PA	ativador de plasminogênio de tecido humano
WNV	Vírus do Oeste do Nilo (do inglês *West Nile Virus*)

RESUMO

O vírus da dengue (DENV) consiste de quatro sorotipos antigenicamente relacionados: DENV-1, DENV-2, DENV-3 e DENV-4. Apesar dos diversos esforços para o desenvolvimento de uma vacina contra dengue, ainda não há nenhuma comercialmente disponível. As proteínas não estruturais 1 e 3 (NS1 e NS3) são indicadas como antígenos promissores para o desenvolvimento de uma vacina contra DENV. Segundo alguns estudos, a proteína NS1 é capaz de induzir uma resposta protetora de anticorpos com atividade de fixação do complemento. A proteína NS3, que realiza reações enzimáticas essenciais para a replicação viral, parece ser imunogênica, contendo um predomínio de epítopos para linfócitos T CD4+ e CD8+. No presente trabalho nós avaliamos o potencial de vacinas de DNA baseadas nas proteínas NS1 e NS3 de DENV-2. Foram construídos cinco plasmídeos, pcTPANS3, pcTPANS3H, pcTPANS3P, pcTPANS3N e pcTPANS3C, contendo a seqüência que codifica o peptídeo sinal do ativador de plasminogênio de tecido humano (t-PA) fusionado ao gene NS3 inteiro ou partes destes. Todos estes plasmídeos mediaram a expressão das proteínas recombinantes *in vitro* em células eucarióticas. Camundongos foram inoculados com estes plasmídeos e desafiados com DENV-2 por via intracerebral (i.c.). Nenhuma destas construções induziu níveis satisfatórios de proteção. Além dos plasmídeos com NS3, foram construídas quatro vacinas de DNA baseadas no gene NS1: 1 - pcENS1, que codifica a região C-terminal da proteína E fusionada à NS1, 2 - pcENS1ANC, similar ao pcENS1 com a adição da porção N-terminal da NS2A (ANC), 3 - pcTPANS1, que codifica o peptídeo sinal t-PA fusionado à NS1 e 4 - pcTPANS1ANC, semelhante ao pcTPANS1 com a adição da seqüência ANC. A proteína NS1 recombinante foi detectada nos extratos celulares e sobrenadante das culturas de células BHK transfectadas com pcTPANS1, pcENS1 e pcENS1ANC. Tais resultados indicam que as seqüências sinais t-PA e E direcionaram a NS1 para secreção. A proteína NS1 também foi observada associada à membrana plasmática de células transfectadas com pcENS1ANC, demonstrando a importância da seqüência ANC para o seu ancoramento. Todos os camundongos imunizados com pcTPANS1 ou pcENS1 produziram altos níveis de anticorpos, direcionados principalmente para epítopos conformacionais da NS1, enquanto que somente metade dos animais inoculados com pcENS1ANC apresentaram níveis detectáveis de anticorpos. A resposta de anticorpos se mostrou duradoura (até 56 semanas após a primeira dose das vacinas), e os animais apresentaram uma rápida resposta secundária após um reforço de DNA. Camundongos imunizados com os plasmídeos pcTPANS1 e pcENS1 se mostraram protegidos contra desafios com DENV-2 por via i.c., sendo o pcTPANS1 levemente mais protetor. Estes dois plasmídeos ativaram a produção de diferentes subclasses de IgG específicas contra NS1. Não foi observada proteção interespecífica quando camundongos imunizados com pcTPANS1 foram desafiados por via i.c. com DENV-1. Os animais imunizados com o pcTPANS1 foram desafiados com DENV-2 por via intraperitoneal e também se mostraram protegidos. Neste modelo de desafio, foi observada uma diminuição dos efeitos histopatológicos do vírus no fígado dos animais vacinados. Resultados preliminares sugerem à lise de células infectadas com DENV-2, dependente do complemento, na presença dos anticorpos direcionados contra NS1.

ABSTRACT

Dengue virus (DENV) consists of four antigenically related serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. Although considerable research has been conducted towards the development of a DENV vaccine, no vaccine is yet commercially available. The non-structural proteins 1 and 3 (NS1 and NS3) have been identified as promising antigens for the development of vaccines against DENV. According to some reports, NS1 can elicit a protective antibody response with complement-fixing activities. NS3, a protein that carries out enzymatic reactions essential for viral replication, appears to be immunogenic, presenting a preponderance of the CD4+ and CD8+ T cell epitopes. In the present work we investigate the potential of DNA vaccines based on the DENV-2 NS1 and NS3 proteins. We constructed five recombinant plasmids, pcTPANS3, pcTPANS3H, pcTPANS3P, pcTPANS3N and pcTPANS3C, which contain the sequence that codes the signal peptide derived from the human tissue plasminogen activator (t-PA) fused to the full or partial length of the DENV-2 NS3 gene. Results indicated that these plasmids promoted the expression of recombinant proteins in eukaryotic cells. Mice were inoculated with these plasmids and challenged by the intracerebral (i.c.) route with DENV-2. None of these constructs induced acceptable protection. Moreover, we constructed four DNA vaccines based on the DENV-2 NS1 gene: 1 - pcENS1, coding the C-terminal of the E protein fused to NS1, 2 - pcENS1ANC, similar to pcENS1 with the addition of the N-terminal of NS2A (ANC), 3 - pcTPANS1, coding the t-PA signal sequence fused to NS1 and 4 - pcTPANS1ANC, similar to pcTPANS1 with the addition of the ANC sequence. The recombinant NS1 protein was detected in cell extracts and culture supernatants from pcTPANS1-, pcENS1- and pcENS1ANC-transfected BHK cells. Such results indicated that the E and t-PA sequences targeted NS1 to secretion. NS1 was also observed in association with plasma membrane of pcENS1ANC-transfected cells, which demonstrated the importance of the ANC sequence for cell anchoring. High levels of antibodies, mainly recognizing surface-exposed conformational epitopes of NS1, were induced in all mice immunized with pcTPANS1 and pcENS1, while only half of pcENS1ANC-inoculated animals presented detectable antibody levels. Long-term antibody response was observed in pcTPANS1 and pcENS1 immunized animals (56 weeks after the first vaccine inoculation) and there was a rapid secondary response after a DNA booster. Protection was elicited in pcTPANS1- and pcENS1-immunized mice challenged with DENV-2 by the i.c. route and the pcTPANS1 seemed to generate a slightly higher protection. Moreover, these two plasmids induced different NS1-specific IgG subclasses. No protection was displayed when pcTPANS1-immunized animals were i.c. challenged with DENV-1. Animals inoculated with pcTPANS1 were also protected when they were challenged with DENV-2 by the intraperitoneal route. Liver tissue from vaccinated animals presented a remarkable decrease of hepatic damages in this challenge mouse model. Preliminary results suggested the complement-mediated lyses of DENV-2 infected cells in the presence of the NS1-specific antibody.

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INTRODUÇÃO

1 - INTRODUÇÃO

A dengue é considerada uma das mais importantes arboviroses humanas, sendo causa de morbidade e mortalidade na maioria das regiões tropicais e subtropicais do mundo, principalmente no sudeste e sul da Ásia, América do Sul, América Central e Caribe (Figura 1.1). Há aproximadamente 2,5 bilhões de pessoas vivendo em locais de risco de infecção, sendo estimado que, anualmente ocorram 100 milhões de casos de dengue, e aproximadamente 500 mil pessoas desenvolvam a forma mais grave da doença, a febre hemorrágica da dengue (*Dengue hemorrhagic fever*, DHF) / síndrome do choque da dengue (*Dengue shock syndrome*, DSS), com uma taxa de casos fatais, que pode variar desde 10-15% em alguns países, a níveis menores que 1% em outros (Gubler, 2002; Kurane, 2007).

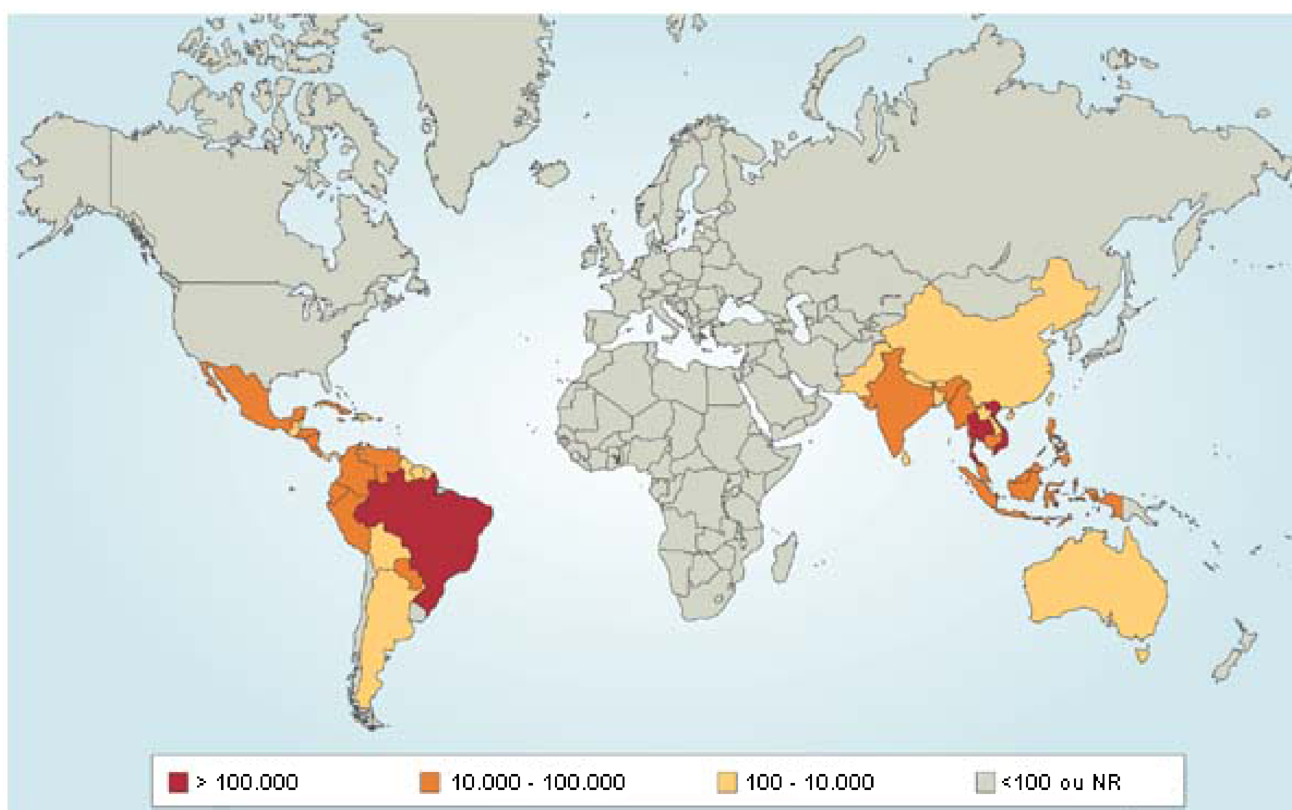

Figura 1.1: Mapa de distribuição da dengue (1995 – 2005). A dengue é endêmica na maioria das regiões tropicais e subtropicais. O mapa indica o número de casos de dengue no período de um ano. A ausência de vigilância na África durante a última década torna difícil acessar o nível endêmico desta região. NR – não relatado (modificado de Whitehead *et al*., 2007).

O agente etiológico da dengue, o vírus da dengue (DENV), pertence ao gênero *Flavivirus,* da família Flaviviridae. Este gênero, que inclui um grande número de agentes causadores de doenças humanas, como febres, encefalites e febres hemorrágicas, compreende aproximadamente 80 membros, cuja maioria é transmitida por artrópodes (Chambers *et al.* 1990; Lindenbach & Rice, 2001). O DENV compreende quatro sorotipos antigenicamente distintos, mas proximamente relacionados (DENV-1, DENV-2, DENV-3 e DENV–4), que são transmitidos ao homem por mosquitos do gênero *Aedes*, tendo como principal vetor o *Aedes aegypti*. Embora nos testes de diagnóstico seja detectada extensa reatividade cruzada entre esses sorotipos, a infecção primária por um sorotipo de DENV induz uma imunidade protetora de longa duração somente ao sorotipo infectante. Tal infecção não é capaz de gerar uma proteção cruzada contra os outros sorotipos virais, podendo ocorrer infecções seqüenciais com os diferentes sorotipos de DENV (Lindenbach & Rice, 2001; Guzmán & Kouri, 2002; Whitehead *et al.*, 2007).

1.1 - Histórico da dengue

Apesar das 3 últimas décadas estarem caracterizadas pela emergência e re-emergência de epidemias de dengue associadas a quadros mais severos da doença, como a DHF, a febre do dengue (*dengue fever*, DF) é uma doença antiga. Não há um consenso de quando esta doença surgiu na população humana, entretanto, existem registros de quadros clinicamente compatíveis com a DF na enciclopédia médica chinesa, editada formalmente no ano de 610 e novamente em 992, onde foram encontradas descrições de uma doença conhecida como “veneno da água”. Na verdade, os primeiros relatos de epidemias na Ásia e nas Américas de uma doença que possivelmente seria dengue ocorreram nos anos 1779-1780. Após esse período aconteceram epidemias intermitentes nestes dois continentes. Nos séculos seguintes, com o auxílio dos transportes marítimos que facilitaram a dispersão do vetor *Aedes aegypti* e de pessoas susceptíveis, o vírus se espalhou pelas regiões tropicais e subtropicais (Monath, 1994; Gubler, 1998; Holmes & Twiddy, 2003; Mackenzie *et al.*, 2004).

Na década de 60, a América conseguiu controlar o *Aedes aegypti* em um programa de controle da febre amarela urbana, outra doença causada por um flavivírus, o vírus da febre amarela, que compartilha o vetor com o vírus da dengue. Esse programa, empregado principalmente durante as décadas de 50 e 60, foi abandonado nos anos 70 levando à dispersão geográfica do mosquito. Com a ampla distribuição do vetor, somado ao crescimento do tráfego entre diferentes países e a densa urbanização, a dengue se difundiu pelo continente

americano, levando a uma elevação tanto da freqüência, quanto da magnitude das epidemias de dengue, com conseqüente surgimento e aumento do número de casos de DHF (Istúriz *et al.*, 2000; Gubler, 2002; Orozco, 2007).

Até 1981, a dengue hemorrágica ocorreu esporadicamente nas Américas, embora tenham acontecido epidemias de febre do dengue, na região do Caribe e América do Sul, nas décadas de 60 e 70. Contudo, em 1981, o surto de dengue hemorrágica ocorrido em Cuba, com 24.000 casos de DHF e 158 mortes, marcou o início da dengue hemorrágica no continente americano. Um evento similar aconteceu na Venezuela, nos anos de 1989/90, com a ocorrência de mais de 3000 casos de DHF (Guzmán *et al.*, 1990; Monath, 1994; Istúriz *et al.*, 2000; Orozco, 2007).

Atualmente, os 4 sorotipos de DENV estão presentes nas Américas, com ocorrência de hiper-endemicidade (co-circulação de múltiplos sorotipos) em muitas regiões (OMS - http://www.paho.org/english/ad/dpc/cd/dengue-cases-2007.htm, acessado em 30/03/2008). Além disso, a dengue é endêmica em mais de 100 países, com casos de DHF sendo relatados em mais de 60 países. Nos últimos anos, essa doença vem se expandindo, levando ao aumento do número de centros urbanos hiper-endêmicos e do número de casos de DHF e consequentemente morte. A Organização Mundial da Saúde (OMS) estima que o número de casos de dengue aumentou cerca de 30% nos últimos 50 anos, com conseqüente elevação da ocorrência da forma mais grave da doença, DHF e mortes (Istúriz *et al.*, 2000; Kurane, 2007; Orozco, 2007; OMS - http://www.who.int/csr/disease/dengue/en/index.html, acessado em 29/01/2008).

1.1.2 - Dengue no Brasil

No Brasil, há relatos de surtos de dengue durante o século XIX e início do século XX. Entretanto, com a campanha da erradicação do mosquito *Aedes aegypti* iniciada por Oswaldo Cruz em 1904, não há descrições de surtos da doença de 1923 até 1981 (Figueiredo, 1996; Nogueira *et al.*, 2007).

Em 1981, foi observado um surto de dengue pelos tipos 1 e 4 em Roraima (Osanai *et al.*, 1983). Quatro anos mais tarde, DENV-1 ressurgiu no Rio de Janeiro e se disseminou em seguida por quase todo o país (Schatzmayr *et al.*, 1986). Em 1990, uma nova epidemia causada por DENV-2 se iniciou no Rio de Janeiro, produzindo centenas de casos de DHF/DSS (Nogueira *et al.*, 1993). Até o ano 2000, ocorreu a circulação simultânea de DENV-1 e DENV-2. Neste mesmo ano, iniciou-se uma epidemia de DENV-3 no Rio de

Janeiro, que novamente se espalhou pela cidade e posteriormente por quase todo o país (Teixeira *et al.*, 2005; Nogueira *et al.*, 2007).

Atualmente, os sorotipos DENV-1, 2 e 3 circulam no Brasil (Figura 1.2). Com isso o número de casos de DHF e consequentemente a taxa de letalidade vêm aumentando no país. Em 2002, com a introdução do DENV-3, foi registrado o maior pico epidêmico da doença até então no Brasil e a taxa de morte foi duas vezes maior que a observada anteriormente, revelando uma maior gravidade na ocorrência da doença. Dessa forma, o Brasil se tornou responsável por 60% dos casos de dengue do continente americano, sendo que nos anos de 2002 e 2003, cerca de 80% dos casos de DHF relatados no continente americano ocorreram aqui (Teixeira *et al.*, 2005; Nogueira *et al.*, 2007; Ministério da Saúde - http://www.saude.to.gov.br/pagina_adm/download/ boletim_dengue _semana18.pdf, acessado em 29/01/2008).

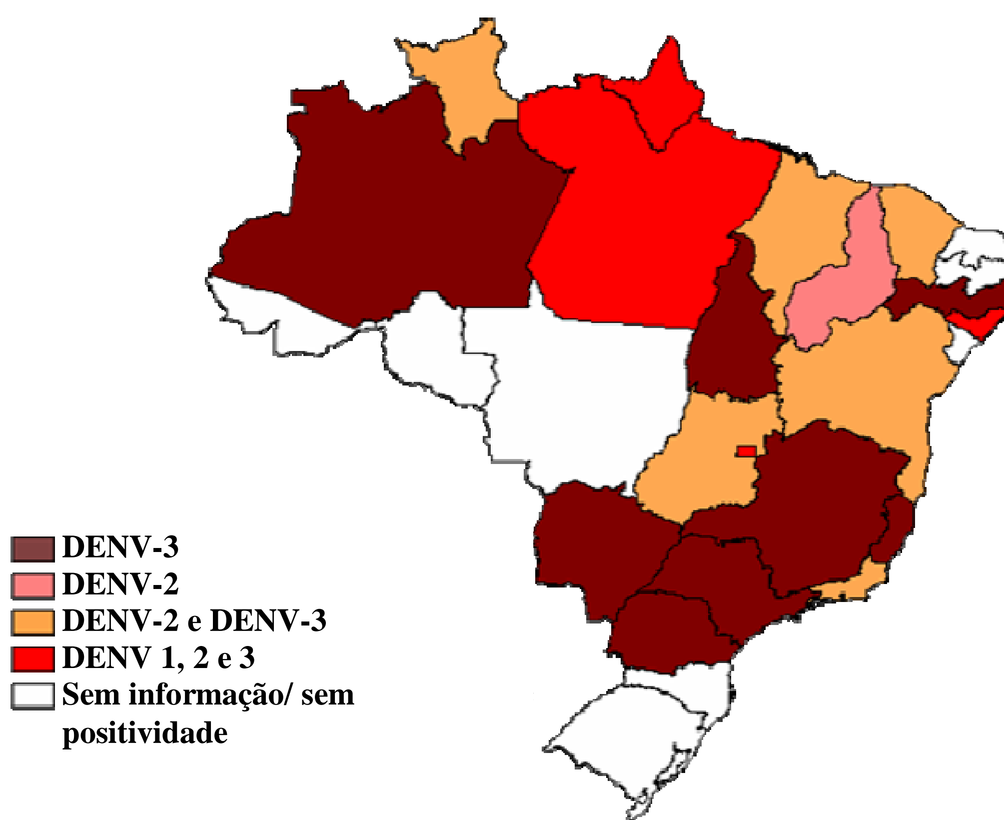

Figura 1.2: Distribuição dos sorotipos de DENV no Brasil, em 2007. O esquema mostra a circulação dos sorotipos de DENV nos estados do Brasil nos quatro primeiro meses de 2007 (Ministério da Saúde - http://www.saude.to.gov.br/pagina_adm/download/boletim_dengue _semana18.pdf, acessado em 29/01/2008)

Em 2007, a Secretaria de Vigilância em Saúde do Ministério da Saúde (SVS/MS) registrou 559.954 casos suspeitos de dengue e 1.541 casos de DHF, com 158 óbitos, o que significa uma taxa de letalidade de 10,2% dos casos de DHF. Dos casos notificados, 79% ocorreram nos 5 primeiros meses do ano, que corresponde a época de maior incidência da doença. Em 2008, já foram registrados 120.000 casos de dengue nos três primeiros meses do ano (boletim de 28 de março), sendo que o estado do Rio de Janeiro registrou um aumento de 211% no número de casos em relação ao mesmo período de 2007, com a ocorrência de 43.523 casos, o que corresponde a 36% das notificações do país (Ministério da saúde - http://portal.saude.gov.br/ portal/arquivos/pdf/boletim_dengue_2803.pdf, acessado em 13/04/2008).

1.2 - O vírus da dengue

Semelhante aos outros flavivírus, DENV possui um envelope viral que consiste em uma bicamada lipídica derivada do retículo endoplasmático, onde estão inseridas as proteínas do envelope (E) e membrana (M). A partícula viral apresenta formato esférico e diâmetro de aproximadamente 50nm. Abaixo do envelope viral há um nucleocapsídeo de aproximadamente 30nm de diâmetro composto por um capsídeo viral de formato icosaédrico, formado pela proteína do capsídeo (C) e complexado a uma molécula de RNA fita simples com polaridade positiva (Figura 1.3 A e C) (Lindenbach & Rice, 2001; Kuhn *et al.* 2002; Qi *et al.*, 2008).

O RNA viral, de aproximadamente 10, 7 kb, é modificado em sua extremidade 5' pela adição da estrutura *cap*, mas é destituído de cauda poli-A na extremidade 3' e compreende um único quadro de leitura aberto que codifica a poliproteína precursora das proteínas flavivirais. Este precursor é clivado por proteases celulares e pela protease viral gerando as três proteínas estruturais: capsídeo, pré-membrana (prM) que quando clivada gera a proteína de membrana, e envelope, além das sete proteínas não estruturais, NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5 (Figura 1.4). As proteínas estruturais traduzidas são incorporadas nas partículas virais durante sua maturação, enquanto as proteínas não estruturais estão envolvidas na replicação e/ou montagem dos virions. As regiões não codificantes 3' e 5' também são importantes para replicação viral (Lindenbach & Rice, 2001; Mukhopadhyay *et al.*, 2005; Qi *et al.*, 2008).

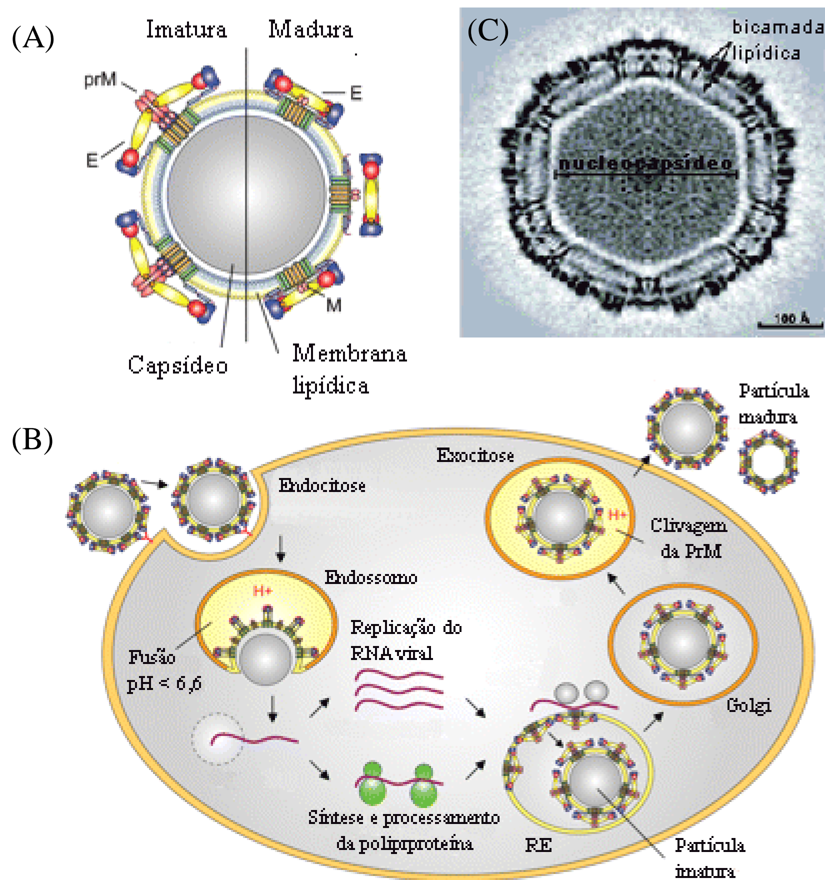

Figura 1.3: Partícula viral e ciclo de replicação do vírus da dengue. (A) Representação esquemática da partícula dos flavivírus imatura e madura e (B) do Ciclo de replicação dos flavivírus: a entrada do vírus ocorre via endocitose mediada por receptor. No pH ácido do endossoma a proteína E sofre mudanças conformacionais que levam a fusão do envelope viral com a membrana da vesícula. O RNA viral é traduzido e inicia-se o processo de replicação. A montagem das partículas virais ocorre no retículo endoplasmático (R.E.), onde são transportadas pelo Golgi para secreção pela célula hospedeira. (C) Imagem do vírus da dengue obtida por microscopia crioeletônica (modificado de Stiasny & Heinz, 2006 e Purdue University, http://physorg.com/news 125846262.html, acessado em 10/04/08).

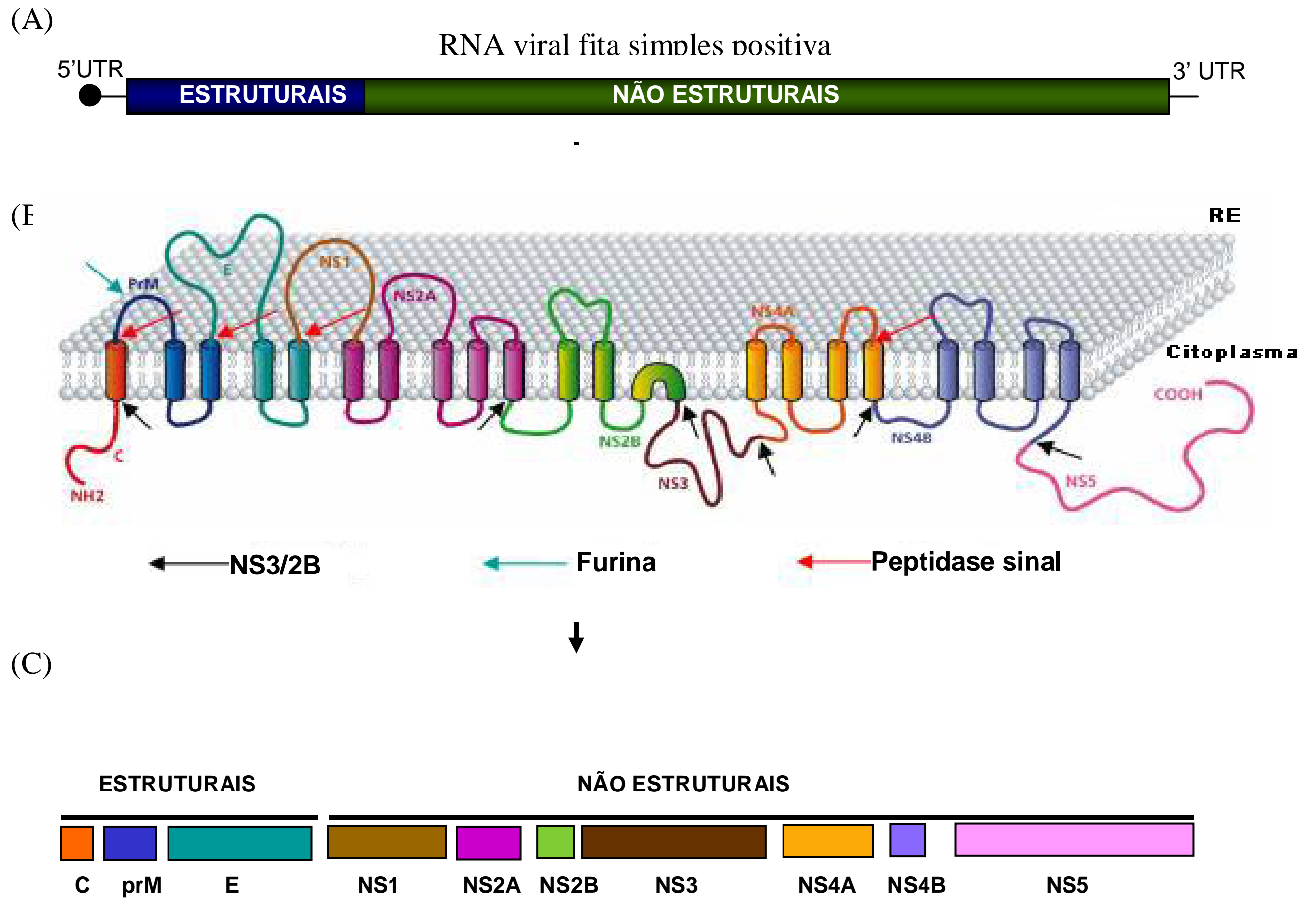

Figura 1.4 – Representação esquemática da tradução e processamento da poliproteína viral. (A) Genoma do vírus da Dengue mostrando a região que codifica as proteínas estruturais e não estruturais, assim como as extremidades 5´ e 3´ não codificantes (do inglês *untranslated region* - UTR). (B) Organização da poliproteína viral na membrana do retículo endoplasmático (RE) e seu processamento pela protease viral NS3/NS2B e por proteases celulares gerando (C) as proteínas estruturais C, prM e E, e as proteínas não estruturais NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5 (modificado de Umareddy *et al*., 2007).

1.2.1 – As proteínas virais

A proteína C, altamente básica, possui cerca de 11 kDa e se associa ao RNA viral formando o nucleocapsídeo. Sua porção central contém domínios hidrofóbicos que interagem com membranas celulares, tendo um papel na montagem da partícula viral (Lindenbach & Rice, 2001; Mukhopadhyay *et al*., 2005).

A glicoproteína prM, de aproximadamente 26 kDa, é gerada pela clivagem do peptídeo sinal na região N-terminal no retículo endoplasmático durante o processamento da poliproteína viral. Aparentemente essa proteína funciona impedindo que a proteína E sofra mudanças conformacionais durante o seu trânsito compartimentos ácidos na fase de maturação do vírus. A porção N-terminal dessa proteína é clivada durante seu transporte pelo Golgi, originando a proteína M presente na partícula madura (Lindenbach & Rice, 2001; Stiasny e Heinz, 2006).

A glicoproteína E é o principal constituinte protéico do envelope viral. Esta proteína de aproximadamente 50 kDa atua nos eventos de interação com o receptor da célula alvo e a fusão entre o envelope viral e a membrana celular (Lindenbach & Rice, 2001; Modis *et al*., 2004; Stiasny & Heinz, 2006) Além disso, a proteína E é um importante alvo da resposta imune humoral em indivíduos infectados pelo DENV (Brinton *et al*., 1998; Lindenbach & Rice, 2001).

A glicoproteína NS1 é essencial para viabilidade do vírus da dengue, contudo, pouco é conhecido sobre o papel desta proteína. Ela possui aproximadamente 45-48 kDa e pode ser encontrada tanto no interior das células infectadas pelo vírus como associada à membrana destas células (Chambers *et al*., 1990; Lindenbach & Rice, 2001) Além disso, ela também é secretada em grandes quantidades, sendo detectada no soro de pacientes na fase aguda da infecção (Young *et al*., 2000). Alguns estudos sugerem que a NS1 está envolvida na replicação viral, provavelmente nos estágios iniciais da replicação do RNA viral (Mackenzie *et al*., 1996; Lindenbach & Rice, 1997; Muylaert *et al*., 1997; Lindenbach & Rice, 1999).

Durante a infecção viral, a NS1 é translocada pela via do retículo endoplasmático utilizando a seqüência sinal localizada na região C-terminal da proteína E. Este peptídeo sinal é clivado por uma peptidase sinal da célula hospedeira gerando a porção N-terminal da proteína NS1 (Falgout *et al.,* 1989; Lindenbach & Rice, 2001). Dentro do retículo endoplasmático, a NS1 é N-glicosilada em dois sítios conservados e a seguir sofre dimerização, essa glicosilação é importante para estabilidade do dímero formado (Winkler *et al*., 1988; Winkler *et al*., 1989; Pryor and Wright, 1993; Pryor and Wright, 1994). Essa proteína é translocada pela via secretora e permanece ancorada a membrana plasmática da célula infectada ou é secretada para o meio extracelular na forma de hexâmeros (Winkler *et al*., 1989; Flamand *et al*., 1999). Alguns estudos já sugeriam que a associação da proteína NS1 à membrana da célula se dava provavelmente via a seqüência hidrofóbica que corresponde à porção C-terminal da proteína NS2A, que parece funcionar como seqüência

sinal para ligação via âncora GPI (Glicosil-Fosfatidil-Inositol) (Pryor and Wright, 1993; Jacobs *et al.*, 2000).

A NS3 é uma proteína de aproximadamente 70 kDa, bastante conservada entre os flavivírus, que participa da replicação do vírus da dengue. Esta proteína apresenta múltiplas atividades enzimáticas incluindo um domínio serino protease localizado na região N-terminal, enquanto a sua porção C terminal apresenta atividades de helicase, Nucleosídeo 5' trifosfatase (NTPase) e RNA trifosfatase 5' terminal (RTPase) (Valle & Fagout, 1998; Lindenbach & Rice, 2001; Xu *et al.,* 2005; Sampath *et al.*, 2006; Qi *et al.,* 2008).

Baseado em comparações de seqüências com outras proteases, nos 180 aminoácidos da porção N-terminal da NS3 foi identificado o domínio serino-protease, sendo os primeiros 167 aminoácidos mapeados como seqüência mínima para esta atividade (Li *et al.*, 1999). Esta enzima associa-se com a proteína NS2B, que funciona como um co-fator para atividade de protease. A NS3-NS2B é responsável pela clivagem da poliproteína viral em sítios específicos (Figura 1.4B) (Valle & Fagout, 1998; Niyomrattanakit *et al.,* 2004; Qi *et al.,* 2008).

A porção C-terminal da proteína NS3 compreende três outras atividades importantes na replicação viral. A função helicase não está completamente esclarecida, mas pode atuar dissociando a estrutura dupla-fita durante a replicação do RNA viral ou desdobrar estruturas secundárias envolvidas no início da síntese de RNA. Esta atividade é dependente de energia, que é fornecida pela atividade NTPase que hidrolisa ATP (Cui *et al.*, 1998; Xu *et al.,* 2005; Sampath *et al.,* 2006). A função RTPase é provavelmente necessária para remoção do grupo fosfato 5'-terminal e a formação da estrutura de *cap* do RNA viral (Murthy *et al.* 1999; Lindenbach & Rice, 2001; Sampath *et al.,* 2006; Qi *et al.*, 2008). Foi também observado em células infectadas por DENV-2 que a proteína NS3 sofre uma clivagem interna dentro da seqüência da helicase, contudo não se sabe a importância deste processamento para o ciclo viral (Arias *et al.*, 1993; Teo & Wright, 1997).

A proteína NS5 apresenta três atividades enzimáticas essenciais para replicação viral. Aproximadamente 320 resíduos da porção N-terminal compreendem uma metiltransferase dependente de S-adenosilmetionina, que apresenta atividade de metiltranferase e guanililtransferase. Esse domínio da NS5 é responsável pelo *cap* e pela metilação da extremidade 5' do RNA viral. A região C terminal da NS5 é uma RNA polimerase dependente de RNA, que atua na síntese do RNA intermediário que serve de molde para transcrição da fita positiva de RNA (Lindenbach & Rice, 2001; Zhou *et al.*, 2007; Qi *et al.*, 2008).

Pouco é descrito sobre a atividade das pequenas proteínas hidrofóbicas NS2A, NS4A e NS4B. Alguns trabalhos demonstram que estas proteínas fazem parte do complexo de replicação. A proteína NS4A aparentemente funciona como âncora da replicase viral à membrana celular. A proteína NS4B parece auxiliar na modulação da replicação viral em associação à NS3. Além disso, essas proteínas poderiam também atuar na inibição da resposta de interferon-α/β do indivíduo infectado (Lindenbach & Rice, 2001; Miller *et al.*, 2006; Umareddy *et al.*, 2006).

1.2.2 - Ciclo de vida

A infecção viral inicia-se após o repasto do mosquito e liberação do vírus. A partícula viral interage com receptores presentes na superfície de células permissivas sendo internalizado via endocitose. Contudo, alguns trabalhos indicam que também pode haver fusão direta do envelope viral com a membrana plasmática das células hospedeiras (Lindenbach & Rice, 2001; Mukhopadhyay *et al.*, 2005).

Vários estudos *in vitro* têm sugerido que o vírus da dengue é capaz de infectar uma diversidade de células. *In vivo*, porém, alguns trabalhos indicam que este vírus tem como alvos primários células dendríticas, monócitos e macrófagos (Wu *et al.*, 2000; Clyde *et al.*, 2006; Kou *et al.*, 2008). Outros estudos sugerem que DENV também se replica em hepatócitos, linfócitos B e T, células endoteliais e células neuronais (Chen *et al.*, 1996; Clyde *et al.*, 2006; Suksanpaisan *et al.*, 2007). A associação inicial do vírus com a célula alvo ocorre pela interação da proteína E com um ou mais receptores celulares. Inicialmente, parece haver uma interação da proteína E com um receptor menos específico, o heparam sulfato, que é um glicosaminoglicano amplamente distribuído nas superfícies celulares, concentrando o vírus na superfície celular. A seguir, a proteína do envelope liga-se a um receptor mais específico, necessário para iniciar a endocitose (Putnak *et al.*, 1997; Clyde *et al.*, 2006; Kou *et al.*, 2008). Há várias moléculas sendo propostas como possíveis receptores para o vírus da dengue, em diferentes linhagens celulares, incluindo as proteínas de choque térmico 70 (Hsp 70) e 90 (Hsp 90) (Reyes-del Valle *et al.*, 2005), GRP78/BiP (*glucose-regulation protein 78*) (Cabrera-Hernandez *et al.*, 2007) e a lectina manose-específica DC-SIGN (*dendritic cell-specific ICAM-grabbing non-integrin*) no caso das células dendríticas (Lozach *et al.*, 2005). Além da interação viral via receptor específico, em uma infecção secundária, a entrada do vírus pode ser também mediada pela ligação de anticorpos direcionados contra DENV com FcRs (receptores para porção Fc de imunoglobulinas) presentes na célula alvo. Este mecanismo

parece aumentar os níveis de infecção e replicação viral, e é denominado ADE (*antibody-dependent enhancement*), podendo ter fortes implicações na patogêsese da dengue e sendo indicado como possível fator para dengue hemorrágica (Halstead, 1979; Wu *et al.*, 2000; Huang *et al.*, 2006; Kou *et al.*, 2008).

Após a endocitose mediada por receptor e acidificação do endossoma, ocorre a trimerização irreversível da proteína E. A modificação conformacional desta proteína expõe o peptídeo de fusão que interage com a membrana vesicular. A fusão do envelope viral com a membrana vesicular culmina na liberação do nucleocapsídeo no citoplasma da célula, levando a uma dissociação da proteína do capsídeo e do RNA (Figura 1.3B) (Chen *et al.*, 1996; Modis *et al.*, 2004; Qi *et al.*, 2008).

Com a exposição do RNA, inicia-se a tradução da poliproteína viral. Este poliprecursor é então clivado por proteases celulares e pela protease viral NS3 associada a NS2B, gerando as proteínas estruturais C, prM e E, e as não-estruturais NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5 (Figura 1.4C). A seguir, fitas de RNA de polaridade negativa são transcritas servindo de molde para polimerização de novas fitas de RNA de polaridade positiva, utilizadas na tradução das proteínas virais e na montagem das novas partículas virais (Markoff *et al.*, 1994; Clyde *et al.*, 2006; Qi *et al.*, 2008). Seqüências hidrofóbicas sinais e de ancoramento direcionam a translocação do poliprecursor através da membrana assim como o ancoramento de algumas proteínas virais (Figura 1.4B) (Lindenbach & Rice, 2001; Mukhopadhyay *et al.*, 2005; Qi *et al.*, 2008).

Análises de ultra-estrutura indicam que a morfogênese viral ocorre em associação às membranas celulares internas, possivelmente dentro do lúmen do retículo endoplasmático. As proteínas do capsídeo interagem com o RNA viral no citoplasma formando o nucleocapsídeo, que adquire o envelope viral por brotamento para o lúmen do retículo endoplasmático. O virion é montado em partículas imaturas que contêm as proteínas prM e E associadas não covalentemente em um complexo heterodimérico. As partículas são transportadas pela via secretora para membrana plasmática em vesículas e liberadas por exocitose (Figura 1.3B) (Lindenbach & Rice, 2001; Mukhopadhyay *et al.*, 2005; Qi *et al.*, 2008). Apesar da presença das proteínas E e prM, as partículas imaturas ainda não são capazes fusionar com membranas da célula hospedeira, necessitando do processamento da prM para se tornarem infecciosas. A clivagem da região N-terminal da glicoproteína prM, que ocorre durante o transporte viral pelo Golgi**,** resulta na proteína M e no rearranjo da proteína E, levando a formação de partículas maduras que são liberadas pela célula (Lindenbach & Rice, 2001; Zhang *et al.,* 2003; Mukhopadhyay *et al.*, 2005; Qi *et al.*, 2008).

1.3 - A dengue

A infecção pelos quatro sorotipos virais, DENV-1, 2, 3 e 4, pode causar sintomas que variam desde quadros inaparentes, até o surgimento de hemorragia e choque, podendo evoluir para o óbito. Contudo, a maioria das infecções é assintomática. O quadro mais comum da doença é a febre do dengue, que apresenta um conjunto de sintomas variáveis como febre alta, cefaléia, mialgia, prostração, dor retro-orbital, náuseas, vômitos, exantema e prurido intenso. Alguns indivíduos também apresentam petéquias e algumas manifestações hemorrágicas. Esses sintomas são geralmente acompanhados por leucopenia e graus variáveis de trombocitopenia (Ministério da Saúde, 2002; Green & Rothman, 2006; Kurane, 2007).

Nos quadros mais graves da doença, a dengue hemorrágica e a síndrome do choque, os sintomas iniciais são semelhantes aos da DF, porém estes evoluem, geralmente na fase de defervescência, para manifestações hemorrágicas com ou sem extravasamento de plasma, instabilidade hemodinâmica e choque. Uma característica importante da doença é a trombocitopenia com hemoconcentração concomitante, sendo que a efusão do plasma é o principal fenômeno fisiopatológico associada à severidade da DHF e se manifesta através de valores crescentes de hematócrito e hemoconcentração (Green & Rothman, 2006; Halstead, 2007; Kurane, 2007; Whitehead *et al.*, 2007). A OMS categoriza a DHF em quatro graus, do menos ao mais severo (grau 1 a 4). Nos graus 3 e 4, em que a efusão de plasma é tão profunda que ocorre o choque, denominamos síndrome do choque da dengue (Ministério da Saúde, 2002; Malavige *et al.*, 2004)

O vírus da dengue pode causar uma diversidade de efeitos clínicos e patológicos, principalmente no sistema hematológico. Há evidências clínicas e experimentais que indicam o envolvimento do fígado nas infecções virais. Os danos no fígado de pacientes com dengue podem ser detectados por alterações nas enzimas séricas aspartato amino transferase (AST) e alanina amino transferase (ALT) e hepatomegalia, esta última sendo mais comum em indivíduos com quadros mais graves da doença, como DHF (Basílio-de-Oliveira *et al.*, 2005; Seneviratne *et al.*, 2006; Ling *et al.*, 2007; de Souza *et al.*, 2007).

Apesar de apenas cerca de 3% dos indivíduos infectados evoluírem para os quadros mais graves da doença, o número de pessoas com DHF é relevante, correspondendo a aproximadamente 500.000 casos anuais, onde a taxa de óbito geralmente varia de 1 a 15%, dependendo do diagnóstico e tratamento médico disponível (Rothman, 2004; Green & Rothman, 2006).

1.3.1 – Fatores de risco para o desenvolvimento de DHF/DSS

A patogênese da dengue hemorrágica é complexa e ainda não está completamente compreendida. Diversos estudos indicam que o maior fator de risco para o desenvolvimento de DHF é a ocorrência de infecção secundária com um sorotipo heterólogo (Halstead, 1970; Guzmán *et al*., 1990; Green & Rothman, 2006,). Além disso, crianças no primeiro ano de vida também apresentam sérios riscos de desenvolvimento de DHF, principalmente as que receberam anticorpos contra DENV passivamente da mãe (Halstead, 1970; Guzmán & Kourí, 2002; Whitehead *et al*., 2007)

Uma das possíveis explicações para o aumento de casos de FDH/DSS em infecções secundárias é o fenômeno de ADE, onde anticorpos não neutralizantes contra dengue facilitariam a entrada do vírus em células permissivas (geralmente da linhagem de fagócitos mononucleares) via FcRs presentes na superfície destas células. Essa interação resultaria em um aumento dos níveis de replicação viral e de células infectadas. Embora esse mecanismo esteja bem estabelecido *in vitro*, ainda são poucas as evidências em humanos e nos modelos animais estudados de que isso resulta no agravamento da doença (Halstead & O´Rourke, 1977; Halstead, 1979; Stephenson, 2005; Huang *et al*., 2006; Halstead, 2007).

O início do extravasamento plasmático na DHF ocorre dias depois da viremia ter reduzido, sugerindo a participação do sistema imune no desenvolvimento desses quadros mais graves. Dessa forma, um mecanismo que vem sendo proposto como envolvido na severidade da doença é a magnitude da resposta dos linfócitos T, incluindo células sorotipo-específicas e células T de memória que apresentam reação cruzada em uma infecção secundária, fenômeno denominado "pecado original" (Mongkolsapaya *et al*., 2003). Durante a infecção, essas células T quando ativadas, proliferariam e produziriam maior quantidade de citocinas pró-inflamatórias como o interferon-γ (INF-γ) e o fator de necrose tumoral-α (TNF-α), entre outras. Essas citocinas podem atuar diretamente nas células endoteliais resultando no extravasamento de plasma e agravamento da doença. Este modelo prediz que pacientes com FDH teriam níveis mais elevados de citocinas no soro, além de maior ativação de células T, sendo que essa ativação seria preferencialmente de células T que apresentassem reação cruzada com o primeiro sorotipo de vírus da dengue (Mongkolsapaya *et al*., 2003; Welsh & Rothman, 2003; Mangada & Rothman, 2005; Stephenson, 2005; Green & Rothman, 2006). Além disso, estudos sugerem que o alto nível de ativação de células T, somado à elevada freqüência de apoptose e a uma resposta imune celular com grande número de células com baixa afinidade ao vírus infectante, resultaria em uma supressão ou atraso na eliminação do

vírus. Consequentemente, a carga viral seria alta e levaria a um agravamento da doença (Welsh & Rothman, 2003; Stephenson, 2005).

Um outro fator que parece relacionar-se com o grau de severidade da doença é a variação genética e antigênica de diferentes cepas virais, favorecendo uma maior virulência. Algumas cepas virais são consideradas mais virulentas que outras. Análises filogenéticas de DENV-2 indicam que enquanto cepas do genótipo americano estão associadas a quadros de febre do dengue, vírus pertencentes ao genótipo do sudeste asiático são mais virulentos e correlacionam-se com os casos mais graves da doença (Holmes & Tiddy, 2003; Cologna, *et al.*, 2005; Stephenson, 2005; Kurane, 2007). Isto pode ser observado durante a introdução do genótipo asiático nas Américas que coincidiu com o aparecimento de dengue hemorrágica nestes países (Rico-Hesse *et al.*, 1997).

Além disso, acredita-se que haja diferenças na predisposição do hospedeiro a desenvolver, ou não dengue hemorrágica. Características como idade, etnia, presença de doença crônica parecem atuar na gravidade da doença (Guzmán *et al.*, 1990; Rothman, 2004; Guzmán, 2005). Uma possível predisposição genética pode ser mediada, entre outros genes, por diferenças nos haplótipos de HLA (*human leucocyte antigen*), entretanto, ainda há relativamente poucos estudos nesta área. Provavelmente a DHF é resultado de um mecanismo complexo, em que estão envolvidos tanto características da cepa viral, quanto do hospedeiro e sua resposta imunológica (Holmes & Twiddy, 2003; Stephenson, 2005).

Atualmente, não há nenhuma droga específica contra esse vírus. A reposição apropriada de líquido, de forma a evitar a hipovolemia tem sido a forma de reduzir a mortalidade por DHF, mas em muitos países a assistência médica é precária. As principais formas de prevenção e controle da dengue são o desenvolvimento de uma vacina e o controle do principal mosquito transmissor. Devido à ausência de uma vacina contra dengue comercialmente disponível, atualmente o único método de evitar a dengue é o controle do seu principal vetor, o *Aedes aegypti*. Infelizmente, nos últimos 30 anos o controle do mosquito foi ineficiente na maioria dos países endêmicos (Monath, 2007; Whitehead *et al.*, 2007; Qi *et al.*, 2008).

Dessa forma, o desenvolvimento de uma vacina contra dengue, que seja capaz de proteger contra infecções dos quatro sorotipos de vírus da dengue é considerada uma das prioridades da OMS (Kinney & Huang 2001; Rothman, 2004; Stephenson, 2005; Monath, 2007).

1.4 - Vacinas contra dengue

A princípio, o desenvolvimento de uma vacina contra dengue deveria ser mais fácil quando comparado a outras doenças virais como a AIDS (do inglês *Acquired Immunodeficiency Syndrome*) e hepatite C, uma vez que a dengue não é uma doença infecciosa crônica. Ao contrário, a replicação viral é controlada em poucos dias e o indivíduo torna-se protegido contra posteriores infecções com vírus do mesmo sorotipo. Entretanto, diversos obstáculos dificultam a construção de uma vacina segura e eficaz contra esse vírus (Green & Rothman, 2006).

O primeiro destes obstáculos é a necessidade do desenvolvimento de uma vacina efetiva capaz de induzir uma resposta imune protetora de longa duração contra os quatro sorotipos virais. Diferentes de outras infecções hemorrágicas, os quadros mais severos de dengue ocorrem geralmente em infecções secundárias (Halstead, 1970; Guzmán *et al.*, 1990; Green & Rothman, 2006). Sendo assim, uma vacina contra dengue que não proteja contra os 4 sorotipos pode aumentar a possibilidade de DHF. Além disso, a vacina ideal contra dengue deve ser disponível para o uso em crianças (Edelman, 2005; Edelman, 2007; Guy & Almond, 2008).

Outra dificuldade para o desenvolvimento de uma vacina contra dengue diz respeito à ausência de um modelo animal que mimetize a infecção viral em humanos. Uma variedade de animais já foi inoculada com DENV, contudo nenhum conseguiu reproduzir os quadros mais graves da doença, mesmo após a inoculação de altas doses de vírus e uso de diferentes vias (Bente & Rico-Hesse, 2006). Camundongos e primatas não humanos são os modelos animais mais utilizados nos testes pré-clínicos dos candidatos à vacina contra dengue. Primatas não humanos podem ser infectados com DENV, mas não desenvolvem sinais clínicos da doença, sendo a avaliação de proteção realizada pela detecção de viremia (Bente & Rico-Hesse, 2006; Freire *et al.*, 2007). O modelo mais utilizado em camundongos para testes pré-clinicos é a inoculação por via intracerebral de vírus neuroadaptado. Além disso, uma variedade de linhagens de camundongos, com diferentes características imunológicas e modificações genética tem sido descrita por diversos grupos, contudo nenhum dos modelos demonstra quadros semelhantes aos da dengue hemorrágica (Bente & Rico-Hesse, 2006; Freire *et al.*, 2007; Kurane, 2007).

Várias estratégias vêm sendo empregadas para o desenvolvimento de uma vacina segura, não reatogênica e imunogênica contra os quatro sorotipos da dengue: vacinas inativadas, vacina atenuadas, vacinas quiméricas, de subunidade protéica, vacinas de DNA,

ou ainda a combinação de várias tecnologias. As vacinas em desenvolvimento, as que apresentam resultados mais adiantados e promissores são as atenuadas e as quiméricas, que já estão em fase de testes clínicos (Chang *et al*., 2004; Hombach *et al*., 2005; Edelman *et al*, 2007; Guy & Almond, 2008).

Seguindo o sucesso de técnicas clássicas para o desenvolvimento de vacinas atenuadas contra outros flavivírus, como por exemplo, a vacina contra a febre amarela, esforços consideráveis vêm sendo realizados para a obtenção de uma vacina tetravalente atenuada contra dengue. Esta vacina deve ser constituída de cepas vacinais que repliquem *in vivo* o suficiente para induzir uma resposta imune equilibrada contra os quatro sorotipos, sem gerar sintomas associados à dengue e sem possibilidade de serem transmitidas pelo vetor *Aedes*. Vários grupos vêm avaliando a produção de vacinas tetravalentes. Uma destas vacinas, desenvolvida pelos grupos da Mahidol University/ Aventis Pasteur apresentou alta taxa de soroconversão após 2 ou 3 doses. Contudo, esta vacina se mostrou reatogênica em adultos e crianças, tendo cessado o seu desenvolvimento pela Aventis Pasteur (Stephenson, 2005; Edelman, 2007). Outra vacina, desenvolvida pela *Walter Reed Army Institute of Research* (WRAIR) nos Estados Unidos em associação com a GlaxoSmithKline, apresentou níveis aceitáveis de imunogenicidade sem efeitos adversos consideráveis em voluntários saudáveis. Embora esses resultados sejam encorajadores, as bases das atenuações não são bem entendidas e, portanto podem levar a interferências na replicação entre os sorotipos e conseqüentemente ao desequilíbrio da resposta imune, gerando riscos para a ocorrência de ADE e o agravamento da doença (Stephenson, 2005; Edelman, 2007).

Outra abordagem empregada para o desenvolvimento de uma vacina atenuada contra o vírus da dengue é a construção de vacinas quiméricas, que utilizam o esqueleto de um vírus e substituem somente alguns genes deste por regiões equivalentes do vírus da dengue. Uma das estratégias empregadas neste caso é a utilização do vírus vacinal da febre amarela YF 17D como esqueleto no qual os genes que codificam as proteínas do envelope prM e E são substituídos pelos genes equivalentes do vírus da dengue. Dessa forma, a partícula viral produzida possui a proteína C, assim como as proteínas não estruturais do vírus da febre amarela, enquanto as proteínas mais externas, prM e E, provêm do vírus da dengue (Caufour *et al,* 2001; Mateu *et al.,* 2007). Estas vacinas foram avaliadas em camundongos e primatas não humanos com resultados promissores de proteção (Caufour *et al,* 2001; Galler *et al.,* 2005). Uma destas vacinas quiméricas, desenvolvida pela Acambis e licenciada pela Sanofi Pasteur (ChimeriVax), já está na fase de testes clínicos. As avaliações indicam que a vacina

foi bem tolerada e gerou soroconversão em 67% dos voluntários para pelo menos 3 sorotipos após a primeira dose da vacina (Edelman, 2007; Monath, 2007).

Outra vacina quimérica em fase avançada de estudo utiliza como esqueleto o próprio DENV-4, sendo que a atenuação neste caso foi direcionada, obtida por deleção de 30 nucleotídeos na região 3' não traduzida. Tais quimeras contêm os genes das proteínas prM e do envelope de DENV-1, -2 e -3. Os testes da formulação monovalente indicaram que a vacina é segura, geneticamente estável e imunogênica após uma inoculação em indivíduos saudáveis (Edelman, 2007).

De um modo geral, as vacinas atenuadas apresentam riscos de infecções por vírus que possam surgir de reversões gênicas ou recombinações, podendo gerar a doença. Isso dificulta ainda mais a formulação de uma vacina viva atenuada multivalente (Lai *et al.* 1998; Chang *et al.* 2001). Além disso, há a possibilidade de interferência homologa ou heteróloga durante a replicação viral. Esta interferência é particularmente importante em termos da dengue, uma vez que o desequilíbrio da resposta imune pode causar o agravamento da doença, caso o indivíduo vacinado adquira a infecção com um dos quatro vírus da dengue que tenha induzido imunidade insuficiente. Além disso, há o risco deste tipo de vacina não ser segura quando administrada em indivíduos imunosuprimidos (Barrett *et al.* 2001; Chang *et al.* 2001; Kinney & Huang 2001; Stephenson, 2005; Edelman, 2007).

Como alternativa, vários grupos vêm avaliando vacinas inativadas ou de subunidade que podem induzir uma resposta imune mais equilibrada contra os 4 sorotipos, sem riscos de reversões. Como desvantagem, essas vacinas necessitam de várias doses para induzir uma imunidade, que geralmente é pouco duradoura e falham em induzir uma forte resposta celular. Testes iniciais com o vírus inativado mostraram que o processo de inativação resulta em perda de imunogenicidade (Kinney & Huang 2001; Hombach *et al.*, 2005; Edelman, 2007).

A vacina de DNA é uma tecnologia promissora que consiste na inoculação de um plasmídeo de expressão contendo o gene de interesse, possibilitando a produção *in vivo* do antígeno desejado e gerando uma resposta imune de amplo espectro e de longa duração. Esta estratégia se mostra apropriada em superar a interferência que pode ocorrer no caso das vacinas atenuadas tetravalentes contra dengue. Alguns grupos têm demonstrado em modelos animais resultados promissores de indução de uma resposta imune protetora contra diversos flavivírus, inclusive o vírus da dengue (Phillpotts *et al.* 1996, Kochel *et al.* 1997, Colombage *et al.* 1998, Porter *et al.* 1998; Putnak *et al.*, 2003; Chang *et al*; 2004; Raviprakashi *et al*; 2006).

1.4.1 - Antígenos escolhidos para construção de uma vacina

Os principais antígenos utilizados em uma vacina contra dengue são as proteínas E e NS1, alvos da resposta imune humoral, e a proteína NS3, que parece ser imunogênica, com preponderância de epítopos de células T (Figura 1.5) (Mathew *et al.*, 1996; Brinton *et al.*, 1998; Kurane *et al.*, 1998; Huang *et al.*, 1999; Rothman, 2004).

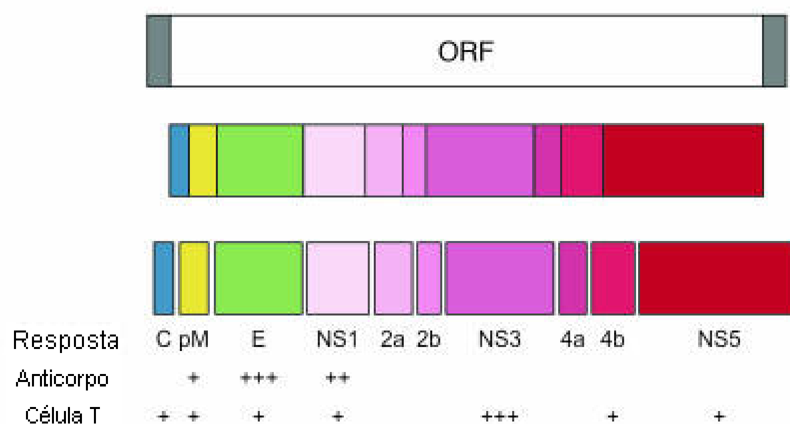

Figura 1.5: Principais alvos da resposta imune contra DENV. Organização do genoma de flavivírus mostrando as proteínas virais e os principais alvos da resposta imune humoral e celular contra DENV. O genoma do DENV contém um quadro de leitura aberto (do inglês *open reading frame* - ORF) que codifica as proteínas virais C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5. O símbolo + indica a intensidade da resposta imune (modificado de Rothman, 2004).

A glicoproteína E, principal constituinte protéico do envelope viral, é um alvo imunodominante da resposta humoral contra o vírus da dengue. Estudos tanto *in vitro* quanto *in vivo*, mostram que anticorpos direcionados contra essa proteína são capazes de neutralizar a infecção viral e protegem animais contra o desafio com o vírus. Além disso, anticorpos contra essa proteína mostram graus variáveis de reatividade cruzada entre os sorotipos de vírus da dengue (Kaufman *et al.*, 1987; Roehrig *et al.*, 1998; Rothman, 2004; Chen *et al.,* 2007b). Entretanto, uma vacina baseada na proteína E, que não proteja completamente contra os 4 sorotipos virais ou cujos anticorpos declinem rapidamente, pode induzir quadros graves da doença se considerarmos que a presença de anticorpos contra epítopos não-neutralizantes ou em concentrações sub-neutralizantes poderiam levar ao fenômeno de ADE (Halstead & O´Rourke, 1977; Halstead, 1979; Stephenson, 2005).

Por outro lado, a utilização de proteínas não estruturais, ausentes da partícula viral, contorna o risco da ocorrência de ADE sendo uma opção vantajosa para o desenho de uma vacina contra dengue.

A proteína NS1 do vírus da dengue foi descrita inicialmente como um antígeno solúvel com atividade de fixação de complemento (Schlesinger *et al.*, 1987; Falgout *et a*l., 1990; Lin *et al.*, 1998). Como informado anteriormente (item 1.2 da introdução), pouco é conhecido sobre a função da proteína NS1, que parece estar envolvida nos estágios iniciais da replicação viral (Mackenzie *et al.*, 1996; Lindenbach & Rice; 1997; Muylaert *et al.*, 1997; Lindenbach & Rice; 1999).

Alguns autores sugerem que a NS1 pode estar envolvida na patogênese viral, contribuindo para um processo autoimune que resultaria na DHF/DSS. Segundo estes trabalhos, anticorpos direcionados contra NS1 poderiam apresentar reação cruzada com plaquetas humanas e antígenos de superfície de células endoteliais (Falconar, 1997; Falconar, 2007). A ligação dos anticorpos a células endoteliais induziria estas a sofrerem apoptose mediada por óxido nítrico. Essa destruição vascular poderia interferir na patogênese da doença (Lin *et al.*, 2002). No entanto, a hipótese de autoimunidade gerada contra NS1 é contestada e perde sua força quando se compara o tempo em que anticorpos contra NS1 circulam no organismo e a natureza transitória dos danos homeostáticos e da permeabilidade vascular (Stephenson, 2005; Halstead, 2007). Outros estudos ainda sugerem que a própria NS1 poderia atuar ativando complemento e formando complexos imunes circulantes envolvidos na patogênese da DHF (Avirutnan *et al.*, 2006).

Por outro lado, diversos trabalhos indicam que a proteína NS1 é um antígeno promissor para o desenvolvimento de uma vacina contra a dengue. Esta proteína é capaz de induzir forte resposta humoral durante a infecção viral (Huang *et al.*, 1999; Libraty *et al.*, 2002). Avaliações iniciais de imunização passiva em camundongos demonstraram a indução de proteção via anticorpo (Gould *et al.*, 1986; Schlesinger *et al.*, 1987; Henchal *et al.*, 1988; Schlesinger *et al.*, 1993). Estudos com a NS1 de diversos flavivírus, expressa em diferentes sistemas recombinantes como *Escherichia coli*, células de inseto, assim como em vacina de DNA, observaram que esta proteína é capaz de induzir uma resposta humoral protetora em modelos animais (Schlesinger *et al.*, 1987; Qu *et al.*, 1993, Schlesinger *et al.*, 1993; Lin *et al.*, 1998; Wu *et al.*, 2003; Hombach *et al.,* 2005; Volpina *et al.*, 2005). Acredita-se que esta proteção é mediada por anticorpos não-neutralizantes com atividade de fixação do complemento, que levariam à lise das células hospedeiras infectadas (Schlesinger *et al.*, 1987; Henchal *et al.*, 1988; Lin *et al.*, 1998).

Já a proteína NS3 parece ser particularmente imunogênica, com uma predominância de epítopos dominantes para linfócitos CD4+ e CD8+, estimulando a resposta de células T vírus-específica e sendo alvo para a ação de células T citotóxicas (Mathew *et al.*, 1996; Kurane *et al.*, 1998; Mathew *et al.*, 1998; Rothman, 2004). Células T específicas para DENV variam em sua habilidade de reconhecer diferentes sorotipos, dependendo do grau de homologia do epítopo. Contudo a reatividade cruzada com múltiplos sorotipos é comum para epítopos de proteínas não estruturais altamente conservadas, como no caso da proteína NS3 (Zivny *et al.*, 1995; Mathew *et al.*, 1996; Kurane *et al.*, 1998; Appanna *et al.*, 2007). Células T CD4+ e CD8+ específicas para dengue produzem predominantemente altos níveis de IFN-γ, TNF-α e TNF-β, assim como a MIP-1β (proteína inibidora de macrófago 1 β) e se mostraram eficientes em lisar células infectadas pelo vírus da dengue *in vitro* (Zivny *et al.*, 1995; Gagnon *et al.*, 1999). Contudo alguns estudos sugerem que NS3 pode induzir apoptose, podendo estar envolvida em mecanismos responsáveis pela patogênese viral (Duarte dos Santos *et al.*, 2000; Shafee & AbuBakar, 2003).

1.5 - Vacina de DNA

A tecnologia da vacinas de ácidos nucléicos teve seu início nos anos 90, quando Wolff e colaboradores (1990) inocularam em camundongos, por via intramuscular, plasmídeos de expressão em células eucarióticas contendo genes marcadores que codificavam as proteínas cloramfenicol acetil transferase, luciferase e β-galactosidade. Este estudo demonstrou que este DNA era capturado pelas células musculares que passavam a sintetizar as proteínas recombinantes codificadas por esses plasmídeos. A seguir, outro grupo evidenciou que um plasmídeo recombinante contendo o gene do hormônio de crescimento humano (hGH) inoculado em camundongos era capaz de induzir uma resposta imune específica contra a proteína codificada por esse gene (Tang *et al*, 1992). No ano seguinte, Ulmer e colaboradores inocularam em camundongos um plasmídeo que codificava a nucleoproteína do vírus influenza e observaram que essa vacina era capaz de induzir células T $CD8^+$ e anticorpos específicos, sendo essa resposta imunológica forte o bastante para proteger o camundongo do desafio com um subtipo diferente de influenza. A partir destes dados, vários pesquisadores começaram a utilizar essa nova tecnologia e demonstraram a eficácia e/ou imunogenicidade das vacinas de DNA, não somente contra doenças infecciosas geradas por diferentes patógenos, mas também em doenças autoimunes, câncer e alergias (Liu, 2003; Guranathan *et al.*, 2000).

Seguindo a injeção do DNA por via intramuscular, células localizadas no sítio de inoculação, como miócitos e queratinócitos, são transfectadas e iniciam a transcrição e tradução do antígeno codificado pelo plasmídeo recombinante. A proteína é processada e apresentada por moléculas do complexo principal de histocompatbilidade I (MHC-I), mas essas células não contêm moléculas co-estimuladoras, necessárias para ativação do linfócito T (Figura 1.6). O antígeno também pode se transferido para células apresentadoras de antígeno profissionais (APC), em um processo denominado de *cross priming*. Além disso, as APCs podem ser também transfectadas diretamente pelo DNA inoculado (Fu *et al.*, 1997; Gurunathan *et al.*, 2000; Srivastava & Liu, 2003).

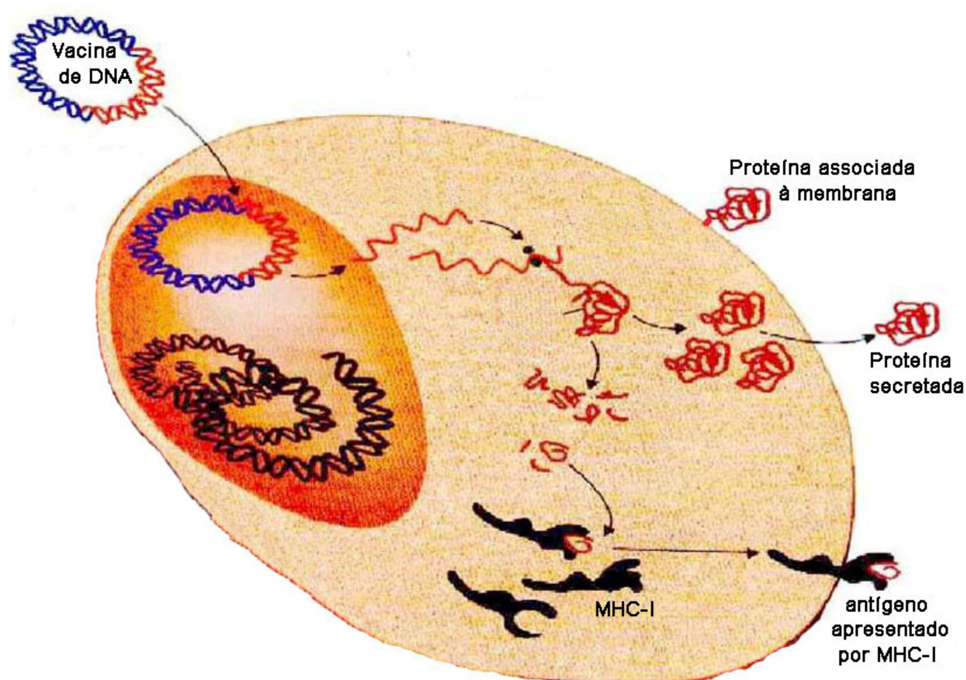

Figura 1.6: Célula transfectada pela vacina de DNA. A célula transfectada pelo plasmídeo recombinante transcreve o RNA mensageiro que é traduzido na proteína recombinante. Esta proteína pode ser processada pela célula hospedeira e ser apresentada pela molécula de MHC-I. Além disso, proteínas contendo seqüências sinalizadoras podem ser translocadas pela via do retículo endoplasmático e se associar à membrana da célula ou ser secretada (modificada de Weiner & Kenned, 1999).

Uma das principais características das vacinas de DNA que estimulou muitos grupos a utilizarem essa nova tecnologia foi a habilidade de levar à expressão endógena do antígeno, semelhante ao que ocorre durante uma infecção viral natural, sendo capaz de ativar uma resposta imune de amplo espectro, humoral e celular, incluindo a ativação de células T citotóxicas. Essa resposta pode ser bastante vantajosa, principalmente no desenvolvimento de vacinas contra patógeno de replicação intracelular como os vírus, em que células citotóxicas reconhecem e lisam células do hospedeiro infectadas, participando do processo de eliminação da infecção. Somado a isso, a produção endógena do antígeno possibilita que a proteína recombinante apresente conformação semelhante à apresentada durante a infecção natural (Ulmer *et al.* 1996, Davis & McCluskie 1999; Srivastava & Liu, 2003).

A indução de uma resposta imune de amplo espectro fornece vantagens em comparação às vacinas clássicas de subunidade ou inativadas que induzem principalmente uma resposta de anticorpos. Por outro lado, as vacinas de DNA não apresentam problemas relacionados às vacinas de organismos atenuados, como a possibilidade de reversão para forma patogênica do agente infeccioso, a inativação térmica e a restrição de uso em indivíduos imuno comprometidos (Davis & Whalen 1995, Dertzbaugh 1998). Somado a isso, as vacinas de DNA são seguras ao serem manipuladas, estáveis a variações de temperatura, de produção barata quando comparada a outras vacinas e permitem uma rápida seleção de seqüências a serem avaliadas (Chang *et al.* 2001).

1.5.1 - Fatores que influenciam a resposta imune

1.5.1.1 – Características do vetor

Os plasmídeos utilizados nas vacinas de DNA contêm basicamente os elementos necessários para sua produção em bactéria, como uma origem de replicação bacteriana e um gene marcador seletivo, que freqüentemente confere resistência a algum antibiótico, e os elementos necessários para expressão do antígeno recombinante na célula hospedeira, como um promotor forte, uma seqüência *enhancer* e seqüências terminais de poliadenilação, além do gene que codifica a proteína de interesse (Lemieux 2002; Srivastava & Liu 2003; Liu & Ulmer, 2005).

Inicialmente, acreditava-se que o plasmídeo funcionaria apenas como um veículo para produção do antígeno recombinante. Contudo, vários estudos demonstram que o DNA plasmidial pode conter seqüências denominadas CpGs que estimulam a imunidade inata,

contribuindo para um aumento na resposta imune específica ao antígeno (Krieg, 2002; Verthelyi & Klinman, 2003; Beláková *et al.*, 2007). Estas seqüências foram inicialmente observadas no gene que confere resistência a ampicilina (Sato *et al.,* 1996). As sequências CpGs não metiladas são reconhecidas principalmente pelos receptores *Toll-Like*-9 (TLR-9) e funcionam como adjuvantes nas vacinas de DNA, além de serem potentes estimuladores de proliferação de células B em estudos *in vitro* (Hemmi *et al.*, 2000; Modlin, 2000; Krieg, 2002; Verthelyi & Klinman, 2003; Beláková *et al.*, 2007).

O desenho básico de uma vacina de DNA é relativamente simples e se baseia em um gene alvo clonado em um vetor de expressão em célula de mamífero. Entretanto, é possível alterar o desenho deste gene retirando regiões prejudiciais à expressão da proteína ou incorporando seqüências que sinalizam para diferentes compartimentos celulares como a membrana plasmática, retículo endoplasmático, núcleo ou secreção para o meio extracelular (Figura 1.6) (Rodriguez & Whitton, 2000). Como conseqüência, essas diferenças afetam a forma de apresentação do antígeno ao sistema imunológico influenciando na resposta imune gerada (Alves *et al*, 1999; Liu, 2003; Donelly *et al.*, 2005). A inclusão de seqüências sinais secretoras, assim como a adição da seqüência LAMP (*lysosomal-associated membrane protein*) que direciona o antígeno para compartimentos especializados contendo MHC-II, leva a um aumento significativo dos níveis de anticorpos específicos (Leitner *et al.*, 1997; Alves *et al*, 1999; Wang *et al.*, 2004a; Anwar *et al.*, 2005). Por outro lado, o direcionamento da proteína para a via de degradação ubiquitina (Ub)/proteosoma através da construção de proteínas fusionadas a ubiquitina, aumenta a resposta T citotóxica contra o antígeno (Rodriguez *et al.*, 1997; Donelly *et al.*, 2005; Dobanõ *et al.*, 2007). Outra metodologia empregada por alguns grupos é a substituição de nucleotídeos, de forma a modificar os códons originais, trocando-os por outros mais utilizados pelo organismo hospedeiro. Essas substituições geralmente levam a um aumento na expressão da proteína recombinante (zur Megede *et al.*, 2000; Lemieux, 2002).

1.5.1.2 – Administração das vacinas de DNA

Outro fator que pode alterar o padrão da resposta imune gerada é a variação na via de administração do plasmídeo recombinante, assim como sua forma de apresentação (DNA descoberto ou incorporado a veículos abióticos ou vivos).

A forma mais utilizada de administração do DNA plasmidial é a via parenteral, que inclui as inoculações intramuscular (i.m.), intradérmica (i.d.) e por biobalística. Esta última

consiste na utilização de partículas de ouro cobertas com o DNA plasmidial, que são empurradas por pressão de Hélio ou CO_2, para dentro da pele através de uma pistola (*Gene Gun)*(Shedlock & Weiner, 2000).

O uso de agulha para injetar uma solução aquosa de DNA plamidial em tecidos é um caminho relativamente simples e efetivo de inoculação das vacinas de DNA. A injeção i.d., principalmente, na transfecção de fibroblastos e queratinócitos, enquanto que na injeção i.m. leva predominantemente a transfecção de miócitos. Nas imunizações i.m. aparentemente poucas células dendríticas são transfectadas diretamente, contudo estas são capazes de apresentar o antígeno, devido ao *cross-priming* a partir dos miócitos transfectados (Dupuis *et al.*, 2000; Gurunathan *et al.*, 2000; Shedlock & Weiner, 2000; Donelly *et al.*, 2005). Durante o bombardeamento da epiderme com DNA plasmidial por *Gene Gun,* ocorre a transfecção direta de queratinócitos, assim como de células de Langerhans. Estas últimas migram rapidamente para os linfonodos locais, o que parece aumentar a eficiência das imunizações por este método, que utiliza cerca de 100 a 1000 vezes menos quantidade de DNA quando comparado às injeções intramusculares e intradérmicas (Robinson & Torres, 1997; Porgador *et al.,* 1998; Shedlock & Weiner, 2000).

Uma variação promissora das técnicas de inoculação parenteral é a eletroporação. Estudos indicam que a eletroporação aumenta cerca de 6-34 vezes a entrada do DNA, quando comparada à injeção i.m., levando a um aumento na expressão da proteína recombinante e nos níveis da resposta imune (Widera *et al.*, 2000; Wang *et al.*, 2004b; Luckay *et al.*, 2007).

A inoculação do plasmídeo recombinate por vias parenterais geralmente não é capaz de ativar de forma eficiente a imunidade de mucosa. Considerando que uma grande parte dos patógenos entra no organismo hospedeiro através das mucosas, vários grupos têm utilizado métodos não invasivos envolvendo a aplicação do DNA em superfícies de mucosa, incluindo a intranasal, a oral e a intravaginal. Vários destes estudos têm demonstrado a indução de resposta antígeno específica local e sistêmica (Kozlowski *et al.*, 1997; Sasaki *et al.*, 1998; McCluskie & Davis, 1999).

Devido à variação na população de células produtoras do antígeno recombinante codificado pelas vacinas de DNA, as diferentes vias de inoculação geram uma resposta imune variada. Estudos indicam que o *gene-gun* induz preferencialmente uma resposta imune humoral, caracterizada por uma progressão rápida de células T helper 2 (Th2), associada à produção de anticorpos IgA e IgG1. Inversamente, a injeção intramuscular geralmente resulta na indução de uma resposta celular, com proliferação preferencialmente de linfócitos Th1, linfócitos citotóxicos e anticorpos IgG2a (Shedlock &Weiner, 2000). Contudo, este é apenas

um dos fatores que influencia no padrão da resposta imune gerada e a combinação de outros parâmetros também pode alterar esse padrão.

Estudos de biodistribuição mostraram que grande parte do plasmídeo inoculado no organismo hospedeiro é rapidamente perdida (Sheets *et al.*, 2006; Donelly *et al.*, 2005). Várias formulações compostas de DNA plasmidial incorporado a veículos abióticos como lipídios catiônicos, lipossomos ou microesferas foram desenvolvidas visando evitar a degradação do DNA e aumentar a eficiência de células do hospedeiro. Estas formulações podem ser liberadas por via parenteral ou na superfície de mucosas (McCluskie & Davis, 1999; Donelly *et al.*, 2005).

Outra estratégia promissora que vem sendo utilizada com sucesso para diferentes patógenos é a combinação de sistemas heterólogos de vacinas denominada *prime-boost*. Esta abordagem consiste em imunizar um organismo contra determinado antígeno utilizando um vetor e depois dar o reforço direcionado contra o mesmo antígeno utilizando outro sistema (Woodland, 2004). Particularmente, o uso da vacina de DNA para "primar" a resposta imune humoral e principalmente celular seguido de uma vacina viral recombinante como reforço vem se mostrando efetiva em induzir uma resposta mais forte e ampla quando comparado com ensaios em que a mesma vacina é administrada nas doses de reforço. Esta combinação vem sendo amplamente utilizada em ensaios clínicos para diferentes patógeno com o objetivo de melhorar a resposta imune gerada em humanos (Shilver *et al.*, 2002; McConkey *et al.*, 2003; Robinson, 2003)

1.5.2 - Ensaios clínicos de vacinas de DNA

Durante a década passada, ensaios de vacinas de DNA em modelos animais, geraram resultados tão promissores que impulsionaram o rápido desenvolvimento de ensaios clínicos. Vários destes ensaios têm investigado a utilidade das vacinas de DNA contra o vírus influenza, o vírus da hepatite B, o papilomavirus humano (HPV), o vírus da imunodeficiência humana (HIV), malária, assim como contra doenças neoplásicas (Rottinghaus *et al.*, 2003; Drape *et al.*, 2006; Burgers *et al.*, 2006; Beláková *et al.*, 2007).

Entretanto, estudos clínicos iniciais utilizando vacinas de DNA inoculadas por via i.m. ou i.d. sugeriram que estas não seriam tão imunogênicas em humanos, quando comparada à resposta gerada em camundongos. Uma das dificuldades observadas diz respeito à baixa eficiência de transfecção das células hospedeiras em humanos, mesmo quando foram utilizadas altas doses de DNA (Liu & Ulmer, 2005). Dessa forma, várias estratégias têm sido

avaliadas na intenção de aumentar a potência das vacinas de DNA como utilização de diferentes métodos de inoculação como, por exemplo, a eletroporação (Widera *et al.*, 2000); co-administração de plasmídeos que codificam citocinas ou uso de adjuvantes (Lemieux, 2002); adição de seqüências imunoestimudoras CpGs (Krieg, 2001); utilização de carreadores como lipossomos catiônicos (Fuller *et al.*, 2006); e a administração combinada de diferentes tipos de vacinas recombinantes em experimentos de dose e reforço (*prime-boost*)(Woodland, 2004).

Até maio de 2007, foram registrados aproximadamente 40 ensaios clínicos de vacinas de DNA. Alguns desses ensaios estão direcionados à avaliação de vacina contra doenças infecciosas como hepatite B, malária, febre hemorrágica do vírus Ebola, inclusive contra a infecção pelo flavivírus WNV, sendo que a maioria avalia vacinas contra o HIV-1 (Beláková *et al.*, 2007). Devido à quantidade cada vez maior de ensaios clínicos baseados nesta tecnologia, torna-se necessária a avaliação de algumas questões relativas à segurança destas vacinas.

Diversos grupos têm avaliado a possibilidade de integração do DNA plasmidial no genoma hospedeiro, em uma variedade de vacinas e modelos animais, mas até o momento praticamente nenhum evento de integração foi detectado (Manam *et al.*, 2000; Kang *et al.*, 2003; Liu & Ulmer, 2005; Coelho-Castelo *et al.*, 2006; Sheets *et al.*, 2006; Pelizon *et al.*, 2007). Somente Wang e colaboradores (2004b) observaram raros eventos de integração quando uma vacina de DNA foi administrada pela técnica de eletroporação, que eleva bastante os níveis de transfecção quando comparada a inoculação por injeção. Outra preocupação é a possibilidade de gerar anticorpos anti-DNA, que poderiam causar quadros de auto-imunidade. Entretanto, não houve relato de indução de auto-anticorpos patogênicos em qualquer um dos modelos avaliados. Além disso, não foi observado indução de tolerância imunológica, indicando que estas vacinas são seguras para uso em humanos (Liu & Ulmer, 2005).

1.5.3 - Vacinas de DNA contra flavivírus

Os flavivirus são um grupo de vírus de grande importância médica, sendo um dos principais alvos de desenvolvimento de vacinas de DNA. Grande parte destes trabalhos utiliza as proteínas de superfície prM e E como antígeno para construção de uma vacina e avalia principalmente a produção de anticorpos neutralizantes contra o vírus, assim como a proteção em modelos animais. Estes estudos têm demonstrado a indução de níveis variáveis de

proteção flavivírus-específica em camundongos, suínos, cavalos e primatas não humanos, dependendo da abordagem estabelecida (Phillpotts *et al.* 1996, Colombage *et al.*1998, Porter *et al.* 1998, Kochel *et al.* 2000, Konishi *et al.* 2000, Kaur *et al.*, 2004; Bharati *et al.*, 2005; Wu *et al.*, 2006; Ishikawa *et al.*, 2007).

Algumas análises também foram realizadas utilizando proteínas não estruturais de flavivírus, incluindo a proteína NS1. Lin e colaboradores (1998) imunizaram camundongos com um plasmídeo recombinante codificando a proteína NS1 do vírus da encefalite japonesa e demonstraram a indução de uma resposta imune protetora contra o desafio com doses letais deste vírus. Estudos de desenvolvimento de vacinas de DNA contra o vírus da encefalite transmitida por carrapato (TBE – *tick-borne encephalitis*) também foram realizados e confirmaram o potencial protetor quando testado em camundongos (Timofeev *et al.*, 2004; Aleshin *et al.*, 2005). Um desses trabalhos utilizando a estratégia de *prime-boost*, com vacina de DNA combinada a vírus vaccinia recombinante mostrou que esta estratégia é capaz de gerar altos níveis de proteção em modelo animal. Tal proteção foi maior quando comparada ao uso das mesmas vacinas em testes separados (Aleshin *et al.*, 2005).

Além disso, estudos com as proteínas NS3, NS4 e NS5 do vírus da hepatite C verificaram a indução de uma resposta celular e proteção dos animais contra a formação e crescimento de tumor causado pela infecção com este vírus (Encke *et al.* 1998, Cho *et al.* 1999). Estes resultados estimulam o estudo destes antígenos flavivirais no desenvolvimento de uma vacina de DNA contra o vírus da dengue.

Em 2006, foi licenciada uma vacina de DNA de uso veterinário em eqüinos contra um flavivírus, o vírus do Oeste do Nilo (do inglês *West Nile Vírus* - WNV), sendo esta a primeira vacina de DNA a ser licenciada (Davis *et al.*, 2001). Esta vacina, baseada nas proteínas prM e E, também tem se mostrado protetora contra a infecção letal do vírus em pássaros (Chang *et al.* 2007), contudo ela ainda não está sendo comercializada (Petersen & Roehring, 2007). Além disso, resultados dos testes clínicos de fase 1 de uma vacina de DNA contra este flavivírus, baseada em um plasmídeo análogo ao utilizado na vacina eqüina licenciada, mostraram a indução de anticorpos neutralizantes nos voluntários, com níveis similares aos identificados como protetores na vacina eqüina (Martin *et al.*, 2007). Tais resultados significam um grande avanço científico na produção de uma vacina de DNA contra um flavivírus (Petersen & Roehring, 2007).

1.5.3.1 – Vacinas de DNA contra a dengue

Semelhante aos outros flavivírus, as vacinas de DNA contra o DENV se baseiam predominantemente nos principais alvos da resposta imune humoral contra DENV, as proteínas E e NS1 (Kinney *et al.*, 2001). Estudos avaliando vacinas de DNA tetravalentes baseadas na proteína E ou somente no domínio III desta proteína, envolvido na interação com o receptor da célula alvo, mostram a indução de anticorpos neutralizantes e proteção contra os 4 sorotipos do vírus em camundongos, apesar desses níveis de proteção contra os diferentes sorotipos variarem (Mota *et al.*, 2005; Konish *et al.*, 2006; Chen *et al.*, 2007b). A inoculação de vacinas de DNA monovalentes, que codicam as proteínas prM e E, em primatas não humanos também tem se mostrado promissora na indução de proteção contra viremia, principalmente em esquemas de imunização do tipo *prime-boost* (Kochel *et al.*, 2000; Putnak *et al.*, 2003; Chen *et al.,* 2007a).

Contudo, devido o risco de anticorpos não-neutralizantes contra a proteína E poderem levar ao aumento da replicação viral, a utilização de proteínas não estruturais, ausentes da partícula viral, seria uma opção vantajosa para o desenho de uma vacina contra dengue.

Até o momento não há nenhum trabalho que utilize o antígeno NS3 de DENV em vacinas de DNA e demonstre que este é capaz de induzir proteção. Por outro lado, alguns grupos têm utilizado NS1 como antígeno na formulação de vacinas de DNA (Wu *et al.*, 2003; Timofeev *et al.*, 2004; Mellado-Sánchez *et al.*, 2005). Em um destes estudos, a imunização de camundongos com um plasmídeo que codifica a proteína NS1 de DENV-2 gerou proteção em camundongos desafiados com doses letais de DENV-2 (Wu *et al.*, 2003).

Baseado nestas evidências, no presente trabalho foram construídos diferentes plasmídeos de expressão eucariótica contendo os genes *ns1* e *ns3* do vírus da dengue, sorotipo 2, com o objetivo de avaliar as respostas imunes induzidas em camundongos e a capacidade protetora destas vacinas de DNA. As construções baseadas na proteína NS1 contêm seqüências sinais que mimetizam a sua produção durante a infecção viral, enquanto que os plasmídeos baseados em NS3 contêm diferentes domínios funcionais desta proteína.

2 – OBJETIVOS

2.1 - Objetivo Geral: Avaliar o potencial de vacinas de DNA que codificam as proteínas NS1 e NS3 na ativação de respostas imunes eficientes e protetoras contra o vírus da dengue.

2.2 - Objetivos específicos:

1 - Clonar a região que codifica a proteína NS1 de DENV-2, fusionada a diferentes seqüências de sinalização celular, nos plasmídeos pcTPA e pcDNA3.

2 – Clonar as seqüências que codificam a proteína NS3 de DENV-2 inteira e seus domínios funcionais (domínio proteolítico, domínio helicase/NTPase/RTPase, e as regiões N e C terminais geradas naturalmente pela clivagem da proteína NS3 durante a infecção viral), no plasmídeo pcTPA.

3 - Analisar a expressão das proteínas recombinantes em culturas de célula de mamífero transfectadas com os diferentes plasmídeos construídos.

4 - Avaliar o perfil da resposta imune humoral em camundongos inoculados com os plasmídeos contendo a seqüência que codifica a proteína NS1.

5 – Avaliar a proteção, intra- e interespecífica gerada pelas diferentes vacinas, pela realização de desafios com os vírus DENV-1 e DENV-2.

6 – Analisar a participação do complemento na proteção gerada pela proteína NS1

MATERIAL E MÉTODOS

3 - MATERIAL E MÉTODOS

A maior parte da metodologia utilizada para obtenção dos resultados referentes ao gene NS1 está descrita nos artigos 1, 2 e 3 da seção “resultados”. Contudo, experimentos iniciais de cálculo de LD_{50} de DENV-1 e DENV-2 inoculados por via intracerebral (i.c.) em camundongos BALB/c, as avaliações de citotoxicidade mediada pelo complemento e por anticorpos direcionados contra a proteína NS1 e o desafio por via i.c. com DENV-1 não foram descritos nos artigos publicados e serão apresentados abaixo.

Os ensaios realizados com o gene NS3, que ainda não estão compilados sob a forma de manuscrito, também estão descritos nesta seção.

3.1 – Meios de cultura e soluções

3.1.1 - Ampicilina (1000 X)

100 mg de ampicilina

1 mL de água deionizada

Esterilizar por filtração (membrana de 0,22 μm)

3.1.2 - Gel de agarose 1%

1 g de agarose em 100 mL de TAE

3.1.3 - Meio 199 com sais de Earle (para inocular em camundongos) (1 L)

100 mL de meio 199 com sais de Earle 10 X (SIGMA)

25 mL de NaHCO3 4,4%, pH 7,0

5% de soro fetal bovino (SFB)

Completar volume com água bidestilada

Todo material usado foi previamente esterilizado

3.1.4 - Meio 199 com sais de Earle completo (1 L)

100 mL de meio 199 com sais de Earle 10 X

25 mL para sistema aberto ou 50 mL para sistema fechado de NaHCO3 4,4%, pH 7,0

10 mL de gentamicina 4 mg/mL

1 mL de fungizona 500 mg/mL

5% de SFB

Completar volume com água bidestilada

Todo material usado foi previamente esterilizado

3.1.5 - Meio DMEM (1 L)

10 g de DMEM (SIGMA)

2,2 g de $Ca(HCO_2)_2$

2 g de HEPES

Água deionizada

Ajustar para pH 7,4

Completar volume com água deionizada

Esterilizar por filtração (membrana de 0,22 μm)

3.1.6 - Meio LB (*Luria Broth*) (1 L)

10 g de triptona

5 g de extrato de levedura

10 g de NaCl

Completar volume com água deionizada

Esterilizar por autoclavação a 120º C por 15 min

3.1.7 - Meio LB sólido

Adicionar 15 g de bacto-agar a cada 1 L de meio LB líquido

Esterilizar por autoclavação a 120º C por 15 min

3.1.8 - Meio TB líquido (1 L)

12 g de triptona

24 g de extrato de levedura

4 mL de glicerol

Completar volume com água deionizada para 900 mL

Esterilizar por autoclavação a 120º C por 15 min

No momento do uso, adicionar 100 mL de solução 0,17 M de KH_2PO_4 e 0,72 M de K_2HPO_4 previamente esterilizado por autoclavação

3.1.9 - PBS 1X (1 L)

8 g de NaCl

0,2 g KCl

1,44 g Na_2HPO_4

0,24 g KH_2PO_4

Ajustar pH 7,4

Completar volume com água deionizada

3.1.10 - Quetamina/xilasina (1 mL)

150 μL de quetamina 10%

40 μL de xilasina 10%

Completar volume com solução salina comercial

3.1.11 - Solução salina BSS (1L)

8 g de NaCl

0,4 g de KCl

0,012 g de $CaCl_2$

0,154 g de $MgSO_4.7H_2O$

0,39 g de $Na_2HPO_4.12H_2O$

0,15 g de KH_2PO_4

1,1 g de glicose

0,0025 g de vermelho de fenol

Ajustar pH 7,4

Completar volume com água Deionizada

Esterilizar por filtração (membrana de 0,22 μm)

3.1.12 - Solução salina BSS-CMF (1 L)

8 g de NaCl

0,4 g de KCl

0,1 g de Na_2SO_4

0,39 g de $Na_2HPO_4.12H_2O$

0,15 g de KH_2PO_4

1,1 g de glicose

0,0025 g de vermelho de fenol

Ajustar pH 7,4
Completar volume com água Deionizada
Esterilizar por filtração (membrana de 0,22 μm)

3.1.13 – TAE (1 L)
4,84 g de Trizma-base
1,14 mL de ácido acético glacial 100%
2 mL/L de EDTA 0,5 M (pH 8,0)
Completar volume com água deionizada
Corado com 0,5 μg/mL de brometo de etídeo

3.1.14 - Tripsina (100 mL)
10 mL de tripsina-EDTA 10x (Invitrogen)
Completar volume com tampão BSS-CMF
Esterilizar por filtração (membrana de 0,22 μm)

3.2 – Células e Vírus

A cepa DH5α de *Escherichia coli* foi utilizada para clonagem e amplificação dos plasmídeos recombinantes e controles nos experimentos referentes à região NS3, de forma semelhante à descrita nos artigos 1 e 3 relativos à NS1.

Células de rim de hamster neonato BHK-21 (ATCC) foram utilizadas para análise da expressão *in vitro* das proteínas recombinantes.

Para os ensaios de lise celular mediada por anticorpos foram utilizadas células epiteliais de rim de macaco verde africano Vero (ATCC). Estas células também foram usadas para titulação de DENV-1 e DENV-2 após os experimentos de desafio por via i.c.

A linhagem de DENV-2 New Guinea (NCG) (GenBank M29095) foi utilizada para clonagem das seqüências que codificam regiões da proteína NS3 de modo semelhante ao descrito nos artigos 1, 2 e 3. Culturas de células do mosquito *Aedes albopictus* (C6/36) infectadas por esses vírus foram cedidas pela Dra. Rita Nogueira do Laboratório de Flavivírus, IOC/FIOCRUZ para extração do RNA viral. Além disso, esta linhagem viral também foi empregada nos experimentos de avaliação da lise celular mediada por anticorpos.

Os ensaios de desafio por via i.c. com DENV-2 foram realizados com a linhagem DENV-2 NGC neuroadaptado a camundongos. Os testes de desafio com DENV-1 foram

realizados com a linhagem Mochizuki (GenBank AB074760), também adaptada a camundongo. Ambos os vírus neuroadaptados foram obtidos no Laboratório de Tecnologia Virológica (LATEV), de BioManguinhos/FIOCRUZ

3.3 – Animais

Todos os experimentos com animais foram realizados com camundongos BALB/c SPF (*specific pathogen free*) machos, provenientes do biotério do Centro Multidisciplinar para Investigação Biológica (CEMIB), Campinas, SP. Os experimentos com os animais foram realizados segundo os princípios éticos determinados pela Comissão de Ética no Uso de Animais (CEUA) da Fiocruz (licença nº P0104-02).

3.4 – Plasmídeos

Para a construção dos diversos plasmídeos recombinantes foram utilizados os plasmídeos pcDNA3 e pcTPA. O plasmídeo pcDNA3 (Invitrogen) contém a região promotora derivada do citomegalovírus humano (CMV), um sítio múltiplo de clonagem, a seqüência de poliadenilação do hormônio de crescimento bovino (do inglês *Bovine Growth Hormone* - BGH) e o gene que confere resistência a neomicina, para a expressão de proteínas heterólogas em células de mamífero. Além disso, este plasmídeo também contém a origem de replicação em células procarióticas (ColE1) e o gene que confere resistência a ampicilina, utilizados para a seleção de bactérias contendo tal plasmídeo.

O plasmídeo pcTPA foi construído anteriormente pelo nosso grupo a partir do pcDNA3, com a inserção da seqüência que codifica o peptídeo sinal do ativador de plasminogênio de tecido humano (t-PA), a jusante da região promotora do CMV (Figura 3.1). A seqüência sinal t-PA foi inserida com o objetivo de direcionar as proteínas recombinantes para a via de secreção.

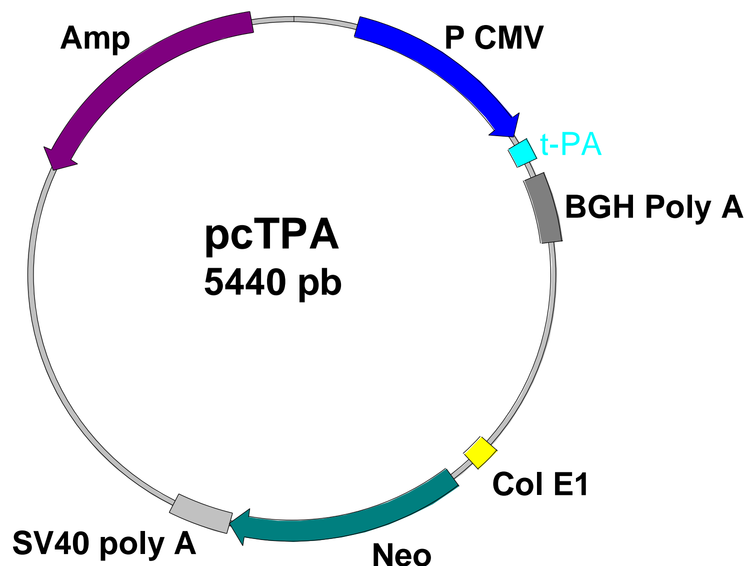

Figura 3.1: Representação esquemática da construção pcTPA. A seqüência que codifica o peptídeo sinal do t-PA, amplificada por PCR foi clonada entre os sítios de reconhecimento das enzimas *Hind* III e *Eco* RV do plasmídeo pcDNA3, originando o plasmídeo pcTPA. P CMV - promotor do CMV; t-PA – seqüência que codifica o peptídeo sinal do ativador de plasminogênio de tecido humano; BGH polyA – região de poliadenilação do BGH; ColE - origem de replicação em células procarióticas; Neo - gene que confere resistência a neomicina; SV40 poly A - região de poliadenilação do vírus símio 40 (do inglês *Simian Virus* 40 - SV 40)

3.5 – Cálculo da LD_{50} de DENV neuroadaptado

A neurovirulência de DENV neuroadaptado foi avaliada em camundongos BALB/c machos com 8 semanas de idade. Grupos de animais (n = 10) foram inicialmente anestesiados com uma mistura de quetamina/xilasina e a seguir inoculados pela via i.c. com 30 µL de DENV diluído (diluições seriadas) em meio 199 com sais de Earle, a partir do estoque viral. Como controle negativo, um grupo de animais foi inoculado somente com o meio de cultura. O inóculo viral foi titulado em células Vero, imediatamente depois do ensaio de desafio, pela equipe do Dr. Marcos Freire, no LATEV em Bio-Manguinhos.

A mortalidade dos animais de cada grupo foi avaliada diariamente durante 21 dias. Após esse período, os animais que sobreviveram foram eutanasiados. A LD_{50} foi determinada pela correlação entre a diluição viral e a razão entre mortos e sobreviventes, de acordo com o método de Reed & Muench (1938).

3.6 – Construção dos plasmídeos contendo seqüências que codificam a proteína NS3 inteira ou somente partes desta

Os experimentos de clonagem das seqüências que codificam a proteína NS3 ou seus domínios, purificação dos plasmídeos e expressão *in vitro* das proteínas recombinantes e desafio por via i.c. foram conduzidos de forma semelhante aos experimentos descritos nos artigos 1 e 3 da seção "resultados". O resumo da metodologia empregada, assim como as variações de protocolo específicas para a região NS3 estão apresentados nos itens abaixo.

3.6.1 – Clonagem das seqüências que codificam a proteína NS3 inteira ou seus domínios

A proteína viral NS3 possui diferentes domínios que atuam no ciclo de replicação do vírus da dengue. Considerando esses domínios e as frações geradas naturalmente pela clivagem de NS3 em células infectadas por DENV, seqüências que codificam diferentes porções desta proteína foram clonadas no plasmídeo de expressão em células eucarióticas, pcTPA (derivado do pcDNA3, Invitrogene), a jusante da seqüência do peptídeo t-PA, que funciona como sinal de secreção.

O RNA total de células C6/36 de *Aedes albopictus* infectadas com DENV-2 Nova Guiné (NGC) foi extraído com Trizol (Invitrogen), de acordo com as recomendações do fabricante e ressuspenso em 20 μL de água livre de RNAse. Uma mistura contendo 4 μL do RNA total e 10 pmoles do oligonucleotídeo iniciador 5'-GGGGGATATCGATAGTGGTT GCGTTG-3', que pareia no início do gene NS1 [(nucleotídeos 2422 a 2437 do genoma de DENV-2 NGC (Irie *et al.*, 1989)] foi incubada por 10 mim a 65° C e a seguir colocada em gelo. A essa mistura foram adicionadas 200 U da enzima transcriptase reversa M-MLV (Invitrogene), 4 μL do tampão 5X (Invitrogene), 2 μL de DTT 0,1M, 8 μL de dNTP 1,25 mM e 0,6 μL do inibidor RNAguard (Invitrogene) e a reação foi incubada por 1 h a 37° C. Após a inativação da enzima a 65° C por 10 minutos, o cDNA foi utilizado como molde para amplificação das construções de NS3 pela reação em cadeia da polimerase (PCR). A tabela 3.1 mostra os oligonucleotídeos senso e anti-senso utilizados na construção de cada plasmídeo recombinante. Uma preparação (50 μL) contendo 10 μL de cDNA, 10 mM de dNTPs, 10 pmoles de cada oligonucleotídeo, 2,5 unidades de Taq DNA polimerase *Platinun* (Invitrogene) e 10 μL do tampão 5X (Invitrogene) foi submetida à PCR no termociclador GeneAmp PCR System (Applied Biosystems). Foi realizada uma incubação inicial de 3 min a

94º C seguida de 30 ciclos de amplificação (2 min a 92º C; 1 min a 55º C; 2 min a 72º C) e incubação final a 72º C por 5 min. Os fragmentos amplificados foram submetidos a eletroforese em gel de agarose 1% dissolvido em tampão TAE 1X corado com brometo de etídeo, de acordo Sambrook e colaboradores (1989). A extração destes fragmentos do gel e sua purificação foram realizadas com o auxílio do kit Geneclean (Bio 101 System), conforme recomendações do fabricante e o DNA foi ressuspenso em 20 μL de água deionizada, livre de nucleases.

Plasmídeo recombinante	Oligonucleotídeos	Região amplificada
pcTPANS3	Senso: 5'-GGGGGATATCGCTGGAGTATTGTGGG-3' *Eco* RV Anti-senso: 5'-GGGGTCTAGA**TTA**CTTTCTTCCAGCTGCA-3' *Xba* I	NS3 inteira (nucleotídeos 4522- 6375)
pcTPANS3H	Senso: 5'-GGGGATATCAGAAAATTGACCATCATGG-3' *Eco* RV Anti-senso: 5'-GGGGTCTAGA**TTA**CTTTCTTCCAGCTGCA-3' *Xba* I	Domínios helicase/NTPase/RTPase (nucleotídeos 5077-6375)
pcTPANS3P	Senso: 5'-GGGGGATATCGCTGGAGTATTGTGGG-3' *Eco* RV Anti-senso: 5'-GGGTCTAGA**TTA**CTTTCGAAAAATGTCATC-3' *Xba* I	Domínio protease (nucleotídeos 4522-5076)
pcTPANS3N	Senso: 5' GGGGGATATCGCTGGAGTATTGTGGG 3' *Eco* RV Anti-senso: 5'GGGGTCTAGA**TTA**TCTTCTTTGTGCTGC 3' *Xba* I	Porção N terminal gerada pela clivagem da NS3 (nucleotídeos 4522-5895)
pcTPANS3C	Senso: 5'GGGGGATATCGGGAGAATAGGAAGAAATC 3' *Eco* RV Anti-senso: 5'GGGGTCTAGA**TTA**CTTTCTTCCAGCTGCA 3' *Xba* I	Porção C terminal gerada pela clivagem da NS3 (nucleotídeos 5896-6375)

Tabela 3.1: Construções dos plasmídeos recombinantes compreendendo as seqüências que codificam a proteína NS3 ou partes desta. A primeira coluna indica o nome das construções recombinantes. Na segunda coluna estão representados os oligonucleotídeos senso e anti-senso utilizados para amplificação dos fragmentos desejados, contendo os sítios de restrição para as enzimas *Eco*RV e *Xba* I, respectivamente. O códon de término de tradução, presente nos oligonucleotídeos anti-senso está representado em negrito. A terceira coluna indica as regiões amplificadas com cada par de oligonucleotídeos. As posições dos oligonucleotídeos foram estabelecidas de acordo com o genoma de DENV-2 NGC (Irie *et al*., 1989).

Os fragmentos amplificados, assim como o plasmídeo pcTPA, foram digeridos com as enzimas *Eco* RV e *Xba* I (Invitrogene) a 37º C por 2 h, de acordo com as indicações da Invitrogene. As amostras digeridas foram novamente purificadas utilizando o sistema Geneclean (Bio 101 System). Para ligação dos fragmentos ao plasmídeo pcTPA foi utilizado o kit Ready-to-go T4 DNA ligase (Amersham Biosciences). A reação de ligação, constituída de 1 μg total de DNA (na proporção de 3 moléculas de insertos : 1 molécula de pcTPA) em volume total de 20 μL, ocorreu a 16º C, por 1 h. Bactérias *Escherichia coli* DH5α, quimicamente competentes, foram transformadas por choque térmico com os produtos da ligação e plaqueadas em meio sólido LB. As colônias obtidas foram crescidas em meio líquido LB contendo 100 μg/ mL de ampicilina e os plasmídeos purificados por preparação em pequena escala utilizando o kit Flexiprep (GE Healthcare). Os clones recombinantes foram selecionados por migração em gel de agarose e digestão com as enzimas de restrição *Eco* RV e *Xba* I, para liberação do inserto.

A clonagem dos diferentes fragmentos da região NS3 foi confirmada por sequenciamento (Sanger *et al* 1992), utilizando 200–250 ng de DNA plasmidial e 3,2 pmoles do oligonucleotídeo apropriado (tabela 3.2.), utilizando o sistema "ABI PRISM dye terminator cycle sequence core kit" (Applied Biosystems), segundo as recomendações do fabricante. As amostras foram seqüenciadas no seqüenciador automático ABI PRISM 3100 (Applied Biosystems) pela Plataforma Genômica – subunidade sequenciamento de DNA (PDTIS-FIOCRUZ).

Os clones recombinantes foram mantidos congelados em nitrogênio líquido, utilizando 250 μL de glicerol 80% (v/v) e 750 μL da cultura de bactéria crescida durante a noite em meio LB líquido com ampicilina.

Oligonucleotídeo	Região de pareamento
5´- GGTGTAACATACCTTACAAG – 3’	Anti-senso, nucleotídeos 4653 - 4672
5´- GTCTTGGCATTGGAGCCTGG – 3´	Senso, nucleotídeos 4811 - 4831
5´- GGAGTGGAGCATATGTGAGT – 3´	Senso, nucleotídeos 4992 - 5011
5´- GAGTATCACTTACACCGTAC – 3´	Anti-senso, nucleotídeos 5306 - 5325
5´- CAATGCACCAATCATGGATG – 3´	Senso, nucleotídeos 5506 - 5525
5´- CACTCTAGTGCAGCACAAAG – 3´	Senso, nucleotídeos 5873 - 5892
5´- GATGCCATTGATGGTGAATAC – 3´	Senso, nucleotídeos 6071 – 6091

Tabela 3.2: **Oligonucleotideos utilizados para o sequenciamento dos fragmentos clonados.** A primeira coluna mostra a seqüência dos oligonucleotídeos usados nas reações de sequenciamento, enquanto a coluna 2 indica a direção de polimerização da cadeia de nucleotídeos e a região de pareamento destes iniciadores. Essas posições foram estabelecidas de acordo com o genoma de DENV-2 NGC (Irie *et al*., 1989).

3.6.2 – Purificação dos plasmídeos em larga escala

Para a produção de grande quantidade dos plasmídeos, inicialmente bactérias *E. coli* transformadas com tais plasmídeos foram crescidas em meio LB contendo 100 μg/ mL de amplicilina a partir dos estoques congelados. As culturas foram incubadas a 37°C, sob agitação (120 rpm), durante a noite (16–18 h). No dia seguinte 25 mL desta cultura foi adicionada a 1 L de meio TB com ampicilina e estas culturas foram mantidas novamente a 37ºC sob agitação, durante a noite.

O DNA plasmidial foi extraído por lise alcalina e purificado em colunas de troca iônica, utilizando “Qiagen Plasmid Giga Kit” (Qiagen), segundo as instruções do fabricante. Foram utilizados 3 L de cultura bacteriana para cada coluna Giga. Os plasmídeos foram ressuspensos em água deionizada e armazenado à – 20 °C até o momento de uso.

O DNA foi quantificado em espectrofotômetro Bio Photometer (Eppendorf) no comprimento de onda de 260 nm. Posteriormente, a concentração destas amostras de DNA foi confirmada por eletroforese em gel de agarose 1% em tampão TAE 1X, corado com brometo de etídeo e visualizado em transiluminador de luz ultravioleta (UV). Para avaliação da

integridade e pureza dos DNAs, os plasmídeos foram digeridos com as enzimas de restrição utilizadas para clonagem dos fragmentos (*Eco* RV e *Xba* I) (Invitrogen), por 1 h a 37° C, em tampão indicado pelo fabricante. Os fragmentos gerados foram analisados por eletroforese em gel de agarose 1%.

3.7 – Transfecção de células de mamífero com os diferentes plasmídeos e detecção das proteínas recombinantes

Células BHK-21 foram mantidas em meio DMEM (SIGMA) adicionado de 5% SFB, a 37º C, sob atmosfera úmida e 5% de CO_2. A monocamada celular era lavada a cada 2 dias com solução salina BSS. Para passagem das células, a monocamada de células era lavada com solução salina BSS-CMF e a monocamada dissociada com solução de tripsina (Invitrogene).

A transfecção das células BHK-21 com os plasmídeos recombinantes e com o plasmídeo controle pcTPA e a detecção da expressão das proteínas recombinantes pela técnica de imunofluorescência foi realizada de forma semelhante a descrita nos materiais e métodos do artigo 3 da seção "resultados".

Para análise por imunofluorescência, 2 x 10^4 células BHK-21 foram plaqueadas em lâminas contendo 8 câmaras (Lab-Tek, NUNC), transfectadas transientemente com 0,2 µg dos plasmídeos recombinantes ou controle e 20 µl de lipofectamina (Invitrogen) em meio OPT-MEM (Invitrogene), de acordo com as recomendações do fabricante. Após 24 horas, as células foram fixadas com 4,0 % de paraformaldeido e permeabilizadas com 0,6 % de saponina. Para detecção da expressão das proteínas recombinantes, as células foram incubadas com fluido ascítico de camundongo para DENV-2 (ATCC) e com conjugado anti-IgG de camundongo marcado com fluoresceína (Southern Biotechnology). As células foram visualizadas em microscópio de fluorescência Nikon Eclipse E600.

3.8 – Imunização de camundongos BALB/c e desafio por via intracerebral

Grupos de camundongos BALB/c (n = 10) foram inoculados com duas doses de cada plasmídeo recombinante ou do plasmídeo controle em um intervalo de duas semanas, por via intramuscular (i.m.) nos quadríceps posteriores, com seringa de insulina e agulha ultrafina 30 G. Em cada dose foram inoculados 100 µg de DNA/100 µl de PBS por animal (sendo 50 µg por pata).

Quatro semanas após a primeira dose das vacinas, os animais foram sangrados por via retro-orbital para obtenção dos soros e posteriormente foram desafiados por via i.c. com 4,32 $\log_{10}$ PFU de DENV-2 NGC neuroadaptado a camundongos em 30 µL de meio 199 com sais de Earle, que corresponde a uma dose letal de 3,8 LD_{50}. O inóculo foi titulado em células Vero logo após o desafio, pela equipe do Dr. Marcos Freire (LATEV).

A morbidade (caracterizada por paralisia dos membros anteriores e/ou posteriores, e pela morte dos animais) e mortalidade foram monitoradas diariamente, durante 21 dias. Após esse período, os animais foram eutanasiados.

O ensaio de proteção cruzada com o plasmídeo pcTPANS1 foi realizado de forma semelhante. Neste caso, quatro semanas após a primeira dose de pcTPANS1, os animais foram desafiados por via i.c com DENV-1, com uma LD_{50} semelhante a utilizada nos experimentos com DENV-2, isto é, foram inoculados com 30 µL de meio contendo 3,2 LD_{50} de DENV-1 Mochizuki.

3.9 – Análise de similaridade de seqüências da proteína NS1

As seqüências de aminoácidos da proteína NS1 das linhagens DENV-2 NGC, DENV-1 Mochizuki assim como de vírus pertencentes aos outros dois sorotipos (DEN-3 e DENV-4) foram alinhadas usando o programa Clustal W (Thompson *et al.*, 1994). O grau de similaridade das seqüências foi analisado pelo programa BioEdit.

3.10 – Ensaio de lise celular mediada pelo complemento na presença de anticorpos contra NS1

Frascos de cultura T-175 cm^2 contendo uma monocamada confluente de células Vero, em meio de manutenção 199 com sais de Earle completo, foram cedidos pelo LATEV. Essas células foram lavadas com 5 mL de verseno/tripsina, dissociadas com 1 mL dessa solução e ressuspensas em 10 mL de meio 199/Earle completo. As células foram plaqueadas em placas de cultura de 96 poços, em uma densidade de 2×10^4 células/ poço/ 200 µL de meio 199/Earle completo adicionado de 5 % de $NaHCO_3$, e incubadas durante a noite a 37° C, com 5 % de CO_2.

No dia seguinte, as células Vero foram infectadas com MOI igual a 1 de DENV-2 NGC a 37° C, com 5 % de CO_2. Após 1 h de incubação, o meio foi trocado e as células

incubadas novamente em meio 199/Earle completo adicionado de 5 % de $NaHCO_3$, nas mesmas condições por 3 dias.

A avaliação da lise celular mediada pelo complemento e por anticorpos contra NS1 foi baseada em Lin *et al*. (1998). A lise celular foi dosada com o auxílio do sistema para detecção da enzima citoplasmática desidrogenase lática (LDH – do inglês *Lactate dehydrogenase*) "Cytotoxicity Detection Kit (LDH)" (Roche).

O soro obtido de camundongos BALB/c inoculados com pcTPANS1, pcENS1 ou pcDNA3 foi aquecido a 56º C por 30 min para inativação do complemento. A seguir, esse soro foi diluído em meio RPMI sem vermelho de fenol disponível comercialmente (Invitrogen), com 1 % de SFB, e incubado com diferentes diluições de complemento de cobaia (Cappel) a 37º C, por 1 h. A monocamada de células Vero infectadas foi lavada duas vezes com meio RPMI sem vermelho de fenol. Uma mistura soro e complemento (200 µL), ou somente soro ou complemento diluídos no mesmo meio, foi adicionada à monocamada em duplicatas. Após 4 horas de incubação a 37º C com 5% de CO_2, a lise celular foi quantificada pela liberação no meio extracelular de lactato desidrogenase (LDH) com "Cytotoxicity Detection Kit", de acordo com as recomendações do fabricante. Resumidamente, 100 µL do sobrenadante foram transferidos a placa de 96 poços MAXSORP (NUNC) e incubados com 100 µL de uma mistura das soluções do Kit; contendo Diaforase/NAD+ e cloreto de iodotetrazólio (INT) e lactato de sódio; a temperatura ambiente por 30 min, no escuro. Após esse tempo foram adicionados 50 µL da solução de inibição da reação (HCl 1 N) e a reação foi lida a 490 nm em espectrofotômetro. A lise máxima foi obtida pela adição de meio RPMI com 1 % de SFB contendo 1 % de Triton X-100, enquanto a lise espontânea foi determinada nos poços contendo somente células. A porcentagem de lise foi calculada segundo a fórmula: 100 x (liberação de LDH no poço testado – lise espontânea) / (lise máxima - lise espontânea).

RESULTADOS

4 – RESULTADOS

Os resultados desta tese estão divididos em duas partes:

Parte I: refere-se aos resultados obtidos com as construções contendo a proteína NS3 ou partes desta. Estes dados não foram ainda publicados na forma de artigo.

Parte II: resultados relativos às construções que codificam a proteína NS1. A maior parte dos dados obtidos a partir de experimentos com as vacinas de DNA contendo o gene NS1 foi compilada em três manuscritos (artigos 1, 2 e 3). Os ensaios de desafio por via i.c. com DENV-1, assim como dados preliminares de lise celular mediada por anticorpos direcionados contra a proteína NS1, não estão incluídos nestes artigos apresentados e também serão descritos na seção II.

PARTE I:

Resultados relativos à proteína NS3

4.I.1 - Construção dos plasmídeos recombinantes contendo a região que codifica a proteína NS3 inteira ou diferentes partes desta

As seqüências amplificadas por PCR foram clonadas no plasmídeo pcTPA, que contém o peptídeo sinal t-PA, clonado entre os sítios das enzimas de restrição *Eco* RV e *Xba* I. Foram construídos cinco plasmídeos recombinantes: pcTPANS3, contendo a região NS3 inteira; pcTPANS3P, contendo somente o domínio protease; pcTPANS3H, contendo o domínio com as atividades helicase/NTPase/RTPase; pcTPANS3N, contendo a região que codifica a porção N-terminal gerada pela clivagem espontânea da proteína NS3; e pcTPANS3C, que compreende a região que codifica a porção C-terminal também obtida após a clivagem espontânea da NS3 (Figura 4.1). Os fragmentos clonados foram seqüenciados, confirmando em todos os casos o quadro de leitura aberta em fase com a seqüência que codifica o peptídeo sinal do t-PA.

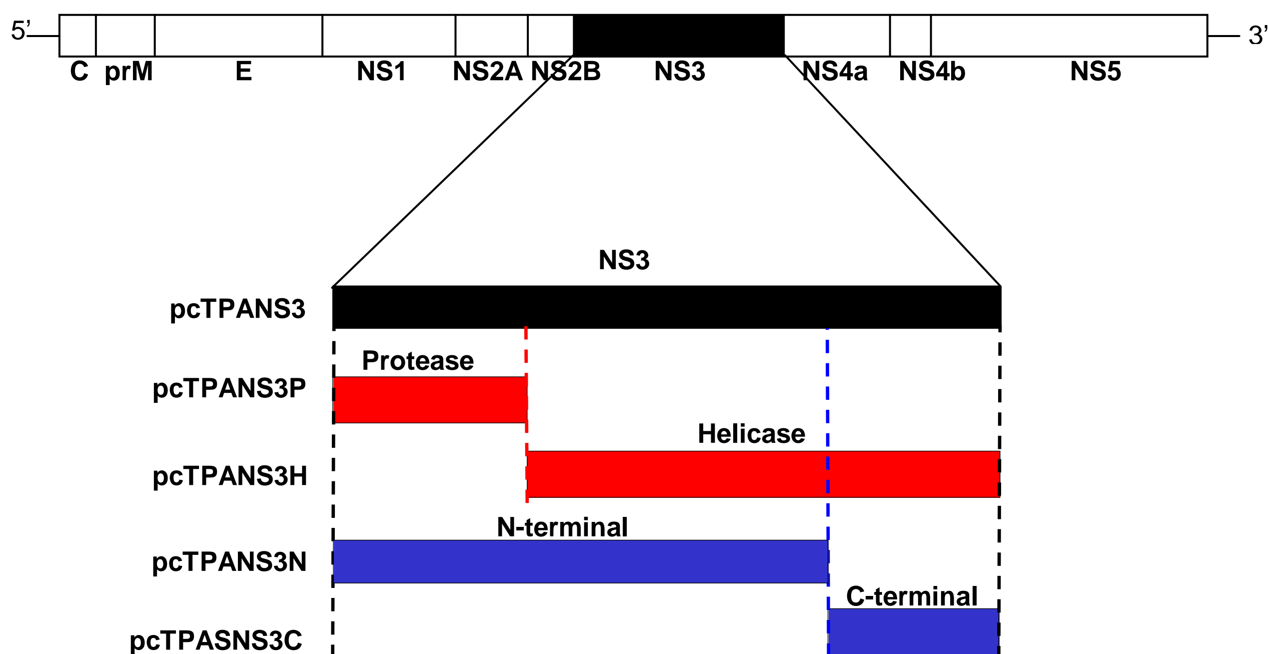

Figura 4.1: Representação esquemática dos fragmentos, contendo o gene NS3 ou seus domínios, amplificados por PCR e clonados no plasmídeo pcTPA. A clonagem destes fragmentos originou os plasmídeos recombinantes pcTPANS3, pcTPANS3H, pcTPANS3P, pcTPANS3N, pcTPANS3C.

4.I.2 - Expressão *in vitro* da proteína NS3

Células BHK-21 foram transfectadas com os plasmídeos recombinantes pcTPANS3, pcTPANS3H, pcTPANS3P, pcTPANS3N, pcTPANS3C, assim como com o plasmídeo controle pcTPA. Vinte e quatro horas após a transfecção, a proteína NS3 foi marcada com fluido ascítico contra DENV-2 e com anti-IgG de camundongo conjugado à fluoresceína. Após marcação, as células foram visualizadas e fotografadas em microscópio de fluorescência. As proteínas NS3 e seus domínios foram detectados no citoplasma das células transfectadas com todos os plasmídeos recombinantes avaliados (Figura 4.2), enquanto as células transfectadas com o plasmídeo controle, pcTPA, apresentaram resultado negativo (dado não mostrado). Estes dados indicam que todos os plasmídeos foram capazes de mediar à expressão das proteínas recombinantes em células de mamífero.

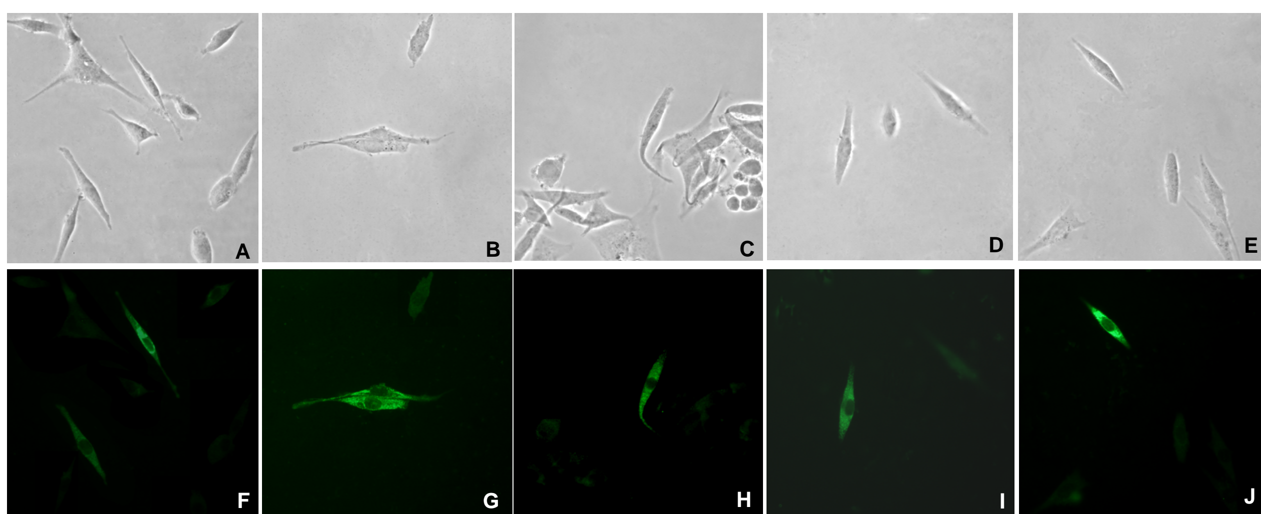

Figura 4.2: Expressão da proteína NS3 ou seus domínios em células BHK-21 transfectadas com os plasmídeos recombinantes. As células, permeabilizadas com saponina e incubadas com fluido ascítico anti-DENV-2 e anticorpo anti-IgG de camundongo conjugado à fluoresceína, foram visualizadas em microscópio de fluorescência. (F, G, H, I e J), assim como em contraste de fase (A, B, C, D e E): (A e F) pcTPANS3; (B e G) pcTPANS3N; (C e H) pcTPANS3C; (D e I) pcTPANS3P; (E e J) pcTPANS3H.

4.I.3 – Desafio de camundongos BALB/c imunizados com os plasmídeos recombinantes contendo as seqüências que codificam a proteína NS3 ou seus domínios

Antes de iniciar os experimentos de desafio intracerebral com DENV-2 NGC neuroadaptado, foi calculada a LD_{50} deste vírus em camundongos BALB/c machos com 8 semanas de idade. Foram realizados 2 experimentos em que o vírus foi diluído de 10^0 a 10^4 vezes e a mortalidade dos animais acompanhada durante 21 dias. Foi estabelecida a utilização de 30 μL da amostra viral sem diluição (10^0) que equivale a 4,32 $\log_{10}$ PFU de DENV-2 e a dose de 3,8 LD_{50}.

Para avaliar a resposta protetora gerada pela inoculação dos diferentes plasmídeos recombinantes, camundongos BALB/c foram imunizados pela via i.m., com duas doses de 100 μg de cada plasmídeo em intervalo de duas semanas, e desafiados quatro semanas após a primeira dose.

Conforme apresentado na Tabela 3, a inoculação com os plasmídeos pcTPANS3, pcTPANS3H, pcTPANS3N, pcTPANS3C gerou níveis muito variados de proteção, enquanto que o pcTPANS3P não induziu uma resposta protetora contra o desafio com DENV-2. Os animais imunizados com os plasmídeos pcTPANS3C e pcTPANS3N apresentaram os maiores índices de sobrevivência (90 % e 80 %, respectivamente). Entretanto, parte dos camundongos deste grupo que sobreviveu ao desafio apresentou fortes sinais de morbidade, com taxas de 60 % e 40 % nos animais inoculados com o pcTPANS3C e o pcTPANS3N, respectivamente.

Ao contrário do esperado, a imunização com o plasmídeo recombinante pcTPANS3, que contém a região NS3 inteira, gerou níveis muito baixos de proteção, com 70 % dos animais apresentando algum sinal clínico e apenas 40 % sobrevivendo ao desafio.

Plasmídeo	% de Morbidade	% de Sobrevivência
-	80	20
pcTPA	80	20
pcTPANS3	70	40
pcTPANS3P	80	20
pcTPANS3H	40	60
pcTPANS3N	40	80
pcTPANS3C	60	90

Tabela 4.1: Porcentagem de sobrevivência e morbidade em camundongos BALB/c imunizados com os plasmídeos pcTPA, pcTPANS3, pcTPANS3C, pcTPANS3N, pcTPANS3P e pcTPANS3H, e desafiados por via i.c. com DENV-2 NGC. Grupos de animais (n = 10) foram inoculados por via intramuscular com duas doses de cada plasmídeo e desafiados 4 semanas após a primeira dose. O grupo controle não imunizado foi desafiado no mesmo dia que os animais inoculados com os diferentes DNAs.

PARTE II

Resultados relativos à proteína NS1:

- **ARTIGO 1:**

Protection against dengue type 2 virus induced in mice immunized with a DNA plasmid encoding the non-structural 1 (NS1) gene fused to the tissue plasminogen activator signal sequence.
Costa, S.M., Paes, M.V., Barreto, D.F., Pinhão, A.T., Barth, O.M., Queiroz, J.L., Armôa, G.R., Freire, M.S., Alves, A.M.
Vaccine (2006) 24(2):195-205.

- **ARTIGO 2:**

DNA vaccine against the non-structural 1 protein (NS1) of dengue 2 virus.
Costa, S.M., Freire, M.S., Alves, A.M.
Vaccine (2006) 24(21):4562-4564.

- **ARTIGO 3:**

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.
Costa, S.M., Azevedo, A.S., Paes, M.V., Sarges, F.S., Freire, M.S., Alves, A.M.
Virology (2007) 358(2):413-423.

- Avaliação da proteção inter-específica através do desafio por via i.c. com DENV-1

- Avaliação de lise de células infectadas com DENV-2 mediada pelo complemento e anticorpos contra a proteína NS1

Abaixo (tabela 4.2) estão descritas as características dos plasmídeos pcTPANS1, pcTPANS1ANC, pcENS1 e pcENS1ANC, que foram utilizados para avaliar as vacinas de DNA contendo o gene NS1.

Plasmídeo original	região amplificada	Plasmídeo recombinante
pcTPA	NS1	**pcTPA**
pcDNA3	sequência que codifica a região C-terminal da proteína E + NS1	**pcENS1**
pcTPA	NS1 + seqüência que codifica a porção N-terminal da proteína NS2A (ANC)	**pcTPANS1ANC**
pcDNA3	sequência que codifica a região C-terminal da proteína E + NS1 + seqüência que codifica a porção N-terminal da proteína NS2A (ANC)	**pcENS1ANC**

Tabela 4.2: Construções dos plasmídeos recombinantes contendo o gene NS1. A primeira coluna mostra os plasmídeo originais, onde o fragmento foi clonado. Na segunda coluna estão descritas as regiões amplificadas por PCR. A última coluna indica os plasmídeos recombinantes construídos.

4.II.1 – Resumo dos resultados dos artigos 1 e 2

Os artigos 1 e 2 desta tese abordam a construção da vacina de DNA pcTPANS1, a avaliação da expressão *in vitro* da proteína NS1, e imunizações de camundongos BALB/c com esta construção.

O plasmídeo pcTPANS1 contém a seqüência que codifica a proteína NS1 de DENV-2 NGC fusionada à seqüência sinal t-PA. Células BHK-21 transfectadas com este plasmídeo foram capazes de expressar a proteína NS1, detectada tanto no interior destas células quanto no sobrenadante das culturas (artigo 1). Posteriormente, foi realizada uma análise mais detalhada da expressão de NS1 mediada pelo pcTPANS1, assim como pelos outros plasmídeos contendo o gene NS1, que será abordada no resumo do artigo 3.

Após a análise da expressão *in vitro* da proteína NS1, camundongos BALB/c foram imunizados com o plasmídeo pcTPANS1 e a resposta imune humoral gerada contra NS1 foi avaliada. Foram realizados diferentes esquemas de inoculação com o plasmídeo pcTPANS1 a fim de se estabelecer um protocolo padrão de imunização das vacinas de DNA. Grupos de camundongos BALB/c (n=10) foram imunizados por via i.m. ou por via intradérmica (i.d.), com 100 µg do plasmídeo pcTPANS1 ou do plasmídeo controle. Nas imunizações por via i.m. foram administradas 2 ou 3 doses de DNA, enquanto que os animais imunizados por via i.d. foram inoculados com 3 doses de DNA, sempre com um intervalo de 2 semanas. Todos os animais, independente da via de inoculação, apresentaram anticorpos específicos contra NS1, detectados por ELISA utilizando, como antígeno de fase sólida NS1, expressa em células de drosófila (Artigos 1 e 2). Entretanto, a da cinética da resposta humoral se mostrou diferente nos três esquemas de imunização. As imunizações por via i.m. induziram níveis mais elevados de anticorpos anti-NS1, sendo os títulos mais altos detectados nos animais inoculados com 3 doses de DNA, com pico da resposta 6 semanas após a primeira imunização. Contudo, foi observada uma diminuição drástica nos níveis destes anticorpos nas semanas subseqüentes, atingindo títulos semelhantes aos detectados com a imunização com duas doses por via i.m., mantendo-se constantes até a 10ª semana (artigo 1). Baseado nestes resultados, o protocolo adotado para as imunizações seguintes foi: 2 doses de DNA por via i.m., com intervalo de duas semanas. Além disso, 56 semanas após a primeira dose de DNA os animais ainda apresentavam níveis detectáveis de anticorpos anti-NS1, e quando foram inoculados com uma dose reforço da vacina mostraram um rápido e significativo aumento da resposta humoral (artigo 1). Tais resultados demonstram a indução de uma resposta imune de longa duração e de memória.

A seguir foram realizados testes de desafio com DENV-2 dos camundongos vacinados com o pcTPANS1, em dois modelos murinos distintos. No primeiro teste, os animais foram desafiados por via i.p. com DENV-2 RJ isolado de um paciente no Rio de Janeiro e não adaptado a camundongos. Neste modelo, os animais não apresentam sinais clínicos aparentes da doença, entretanto a análise de alguns órgãos destes camundongos revelou danos histológicos específicos (anexos 2 e 3). O segundo modelo de desafio se baseia na inoculação por via i.c. de DENV-2 NGC neuroadapatado em camundongos neonatos. Neste modelo, os animais apresentam morbidade, principalmente paralisia dos membros posteriores, podendo culminar em morte.

Os camudongos (n=4) não imunizados ou inoculados com o pcTPANS1 ou pcTPA, foram desafiados com DENV-2 por via i.p. 4 semanas após a primeira dose de DNA e eutanasiados 3 dias após o desafio. A análise histológica do fígado dos animais controles (não imunizados ou inoculados com o pcTPA) demonstrou danos focais como esteatose, tumefação hepatócitária, hemorragia e edema. Por outro lado, nos animais vacinados com o pcTPANS1 tais danos se mostraram reduzidos ou ausentes (artigo 1). Tais análises foram realizadas tanto por microscopia óptica ou microscopia eletrônica.

De modo semelhante, os animais vacinados (n=20) com o pcTPANS1 e desafiados com DENV-2 por via i.c. se mostraram protegidos. Todos os animais imunizados com o pcTPANS1 sobreviveram ao desafio, sendo que apenas 2 animais (10%) apresentaram leve paralisia em uma das patas, enquanto que os animais inoculados com o plasmídeo controle apresentaram 95% de morbidade e apenas 45% de sobrevivência (artigos 1 e 2). Os animais não imunizados que foram inoculados com DENV-2 apresentaram 95% de morbidade e aproximadamente 30% de sobrevivência (artigos 1 e 2).

Os níveis de anticorpos anti-NS1 destes animais foram avaliados antes e depois dos desafios. Os camundongos desafiados por via i.p. não apresentaram um aumento significativo da resposta de anticorpos. Por outro lado, um drástico aumento da resposta humoral foi observado após o desafio por via i.c. (artigo 1).

ARTIGO 1

Protection against dengue type 2 virus induced in mice immunized with a DNA plasmid encoding the non-structural 1 (NS1) gene fused to the tissue plasminogen activator signal sequence.

Costa, S.M., Paes, M.V., Barreto, D.F., Pinhão, A.T., Barth, O.M., Queiroz, J.L., Armôa, G.R., Freire, M.S., Alves, A.M.

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Protection against dengue type 2 virus induced in mice immunized with a DNA plasmid encoding the non-structural 1 (NS1) gene fused to the tissue plasminogen activator signal sequence

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Abstract

Dengue is one of the most important arboviral diseases in humans, and although efforts over the last decades have dealt with the development of a vaccine, this vaccine is not available yet. In order to evaluate the potential of a DNA vaccine based on the non-structural 1 (NS1) protein against dengue virus (DENV), we constructed the pcTPANS1 plasmid which contains the secretory signal sequence derived from human tissue plasminogen activator (t-PA) fused to the full length of the DENV-2 NS1 gene. Results indicate that pcTPANS1 promotes correct expression of NS1 in eukaryotic cells and drives secretion of the recombinant protein to the surrounding medium in a dimeric form. Balb/c mice, intramuscularly inoculated with this plasmid, presented high levels of antibodies, recognizing mainly surface-exposed conformational epitopes present in the NS1 protein expressed by insect cells. Long-term antibody response was observed in animals 56 weeks after the first plasmid inoculation, and a rapid, efficient secondary response was observed after a DNA boost. Vaccinated animals were challenged against DENV-2 in two murine models, based on intracerebral (i.c.) and intraperitoneal (i.p.) virus inoculations, and in both cases, pcTPANS1-immunized mice were protected. Overall, these results provide further support for the use of such a plasmid in a possible approach for the development of a vaccine against DENV.
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Keywords: DNA vaccine; Dengue virus; NS1

1. Introduction

Dengue viruses (DENV) belong to the *Flaviviridae* family and consist of four distinct antigenic types (DENV-1 to 4), transmitted primarily by the *Aedes aegypti*. Infection by these viruses is a global public health problem, with recurring epidemics in tropical and subtropical regions of the world [1]. The resulting disease is mostly asymptomatic or a mild self-limiting acute febrile illness, dengue fever (DF). However, it can also produce a life threatening severe illness, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), with minor or major bleeding from different sites [1]. It has been estimated that between 50,000,000 and 100,000,000 cases of DF occur annually, of which about 250,000 and 500,000 cases lead to DHF [2]. The pathogenesis of DHF is not well understood, but a remarkable feature is that most cases of DHF occur in individuals experiencing a secondary infection with a heterologous serotype, which may be associated with the phenomenon of antibody dependent enhancement (ADE) of the infection [3,4]. Evidence suggests that ADE is caused by non-neutralizing antibodies

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reacting with the virus envelope protein (E), although this hypothesis is mainly supported by in vitro experiments and has not been proved in vivo [3]. Regardless whether or not the hypothesis is correct, the most important risk factor for DHF is the increased incidence of dengue infection.

The control of the DENV vector has proven to be difficult and costly to sustain over time [5]. Consequently, the World Health Organization has prioritized the development of a DENV vaccine [6]. Although considerable research has been done, no effective vaccine against DENV is yet available. Traditional immunization approaches, such as virus inactivation and the use of live attenuated viruses or subunit vaccines, which have yielded successful vaccines against other flaviviruses, revealed to be less effective against DENV [7]. Some evidence points to DENV structural prM, M, E, and non-structural 1 (NS1) and 3 (NS3) proteins as antigens that may elicit a protective immune response [8]. Apparently, immunization with the NS1 protein can confer protection without risks of the induction of anti-virion antibody-mediated ADE [9,10].

DNA-based vaccines have been shown to induce long-lasting cellular and humoral immunity against several pathogens including flaviviruses [11–18]. One of the potential advantages of applying these vaccines to viral diseases is the expression of viral proteins in situ after DNA immunization, leading to proper folding, which includes the presence of posttranslational modifications such as glycosylations that occur during the course of an infection [12]. Based on this evidence, in the present work we construct the pcTPANS1 plasmid, which contains the secretory signal sequence derived from human tissue plasminogen activator (t-PA) fused to the full length of the DENV-2 NS1 gene. Balb/c mice were immunized with this plasmid and the antibody response elicited, as well as the capacity to confer protection in animals challenged with dengue virus, was analyzed. Results demonstrated that inoculation with pcTPANS1 induced high levels of antibodies, which mainly recognize surface-exposed conformational epitopes present in the NS1 protein expressed in insect cells. Furthermore, immunization with such a plasmid was able to induce protection in two murine challenge models, based on intracerebral and intraperitoneal virus inoculations.

2. Materials and methods

2.1. Viruses and cell lines

The dengue 2 virus (DENV-2) strain New Guinea C (NGC DENV-2) was used for cloning the NS1 gene and for challenges with intracerebral virus inoculations. Another DENV-2 (provided by Dr. R. Nogueira, Department of Virology, Fiocruz, RJ, Brazil) and isolated from a patient in Rio de Janeiro, Brazil [19] (RJ DENV-2), was used in an intraperitoneal virus challenge model. NGC DENV-2 and RJ DENV-2 propagations were carried out in Vero cells, in medium 199 with Earle salts (E199) buffered with sodium bicarbonate (Sigma, USA) and in C6/36 cells of *Aedes albopictus* in L15 medium (Sigma), respectively, both supplemented with 10% fetal bovine serum (FBS). In vitro expression of the NS1 recombinant protein was detected in baby hamster kidney cells (BHK-21) in Optimem medium (Invitrogen, Life Technologies, USA).

2.2. Plasmid construction

The dengue NS1 gene was amplified by PCR and cloned in the pcTPA mammalian expression plasmid, a modified pcDNA3 vector (Invitrogen), which contains the human tissue plasminogen activator signal sequence. For the NS1 gene amplification, total RNA from cells infected with DENV-2 was extracted with Trizol (Invitrogen), according to the manufacturer protocol, and the RNA was used as template for the synthesis of a cDNA carried out with the antisense primer 5′-CAT AAG CTT ACA GAG GTT CCC CCA TG-3′, which hybridize to nucleotides 1422–1438 in the NS3 gene. Such cDNA was then used for amplification of the NS1 gene sequence with two primers, sense 5′-GGG GGA TAT CGA TAG TGG TTG CGT TG-3′ and antisense 5′-GGG GCT CGA GTT AGG CTG TGA CCA AG-3′, containing restriction sites for *Eco*RV and *Xho*I, respectively. The amplified products were electrophoresed on a 1% agarose gel, recovered with glass beads, geneclean (Stratagene, La Jolla, USA), restricted with *Eco*RV and *Xho*I, and ligated to pcTPA previously digested with the same enzymes. Recombinants were screened by restriction mapping and sequencing by ABI PRISM dye terminator cycle sequencing core kit (Applied Biosystems, USA). The recombinant plasmid named pcTPANS1 contains the NS1 gene in frame with the t-PA signal sequence, which targets secretion of the encoded protein to extracellular space. Large-scale preparations of pcTPA and pcTPANS1 plasmids were produced on transformed *Escherichia coli* DH5α, grown in ampicilin-containing LB medium, and purification were conducted by alkaline lysis and Qiagen Plasmid Giga Kit (Qiagen, Germany), according to manufacture'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.

2.3. In vitro expression of NS1 in mammalian cells

BHK-21 cells were transiently transfected with either pcTPANS1 or pcTPA. Briefly, cells (5×10^5) were plated in 25 cm^2 bottles with Optimen medium (Invitrogen) and transfected with 2 μg of DNA using lipofectamine (Invitrogen) under conditions suggested by the manufacturer. Whole-cell extracts and culture supernatants, concentrated with Centricon YM-3 (Millipore Corporation, USA) were harvested 24 h following transfection, suspended in SDS-PAGE sample buffer [20] and boiled for 5 min. For detection of monomeric and dimeric forms of NS1, samples were

suspended in phosphate buffer saline (PBS), pH 7.2 with aprotinin (4 μg/mL), leupeptin (4 μg/mL), phenylmethanesulfonyl fluoride (0.1 mg/mL) without 2-mercaptoethanol, submitted or not to heat-treatment (boiled for 5 min). Proteins were sorted in SDS-PAGE (12.5% total acrylamide concentration) followed by Western blotting. Nitrocellulose membranes were blocked overnight at 4 °C with 1% BSA in 0.05% Tween 20-PBS (PBST), washed in PBST and incubated for 1 h at room temperature with DENV-2 hyperimmune mouse ascitic fluid (ATCC, Washington, USA) at a dilution of 1:1000 in PBST. Membranes were then washed, incubated for 1 h at room temperature with rabbit anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Southern Biotechnology, USA) diluted 1:4000 in PBST and washed again with PBST. Membranes were developed with the ECL kit (Amersham Biosciences, Buckinghamshire, UK) and exposed to Kodak X Omat films for 1–2 min.

2.4. *Immunization procedure*

All 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. Four- to six-week-old male Balb/c mice were immunized either by intramuscular (i.m.) or intradermal (i.d.) injection, using 27-gauge needles. In i.m. immunizations, tibialis posterior muscles of each limb were injected with 50 μg of plasmid dissolved in 50 μL of PBS (100 μg/mice), and in i.d. inoculations 100 μg of DNA (in 100 μL of PBS) were injected in the base of the tail. For the evaluation of NS1-specific antibody response kinetics, animals (six mice in each group) were immunized with two or three DNA doses given 2 weeks apart and bled by retro-orbital puncture, before inoculation (preimmune sera) and at various time intervals after immunization. For challenge experiments, mice were immunized by i.m. route with two DNA doses given 2 weeks apart and bled 4 weeks after the first DNA dose and at the end of experiments, when animals were sacrificed. Initially, serum samples were individually tested for reactivity against the NS1 protein and then pooled and stored at −20 °C for subsequent analyses.

2.5. *Detection of NS1-specific antibodies by ELISA*

Enzyme-linked immunosorbent assay (ELISA) was carried out with heat-denatured (boiled for 5 min) or intact recombinant DENV-2 NS1 protein as solid-phase bound antigen, expressed either in insect cells or in *E. coli*. The former recombinant NS1 protein was expressed in *Drosophila melanogaster* strain Schneider (S2) cells [21] and purified by immunoaffinity chromatography (Hawaii Biotechnology Group Inc., USA). For expression of the recombinant protein in bacteria cells, the NS1 gene from DENV-2 strain NGC was cloned into the pET23b vector (Novagen, USA), which was used for transforming the *E. coli* strain BL21 and the protein was purified by Ni-NTA column (Qiagen) as described elsewhere [22]. Wells of MaxiSorp plates (Nunc, Denmark) were coated at 37 °C for 1 h with 0.1 μg of the NS1 protein in 100 μL PBS and blocked overnight at 4 °C with 2% skim milk in PBST. After multiple washes in PBST, serial diluted sera were added to wells, followed by incubation for 1 h at 37 °C, subsequent washing and incubation with goat anti-mouse IgG conjugated with horseradish peroxidase (Southern Biotechnology) for 1 h at 37 °C and washed again with PBST. Reactions were visualized, after 20 min at room temperature, with ortho-phenylenediamine dihydrochloride (Sigma) and H_2O_2 as substrate and with a 9N H_2SO_4 stopping solution, and measured at a 450 nm. Titers were established as the reciprocal of serum dilution, which gave an absorbance above that of the respective preimmune serum.

2.6. *DENV-2 challenge in mice*

Two weeks after the second DNA dose, animals i.m. immunized as described above were tested in two challenge mouse models, by i.p. or i.c. DENV inoculations. For challenge with i.p. inoculation, it was used 100 $TCID_{50}$/0.2 mL of RJ DENV-2, which has not undergone any passage in mouse brain. Mice were separated in four groups ($n = 4$): pcTPANS1-immunized animals challenged with DENV-2; pcTPA-inoculated animals challenged with DENV-2; non-immunized control group challenged with DENV-2; and another mouse group not inoculated with either DNA or DENV-2. Three days after challenge, animals were anesthetized i.p. with 4% chloral hydrate (0.4 mL/25 g of animal) and liver fragments were collected as well as serum samples. For observations in light microscopy, liver tissue fragments were fixed by immersion in Millonig's fixative, dehydrated with ethanol and paraffin-embedded. Sections 5 μm thick were stained with haematoxilyn and eosin (HE). For electron microscopy, fragments were immersed in glutaraldehyde in cacodylate buffer (0.2 M, pH 7.2), dehydrated in acetone, post-fixed with 1% buffered osmium tetroxide, embedded in epoxy resin and polymerized at 60 °C during 3 days. Ultra-thin sections, 60 nm thick, were obtained using a diamond knife adapted to a Reichert–Jung Ultracut E microtome. Sections were stained with uranyl acetate and lead citrate [23] and observed in a Zeiss EM-900 electron microscope. For i.c. challenge, each mouse was inoculated with 4.32 $\log_{10}$ PFU (5.84 $\log_{10}$ PFU/mL) of a mouse brain-adapted virus, the NGC DENV-2, in 30 μL of E199 medium supplemented with 5% of FCS. It was used the sample stock of NGC DENV-2, which was not diluted and corresponded to 3.8 LD_{50} in adult Balb/c mice, that are naturally less susceptible to i.c. DENV challenge as compared to newborn mice. Inoculum was back-titered in Vero cells as described previously [24], immediately after the challenge procedure. Before virus injection, animals were anesthetized with a mixture of ketamine-xylazine [25]. Mice were separated in three groups ($n = 20$): pcTPANS1- and pcTPA-immunized mice and non-immunized animals, all challenged with the same virus sample. Animals were monitored for 21 days and

morbidity, mainly the appearance of hind leg paralysis, and deaths were recorded. Moribund animals were euthanized by placing them in a lethal chamber containing carbon dioxide. After 21 days, survived animals were sacrificed and blood samples were collected.

3. Results

3.1. In vitro expression of the pcTPANS1-encoded protein

The NS1 gene from NGC strain of DENV-2 was cloned into the pcTPA vector to make the pcTPANS1 plasmid vaccine. Expression of the DENV-2 NS1 protein was evaluated by Western blot with cell lysates and culture supernatants of transient transfected BHK-21 cells. A protein of approximately 48 kDa was detected in pcTPANS1-transfected cell lysates and culture supernatants while cells transfected with pcTPA alone did not present any corresponding reaction (Fig. 1a), confirming that the 48 kDa protein detected in pcTPANS1-transfected cells is, in fact, the NS1 protein. Moreover, these results indicate that the pcTPANS1 promotes the secretion of most part of the NS1 protein to extracellular medium due to the t-PA signal peptide. In order to evaluate whether the NS1 protein secreted by pcTPANS1-transfected cells was in monomeric or dimeric form, samples collected from culture supernatants, suspended in non-reducing conditions and submitted or not to heat-treatment, were analyzed in Western blot and compared to the NS1 protein expressed in insect cells. The predominance of an approximately 96 kDa protein was observed in non-denatured NS1 expressed in BHK-21 cells while a 48 kDa protein was mainly detected when these sample were heat-submitted (Fig. 1b). Such results indicate that pcTPANS1-transfected cells secrete mainly dimeric forms of NS1 and suggest that the protein is glycosylated, since stability of dimmer seems to be influenced by addition of sugar groups to this protein. Slight size differences were observed between the NS1 protein expressed in insect and BHK-21 cells (Fig. 1b), which may be due to differences in glycosylation patterns.

3.2. Time course of the humoral immune response in pcTPANS1-immunized mice

All Balb/c mice immunized with pcTPANS1 were tested individually, 6 weeks after the first DNA injection, and presented a significant humoral immune response against the NS1 protein expressed in insect cells. At this time point, individual NS1-specific antibody titers ranged from 6000 to 20,000 in mice i.d. inoculated and from 21,000 to 32,000 or from 62,000 to 108,000 in animals i.m. immunized with two or three doses, respectively (data not shown). Time course of serum NS1-specific IgG response were then analyzed in pooled samples. Animals i.m. immunized presented higher levels of NS1-specific antibodies when compared to i.d. inoculations in all tested time (Fig. 2). Two weeks after the first immunization, all mice presented NS1 antibody response although in lower levels (mean of 5000). The response increased significantly after the second DNA dose and peak level of NS1-specific antibodies was observed 6 weeks after the first immunization in animals receiving three doses of the pcTPANS1 (titers of 92,000 for i.m. and of 17,000 for i.d. inoculations), while mice i.m. inoculated with two DNA doses showed higher antibody titers 4 weeks after the first immunization (titer of 33,000) (Fig. 2). Although mice i.m. immunized with three plasmid doses presented higher antibody titers in the sixth week, these levels decreased significantly 2 weeks later, attaining titers similar to those observed in animals receiving two DNA doses, in which

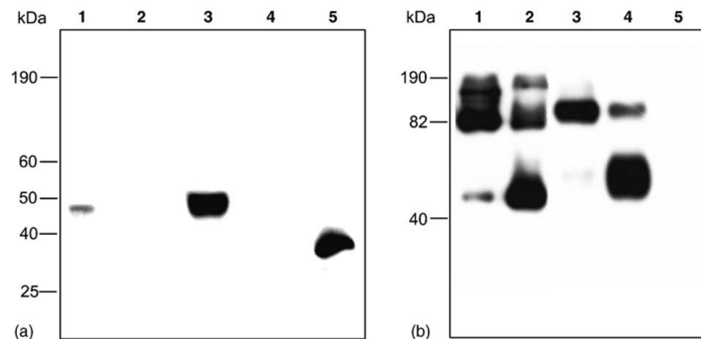

Fig. 1. pcTPANS1-driven production of the recombinant NS1 protein detected in SDS-PAGE and Western blots with DENV-2 hyperimmune mouse ascitic fluid. Samples were suspended in SDS-PAGE buffer with heat-treatment (a) or in phosphate buffer with protease inhibitors without 2-mercaptoethanol, submitted or not to heat-treatment (b). (a) Whole-cell extract (lanes 1 and 2) and culture supernatants (lanes 3 and 4) harvested from BHK-21 cells transfected with pcTPANS1 (lanes 1 and 3) or pcTPA (lanes 2 and 4) and control NS1 protein expressed in *E. coli* (lane 5). (b) Purified NS1 protein expressed in insect cells (lanes 1 and 2) and culture supernatants harvested from BHK-21 cells transfected with pcTPANS1 (lanes 3 and 4) or with pcTPA (lane 5) not submitted to heat-treatment (lanes 1, 3 and 5) or boiled for 5 min (lanes 2 and 4).

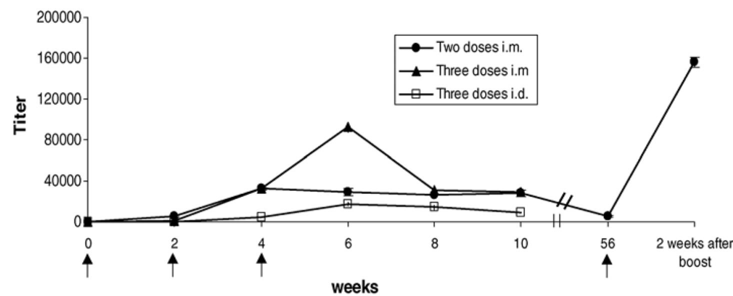

Fig. 2. Time course of serum IgG response to NS1 protein from Balb/c mice immunized with pcTPANS1. Each animal group (n = 6) was injected i.d. or i.m. with two or three doses of DNA (100 μg each). Samples were taken at indicated times and titers of NS1-specific antibodies were determined in ELISA with purified 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 in each group. Arrows indicate time point of DNA inoculations. Bars represent standard deviations of the mean of duplicates.

NS1-specific antibody levels remained stable from 4 until 10 weeks after the first inoculation (titers around 30,000). Consequently, all the following experiments were performed with i.m. immunization of Balb/c mice with two DNA doses. Long-term humoral response was also analyzed in mice i.m. immunized, which still showed specific-NS1 antibodies 56 weeks after the first plasmid inoculation (titers of 5000). Moreover, a significant increase in specific-NS1 antibody levels were observed after a booster DNA dose, given more than one year after the beginning of immunizations, with titer of 156,000 (Fig. 2), which were comparable to those detected in hyperimmune antisera (titer of 298,000) (data not shown). Epitope specificity of pcTPANS1-induced antibodies was analyzed in ELISA using the NS1 protein expressed either in insect cells, in the heat-denatured and intact forms, or in *E. coli* as solid-phase bound antigen. Antibodies recognized mainly the intact form of NS1 expressed in insect cells (Fig. 3). The reactivity of pcTPANS1-elicited antibodies was significantly reduced when the heat-denatured protein was used (approximately 30-fold lower when compared to the non-denatured NS1 during peak antibody level) and no reaction was observed when a NS1 protein expressed in *E. coli* was used in ELISA plates (Fig. 3). These results suggest that pcTPANS1-induced antibodies predominantly recognized conformational surface-exposed epitopes and that such epitopes were absent in the protein expressed in bacteria cells. Sera from animals inoculated with control vector, pcTPA, or preimmune sera did not react with either the NS1 protein forms (data not shown).

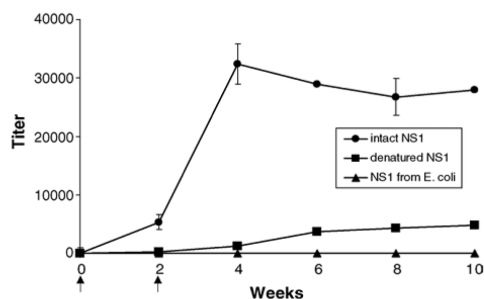

Fig. 3. Time course of serum IgG response to NS1 protein from Balb/c mice (n = 6) i.m. immunized with two doses of the pcTPANS1 (100 μg each). Titers of NS1-specific antibodies were determined in ELISA with purified non-denatured or heat-denatured NS1 protein expressed in insect cells or the NS1 protein expressed in *E. coli* 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. Bars represent standard deviations of the mean of duplicates.

3.3. Immunization with the pcTPANS1 protects mice against a subsequent challenge with DENV-2

To assess the efficacy of pcTPANS1 immunization against dengue virus infection, groups of mice were i.m. inoculated with this plasmid or with control vector and challenged with DENV-2 two weeks after the second DNA dose, when peak NS1-specific antibody levels were identified. Two different challenge mouse models were used with i.p. and i.c. virus inoculations. In the first challenge model, it was used the RJ DENV-2 sample, which is a non-mouse adapted virus isolated from a human patient and therefore, as expected, i.p. inoculation with this virus did not lead to mortality in mice. However, tissue injuries were evident in fragments collected from livers of non-immunized or control vector-inoculated animals. In non-immunized and non-challenged mice, liver tissues presented a regular structured hepatic parenchyma around a medzonal area (Zone II) (Fig. 4a), observed in light microscopy. In contrast, 72 h post RJ DENV-2 infection, focal zones of hepatic injury were observed either in non-immunized or pcTPA-inoculated animals (Fig. 4b and d). Micro and macro vacuolization in sinusoidal pole and tumefaction of hepatocytes were detected around a medzonal area (Zone II) (Fig. 4b) and in liver portal space (Fig. 4d). Vacuolization was also noted in endothelium of central lobular

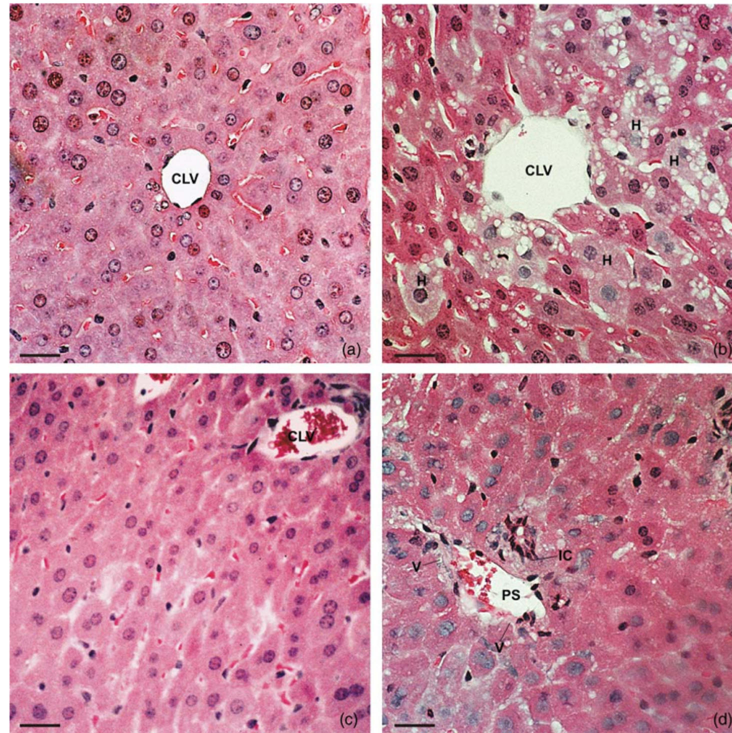

Fig. 4. Histopathological observation of liver tissue from Balb/c mice challenged with i.p. inoculation of RJ DENV-2 (100TCID$_{50}$) and HE stained. Each mouse group ($n = 4$) was i.m. injected with two DNA doses (100 μg each), challenged 4 weeks after the first immunization and sacrificed 3 days after virus infection. (a) Non-infected mouse liver tissue; bar = 33 μm. (b) Liver tissue from pcTPA-inoculated mouse challenged with DENV-2. Note tumefaction and vacuolization of hepatocytes placed around the medzonal area; bar = 23 μm. (c) Normal aspect of hepatic tissue of mouse immunized with the pcTPANS1 and challenged with DENV-2; bar = 23 μm. (d) Mouse liver tissue infected with RJ DENV-2 and sacrificed 72 h post-infection. Note tumefation of hepatocytes around the portal space, inflammatory cells around the biliar duct and vacuolization in endothelial portal cells, bar = 15 μm. CLV: central lobular vein; H: hepatocyte; PS: portal space; V: vacuolization; IC: inflammatory cells.

vein as well as sinusoid portal (Fig. 4b and d). Mice vaccinated with the pcTPANS1, on the other hand, presented a significant decrease of such a damage, either in the number of hepatic injury focus or in magnitude of tissue damages. Analysis in light microscopy of pcTPANS1-vaccinated mice liver showed normal structured parenchyma, hepatocytes and sinusoid capillars in most of the studied area (Fig. 4c). Injury in liver caused by RJ DENV-2 inoculation was more evident in electron micrograph. Analysis in non-infected mice revealed hepatocytes with numerous mitochondria, glycogen, rough and smooth endoplasmic reticulum (Fig. 5a) and sinusoidal borders presented short finger-like cytoplasmic extensions (Fig. 5b). Hepatocyte in DENV-2 infected mice presented cytoplasm rarefaction causing alterations in organels, such as mitochondrial swelling and dilatation in rough endoplasmic reticulum (Fig. 5c). Damages were also noted in sinusoidal wall with endothelial capillar break (Fig. 5d). In pcTPANS1-immunized animals, normal hepatocytes and sinusoid capillars with preserved endothelium were observed (Fig. 5e and f).

Mice challenged by i.c. inoculation with the mouse brain-adapted NGC DENV-2 was monitored the following 21 days. This challenge model is a well-established mice model for vaccine tests against DENV, in which morbidity, regarding mainly the development of hind leg paralysis, and mortality is recorded. In the non-immunized animal group, the first paralyzed mice were detected 8 days after virus inoculation and on the 13th day 95% of mice presented paralysis or dyed (Fig. 6a). In the pcTPA-inoculated mouse group morbidity was observed 7 days after virus challenge and on the 13th day also 95% of animals were paralyzed or dead (Fig. 6a). In contrast, in pcTPANS1-immunized animal group only two mice (10% of animals) presented paralysis. One mouse presented a temporary paralysis from the 10th to the 13th day and the other animal was permanent paralyzed from the 12th day (Fig. 6a). Morbidity rate differences observed between

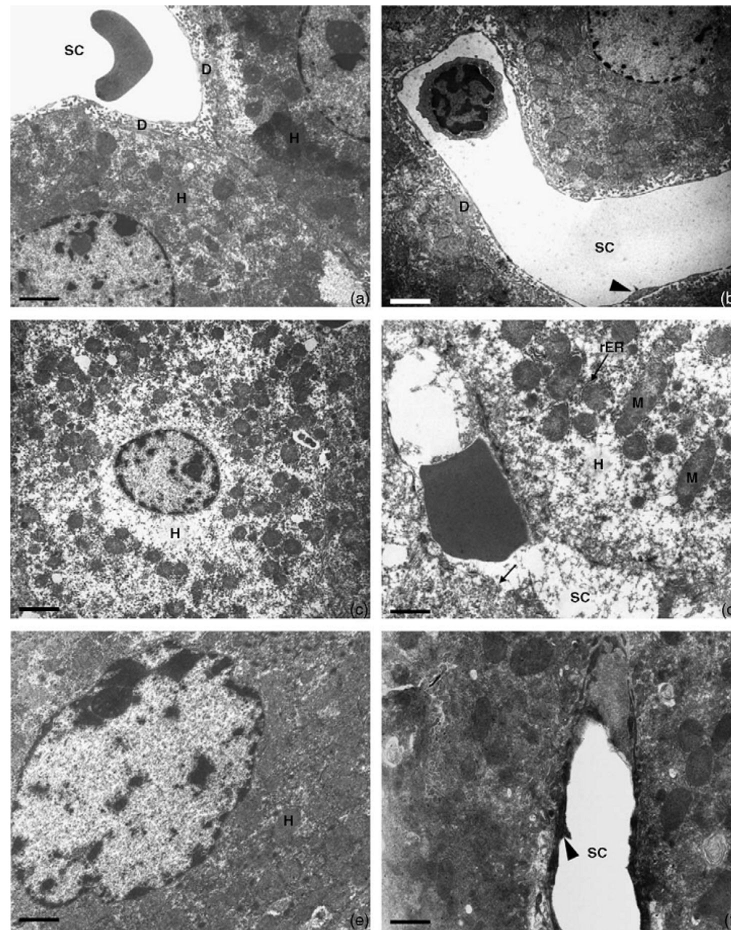

Fig. 5. Ultrathin section analysis of liver tissue from Balb/c mice challenged with i.p inoculation of RJ DENV-2 (100TCID$_{50}$). Mice were treated as described in Fig. 4. (a) and (b) Non-infected liver hepatocytes showing dense cytoplasm, normal aspect of cell organelles and regular structured sinusoidal border with fenestrated endothelium (arrow head); bar = 2.5 μm. (c and d) Hepatocytes of RJ DENV-2 infected mice. Note general tumefaction, cytoplasm rarefaction, alteration of cell organelles, such as mitochondrial swelling, dilatation of rough endoplasmic reticulum and capillar endothelial break (arrow); bar = 2.5 μm (c) and 1.2 μm (d). (e and f) Normal aspect of hepatocytes and sinusoid capillar with fenestrated endothelium (arrow head) of mice immunized with the pcTPANS1 and challenged with RJ DENV-2; bar = 1.4 μm (e) and 1.0 μm (f). H: hepatocyte; D: Disse's space; SC: sinusoid capillar; M: mitochondria; rER: rough endoplasmic reticulum.

pcTPANS1-immunized mice and control animals were in accordance to survival rate detected in this animal group, which was remarkably different from the two other groups. The pcTPANS1-immunized animals were 100% protected against DENV-2 i.c. challenge, while only 30% and 45% of non-immunized or pcTPA-inoculated mice, respectively, survived to virus inoculation (Fig. 6b).

Levels of NS1-specific antibodies were analyzed prior and after challenges. No significant increase of antibody titers against the NS1 protein was observed 3 days following i.p. challenge when pcTPANS1-immunized mice were sacrificed (Fig. 7a). Non-immunized or pcTPA-inoculated mice i.p. challenged with RJ DENV-2 did not present detectable NS1-specific antibodies (data not shown). On the other hand, NS1-specific antibody levels increased significantly after i.c. challenge with NGC DENV-2 in pcTPANS1-vaccinated mice (titers ranging from 30,000 before challenge to 185,000 after virus inoculation), while pcTPA-inoculated or

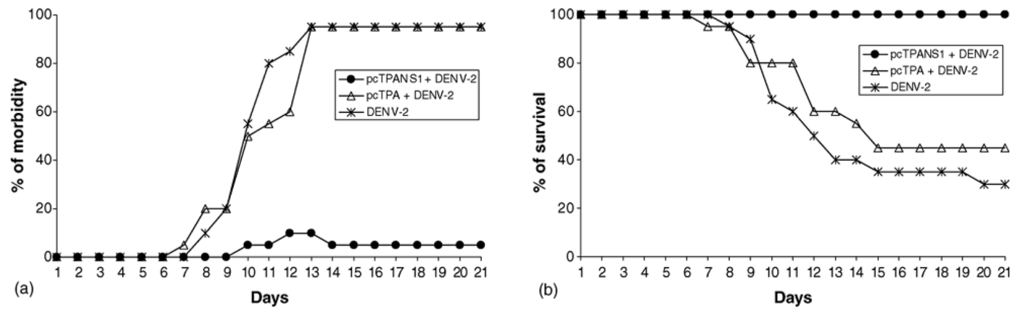

Fig. 6. Percentage of morbidity (a) and survival (b) of Balb/c mice immunized with pcTPANS1 or pcTPA and i.c. challenged with NGC DENV-2 (4.32 $\log_{10}$ PFU). Groups of mice (n = 20) were i.m. immunized with two DNA doses (100 μg each) and challenged 4 weeks after the first plasmid inoculation. Non-immunized control mice (n = 20) followed the same virus infection procedure. Mice were daily monitored and pathological symptoms, mainly hind leg paralysis, were recorded.

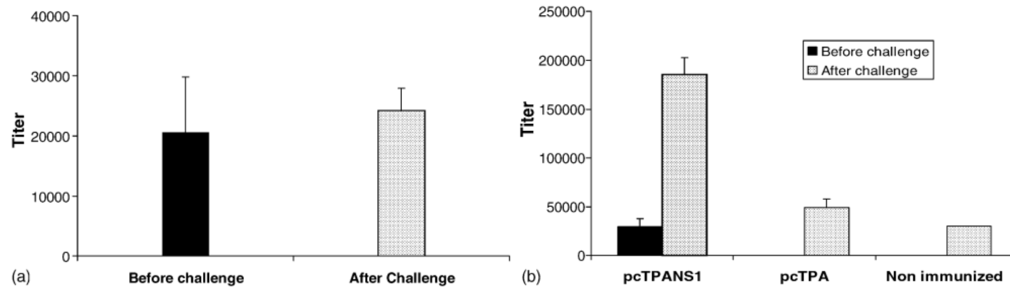

Fig. 7. NS1-specific antibody response in Balb/c mice after challenges DENV-2. Mice were i.m. immunized with two DNA doses and challenged 4 weeks after the first plasmid inoculation. (a) Mice immunized with pcTPANS1 and sacrificed 3 days after i.p. inoculation of RJ DENV-2 (n = 4). (b) Mice inoculated with pcTPANS1, pcTPA or non-immunized animals and i.c. challenged with NGC DENV-2. Twenty-one days after challenges, survived animals were sacrificed and serum samples were collected (n = 20 for pcTPANS1-immunized, n = 9 for pcTPA-injected and n = 6 for non-immunized mice). Titers of NS1-specific antibodies were determined in ELISA with purified NS1 protein expressed in insect cells as solid-phase bound antigen. Data represent the mean of individual values in each animal group tested in duplicate and bars are standard deviation of the mean.

non-immunized survived animals presented remarkable lower NS1-specific antibody titers after challenge (mean of 50,000 and 30,000, respectively) (Fig. 7b).

4. Discussion

In the present report a plasmid containing the NS1 gene from DENV-2 fused to the t-PA signal sequence (pcTPANS1) was constructed in order to evaluate the potential of such an approach to induce an NS1-specific antibody response as well as to confer protection in mice. Results indicate that pcTPANS1 drives secretion of the recombinant NS1 protein in a dimeric form to the surrounding medium, as revealed by Western blot analysis of pcTPANS1-transfected BHK cells. According to previous observations, NS1 is secreted from DENV infected mammalian cells as a dimmer [26,27] and can then be organized as hexamers [28]. Stability of the dimmer is influenced by glycosylation of the protein, which contains two conserved *N*-glycosylation sites [27,28]. Therefore, the secretion of NS1 as a dimmer by in vitro pcTPANS1-transfected cells suggests that this protein is glycosylated and that the NS1 synthesized in vivo after inoculation of such a plasmid might have a tri-dimensional structure similar to the native viral protein.

Several reports indicate that the efficiency and magnitude of immune responses elicited by DNA vaccines are influenced by the ability of the expressed antigen to be secreted [29,30]. In agreement with such observations, our current work has demonstrated that mice immunized with the pcTPANS1 plasmid presented high levels of NS1-specific antibodies, especially in animals i.m. inoculated. Levels of pcTPANS1-induced antibodies were higher than those detected in serum samples of human patients with primary or secondary DENV infections, as described by Lemes et al. [22] in ELISA assays similar to those presented herein,

using the NS1 protein expressed in insect cells as the solid-phase bound antigen. Furthermore, a long-term humoral immune response was observed in pcTPANS1-inoculated animals presenting specific-NS1 antibodies even 56 weeks after the first plasmid inoculation, with a significant increase after a booster. Such results indicate the activation of an immunological memory with a rapid secondary antibody response, similar to other investigations with DNA vaccines against different pathogens [31–33]. Moreover, antibodies generated after immunization with the pcTPANS1 plasmid strongly recognized the intact NS1 expressed in insect cells, while the reaction was weakly detected when this protein was heat-denatured. Both observations indicate that such antibodies were mainly directed to conformational surface-exposed epitopes. Consistent with these findings, no reaction was observed between pcTPANS1-elicited antibodies and the recombinant NS1 expressed in *E. coli*, which is unable to make posttranslational modifications, such as glycosylations, and consequently might synthesize a protein with a tri-dimensional structure remarkably different from the native viral protein.

Immunization with the pcTPANS1 plasmid provided protection against challenges with DENV-2 in two different models. Experiments with i.p. inoculation of a DENV-2 isolated from a patient displayed that non-immunized or control plasmid (pcTPA) inoculated mice presented hepatic injuries in focal zones, characterized by micro and macro vacuolization of the central lobular vein and sinusoidal portal endothelia, in conjunction with swelling of hepatocytes presenting cytoplasm rarefaction and altered organelles. Similar liver tissue damages have previously been reported in other studies with DENV infected mouse models [34,35]. In contrast, tissue injuries were significantly reduced or absent in pcTPANS1-vaccinated mice, revealing a protective immune response against a non-adapted virus, which circulates in human populations. Protection generated by pcTPANS1 was also confirmed in challenge experiments with i.c. inoculation of NGC DENV-2, a mouse adapted virus. All pcTPANS1-vaccinated mice survived DENV-2 inoculation, while 70% and 55% of non-immunized and pcTPA-injected animals, respectively, died after virus challenge. Experiments were performed in adult Balb/c mice that are naturally less susceptible to i.c. DENV inoculation as compared to newborn mice, which may explain the survival rates observed in control animals. Besides, the difference in death rate detected between non-immunized and pcTPA-inoculated animals could be explained by the presence of bacterial derived CpG motifs in this plasmid, which has shown to be an immunostimulator factor, acting as a non-specific host innate immune response inductor [36,37]. However, severe morbidity was observed in both control mouse groups after challenges with NGC DENV-2, with 95% of hind leg paralysis or death. Morbidity rate in pcTPANS1-vaccinated animals, on the other hand, was substantially lower. Only two mice (10%) presented hind leg paralysis, one of which completely recovered normal function after 4 days. Most pathologies observed in mice submitted to i.c. DENV inoculations were concentrated in nervous system although the engagement of hepatic tissue is also possible. Further histopathological analysis will be performed in tissues of survived animals in order to evaluate the damage extension in several organs.

Another DNA vaccine based on the NS1 protein [38] was also reported, in which a protection of C3H mice i.m. injected with a DNA vaccine and challenged with intravenous inoculation of a DENV-2 isolated in Taiwan was observed, although 28% of the vaccinated animals died post challenge and 30% developed hind paralysis [38]. Another difference between our studies and those from Taiwan [38] is the magnitude of antibody response elicited by both DNA vaccines. Mice immunized with pcTPANS1 produced high levels of NS1-specific antibodies, while in the Wu et al. [38] results, NS1-specific antibodies could only be detected after virus challenge. One possible explanation for these differences is the presence of the t-PA signal sequence in the pcTPANS1 plasmid, which is a known efficient peptide promoting the secretion of recombinant proteins to extracellular medium in other DNA vaccines [39–41]. Animals vaccinated with pcTPANS1 exhibited a significant increase of NS1-specific antibody titers 21 days after i.c. challenge, which indicates a strong secondary immune response. This titer increase also demonstrated that virus was still replicating in host cells, although a significant protection was observed in these challenged animals. Such results were expected, since NS1 is not present in the virion particle and, consequently, the immune response against this protein can only be protective after some initial virus replication in host cells. Animals challenged with i.p. virus inoculation did not show significant increase in NS1-specific antibody levels. These animals were sacrificed 3 days after challenge, which may not be sufficient time for a secondary response.

NS1 is a particularly interesting protein for vaccine studies, since it is highly immunogenic, evoking a strong antibody response in recovered dengue patients. Antibodies against NS1 may confer protection due to its Fc portion with complement fixing activity, which seems to kill infected target cells without the risk of ADE virus replication [9,10,38,42]. Immunization with the pcTPANS1 plasmid induced high levels of NS1-specific antibodies and conferred protection against virus challenge. These antibodies probably have a role to play in the protection conferred by the pcTPANS1 immunization, although the possibility of a cellular immune response elicited with this DNA vaccine, which may also contribute for such protection, cannot be excluded and further studies will be necessary in order to evaluate this question.

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References

[1] Monath TP, Heinz FX. Flaviviruses. In: Fields BN, Knipe DM, Howley PM, editors. Fields virology. New York: Raven Press; 1996. p. 961–1034.

[2] Rigau-Pérez JG, Clark GG, Gubler DJ, Reiter P, Sanders EJ, Vorndam AV. Dengue and dengue haemorrhagic fever. Lancet 1998;352:971–7.

[3] Halstead SB, O'Rourke EJ. Dengue viruses and mononuclear phagocytes I. Infection enhancement by non-neutralizing antibody. J Exp Med 1977;146:201–29.

[4] Rothman AL, Ennis FA. Immunopathogenesis of dengue hemorrhagic fever. Virology 1999;257:1–6.

[5] Gubler DJ, Trent DW. Emergence of epidemic dengue/dengue hemorrhagic fever as a public health problem in the Americas. Infect Ag Dis 1994;2:383–93.

[6] Roberstson SE, Mayans MV, El-Husseiny A, Clemens JD, Ivanoff B. The WHO vaccine trial registry. Vaccine 2002;20:31–41.

[7] Chambers TJ, Tsai TF, Pervikov Y, Monath TP. Vaccine development against dengue and Japanese encephalitis: report of a World Health Organization meeting. Vaccine 1997;15:1494–502.

[8] Putnak R. Progress in the development of recombinant vaccinates against dengue and other arthropod-borne flaviviruses. In: Kurstak E, editor. Modern vaccinology. New York: Plenum Medical; 1994. p. 231–52.

[9] Schlesinger JJ, Brandriss MW, Walsh EE. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. J Gen Virol 1987;68:853–7.

[10] Henchal EA, Henchal LS, Schlesinger JJ. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol 1988;69:2101–7.

[11] Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993;259:1745–9.

[12] Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Ann Rev Immunol 1997;15:617–48.

[13] Colombage G, Hall R, Pavy M, Lobigs M. DNA-based and alphavirus-vectored immunisation with PrM and E proteins elicits long-lived and protective immunity against the flavivirus, murray valley encephalitis virus. Virology 1998;250:151–63.

[14] Schultz J, Dollenmaier G, Mölling K. Update on antiviral DNA vaccine research (1998–2000). Intervirology 2000;43:197–217.

[15] Konishi E, Yamaoka M, Kurane I, Mason PW. A DNA vaccine expressing dengue type 2 virus premembrane and envelope genes induces neutralizing antibody and memory B cells in mice. Vaccine 2000;18:1133–9.

[16] Kochel TJ, Raviprakash K, Hayes CG, Watts DM, Russell KL, Gonzalo AS, et al. A dengue virus serotype-1 DNA vaccine induces virus neutralizing antibodies and provides protection from viral challenge in Aotus monkeys. Vaccine 2000;18:3166–73.

[17] Chang GJJ, Davis BS, Hunt AR, Holmes DA, Kuno G. Flavivirus DNA vaccines: current status and potential. Ann N Y Acad Sci 2002;951:272–85.

[18] Putnak R, Fuller J, VanderZanden L, Innis BL, Vaughn DW. Vaccination of rhesus macaques against dengue-2 virus with a plasmid DNA vaccine encoding the viral pre-membrane and envelope genes. Am J Trop Med Hyg 2003;68:469–76.

[19] Nogueira RMR, Miagostovich MP, Lampe E, Souza RW, Zagne SMO, Schatzmayr HG. Dengue epidemic in the state of Rio de Janeiro, Brazil, 1990–1: co-circulation of dengue 1 and dengue 2 serotypes. Epidemiol Infect 1993;111:163–70.

[20] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1989.

[21] Schneider I. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. J Embryol Exp Morphol 1972;27:353–65.

[22] Lemes EM, Miagostovicsh MP, Alves AM, Costa SM, Fillipis AM, Armoa GR, Araujo MA. Circulating human antibodies against dengue NS1 protein: potential of recombinant D2V-NS1 proteins in diagnostic tests. J Clin Virol 2005;32:305–12.

[23] Reynolds E. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 1963;17:208–12.

[24] Caufour PS, Motta MCA, Yamamura AMY, Vazques S, Ferreira II, Jabor AV, et al. Construction, characterization and immunogenicity of recombinant yellow fever 17D-dengue type 2 viruses. Virus Res 2001;79:1–14.

[25] Erhardt W, Hebestedt A, Aschenbrenner G, Pichotka B, Blumel G. A comparative study with various anesthetics in mice (pentobarbitone, ketamine-xylazine, carfentanyl-etomidate). Res Exp Med (Berl) 1984;184:159–69.

[26] Pryor MJ, Wright PJ. The effects of site-directed mutagenesis on the dimerization and secretion of the NS1 protein specified by dengue virus. Virology 1993;194:769–80.

[27] Pryor MJ, Wright PJ. Glycosylation mutants of dengue virus NS1 protein. J Gen Virol 1994;75:1183–7.

[28] Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble examer in a glycosylation-dependent fashion. J Virol 1999;73:6104–10.

[29] Boyle JS, Koniaras C, Lew AM. Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. Int Immunol 1997;9:1897–906.

[30] Inchauspe G, Vitvitski L, Major ME, Jung G, Spengler U, Maisonnas M, Trepo C. Plasmid DNA expressing a secreted or a nonsecreted form of hepatitis C virus nucleocapsid: comparative studies of antibody and T-helper responses following genetic immunization. DNA Cell Biol 1997;16:185–95.

[31] Davis HL, Mancini M, Michel ML, Whalen RG. DNA-mediated immunization to hepatitis B surface antigen: longevity of primary response and effect of boost. Vaccine 1996;14:910–5.

[32] Deck RR, DeWitt CM, Donnelly JJ, Liu MA, Ulmer JB. Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. Vaccine 1997;15:71–8.

[33] Alves AMB, Lásaro MO, Pyrrho AS, Gattass CR, Almeida DF, Ferreira LCS. Antibody response in mice immunized with plasmid DNA encoding the colonization factor antigen I (CFA/I) of enterotoxigenic *Escherichia coli* (ETEC). FEMS Immunol Med Microbiol 1999;23:321–30.

[34] An J, Kimura-Kuroda J, Hirabayashi Y, Yasui K. Development of a novel mouse model for dengue virus infection. Virology 1999;263:70–7.

[35] Paes MV, Pinhão AT, Barreto DF, Costa SM, Oliveira MP, Nogueira AC, et al. Liver injury and viremia in mice infected with Dengue-2 virus. Virology 2005;338:236–46.

[36] Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 1996;273:352–4.

[37] Horner AA, Datta SK, Takabayashi K, Belyakov IM, Hayashi T, Cinman N, et al. Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosal sites. J Immunol 2001;167:1584–91.

[38] Wu SF, Liao CL, Lin YL, Yeh CT, Chen LK, Huang YF, et al. Evaluation of protective efficacy and immune mechanisms of using a non-structural protein NS1 in DNA vaccine against dengue 2 virus in mice. Vaccine 2003;21:3919–29.

[39] Li Z, Howard A, Kelley C, Delogu G, Collins F, Morris S. Immunogenicity of DNA vaccines expressing tuberculosis proteins fused

to tissue plasminogen activator signal sequences. Infect Immun 1999;67(9):4780–6.

[40] Alves AM, Lasaro MO, Almeida DF, Ferreira LC. DNA immunisation against the CFA/I fimbriae of enterotoxigenic *Escherichia coli* (ETEC). Vaccine 2001;19:788–95.

[41] Ashok MS, Rangarajan PN. Protective efficacy of a plasmid DNA encoding Japanese encephalitis virus envelope protein fused to tissue plasminogen activator signal sequences: studies in a murine intracerebral virus challenge model. Vaccine 2002;20:1563–70.

[42] Srivastava AK, Putm, Putnak JR, Warren RL, Hoke Jr CH. Mice immunized with a dengue type 2 virus E and NS1 fusion protein made in *Escherichia coli* are protected against lethal dengue virus infection. Vaccine 1995;13:1251–8.

ARTIGO 2

DNA vaccine against the non-structural 1 protein (NS1) of dengue 2 virus

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DNA vaccine against the non-structural 1 protein (NS1) of dengue 2 virus

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Abstract

Dengue is one of the most important mosquito-borne viral disease causing dengue fever and/or dengue shock syndrome/haemorrhagic fever. In some reports, the non-structural protein 1 (NS1) has been identified as a promising antigen for the development of vaccines against dengue virus (DENV). Apparently, it can elicit a protective antibody response with complement-fixing activities. In order to investigate the potential of a DNA vaccine based on the NS1 protein against DENV, we used the plasmid pcTPANS1, which contains the secretory signal sequence derived from human tissue plasminogen activator (t-PA) fused to the full length of the DENV-2 NS1 gene. All Balb/c mice intramuscularly inoculated with the pcTPANS1 presented high levels of NS1-specifc antibodies. Vaccinated animals were challenged with intracerebral DENV-2 virus inoculations and a 100% survival was observed. In general, results demonstrate that the pcTPANS1 plasmid is able to induce protection in mice, and then may be used as a vaccination approach against DENV in further assays.
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Keywords: DNA vaccine; Dengue; NS1

1. Introduction

Dengue viruses consist of four distinct antigenic types (DENV-1–4) causing one of the most important arboviral disease. Infection with DENV is a global public health problem, with recurring epidemics in tropical and subtropical regions of the world [1]. The disease presents a wide range of clinical symptoms, including a mild self-limiting acute febrile illness, the dengue fever (DF), and hemorrhagic fever and/or shock syndrome (DHF/DSS) [2]. Although considerable research has been done towards the development of a DENV vaccine, no vaccine is yet available for clinical use [3].

Several reports have shown that the non-structural 1 protein (NS1) from DENV is highly immunogenic and may induce protective antibodies with complement fixing activity, which kills infected target cells [4,5]. Besides, the NS1-elicited immune response seems to be effective without the risk of the phenomenon of antibody dependent enhancement (ADE) of the infection, probably caused by non-neutralizing antibodies reacting to the virus envelope protein (E) [6].

DNA-based vaccines have been shown to induce long lasting cellular and humoral immunity against several pathogens including flaviviruses [7,8]. Based on these evidences, in the present work, we investigated the use of a plasmid encoding the NS1 DNA sequence from DENV-2, fused to the human tissue plasminogen activator (t-PA) signal sequence, as a DNA vaccine. Mice immunized with this plasmid presented high levels of NS1-specific antibodies and were protected against DENV-2 challenge.

2. Materials and methods

2.1. Immunization procedure

The pcTPANS1, a pcDNA3 modified plasmid, which contains the NS1 region from DENV-2 fused to the t-PA secretory signal sequence, was used for immunizations. Control experiments were performed with the pcDNA3 vector. Plasmids

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were purified from *Escherichia coli* by Qiagen Plasmid Giga kits (Qiagen). Male Balb/c mice, 4–6 weeks old, were intramuscularly (i.m.) injected in each hind limb tibialis anterior muscle with 50 μg of the pcTPANS1 or pcDNA3 plasmids dissolved in 50 μl of PBS (100 μg/dose/mice). Mice were inoculated with two DNA doses, given two weeks apart, and bled before immunization (pre-immune) and 4 weeks after the first DNA dose.

2.2. Detection of NS1-specific antibodies by ELISA

ELISA was carried out with the recombinant DENV-2 NS1 protein, as solid-phase bound antigen, expressed in mosquito cells (Hawaii Biotechnology Group Inc.). Wells of MaxiSorp plates (Nunc) were coated at 37 °C for 1 h with 0.1 μg of NS1 protein in 100 μL PBS. ELISA was then performed as described elsewhere [9] with goat anti-mouse IgG conjugated with horseradish peroxidase (Southern Biotechnology). Titres were established as the reciprocal of serum dilution, which gave an absorbance above that of the respective pre-immune serum.

2.3. DENV-2 challenge in mice

The mouse brain-adapted DENV-2, strain New Guinea C (NGC), was used for challenge experiments. Two weeks after the second DNA dose, each immunized mouse was challenged with intracerebral (i.c.) inoculation with 30 μL of 4.32 $\log_{10}$ PFU (5.84 $\log_{10}$ PFU/mL) of DENV2. Inoculum was back-tittered in Vero cells immediately after the challenge procedure, as described previously [10]. Mice were separated in three groups: pcTPANS1- and pcTPA-immunized mice and non-immunized animals, all challenged with the same virus sample. Animals were monitored for 21 days and deaths were recorded.

3. Results and discussion

The pcTPANS1 plasmid was tested as a DNA vaccine against DENV in a murine model. Balb/c mice were i.m. immunized with this plasmid and the humoral immune response was analysed in ELISA tests. Serum samples were individually screened for reactivity against the NS1. All pcTPANS1-immunized mice ($n = 10$) presented a significant antibody response, with titres ranging from 21,000 to 32,000 (Fig. 1). Control animals inoculated with the pcDNA3 ($n = 10$) did not present antibodies recognizing the NS1 protein (Fig. 1). These results indicate that the NS1 protein is expressed in vivo after the pcTPANS1 inoculation and that such expression is able to induce a strong antibody response.

The efficacy of such vaccine in inducing a protective immunity was analyzed. pcTPANS1- and pcDNA3-inoculated animals were challenged 4 weeks following the first immunization, which corresponds to peak level of NS1-specifc IgG antibodies. Two independent challenge experiments were performed at the same conditions. In both experiments, all pcTPANS1-immunized mice survived to virus challenge (Table 1). In contrast to the 100% survival of vaccinated mice, control groups presented a significant reduced survival rates. Animals inoculated with the pcDNA3 vector showed 50% (5/10) and 40% (4/10) survival rates, while in non-immunized control group, 25% (2/8) and 30% (3/10) of animals survived to virus challenge in the first and second experiments, respectively (Table 1). Differences in survival rates observed between pcDNA3- and non-immunized animals might be explained by the presence of CpG motifs in the background of the pcDNA3 plasmid, which is a known immunostimulator factor [11] and could promote a non-specific protection. However, protection conferred by the pcTPANS1 immunization is undoubtedly specific to the NS1 protein.

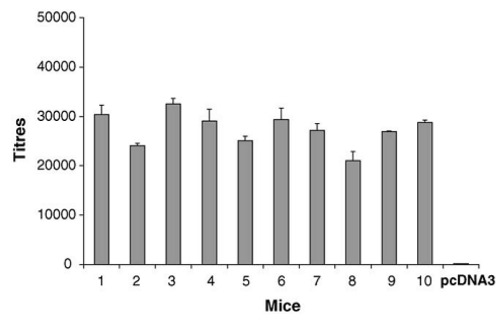

Fig. 1. Individual NS1-specific IgG response of pcTPANS1-immunized mouse sera. Animals were i.m. inoculated with two DNA doses and blood samples were taken 4 weeks after the first immunization for ELISA. Samples of pcDNA3-inoculated mice were pooled. Titres were established as the reciprocal of the serum dilution that gave an absorbance above that of pre-immune sera. Data represent the mean of duplicate values for each sample.

Another DNA vaccine based in the NS1 protein was also reported [12], in which vaccinated C3H mice were challenged intravenously with a DENV-2 isolated in Taiwan. However, protection was not complete and the NS1-specific antibody response was marginal [12]. In general, results here presented demonstrate that the pcTPANS1 plasmid is a promising DNA vaccine and might be used in further approach for the development of a vaccine against DENV.

Table 1
Protection of Balb/c mice i.c. challenged with the mouse brain-adapted DENV-2 NGC

Independent experiment	No. of survivors/no. total (% survivors)		
	pcTPANS1[a]	pcDNA3[a]	DENV-2[b]
1	10/10(100%)	5/10 (50%)	2/8 (25%)
2	10/10(100%)	4/10 (40%)	3/10 (30%)

[a] Mice i.m. immunized with two DNA doses and challenged with DENV-2 NGC 4 weeks after the first immunization.
[b] Non-immunized mice challenged with DENV-2 NGC.

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References

[1] Monath TP, Heinz FX. Flaviviruses. In: Fields BN, Knipe DM, Howley PM, editors. Fields Virology. New York: Raven Press; 1996. p. 961–1034.
[2] Halstead SB. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascate. Rev Infect Dis 1989;11: 830–9.
[3] Roberstson SE, Mayans MV, El-Husseiny A, et al. The WHO vaccine trial registry. Vaccine 2002;20:31–41.
[4] Schlesinger JJ, Brandriss MW, Walsh EE. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. J Gen Virol 1987;68: 853–7.
[5] Henchal EA, Henchal LS, Schlesinger JJ. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol 1988;69(Pt 8):2101–7.
[6] Halstead SB, O'Rourke EJ. Dengue viruses and mononuclear phagocytes I. Infection enhancement by non-neutralizing antibody. J Exp Med 1977;146:201–29.
[7] Schultz J, Dollenmaier G, Mölling K. Update on antiviral DNA vaccine research. Intervirology 2000;43:197–217.
[8] Chang GJJ, Davis BS, Hunt AR, et al. Flavivirus DNA vaccines: currant status and potential. Annals New York Academy of Science 2002;951:272–85.
[9] Alves AM, Lasaro MO, Almeida DF, et al. DNA immunisation against the CFA/I fimbriae of enterotoxigenic *Escherichia coli* (ETEC). Vaccine 2001;19(7–8):788–95.
[10] Caufour PS, Motta MCA, Yamamura AMY, et al. Construction, characterization and immunogenicity of recombinant yellow fever 17D-dengue type 2 viruses. Virus Res 2001;79:1–14.
[11] Sato Y, Roman M, Tighe H, et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 1996;273:352–4.
[12] Wu SF, Liao CL, Lin YL, et al. Evaluation of protective efficacy and immune mechanisms of using a non-structural protein NS1 in DNA vaccine against dengue 2 virus in mice. Vaccine 2003;21:3919–29.

4.II.2 – Resumo dos resultados do artigo 3

O artigo 3 compara os resultados obtidos com os plasmídeos recombinantes pcENS1, pcTPANS1, pcENS1ANC e pcTPANS1ANC quanto a expressão da proteína NS1 *in vitro*, a indução da resposta imune humoral em camundongos BALB/c e a proteção contra o desafio com DENV-2.

Primeiramente, foram construídos quatro plasmídeos recombinantes contendo o gene NS1: pcENS1, que contém a seqüência de 63 nucleotídeos que codifica a região C-terminal da proteína E fusionda ao gene NS1 clonado no plasmídeo pcDNA3; pcENS1ANC semelhante ao pcENS1 com a adição da seqüência de 138 nucleotídeos que codifica a porção N-terminal da proteína NS2A (ANC) a jusante do gene NS1, clonado no plasmídeo pcDNA3; pcTPANS1 que contém o gene NS1 clonado a jusante da seqüência que codifica o peptídeo sinal t-PA; pcTPANS1ANC, que compreende a seqüência t-PA, seguida do gene NS1 e da seqüência ANC. A integridade das construções foi confirmada por seqüenciamento.

A expressão da proteína NS1 a partir destes plasmídeos foi avaliada pela transfecção de células BHK-21. A proteína recombinante NS1 foi detectada por imunofluorescência, no citoplasma das células transfectadas com os plasmídeos pcTPANS1, pcENS1 e pcENS1ANC. Contudo, tal proteína não foi observada nas células transfectadas com o plasmídeo pcTPANS1ANC, assim como nos controles. Além disso, a expressão de NS1 foi confirmada por *western-blot*. Uma proteína de aproximadamente 48 kDa, que corresponde à NS1, foi detectada no extrato celular e no sobrenadante das células transfectadas com os plasmídeos pcTPANS1, pcENS1 e pcENS1ANC, indicando que as duas seqüências sinais utilizadas (t-PA e porção C-terminal da proteína E) foram capazes de direcionar a proteína recombinante para via do retículo endoplasmático e sua posterior secreção. Mais uma vez, não foi observada a expressão de NS1 nos extratos das células transfectadas com o plasmídeo pcTPANS1ANC, o que sugere uma incompatibilidade das seqüências hidrofóbicas t-PA e ANC em uma mesma construção. A proteína NS1 detectada nos sobrenadantes das culturas apresentou peso molecular de 96 kDa, quando as amostras não foram desnaturadas, indicando que tal proteína está sendo secretada na forma de dímeros.

A análise por microscopia eletrônica das células BHK-21 transfectadas com os plasmídeos recombinants pcTPANS1, pcENS1 e pcENS1ANC evidenciou a presença da proteína NS1 em vesículas. Entretanto, somente nas células transfectadas com pcENS1ANC, a proteína recombinante também foi observada associada à membrana plasmática. Tais

resultados confirmam as suposições de que a região C-terminal da proteína NS2A é essencial para a associação da proteína NS1 à membrana citoplasmática da célula hospedeira.

A seguir, a resposta imune humoral gerada pela imunização de camundongos BALB/c com os diferentes plasmídeos foi avaliada por ELISA. Grupos de camundongos (n = 10) foram imunizados com os plasmídeos recombinantes pcTPANS1, pcTPANS1ANC, pcENS1 e pcENS1ANC, e com os plasmídeos controles. Todos os animais imunizados com os plasmídeos pcTPANS1 e pcENS1 apresentaram níveis de anticorpos anti-NS1 homogêneos e significantemente maiores do que o controle. Por outro lado, a inoculação com o pcENS1ANC induziu uma resposta bastante heterogênea, em que somente metade dos animais apresentou anticorpos anti-NS1, e em níveis mais baixos que os gerados com o pcENS1 ou o pcTPANS1. Conforme o esperado, a inoculação com o pcTPANS1ANC não induziu uma resposta contra NS1.

A cinética da resposta de IgG específica contra NS1 induzida nas imunizações com os plasmídeos pcTPANS1 e pcENS1 foi bastante semelhante, com o pico da resposta ocorrendo na 4ª semana após a primeira inoculação e mantendo-se estável nas 6 semanas seguintes. A inoculação do plasmídeo pcENS1ANC induziu um padrão semelhante de resposta humoral, contudo com níveis de anticorpos foram bem mais baixos. Nas três imunizações, os anticorpos gerados reconheceram predominantemente epítopos conformacionais da proteína NS1, expressa em células de drosófila, uma vez que as reações detectadas se mostraram muito menores quando tal proteína foi desnaturada por calor e utilizada nas placas de ELISA.

Considerando que os plasmídeos pcTPANS1 e pcENS1 geraram uma resposta imune humoral homogênea, a proteção gerada pela imunização com esses plasmídeos foi avaliada em experimentos de desafio intracerebral com DENV-2.

Camundongos (n = 30) imunizados com pcTPANS1, pcENS1 e controles foram desafiados com DENV-2 por via i.c., quatro semanas após a primeira dose de DNA. As duas vacinas geraram níveis de proteção elevados, embora os animais imunizados com o pcTPANS1 tenham apresentado uma taxa de sobrevivência (97%) maior do que os inoculados com o plasmídeos pcENS1 (87%). Por outro lado, nos grupos controles apenas 33% dos animais sobreviveram ao desafio. A análise dos sinais clínicos observados nos camundongos dos diferentes grupos corrobora estes dados. Enquanto a taxa de morbidade dos grupos controles foi de 90%, apenas 10% dos animais inoculados com o pcTPANS1 e 27% dos imunizados com o pcENS1 apresentaram algum tipo de sinal clínico. Estes resultados indicam que a inoculação com o vetor pcTPANS1 foi levemente mais protetora do que a imunização com pcENS1, embora os dados não sejam estatisticamente significativos.

A análise dos níveis de anticorpos anti-NS1 no soro de camundongos inoculados com o plasmídeo pcTPANS1 ou pcENS1 mostrou um aumento de mais que 5 vezes nos títulos destes anticorpos após o desafio com o vírus. A avaliação do perfil das subclasses de anticorpos IgG1 e IgG2a específicos contra NS1 mostrou que camundongos inoculados com o plasmídeo pcTPANS1 apresentaram principalmente anticorpos IgG1, antes e depois do desafio com o vírus, enquanto que os títulos de IgG1 e IgG2a foram semelhantes em animais imunizados com pcENS1. Essas diferenças podem estar relacionadas à proteção, levemente maior, gerada pela imunização com o plasmídeo pcTPANS1. Já os animais controles (inoculados com pcTPA ou não imunizados), que sobreviveram ao desafio apesar de exibirem fortes sinais clínicos, apresentaram anticorpos anti-NS1 predominantemente do tipo IgG2a.

Também foram realizados ensaios de soroneutralização em células Vero, utilizando o soro de animais vacinados com estes plasmídeos. Não foi observado nenhum nível de soroneutralização nestes testes.

Em adição aos dados deste artigo, foi avaliada a resposta imune humoral de longa duração em animais imunizados com pcENS1. Semelhante ao observado com os animais vacinados com pcTPANS1 (artigo1), camundongos inoculados com o plasmídeo pcENS1 ainda exibiam níveis detectáveis de anticorpos específicos contra NS1 na 56ª semana (títulos de aproximadamente 6.500). Após uma dose reforço nestes animais houve um rápido e significativo aumento nos níveis de anticorpos anti-NS1 (títulos de aproximadamente 120.000) (Figura 11), indicando que a inoculação com estas vacinas de DNA induz uma resposta imune de longa duração e de memória.

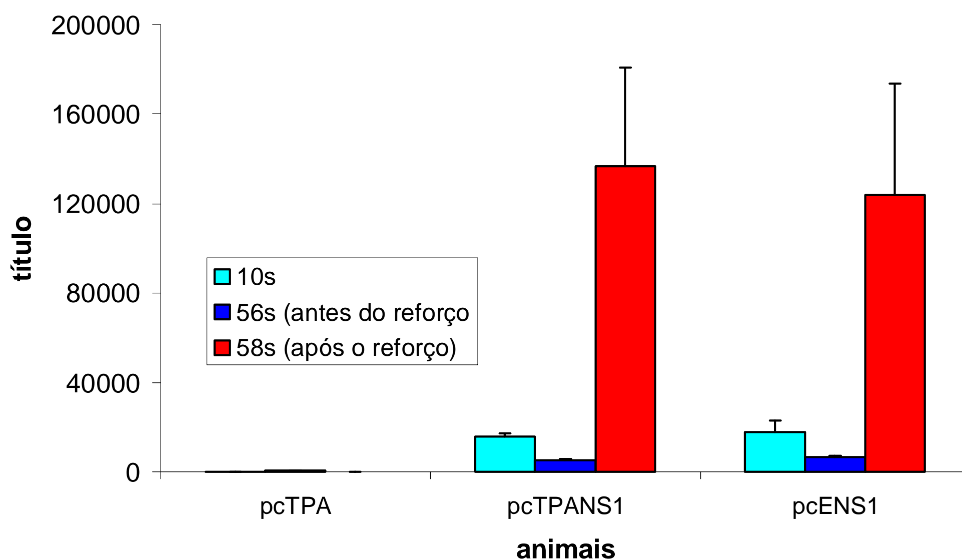

Figura 4.3: Resposta imune humoral de longa duração em camundongos BALB/c imunizados com os plasmídeos pcTPANS1 e pcENS1, avaliada antes e depois do reforço. Os animais foram sangrados 56 semanas após o início do experimento e 2 semanas após o reforço com a terceira dose de DNA (58 semanas). Os níveis de anticorpos IgG foram avaliados por ELISA utilizando proteína NS1 expressa em células de drosófila em sua forma intacta.

ARTIGO 3

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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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.
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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

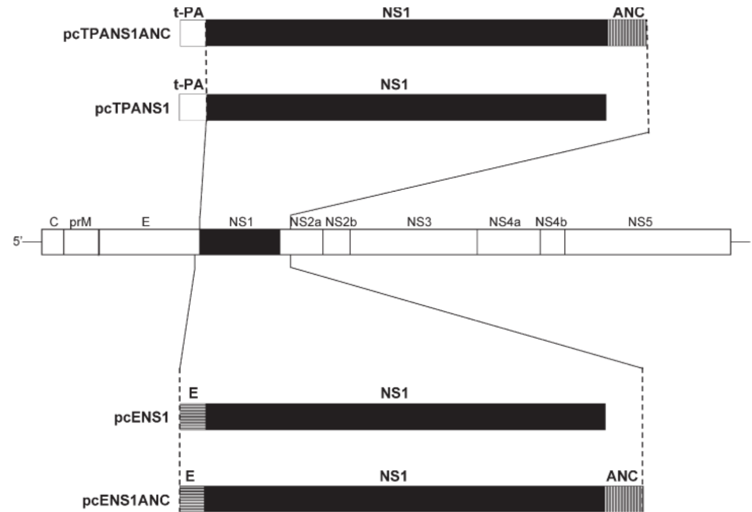

Fig. 1. Schematic diagram of DENV-2 genome map and plasmid constructs. Black area represents the NS1 region; hatched boxes represent signal sequences from the C-terminal of E protein (E) and the N-terminal of NS2a protein (ANC); open box represents the signal peptide derived from the human tissue plasminogen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

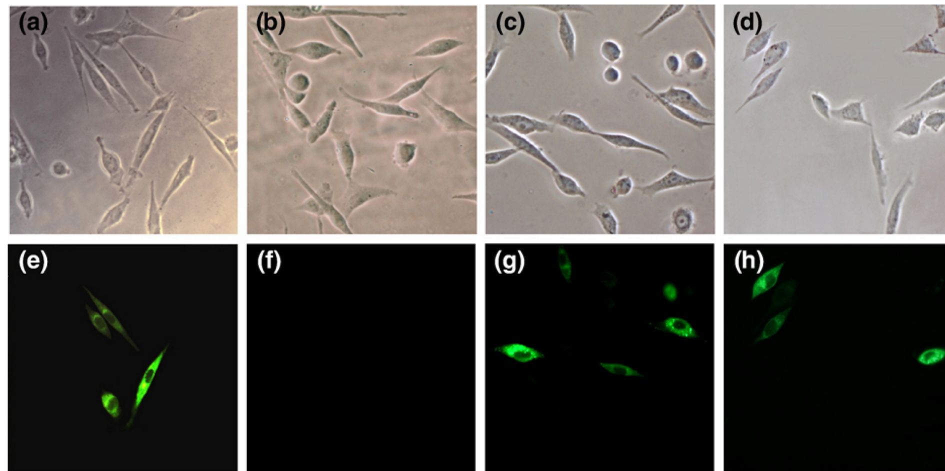

Fig. 2. BHK-21 cells transfected with the different recombinant plasmids: pcTPANS1 (a, e), pcTPANS1ANC (b, f), pcENS1 (c, g) and pcENS1ANC (d, h). Cells were permeabilized, fixed and treated with DENV-2 hyperimmune mouse ascitic fluid and anti-mouse fluorescein-conjugated goat IgG. Phase contrast (a–d);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

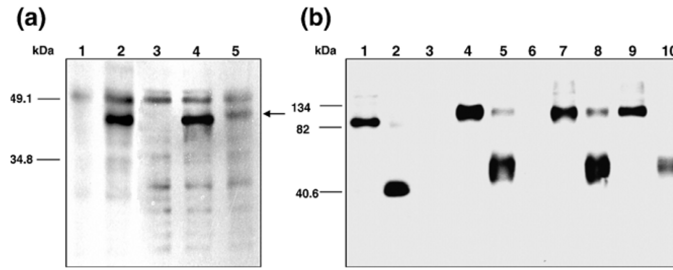

Fig. 3. NS1 protein expressed from recombinant plasmids in transfected BHK cells and detected in Western blots of SDS-PAGE, using DENV-2 hyperimmune mouse ascitic fluid. Samples were suspended in SDS-PAGE buffer with heat treatment (a) or in phosphate buffer with protease inhibitors without 2-mercaptoethanol, 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

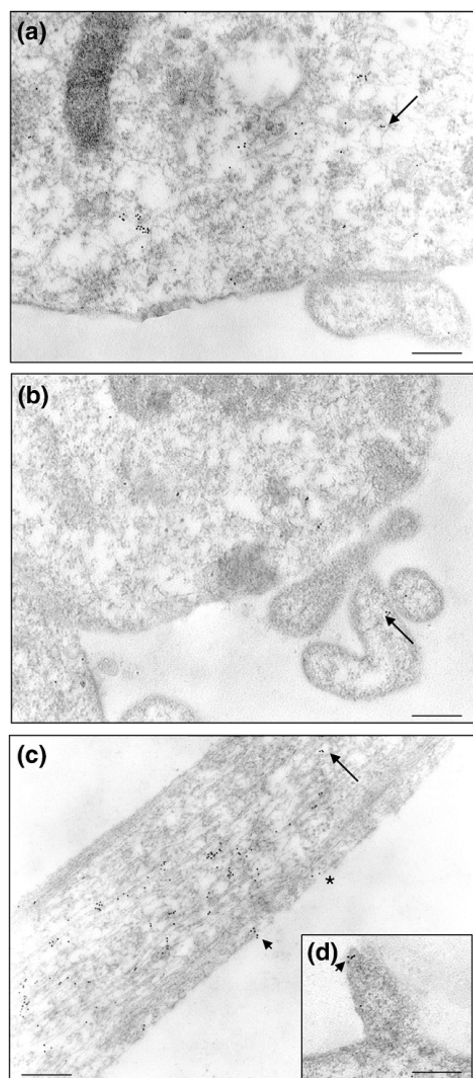

Fig. 4. Electron microscopy of transfected BHK cells, immunolabeled with DENV-2 hyperimmune mouse ascitic fluid and anti-mouse colloidal gold conjugated IgG. Cells transfected with: (a) pcTPANS1, scale bar=0.45 μm; (b) pcENS1, scale bar=0.22 μm; (c) pcENS1ANC, scale bar=0.21 μm; (d) pcENS1ANC, scale 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).

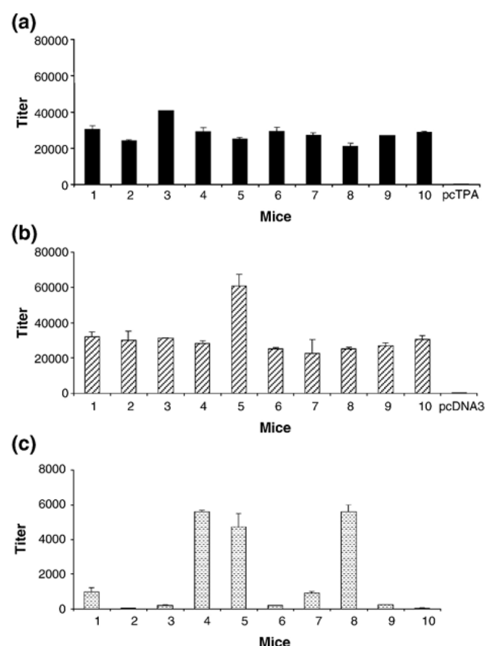

Fig. 5. Individual NS1-specific IgG response elicited in mice immunized with (a) pcTPANS1, (b) pcENS1 and (c) pcENS1ANC. Animals were i.m. inoculated with two DNA doses, blood samples were taken 4 weeks after the first immunization and tested by ELISA. Serum samples harvested from mice inoculated with 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.

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.

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-

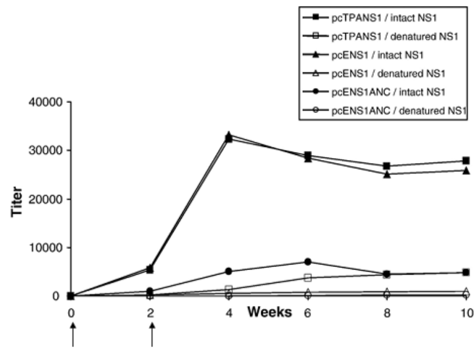

Fig. 6. Time course of serum IgG response to NS1 protein from Balb/c mice i.m. immunized with two doses of pcTPANS1, pcENS1 or pcENS1ANC. Titers were determined in ELISA using purified intact or heat-denatured NS1 protein expressed in insect cells, as solid-phase bound antigen. Data represent the mean 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

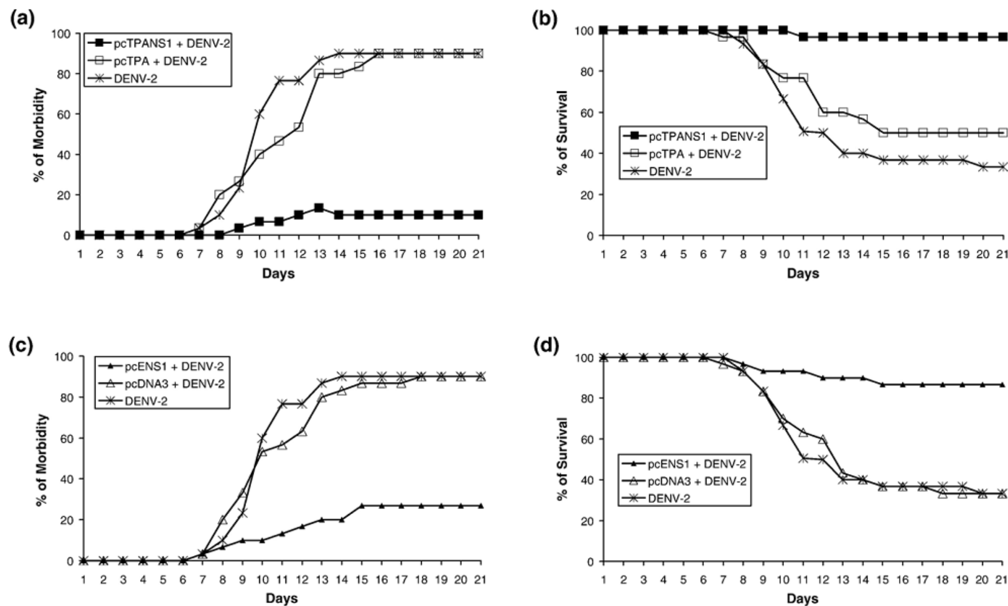

Fig. 7. Percentage of morbidity (a, c) and survival (b, d) of Balb/c mice immunized with pcTPANS1 (a, b) and pcENS1 (c, d) and i.c. challenged with NGC DENV-2. Mice were i.m. immunized with two DNA doses and challenged 4 weeks after the first plasmid inoculation. Non-immunized and pcTPA or pcDNA3-injected 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).

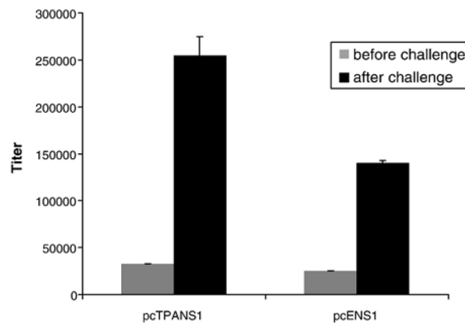

Fig. 8. NS1-specific antibody response in pcTPANS1- and pcENS1-immunized mice, before and after challenges with DENV-2. Mice were i.m. injected with two DNA doses and challenged 4 weeks after the first plasmid inoculation. Serum samples were collected 4 weeks after the first DNA immunization (before 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

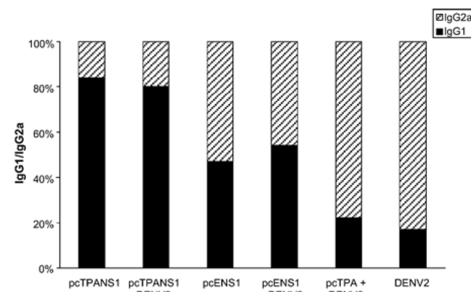

Fig. 9. NS1-specific IgG subclass responses in mice immunized with pcTPANS1 and pcENS1, before and after challenge with DENV-2. Animals were inoculated with recombinant plasmids or control vector (pcTPA), and serum samples were harvested and pooled as depicted in Fig. 8. Each column represents the relative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

Alves, A.M.B., Lásaro, M.O., Almeida, D.F., Ferreira, L.C.S., 1998a. Epitope specificity of antibodies raised against enterotoxigenic *Escherichia coli* CFA/I fimbriae in mice immunized with naked DNA. Vaccine 16, 9–15.

Alves, A.M.B., Lásaro, M.O., Almeida, D.F., Ferreira, L.C.S., 1998b. Immunoglobulin G subclass responses in mice immunized with plasmid DNA encoding the CFA/I fimbria of enterotoxigenic *Escherichia coli*. Immunol. Lett. 62, 145–149.

Alves, A.M.B., Lásaro, M.O., Almeida, D.F., Ferreira, L.C.S., 1999. New vaccine strategies against enterotoxigenic *Escherichia coli*. I: DNA vaccines against the CFA/I fimbrial adhesin. Braz. J. Med. Biol. Res. 32, 223–229.

Alves, A.M.B., Lásaro, M.O., Almeida, D.F., Ferreira, L.C., 2001. DNA immunisation against the CFA/I fimbriae of enterotoxigenic *Escherichia coli* (ETEC). Vaccine 19, 788–795.

Ashok, M.S., Rangarajan, P.N., 2002. Protective efficacy of a plasmid DNA encoding Japanese encephalitis virus envelope protein fused to tissue plasminogen activator signal sequences: studies in a murine intracerebral virus challenge model. Vaccine 20, 1563–1570.
Brinton, M.A., Kurane, I., Mathew, A., Zeng, L., Shi, P.Y., Rothman, A., Ennis, F.A., 1998. Immune mediated and inherited defences against flaviviruses. Clin. Diagn. Virol. 10, 129–139.
Caufour, P.S., Motta, M.C.A., Yamamura, A.M.Y., Vazques, S., Ferreira, I.I., Jabor, A.V., Bonaldo, M.C., Freire, M.S., Galler, R., 2001. Construction, characterization and immunogenicity of recombinant yellow fever 17D-dengue type 2 viruses. Virus Res. 79, 1–14.
Chambers, T.J., Hahn, C.S., Galler, R., Rice, C.M., 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. 44, 649–688.
Chung, K.M., Nyabakken, G.E., Thompson, B.S., Engle, M.J., Marri, A., Fremont, D.H, Diamond, M.S., 2006. Antibodies against West Nile Virus nonstructural protein NS1 prevent lethal infection through Fc gamma receptor-dependent and -independent mechanisms. J. Virol. 80, 1340–1351.
Costa, S.M., Freire, M.S., Alves, A.M.B., 2006a. DNA vaccine against the non-structural 1 protein (NS1) of dengue 2 virus. Vaccine 24, 4562–4564.
Costa, S.M., Paes, M.V., Barreto, D.F., Pinhao, A.T., Barth, O.M., Queiroz, J.L., Armoa, G.R., Freire, M.S., Alves, A.M.B., 2006b. Protection against dengue type 2 virus induced in mice immunized with a DNA plasmid encoding the non-structural 1 (NS1) gene fused to the tissue plasminogen activator signal sequence. Vaccine 24, 195–205.
Donnelly, J.J., Wahren, B., Liu, M.A., 2005. DNA vaccines: progress and challenges. J. Immunol. 175, 633–639.
Edelman, R., 2005. Dengue and dengue vaccines. J. Infect. Dis. 191, 650–653.
Erhardt, W., Hebestedt, A., Aschenbrenner, G., Pichotka, B., Blumel, G., 1984. A comparative study with various anesthetics in mice (pentobarbitone, ketamine-xylazine, carfentanyl-etomidate). Res. Exp. Med. (Berl) 184, 159–169.
Falgout, B., Chanock, R., Lai, C.J., 1989. Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. J. Virol. 63, 1852–1860.
Falgout, B., Bray, M., Schlesinger, J.J., Lai, C.J., 1990. Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. J. Virol. 64, 4356–4363.
Flamand, M., Megret, F., Mathieu, M., Lepault, J., Rey, F.A., Deubel, V., 1999. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J. Virol. 73, 6104–6110.
Guzmán, C.A., Kourí, G., 2002. Dengue: an update. Lancet Infect. Dis. 2, 33–42.
Halstead, S.B., Deen, J., 2002. The future of dengue vaccines. Lancet 360, 1243–1245.
Halstead, S.B., O'Rourke, E.J., 1977. Dengue viruses and mononuclear phagocytes I. Infection enhancement by non-neutralizing antibody. J. Exp. Med. 146, 201–229.
Henchal, E.A., Henchal, L.S., Schlesinger, J.J., 1988. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J. Gen.Virol. 69 (Pt 8), 2101–2107.
Irie, K., Mohan, P.M., Sasaguri, Y., Putnak, R., Padmanabhan, R., 1989. Sequence analysis of cloned dengue virus type 2 genome (New guinea-C strain). Gene 75, 197–211.
Jacobs, M.G., Robinson, P.J., Bletchly, C., Mackenzie, J.M., Young, P.R., 2000. Dengue virus nonstructural protein 1 is expressed in a glycosyl-phosphatidylinositol-linked form that is capable of signal transduction. FASEB J. 14, 1603–1610.
Kinney, R.M., Huang, C.Y.H., 2001. Development of new vaccines against dengue fever and Japanese encephalitis. Intervirology 144, 176–197.
Leatherbarrow, R.M., Dwek, R.A., 1984. Binding of complement subcomponent C1q to mouse IgG1 m IgG2a and IgG2b: a novel C1q binding assay. Mol. Immunol. 21, 321–327.
Li, Z., Howard, A., Kelley, C., Delogu, G., Collins, F., Morris, S., 1999. Immunogenicity of DNA vaccines expressing tuberculosis proteins fused to tissue plasminogen activator signal sequences. Infect. Immun. 67, 4780–4786.
Lin, Y.L., Chen, L.K., Liao, C.L., Yeh, C.T., Ma, S.H., Chen, J.L., Huang, Y.L., Chen, S.S., Chang, H.Y., 1998. DNA immunization with Japanese Encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. J. Virol. 72, 191–200.
Lindenbach, B.D., Rice, C.M., 2001. Flaviviridae: the viruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincott Williams and Wilkins, Philadelphia, pp. 991–1041.
Lu, Y., Raviprakash, K., Leao, I.C., Chikhlikar, P.R., Ewing, D., Anwar, A., Chougnet, C., Murphy, G., Hayes, C.G., August, T.J., Marques Jr., E.T., 2003. Dengue 2 PreM-E/LAMP chimera targeted to the MHC class II compartment elicits long-lasting neutralizing antibodies. Vaccine 21, 2178–2189.
Lucisano, Y.M., Lachmann, P.J., 1991. The effect of antibody isotype and antigenic epitope density on the complement-fixing activity of immune complexes: a systematic study using chimaeric anti-NIP antibodies with human Fc regions. Clin. Exp. Immunol. 84, 1–8.
Mackenzie, J.M., Jones, M.K., Young, P.R., 1996. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology 220, 232–240.
Pryor, M.J., Wright, P.J., 1993. The effects of site-directed mutagenesis on the dimerization and secretion of the NS1 protein specified by dengue virus. Virology 194, 769–780.
Pryor, M.J., Wright, P.J., 1994. Glycosylation mutants of dengue virus NS1 protein. J. Gen.Virol. 75 (Pt 5), 1183–1187.
Rothman, A.L., 2004. Dengue: defining protective versus pathologic immunity. J. Clin. Invest. 113, 946–951.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
Schlesinger, J.J., Brandriss, M.W., Walsh, E.E., 1987. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. J. Gen. Virol. 68, 853–857.
Schlesinger, J.J., Foltzer, M., Chapman, S., 1993. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. Virology 192, 132–141.
Winkler, G., Maxwell, S.E., Ruemmler, C., Stollar, V., 1989. Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. Virology 171, 302–305.
Wu, S.F., Liao, C.L., Lin, Y.L., Yeh, C.T., Chen, L.K., Huang, Y.F., Chou, H.Y., Huang, J.L., Shaio, M.F., Sytwu, H.K., 2003. Evaluation of protective efficacy and immune mechanisms of using a non-structural protein NS1 in DNA vaccine against dengue 2 virus in mice. Vaccine 21, 3919–3929.
Young, P.R., Hilditch, P.A., Bletchly, C., Halloran, W., 2000. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J. Clin. Microbiol. 38, 1053–1057.

4.II.3 – Resultados não publicados

Conforme observado nos artigos 1, 2 e 3 desta seção, a imunização com o plasmídeo pcTPANS1 foi capaz de gerar proteção intraespecífica em camundongos BALB/c imunizados com este plasmídeo e desafiados por via intracerebral e intraperitoneal com DENV-2. Assim, nós também analisamos se a inoculação com este plasmídeo é capaz de proteger camundongos BALB/c contra o desafio intracerebral com DENV-1. Para fundamentar este teste, a similaridades da seqüência de aminoácidos das linhagens de DENV-1 e DENV-2 utilizadas, foram comparadas.

4.II.3.1 - Desafio com DENV de camundongos imunizados com o plasmídeo pcTPANS1

Inicialmente foi calculada a LD_{50} da cepa neuroadaptada Mochizuki de DENV-1 inoculada por via i.c. em camundongos BALB/c machos com 8 semanas de idade, para que os resultados de desafio com esta amostra pudessem ser comparados com os obtidos com DENV-2 NGC. O vírus foi inoculado por via i.c. nas diluições de 10^2 a 10^6 e a mortalidade dos animais foi acompanhada durante 21 dias. Os cálculos indicaram que a amostra de DENV-1 diluída 10^4 vezes corresponde a 3,2 LD_{50}. Foi estabelecida, então, a utilização de 30 μL da amostra de DENV-1 diluída 10^4 vezes, para os experimentos de desafio por via i.c..

Sendo assim, camundongos BALB/c (n = 10) foram inoculados por via i.m. com os plasmídeos pcTPANS1, ou pcTPA e desafiados por via i.c. com DENV-1, linhagem Mochizuki. Diferente do observado com DENV-2, os níveis de morbidade e sobrevivência dos animais vacinados e controles desafiados com DENV-1 foram semelhantes, sendo que todos os animais que apresentaram paralisia, posteriormente morreram. Somente 20% dos animais vacinados com pcTPANS1, sobreviveram ao desafio, semelhante ao detectado nos outros grupos (20% e 10% de sobrevivência nos camundongos inoculados com pcTPA e não imunizados, respectivamente) (Figura 4.4). A cinética do aparecimento dos sinais clínicos e mortalidade nos três grupos testados também se mostrou semelhante, com início da morbidade no 8º dia pós-desafio. A partir destes dados, concluímos que a inoculação com o plasmídeo pcTPANS1 não foi capaz de gerar uma resposta imune protetora contra DENV-1, sugerindo que o antígeno NS1 de DENV-2 não é capaz de promover uma proteção interespecífica contra DENV-1.

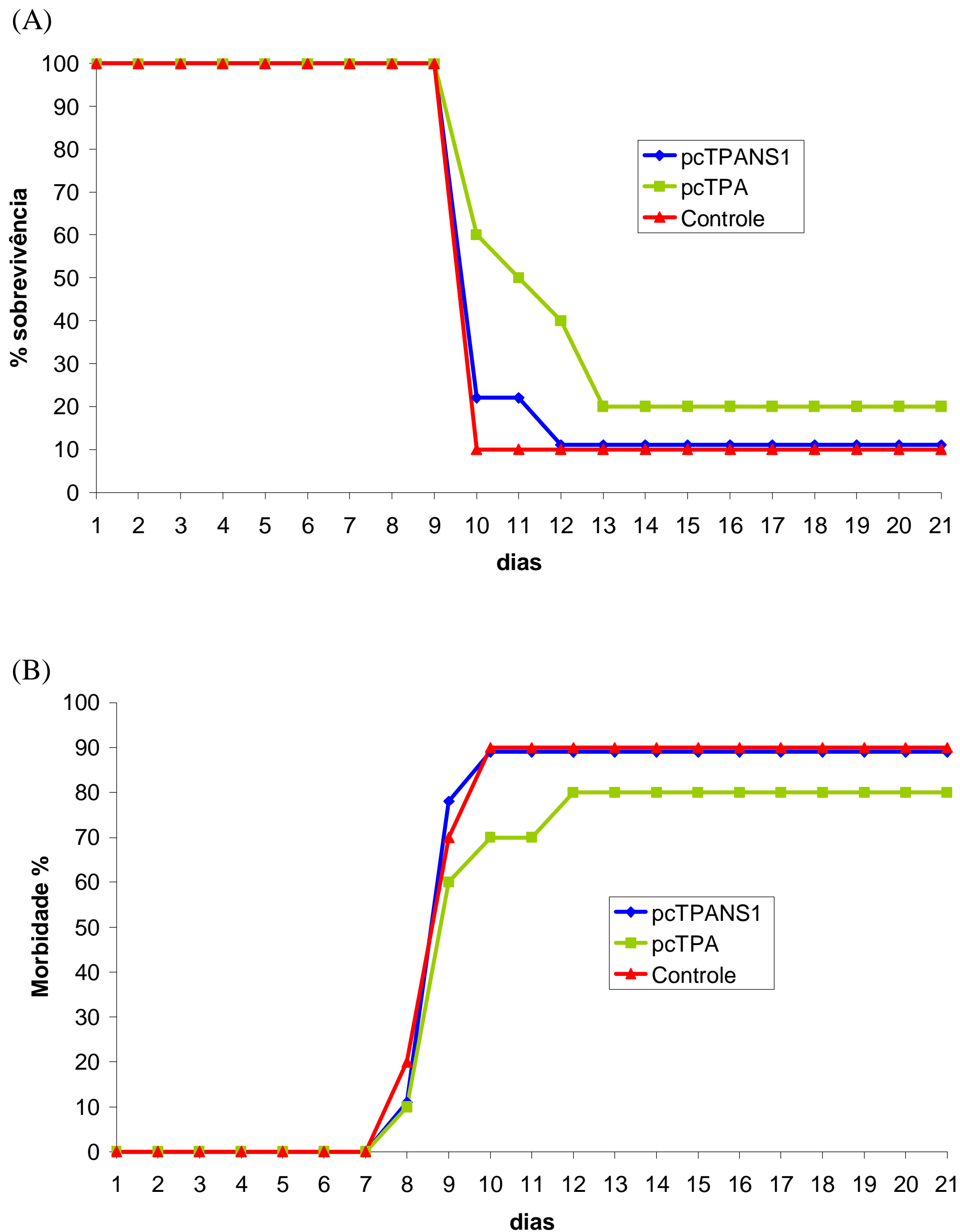

Figura 4.4: Porcentagem de sobrevivência (A) e morbidade (B) em camundongos BALB/c imunizados com o plasmídeo pcTPANS1 e desafiados por via i.c. com DENV-1. Grupos de camundongos (n = 10) foram inoculados por via intramuscular com duas doses de pcTPANS1 ou pcTPA e desafiados 4 semanas após a primeira dose. O controle representa os animais que foram somente inoculados com DENV-1.

4.II.3.2 – Análise do grau de similaridade entre seqüências da proteína NS1

A seqüência de aminoácidos da proteína NS1 da linhagem utilizada para clonagem do gene NS1, DENV-2 NGC, e da linhagem de DENV-1 Mochizuki foram alinhadas pelo programa Clustal W (Figura 4.5). Além destas seqüências, outras linhagens de DENV-1 e DENV-2, assim como linhagens de DENV-3 e DENV-4 foram alinhadas e o grau de similaridade entre as seqüências calculado.

```
DENV-1   DSGCVVNWKGRELKCGSGIFVTNEVHTWTEQYKFQADSPKRLSAAIGKAWEEGVCGIRSA 60
DENV-3   DMGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRVATAIAGAWENGVCGIRST 60
DENV-2   DSGCVVSWKNKELKCGSGIFITDNVHTWTEQYKFQPESPSKLASAIQKAHEEGICGIRSV 60
DENV-4   DMGCVASWSGKELKCGSGIFVVDNVHTWTEQYKFQPESPARLASAILNAHKDGVCGIRST 60
         *.***. *.  *********  .  ***********  **  .   ** .* ..* *****

DENV-1   TRLENIMWKQISNELNHILLENDMKLTVVVGDVSGILAQGKKMIGPQPMEHKYSWKSWGK 120
DENV-3   TRMENLLWKQIANELNYILWENDIKLTVVVGDITGVLEQGKRTLTPQPMELKYSWKTWGL 120
DENV-2   TRLENLMWKQITPELNHILSENEVKLTIMTGDIKGIMQAGKRSLQPQPTELKYSWKTWGK 120
DENV-4   TRLENVMWKQITNELNYVLWEGGHDLTVVAGDVKGVLTKGKRALTPPVSDLKYSWKTWGK 120
         **.** .****  ***..* *.  .**   **  *.   **    *.. . ***** **.

DENV-1   AKIIGADVQNTTFIIDGPNTPECPDDQRAWNIWEVEDYGFGIFTTNIWLKLRDSYTQVCD 180
DENV-3   AKIVTAETQNSSFIIDGPSTPECPSASRAWNVWEVEDYGFGVFTTNIWLKLREVYTQLCD 180
DENV-2   AKMLSTESHNQTFLIDGPETAECPNTNRAWNSLEVEDYGFGVFTTNIWLKLREKQDVFCD 180
DENV-4   AKIFTPEARNSTFLIDGPDTSECPNERRAWNSLEVEDYGFGMFTTNIWMKFREGSSEVCD 180
         **       * .* **** * ***   ****  ******** ******.*.*      **

DENV-1   HRLMSAAIKDSRAVHADMGYWIESEKNETWKLARASFIEVKTCVWPKSHTLWSNGVLESE 240
DENV-3   HRLMSAAVKDERAVHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWSNGVLESD 240
DENV-2   SKLMSAAIKDNRAVHADMGYWIESALNDTWKIEKASFIEVKSCHWPKSHTLWSNGVLESE 240
DENV-4   HRLMSAAIKDQKAVHADMGYWIESSKNQTWQIEKASLIEVKTCLWPKTHTLWSNGVLESQ 240
         .******.** .************  * .*.   **.**** * ***.***********

DENV-1   MIIPKIYGGPISQHNYRPGYSTQTAGPWHLGKLELDFDLCEGTTVVVDEHCGNRGPSLRT 300
DENV-3   MIIPKSLAGPISQHNHRPGYHTQTAGPWHLGKLELDFNYCEGTTVVISENCGTRGPSLRT 300
DENV-2   MIIPKNFAGPVSQHNYRPGYHTQTAGPWHLGKLEMDFDFCEGTTVVVTEDCGNRGPSLRT 300
DENV-4   MLIPKSYAGPFSQHNYRQGYATQTVGPWHLGKLEIDFGECPGTTVTIQEDCDHRGPSLRT 300
         *****.  ** ****.*.** ***.********* **. *.****.. * *..*******

DENV-1   TTVTGKVIHEWCCRSCTLPPLRFKGEDGCWYGMEIRPVKDKEENLVKSMVSA 352
DENV-3   TTVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPINEKEENMVKSLASA 352
DENV-2   TTASGKLITEWCCRSCTLPPLRYRGEDGCWYGMEIRPLKEKEENLVNSLVTA 352
DENV-4   TTASGKLVTQWCCRSCTMPPLRFLGEDGCWYGMEIRPLSEKEENMVKSQVTA 352
         **  ** . .*******.****  *************   ****.* * . *
```

Figura 4.5: Alinhamento da seqüência de aminoácidos de NS1 dos quatro sorotipos virais. As seqüências foram obtidas no GenBank, e alinhadas com o programa Clustal. Aqui estão representadas somente 4 seqüências de cada sorotipo. DENV-1 Mochizuki (BAB72261); DENV-2 NGC (M29095); DENV-3 (M93130); DENV-4 (AF326825). O símbolo (∗) representa os aminoácidos conservados nas 4 seqüências analisadas, enquanto (**.**) representa os aminoácidos semelhantes nas seqüências de DENV-1 e DEN-2.

As seqüências DENV-1 Mochizuki e DENV-2 NGC apresentaram similaridade de 74% (Figura 4.6). O grau de similaridade entre as seqüências de diferentes sorotipos variou entre 69% a 78%. Na figura 4.6 está representada apenas uma seqüência de cada sorotipo de DENV.

	DENV -1	DENV-2	DENV-3	DENV-4
DENV-1	**-**	**74**	**79**	69
DENV-2	**74**	**-**	**73,5**	73
DENV-3	**79**	**73,5**	**-**	73
DENV-4	69	73	73	-

Tabela 4.3: Matriz de similaridade entre as seqüências de aminoácidos da proteína NS1 dos 4 sorotipos de DENV. A matriz de similaridade foi obtida com o programa BioEdit. Os números indicam o grau de similaridade (%) entre as seqüências de aminoácidos de DENV-1 Mochizuki (BAB72261); DENV-2 NGC (M29095); DENV-3 (M93130); DENV-4 (AF326825).

4.II.3.3 – Indução de lise celular mediada pelo complemento na presença de anticorpos contra NS1

Para verificar se anticorpos contra NS1 poderiam causar a lise celular de células infectadas com DENV-2 através da interação com proteínas do complemento, foi realizado um experimento piloto de citotoxicidade. Duas amostras de soro contendo anticorpos contra NS1 (uma amostra obtida com a imunização com o pcTPANS1 e outra com o pcENS1) foram utilizadas com diferentes concentrações de proteínas do complemento, obtido em cobaia, e incubadas posteriormente com células Vero infectadas com DENV-2. Além disso, o soro de um camundongo inoculado com o pcDNA3 também foi utilizado como controle das reações. A porcentagem de lise das células Vero se mostrou maior quando estas foram colocadas em contato com as proteínas do complemento incubadas previamente com os soros anti-NS1 (maiores valores correspondente a aproximadamente 55% de lise) em comparação com a lise

ocorrida nas células mantidas somente com o complemento de cobaio (maiores valores correspondente a cerca de 40% de lise) (Figuras 4.5A e 4.5B). Em contraste, aparentemente a lise das células incubadas com soro de animais inoculados com o controle pcDNA3 foi semelhante na ausência ou presença de soro, indicando que esta não foi específica (Figura 4.5C). Resultados semelhantes foram obtidos com duas outras amostras de soro de animais imunizados com o pcTPANS1 e pcENS1 (dados não mostrados). Tais dados sugerem a participação do sistema complemento na proteção gerada pela imunização com estes plasmídeos. Entretanto, novos experimentos serão necessários, com um maior número de amostras de soro, para a comprovação de tais dados.

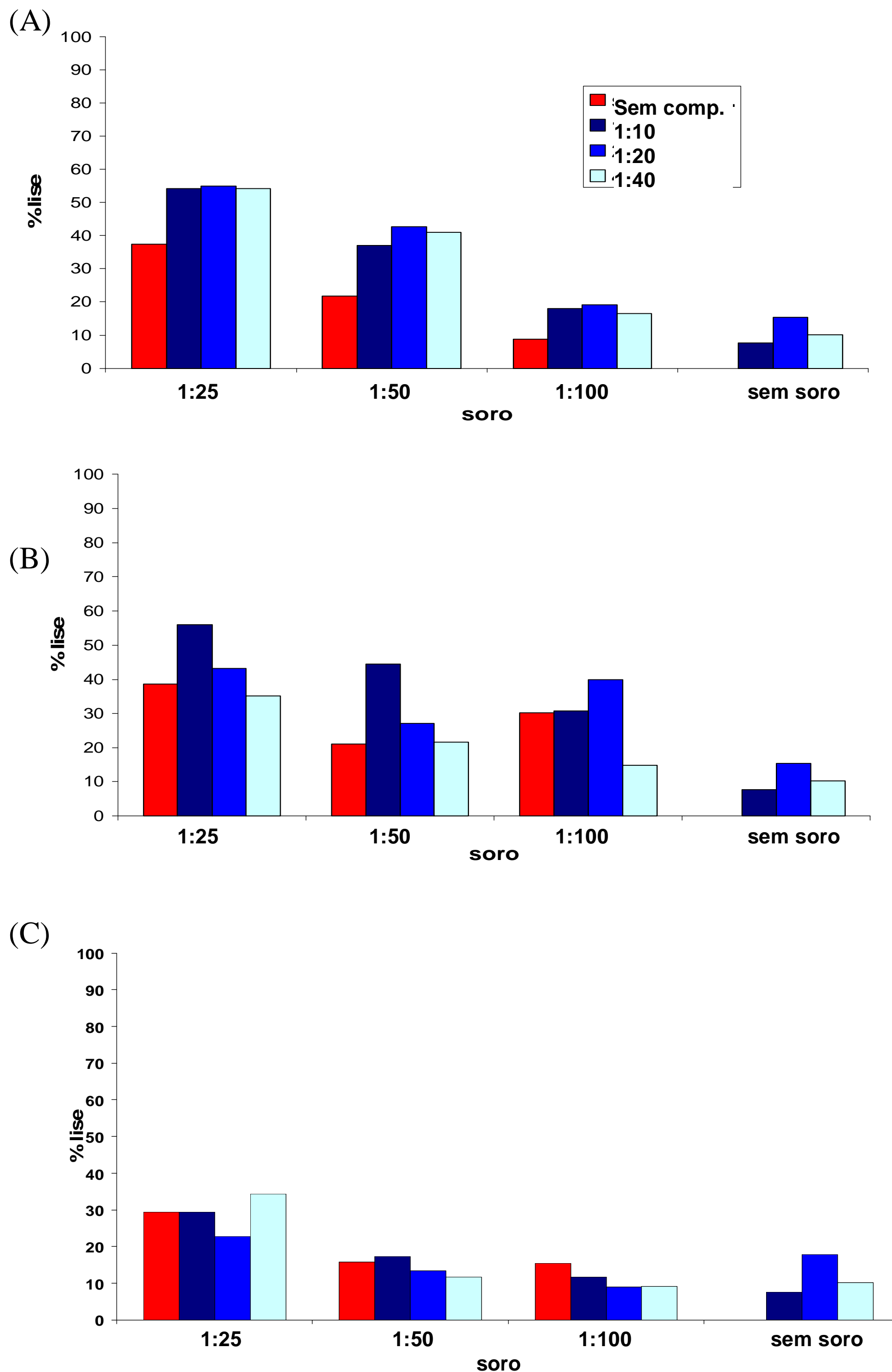

Figura 4.6: Porcentagem de lise de células Vero infectadas com DENV-2 mediada pelo complemento. Diluições seriadas do soro de animais inoculados com pcTPANS1 (A), pENS1 (B) ou pcDNA3 (C) foram incubadas com diferentes diluições do complemento de cobaio. A seguir, esta mistura foi incubada com Células Vero foram infectadas com DENV-2. A lise foi avaliada por liberação da enzima citoplasmática LDH. O eixo do X indica as diluições de soro utilizadas. A barra vermelha representa a amostra sem complemento, enquanto as outras barras referem-se ao soro incubado com diferentes diluições de complemento.

DISCUSSÃO

5 – DISCUSSÃO

Atualmente não há nenhuma vacina contra dengue comercialmente disponível capaz de gerar proteção contra os quatro sorotipos virais, apesar dos esforços de vários grupos.

As vacinas de DNA compreendem a inoculação de um vetor de expressão eucariótica contendo o antígeno de interesse, que é sintetizado *in vivo* pelas células do organismo inoculado, ativando uma resposta imune humoral e celular específicas e de longa duração (Ulmer *et al.* 1996; Robinson 1997; Liu & Ulmer, 2005). Uma das vantagens destas vacinas é a adição de seqüências sinalizadoras que direcionam a produção da proteína recombinante para diferentes compartimentos celulares, afetando assim a apresentação do antígeno e consequentemente alterando a resposta imune gerada específica (Rodriguez *et al.*, 1997; Alves *et al*, 1999; Anwar *et al.*, 2005; Donelly *et al.*, 2005; Dobanõ *et al.*, 2007).

Muitos grupos têm utilizado as vacinas de DNA como estratégia para o desenvolvimento de uma vacina contra dengue. Grande parte destes trabalhos é direcionada para a construção de uma vacina baseada na proteína E, que induza anticorpos neutralizantes contra os quatro sorotipos virais (Kochel *et al.*, 2000; Putnak *et al.*, 2003; Mota *et al.*, 2005; Konish *et al.*, 2006; Chen *et al.*, 2007b).

Neste trabalho, foram avaliadas vacinas de DNA baseadas em dois outros antígenos de DENV, as proteínas NS1 e NS3. A resposta imune humoral contra os flavivírus é direcionada predominantemente contra as proteínas virais E e NS1 (Chambers *et al.*, 1990; Brinton *et al.*, 1998). Contudo, uma vantagem em usar proteínas não estruturais no desenvolvimento de uma vacina seria a não indução de anticorpos envolvidos no fenômeno de aumento da replicação viral mediado pela resposta imune, que parecem ser direcionados contra a proteína E (Halstead & O´Rourke, 1977; Halstead, 1979; Stephenson, 2005). Assim, nós optamos pelo desenho de vacinas de DNA que codifiquem a proteína NS1, importante alvo da resposta imune humoral, e a proteína NS3, que parece ser particularmente imunogênica, com uma predominância de epítopos dominantes para linfócitos T CD4+ e CD8+, sendo alvo para a ação de células T citotóxicas (Mathew *et al.*, 1998; Rothman, 2004). Dessa forma, esta tese se baseou na hipótese de que o uso destas proteínas na constituição de uma vacina para dengue poderia gerar a combinação de uma resposta imune celular e humoral protetora contra a infecção pelo vírus da dengue.

Neste trabalho foram construídas vacinas de DNA baseadas nas proteínas NS1 e NS3 de DENV-2. Apesar da análise dos plasmídeos recombinantes indicarem que os plasmídeos que contêm a região NS3, assim como três das construções contendo o gene NS1 são capazes de direcionar a expressão *in vitro* destes antígenos, foram obtidos resultados mais promissores com a proteína NS1. Deste modo, os experimentos relativos à NS1 progrediram mais rapidamente do que a avaliação do antígeno NS3, compreendendo a maior parte dos resultados desta tese. Sendo assim, esta seção será dividida em duas partes: a parte 1 aborda os resultados relativos à NS3, enquanto que a parte 2 compreende os resultados obtidos com o antígeno NS1.

5.1 – Antígeno NS3

A estratégia de criação de vacinas de DNA baseadas na proteína NS3 levou em consideração a presença dos domínios funcionais desta proteína que atuam durante o ciclo de replicação do vírus da dengue. Na porção C-terminal da NS3 estão localizados os domínios helicase/NTPase/RTPase, enquanto que sua região N-terminal compreende um domínio serino protease que, quando associado à proteína viral NS2B, promove a clivagem de alguns sítios da poliproteína viral precursora durante a replicação (Valle & Falgout, 1998; Li *et al.*, 1999; Murthy *et al.* 1999; Niyomrattanakit *et al.,* 2004; Xu *et al.,* 2005; Sampath *et al.,* 2006; Qi *et al.*, 2008). Além disso, a proteína NS3 pode ser clivada naturalmente em células infectadas pelo vírus da dengue (Arias *et al.*, 1993; Teo & Wright, 1997). Baseado nestas informações e na possibilidade de alguns destes domínios poderem modificar o metabolismo da célula hospedeira, foram construídos 5 plasmídeos recombinantes contendo seqüências que codificam a proteína NS3 inteira de DENV-2 ou partes desta, visando estudar o seu potencial imunogênico e protetor.

De acordo com a análise por imunofluorescência de células transfectadas com as construções pcTPANS3, pcTPANS3P, pcTPANS3H, pcTPANS3N e pcTPANS3C, esses plasmídeos foram capazes de mediar a expressão das proteínas recombinantes. Contudo, apesar de não termos quantificado tais expressões, em todas as transfecções observamos, por imunofluorescência, um número reduzido de células expressando NS3, quando comparado aos resultados obtidos com NS1 (dados não mostrados). Tal fato pode estar relacionado a uma toxicidade destas proteínas contendo domínios funcionais capazes de alterar a fisiologia das células transfectadas, ocasionando a sua morte. De fato, alguns trabalhos sugerem que a proteína NS3 pode induzir apoptose em células

infectadas (Duarte dos Santos *et al.*, 2000; Shafee & AbuBakar, 2003). Esses plasmídeos podem futuramente serem utilizados em estudos para avaliação da participação da proteína NS3 na apoptose celular.

Os plasmídeos contendo o gene NS3 geraram diferentes níveis de proteção contra DENV-2 nos testes de desafio por via i.c. Entretanto, nenhuma dessas construções induziu níveis aceitáveis de proteção, quando os parâmetros de morbidade foram considerados. A inoculação dos plasmídeos pcPTANS3N e pcTPANS3C, que correspondem às regiões N-terminal e C-terminal da proteína NS3 geraram os maiores níveis de sobrevivência, contudo grande parte destes animais apresentou quadros de paralisia. Resultados semelhantes foram observados com o plasmídeo pcTPANS3H, sendo que os níveis de sobrevivência foram ainda menores. Por outro lado, quando avaliamos a construção pcTPANS3, que codifica a proteína NS3 inteira, os níveis de proteção, tanto em termos de morbidade quanto de mortalidade, foram bem mais baixos. O plasmídeo pcTPANS3P, que codifica o domínio protease na porção N-terminal da proteína, não gerou qualquer nível de proteção, uma vez que os valores de morbidade e sobrevivência dos animais inoculados com este plasmídeo foram semelhantes aos obtidos nos animais controle, sugerindo que determinantes de proteção concentram-se principalmente na porção contendo os domínios helicase/NTPase/RTPase. Essa construção contém somente os 185 aminoácidos do N-terminal da proteína NS3 e pode não ser tão efetiva na indução de uma resposta imune. De fato, segundo Brinton e colaboradores (1998), a maioria dos epítopos para células B e T, em humanos, está localizada a partir do aminoácido 200 de NS3.

Os níveis insatisfatórios de proteção gerados pelas vacinas de DNA com NS3 podem estar relacionados à baixa eficiência de expressão das proteínas recombinantes *in vivo*, semelhante ao que observamos *in vitro,* o que levaria a uma fraca resposta imune no hospedeiro. Além disso, esses plasmídeos contêm a seqüência do peptídeo sinal t-PA, que leva à secreção das proteínas recombinantes. Apesar da secreção de proteínas mediada por vacinas de DNA ser eficiente para induzir uma resposta imune humoral, tais vacinas podem não ser ideais para a ativação de uma resposta imune celular. Portanto, como a NS3 é uma proteína que se localiza no citoplasma das células infectadas e possui principalmente epítopos indutores de linfócitos T (Kurane *et al.*, 1998; Mathew *et al.*, 1996; Mathew *et al.*, 1998; Rothman, 2004), os plasmídeos construídos no presente trabalho podem não ser os mais adequados para mediar a expressão da NS3 em um compartimento celular, de maneira a induzir uma resposta

imunológica apropriada. Além destes plasmídeos, também construímos outros vetores semelhantes aos primeiros sem a seqüência sinal t-PA. Entretanto, não foi possível detectar a expressão *in vitro* de NS3 mediada por tais plasmídeos, provavelmente, devido às características tóxicas desta proteína (dados não mostrados).

Em um estudo realizado com uma vacina de DNA com o gene NS3 do flavivírus TBE também não foi observada proteção em camundongos, apesar de terem sido detectados anticorpos contra NS3 (Morozova *et al.,* 1999). Em outro estudo, de imunização passiva com anticorpos anti-NS3 de DENV-1, Tan e colaboradores (1990) observaram um nível muito baixo de proteção, baseado no aumento do tempo de sobrevida de camundongos. Em contradição com a maioria dos estudos com NS3, que indicam que esta proteína induz primordialmente uma resposta imune celular, esses dois trabalhos se basearam na proteção mediada por anticorpos, o que parece não ser a melhor abordagem.

Portanto, para uma maior compreensão de uma possível proteção mediada pela proteína NS3, serão necessários novos experimentos visando a avaliação das respostas imunes celulares induzidas pelas vacinas de DNA descritas na presente tese.

5.2 – Antígeno NS1

A proteína NS1 é indicada como um antígeno promissor para o desenvolvimento de uma vacina contra a dengue. Estudos com NS1 de diferentes flavivírus, expressa *in vitro* em *E. coli* e baculovírus, ou *in vivo*, por vírus recombinantes e vacinas de DNA, observaram que esta proteína é capaz de induzir uma resposta imune humoral protetora mediada por anticorpos não-neutralizantes (Qu *et al.*, 1993; Lin *et al.*, 1998, Aleshin *et al.*, 2005; Chung *et al.*, 2006; Lin *et al.*, 2008). Os dados apresentados no presente trabalho indicam que a imunização com uma vacina de DNA baseada na proteína NS1 de DENV-2 é capaz de gerar altos níveis de proteção intraespecífica em camundongos.

Durante a infecção viral, a glicoproteína NS1 é encontrada tanto no interior das células infectadas quanto associada à membrana plasmática destas células, na forma de dímeros. Além disso, a NS1 também é secretada para o meio extracelular sendo encontrada em formas oligoméricas como hexâmeros. A região C-terminal da proteína E atua como peptídeo sinal para translocação da NS1 pelo retículo endoplasmático (Winkler *et al* 1989; Pryor and Wright, 1993; Flamand *et al.*, 1999; Young *et al.*, 2000). Por outro lado, antes dos nossos achados, alguns estudos já sugeriam que a associação

da proteína NS1 à membrana da célula se dava provavelmente via a seqüência hidrofóbica que corresponde à porção C-terminal da proteína NS2A, que parece funcionar como seqüência sinal para ligação via âncora GPI (Falgout *et al.*, 1989; Jacobs *et al.*, 2000). Com o objetivo de mimetizar as formas de NS1 produzidas durante a infecção do vírus da dengue, foram construídos 4 plasmídeos recombinantes contendo o gene NS1 fusionado a diferentes seqüências sinalizadoras a jusante e a montante deste gene. Duas construções codificam o peptídeo sinal natural utilizado pela proteína NS1 para sua secreção durante a infecção viral, pcENS1 e pcENS1ANC. Uma destas construções contém, a jusante ao gene NS1, a seqüência que codifica os primeiros 46 aminoácidos da proteína NS2A (pcENS1ANC). Os outros dois plasmídeos recombinantes (pcTANS1 e pcTANS1ANC) são semelhantes aos primeiros, porém contêm a seqüência que codifica o peptídeo sinal heterólogo t-PA, ao invés da seqüência que codifica a região C-terminal da proteína E.

A expressão *in vitro* da proteína NS1 codificada por esses plasmídeos variou bastante. Somente 3 construções (pcTPANS1, pcENS1 e pcENS1ANC) foram capazes de mediar a expressão da proteína NS1. Por outro lado, aparentemente a presença das seqüências hodrofóbicas t-PA e ANC em uma mesma construção (pcTPANS1ANC) se mostrou incompatível. É possível que estas duas seqüências levem a uma conformação que impossibilite o seu processamento correto e conseqüente esta proteína se acumule na célula, tornando-se tóxica. Foi observado que a proteína NS1 codificada pelo pcENS1ANC migrou um pouco mais lentamente no gel contendo o extrato das células transfectadas. Tal diferença deve-se à presença da seqüência ANC nesta proteína recombinante.

Os plasmídeos pcTPANS1, pcES1 e pcENS1ANC também foram capazes de mediar a secreção da proteína recombinante, encontrada na forma dimérica no sobrenadante das culturas celulares. Estes resultados demonstram que tanto o peptídeo sinal natural da NS1 quanto o peptídeo heterólogo t-PA foram eficientes em direcionar a proteína NS1 pela via do retículo endoplasmático e sua posterior secreção. De fato, estudos mostram que a secreção desta proteína é dependente da sua dimerização Somado a isso, a formação de dímeros da proteína NS1 sugere que esta proteína foi corretamente glicosilada, uma vez que a estabilidade destes dímeros é influenciada pela adição dos grupamentos de açúcar (Pryor & Wright, 1993; Pryor & Wright, 1994; Flamand *et al.*, 1999). As análises por microscopia eletrônica demonstraram a presença de NS1 na membrana plasmática somente nas células transfectadas com o plasmídeo

pcENS1ANC, confirmando que a região N-terminal de NS2A é importante para a associação de NS1 à membrana plasmática de células infectadas por DENV.

Para avaliar o potencial imunogênico destas vacinas, camundongos BALB/c foram inoculados com os plasmídeos recombinantes. Inicialmente, o protocolo a ser adotado nos experimentos de imunização e desafio foi avaliado, utilizando imunizações pelas vias i.d. ou i.m., com duas ou três doses do plasmídeo pcTPANS1. As imunizações por via i.m. induziram maiores níveis de anticorpos, com um menor número de doses (2 doses). Apesar da administração i.m. de três doses de DNA ter gerado títulos maiores de anticorpos durante o pico da resposta imune humoral (6ª semana), a partir da 8ª semana do experimento, os níveis de anticorpos gerados com duas ou três doses, se mostraram semelhantes e permaneceram estáveis até a 10ª semana. Deste modo, optamos por administrar duas doses de DNA por via i.m., utilizando assim menor quantidade de DNA.

A inoculação dos plasmídeos pcTPANS1 e pcENS1 induziu uma resposta imune humoral homogênea, com altos títulos de IgG contra NS1 em todos os camundongos imunizados. Os níveis desses anticorpos foram mais altos do que os observados por Lemes e colaboradores (2005) no soro de pacientes com infecção primária ou secundária por DENV, utilizando o mesmo antígeno (proteína NS1 produzida em cultura de células de drosófila) e protocolo semelhante de ELISA (Anexo 1). Esses dados estão de acordo com estudos que indicam que a presença de peptídeos sinais, que direcionam a proteína recombinante para via do retículo endoplasmático e a sua secreção, induzem altos níveis de anticorpos (Inchauspe *et al.*, 1997; Alves *et al.*, 1999, Alves *et al.*, 2000).

Por outro lado, os camundongos inoculados com pcENS1ANC apresentaram uma resposta imune humoral mais baixa e heterogênea, em que somente metade dos animais produziram níveis detectáveis de anticorpos. Essa diferença na resposta de anticorpos contra NS1 pode estar relacionada às características de expressão da proteína NS1 em células transfectadas pelo pcENS1ANC. Parte da proteína NS1 produzida por estas células é retida na membrana plasmástica, conforme observado nos experimentos de expressão *in vitro*, disponibilizando assim uma quantidade menor de proteína solúvel. Além disso, apesar de não ter sido realizada a quantificação de proteínas nestes experimentos, foi perceptível que a quantidade de NS1 presente no extrato celular e sobrenadante da cultura de células transfectadas com pcENS1ANC se mostrou menor

do que a observada, nas mesmas condições, para os outros dois plasmídeos recombinantes, pcTPANS1 e pcENS1.

A cinética de resposta imune humoral após a imunização com os três plasmídeos recombinantes mostrou-se semelhante, com picos da resposta duas semanas após a segunda dose da vacina, seguida de níveis constantes de anticorpos até a décima semana do experimento. Contudo, conforme esperado, os níveis de anticorpos induzidos com pcENS1ANC foram menores do que com os gerados com pcTPANS1 e pcENS1. Em todas as imunizações, os anticorpos gerados reconheceram principalmente epítopos conformacionais da proteína NS1, uma vez que a reação contra NS1 desnaturada foi cerca de 30 vezes mais baixa quando comparada ao reconhecimento da proteína nativa. Resultados semelhantes foram observados quando avaliamos a reatividade dos anticorpos presentes no soro de animais imunizados com o plasmídeo pcTPANS1 contra a proteína NS1 produzida em *E. coli*. Neste sistema de expressão não ocorre a glicosilação das proteínas e consequentemente a NS1 produzida não adquire a conformação semelhante à observada durante a infecção viral. A NS1 produzida por células infectadas com o vírus da dengue é uma glicoproteína, cuja conformação tri-dimensional e secreção são influenciadas pela sua correta glicosilação (Pryor & Wright, 1994). Portanto, nossos resultados sugerem que a NS1 expressa em camundongo, mediada pela transfecção de células do hospedeiro com tais plasmídeos, deve estar sendo glicosilada e apresentada ao sistema imune de forma similar a que ocorre na infecção por DENV.

A detecção de anticorpos em camundongos inoculados com os plasmídeos pcTPANS1 e pcENS1 56 semanas após o início do experimento, mostra que a imunização com estes plasmídeos é capaz de gerar uma resposta imune de longa duração. Somado a isso, a inoculação de uma dose reforço induziu um aumento de mais de 17 vezes nos títulos de anticorpos, indicando a presença de células de memória.

A ausência de um modelo animal que reflita as formas clássica e severa da dengue tem sido um obstáculo para estudar alguns aspectos da patogênese da doença e a capacidade protetora de vacinas. A adaptação do vírus ao modelo animal é uma forma útil para avaliar a proteção conferida por candidatos a vacina. Assim, as fases iniciais de testes pré-clínicos com vacinas contra dengue e outros flavivírus utilizam principalmente desafios por via intracerebral com vírus neuroadaptados (Gould *et al.*, 1986, Falgout *et al.*, 1990; Lin *et al.*, 1998; Wu *et al.*, 2003; Bente & Rico-Hesse, 2006; Chen *et al.*, 2007; Guy & Almond, 2007; Lazo *et al,* 2007). Apesar da utilidade deste

modelo, os sinais clínicos avaliados, como paralisia dos membros posteriores, não refletem os sintomas ocorridos durante a infecção viral por DENV. Neste trabalho, nós avaliamos a indução de proteção pela inoculação da vacina pcTPANS1 tanto pelo desafio de camundongos por via intracerebral, quanto através de um modelo proposto por Paes e colaboradores (2006) (Anexo 2), em que camundongos BALB/c foram inoculados por via intraperitoneal com DENV-2 não adaptado a camundongos. Neste modelo, os animais apresentam danos focais no fígado, caracterizado por análises histopatológicas e bioquímicas, assim como em outros órgãos, com o pico da viremia ocorrendo no sétimo dia de infecção. Esses danos correlacionam-se com o observado na infecção em humanos (Anexos 2 e 3).

A imunização com o pcTPANS1 foi capaz de gerar proteção nos dois modelos murinos avaliados. No desafio por via intraperitoneal, os dados histopatólógicos evidenciaram uma diminuição tanto na intensidade quanto na extensão dos danos no fígado dos animais vacinados, em comparação com os controles. Tais resultados indicam que a vacina de DNA pcTPANS1, que contém o gene NS1 da cepa Nova Guiné de DENV-2, é capaz de induzir proteção contra uma linhagem de vírus circulante no Brasil.

Nos testes de desafio por via i.c., além do pcTPANS1, também foi avaliada a proteção gerada pelo pcENS1. O plasmídeo pcENS1ANC não foi utilizado devido ao fato deste DNA ter gerado uma resposta imune humoral muito heterogênea. A imunização com os plasmídeos pcENS1 ou pcTPANS1 foi capaz de gerar uma resposta imune protetora contra DENV-2. Observamos que cerca de 87% e 97% dos camundongos vacinados com pcENS1 e pcTPANS1, respectivamente, sobreviveram ao desafio. O alto nível de proteção também foi observado quando avaliamos a morbidade. Somente 10% e 27% dos animais imunizados com pcTPANS1 e pcENS1, respectivamente, apresentaram algum sinal clínico. Essas freqüências foram bem diferentes das observadas nos animais controles, em que 90% dos camundongos apresentaram paralisia ou morreram após o desafio. Além disso, a gravidade da paralisia desenvolvida pelos animais controles foi muito maior, com acometimento das duas patas posteriores, quando comparada com a observada nos animais vacinados, em que geralmente só um dos membros posteriores era levemente afetado (dados não mostrados). Houve também um atraso no aparecimento dos sinais clínicos dos animais imunizados com pcTPANS1 (9º dia após o desafio), em relação ao grupo controle e aos animais vacinados com pcENS1 (7º dia). Esses dados sugerem que a imunização com o

pcTPANS1 é levemente mais protetora do que a inoculação com o pENS1, apesar das diferenças entre os dois plasmídeos não terem sido estatisticamente significantes.

Wu e colaboradores (2003) também avaliaram uma vacina de DNA baseada no gene NS1 de uma linhagem de DENV-2 isolada de Taiwan e demonstraram proteção em experimentos de desafio. Neste trabalho, camundongos C3H foram inoculados com uma vacina semelhante ao pcENS1, diferindo deste pela ausência de dois aminoácidos no peptídeo sinal correspondente a porção C-terminal da proteína E. Além disso, o protocolo de experimentação utilizado por tais autores foi um pouco diferente, com a inoculação por via i.m. de 3 doses de 80μg do plasmídeo recombinante, no intervalo de 1 semana, e desafio por via intravenosa com DENV-2. Os autores observaram que a inoculação com a vacina de DNA induziu 82% de sobrevivência, em contraste com 30% detectado no grupo controle. Tais valores se mostraram semelhante ao observado nos nossos experimentos com o pcENS1.

A avaliação da resposta imune humoral, antes e após o desafio com DENV-2, por via i.c., mostrou que houve um aumento de mais de 5 vezes nos títulos de IgG contra NS1 após o desafio. Esse aumento sugere que ocorreu algum nível de replicação viral nos animais desafiados. Na realidade, é esperado que a proteção via NS1 não seja neutralizante, visto que esta proteína não está presente na partícula viral e só é produzida após a célula ter sido infectada pelo vírus. Consequentemente, a proteção via NS1 só pode ocorrer após a replicação inicial do vírus. De fato, conforme o esperado, os soros dos animais vacinados com pcENS1 ou pcTPANS1 não foram capazes de neutralizar a entrada de DENV-2 em células Vero (dados não mostrados).

A análise do perfil de imunoglobulinas antes e após o desafio com DENV-2 mostra a indução de perfis diferentes de resposta imume induzida pelos plasmídeos pcENS1 e pcTPANS1. Enquanto o pcTPANS1 induziu preferencialmente anticorpos anti-NS1 da subclasse IgG1, a inoculação com pcENS1 levou a um equilíbrio de IgG1 e IgG2a. Por outro lado, os animais que receberam somente o vírus e sobreviveram ao desafio, a maioria destes apresentando grave paralisia dos membros posteriores, produziram níveis maiores de IgG2a. Esta variação na resposta imune induzida pelas diferentes vacinas pode estar relacionada a maior proteção gerada pelo pcTPANS1.

Também foi avaliada a indução de proteção interespecifica em camundongos imunizados com o plasmídeo recombinante pcTPANS1 (DENV-2) e posteriormente desafiados por via i.c. com DENV-1. A cinética de morbidade e mortalidade nos grupos de animais vacinados e controles se mostraram semelhantes, com o início dos sinais

clínicos e mortalidade sendo detectados concomitantemente em ambos os grupos. Esse resultado demonstra que a inoculação com o plasmídeo pcTPANS1 não foi capaz de gerar uma resposta imune protetora contra DENV-1, sugerindo que uma vacina de DNA utilizando como antígeno a proteína NS1 e baseada em apenas um sorotipo viral não é capaz de proteger contra todos outros sorotipos do vírus da dengue. Por outro lado, tais dados indicam que, apesar de não protetora, a vacina pcTPANS1 não levou ao agravamento da doença gerada por DENV-1.

A NS1 é uma proteína conservada entre os flavivírus. Se não considerarmos possíveis mudanças que possam ter ocorrido na seqüência desta proteína durante as passagens de DENV-1 pelo cérebro de camundongos, o alinhamento das seqüências de aminoácidos de NS1 das linhagens DENV-2 Nova Guiné e DENV-1 Mochizuki mostra que estas proteínas apresentam 74% de similaridade. Tal nível de variabilidade é encontrado geralmente entre seqüências de NS1 dos diferente sorotipos de dengue conforme observado neste trabalho e descrito por Deubel *et al.* (1988). Portanto, é esperado que também não ocorra proteção cruzada em experimentos com outros sorotipos virais. Nossos resultados estão de acordo com Schlesinger e colaboradores (1987), que mostraram que camundongos imunizados com a proteína NS1 de DENV-2 não apresentaram proteção em testes de desafio com DENV-1.

Alguns trabalhos sugerem que anticorpos contra NS1 podem ter um papel patogênico na infecção por DENV. O complexo formado por NS1 e anticorpos contra esta proteína em uma infecção secundária poderiam ativar o complemento de forma a levar ao extravasamento vascular. Esses anticorpos poderiam também reagir de forma cruzada com antígenos presentes na superfície de células endoteliais e induzir apoptose, levando a danos vasculares que favoreceriam à patogênese da doença (Avirutam *et al.*, 1998; Falconar, 1999). Contudo nos experimentos de desafio por via i.p., em que as vacinas de DNA induziram altos níveis de anticorpos conta NS1, não foi observado um agravamento dos danos vasculares nos animais imunizados somente com o plasmídeo pcTPANS1. Além disso, a cinética da presença de anticorpos contra NS1 no soro de pacientes infectados por DENV durante e após a fase aguda da infecção viral não se correlaciona com o tempo de extravasamento de plasma, que ocorre nos casos mais severos da doença (Stephenson, 2005; Green & Rothman, 2006). Portanto, a hipótese de que anticorpos contra NS1 que possam levar a danos vasculares não se mantém, quando levamos em consideração esses dados.

Ainda não está estabelecido de que forma a proteína NS1 é capaz de gerar proteção nos modelos animais. Diversos estudos de imunização passiva demonstram que anticorpos contra NS1 de dengue ou de outros flavivírus são capazes de proteger contra desafios com doses letais desses flavivírus (Schlesinger *et al.*, 1986, Schlesinger *et al.*, 1987; Gould *et al.*, 1986; Falgout *et al.*, 1990; Jacobs *et al.*, 1992; Lin *et al.*, 1998; Aleshin *et al.*, 2005; Chung *et al.*, 2006; Lin *et al.*, 2008). Esses trabalhos evidenciam que a resposta de anticorpos contra a proteína NS1 tem papel importante na proteção. Entretanto, não ainda se sabe o mecanismo de proteção desempenhado por esses anticorpos.

A lise de células infectadas mediada pelo complemento é o mecanismo mais aceito para explicar proteção gerada por anticorpos contra NS1. Tal explicação é baseada nas observações iniciais de que anticorpos com atividade de fixação do complemento da febre amarela podem proteger parcialmente camundongos contra o desafio com o vírus (Schlesinger *et al.*, 1985). Outros trabalhos utilizando proteínas NS1 de diferentes flavivirus confirmaram a participação do sistema complemento na proteção gerada por esta proteína (Schlesinger *et al.*, 1987; Falgout *et al.*, 1990; Qu *et al.*, 1993; Lin *et al.*, 1998). Contudo, alguns estudos de imunização passiva em animais deficientes de proteínas que fazem parte do sistema complemento, utilizando os vírus TBE, febre amarela e WNV, sugerem que a proteção via anticorpos contra NS1 não depende do complemento (Schlesinger *et al.*, 1993; Jacobs *et al.* 1994; Chung *et al*, 2006a).

Portanto, com o objetivo de estudar se anticorpos presentes no soro dos animais imunizados com pcENS1 ou pcTPANS1 podem levar à lise celular dependente do complemento, foi realizado um teste inicial de avaliação de lise celular, baseado no trabalho de Lin e colaboradores (1998). O estudo destes autores mostrou que o soro de animais imunizados com uma vacina de DNA contra o vírus da encefalite japonesa (JEV) baseada na proteína NS1 apresentou atividade citolítica contra as células infectadas, de maneira dependente do complemento. Em nosso experimento piloto foi observada a lise celular, quando o soro de animais inoculados com o pcENS1 ou o pcTPANS1 foi colocado em contato com células Vero infectadas com DENV-2, na presença do complemento de cobaio. Por outro lado, o soro dos animais controles não gerou lise específica na presença deste complemento. Tais resultados sugerem que a lise de células infectadas, mediada por anticorpos contra NS1 e dependente do sistema complemento, pode ser um dos mecanismos envolvidos na proteção gerada pela

imunização com os plasmídeos pcENS1 e pcTPANS1. Entretanto, são necessários novos experimentos, com um número maior de amostras de soro, para confirmar estes dados.

Por outro lado, Chung e colaboradores (2006b) demonstraram que a NS1 de WNV se liga a uma proteína reguladora do complemento, proteína fH, interferindo com a função de C3b. Os autores sugerem que este pode ser um mecanismo utilizado pelo patógeno para evadir do sistema imune do hospedeiro. Se tal mecanismo realmente ocorrer, anticorpos contra NS1 podem bloquear a interação desta proteína com fH, minimizando uma possível função imunomoduladora da NS1 (Schlesinger, 2006). Apesar de aparentemente anticorpos contra NS1 de diferentes flavivirus terem ação protetora, não se sabe se outros mecanismos poderiam também estar envolvidos nesta proteção, como por exemplo, a ativação de uma resposta imune celular envolvendo células T citotóxicas.

Em resumo, nosso trabalho demonstrou que as vacinas de DNA pcENS1 e pcTPANS1, baseadas na proteína NS1 de DENV-2, são capazes de induzir altos títulos de anticorpos específicos contra essa proteína e conferem proteção intra-específica em camundongos desafiados com DENV-2. Os resultados estimulam a utilização da proteína NS1 em uma abordagem utilizando vacinas de DNA contra a dengue. Sendo assim, temos como perspectivas para a continuação deste trabalho, a construção e análise de vacinas de DNA contendo o gene NS1 dos outros sorotipos virais, assim como a avaliação dos mecanismos de proteção gerados por estas vacinas.

CONCLUSÕES

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- Todas as vacinas de DNA codificando a proteína NS3 ou seus domínios foram capazes de mediar a expressão *in vitro* das proteínas recombinantes.
- As vacinas de DNA baseadas no antígeno NS3 não foram capazes de gerar uma proteção satisfatória em camundongos desafiados por via i.c. com DENV-2
- A maioria das vacinas de DNA baseadas em NS1 (pcTPANS1, pcENS1 e pcENS1ANC) foram capazes de mediar a expressão *in vitro* da proteína NS1.
- O plasmídeo que codifica a NS1 franqueada pela seqüência sinal t-PA e a seqüência N-terminal da NS2A (pcTPANS1ANC) não foi capaz de mediar a expressão *in vitro* de NS1.
- As seqüências t-PA e E funcionaram como peptídeos sinais nas construções pcTPANS1, pcENS1 e pcENS1ANC, direcionando a secreção da proteína NS1 para o meio extracelular na forma de dímeros.
- A expressão de NS1 associada à membrana plasmática das células transfectadas foi obtida somente com a construção pcENS1ANC confirmando a importância da seqüência N-terminal da proteína NS2A nesta interação.
- As inoculações com pcTPANS1 ou pcENS1 foram capazes de induzir altos níveis de anticorpos específicos contra epítopos conformacionais da proteína NS1, em todos os animais imunizados.
- A resposta de anticorpos gerada com o plasmídeo pcENS1ANC se mostrou muito heterogênea, e somente metade dos animais apresentou níveis detectáveis de anticorpos contra NS1.
- A imunização com o pcTPANS1 conferiu proteção em camundongos inoculados por via i.p. com uma linhagem de DENV-2 circulante no Brasil, não adaptada a camundongos.
- A imunização com o plasmídeo pcTPANS1 ou pcENS1 foi capaz de induzir proteção contra doses letais de DENV-2 neuroadaptado inoculado por via i.c. Estes plasmídeos induziram a produção de subclasses diferentes de IgG contra NS1.

- A vacina de DNA baseada em NS1 de DENV-2 pcTPANS1 não promoveu resposta protetora cruzada para DENV-1
- Os anticorpos gerados com as imunizações com o pcTPANS1 ou o pcENS1 foram capazes de levar à lise de células infectadas com DENV-2, em um mecanismo dependente de proteínas do complemento.

REFERÊNCIAS

7 - REFERÊNCIAS BIBLIOGRÁFICAS

Aleshin SE, Timofeev AV, Khoretonenko MV, Zakharova LG, Pashvykina GV, Stephenson JR, Shneider AM, Altstein AD. 2005. Combined prime-boost vaccination against tick-borne encephalitis (TBE) using a recombinant vaccinia virus and a bacterial plasmid both expressing TBE virus non-structural NS1 protein. *BMC Microbiol*. 5: 45.

Alves AM, Lásaro MO, Almeida DF, Ferreira LC. 1999. New vaccine strategies against enterotoxigenic Escherichia coli. I: DNA vaccines against the CFA/I fimbrial adhesin. *Braz J Med Biol Res.* 32(2): 223-229.

Alves AM, Lásaro MO, Almeida DF, Ferreira LC. 2000. DNA immunisation against the CFA/I fimbriae of enterotoxigenic Escherichia coli (ETEC). Vaccine. 19(7-8): 788-795.

Anwar A, Chandrasekaran A, Ng ML, Marques E, August JT. 2005. West Nile premembrane-envelope genetic vaccine encoded as a chimera containing the transmembrane and cytoplasmic domains of a lysosome-associated membrane protein: increased cellular concentration of the transgene product, targeting to the MHC II compartment, and enhanced neutralizing antibody response. *Virology*. 332(1): 66-77.

Appanna R, Huat TL, See LL, Tan PL, Vadivelu J, Devi S. 2007. Cross-reactive T-cell responses to the nonstructural regions of dengue viruses among dengue fever and dengue hemorrhagic fever patients in Malaysia. *Clin Vaccine Immunol.* 14(8): 969-977.

Arias CF, Preugschat F, Strauss JH. 1993. Dengue 2 virus NS2B and NS3 form a stable complex that cleave NS3 within the helicase domain. *Virology* 193: 888-899.

Avirutnan P, Malasit P, Seliger B, Bhakdi S, Husmann M. 1998. Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis. *J Immunol* 161(11): 6338-6346.

Avirutnan P, Punyadee N, Noisakran S, Komoltri C, Thiemmeca S, Auethavornanan K, Jairungsri A, Kanlaya R, Tangthawornchaikul N, Puttikhunt C, Pattanakitsakul SN, Yenchitsomanus PT, Mongkolsapaya J, Kasinrerk W, Sittisombut N, Husmann M, Blettner M, Vasanawathana S, Bhakdi S, Malasit P. 2006. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. *J Infect Dis* 193(8): 1078-1088.

Barrett AD. 2001. Current status of flavivirus vaccines. *Ann N Y Acad Sci*. 951: 262-271.

Basílio-de-Oliveira CA, Aguiar GR, Baldanza MS, Barth OM, Eyer-Silva WA, Paes MV. 2005. Pathologic study of a fatal case of dengue-3 virus infection in Rio de Janeiro Brazil. *Braz J Infect Dis*. 9(4): 341-347.

Beláková J, Horynová M, Krupka M, Weigl E, Raska M. 2007. DNA vaccines: are they still just a powerful tool for the future? *Arch Immunol Ther Exp* (Warsz). 55(6): 387-398.

Bente DA, Rico-Hesse R. 2006. Models of dengue virus infection. *Drug Discov Today Dis Models*. .3(1): 97-103.

Bharati K, Appaiahgari MB, Vrati S. 2005. Effect of cytokine-encoding plasmid delivery on immune response to Japanese encephalitis virus DNA vaccine in mice. *Microbiol Immunol*. 49(4): 349-353.

Brinton MA, Kurane I, Mathew A, Zeng L Shi PY, Rothman A, Ennis FA. 1998. Immune mediated and inherited defences against flaviviruses. *Clin Diagn Virol.* 10(2-3): 129-139.

Burgers WA, van Harmelen JH,Shephard E, Adams C, Mgwebi T, Bourn W, Hanke T, Williamson AL, Williamson C. 2006. Design and preclinical evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. *J Gen Virol* 87(Pt 2): 399-410.

Cabrera-Hernandez A, Thepparit C, Suksanpaisan L, Smith DR. 2007. Dengue virus entry into liver (HepG2) cells is independent of hsp90 and hsp70. *J Med Virol*. 79(4): 386-392

Caufour PS, Motta MCA, Yamamura AMY, Vazquez S Ferreira II, Jabor AV, Bonaldo MC, Freire MS, Galler R. 2001. Construction, characterization and immunogenicity of recombinant yellow fever 17D-dengue type 2 viruses. *Virus Res.* 79: 1-14.

Chambers TJ, Hahn CS, Galler R, Rice CM. 1990. Flavivirus genome organization, expression, and replication. *Annu. Rev. Microbiol.* 44: 649-688.

Chang GJ, Davis BS, Hunt AR, Holmes DA, Kuno G. 2001. Flavivirus DNA vaccines: current status and potential. *Ann N Y Acad Sci.* 951: 272-285.

Chang GJ, Kuno G, Purdy DE, Davis BS. 2004. Recent advancement in flavivirus vaccine development. *Expert Rev Vaccines*. 3(2):199-220.

Chang GJ, Davis BS, Stringfield C, Lutz C. 2007. Prospective immunization of the endangered California condors (Gymnogyps californianus) protects this species from lethal West Nile virus infection. *Vaccine*. 25(12): 2325-2330.

Chen L , Ewing D, Subramanian H, Block K, Rayner J, Alterson KD, Sedegah M, Hayes C, Porter K, Raviprakash K A heterologous. 2007a. DNA prime-Venezuelan equine encephalitis virus replicon particle boost dengue vaccine regimen affords complete protection from virus challenge in cynomolgus macaques. *J Virol*. 81(21): 11634-11639.

Chen S, Yu M, Jiang T, Deng Y, Qin C, Qin E. 2007b. Induction of tetravalent protective immunity against four dengue serotypes by the tandem domain III of the envelope protein. *DNA Cell Biol* 26(6): 361-367.

Chen Y, Maguire T, Marks RM. 1996. Demonstration of binding of dengue virus envelope protein to target cells. *J Virol*. 70(12): 8765 - 8772.

Cho JH, Lee SW, Sung YC. 1999. Enhanced cellular immunity to hepatitis C virus nonstructural proteins by codelivery of granulocyte macrophage-colony stimulating factor gene in intramuscular DNA immunization. *Vaccine* 17(9-10): 1136-1144.

Chung KM, Nybakken GE, Thompson BS, Engle MJ, Marri A, Fremont DH, Diamond MS. 2006a. Antibodies against West Nile Virus nonstructural protein NS1 prevent lethal infection through Fc gamma receptor-dependent and -independent mechanisms. *J Virol*. 80(3): 1340-1351.

Chung KM, Liszewski MK, Nybakken G, Davis AE, Townsend RR, Fremont DH, Atkinson JP, Diamond MS. 2006b. West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. *Proc Natl Acad Sci U S A*. 103(50): 19111-19116.

Clyde K, Kile JL, Harris E. 2006. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. *J. Virol* 80 (23): 11418–11431.

Coelho-Castelo AA, Trombone AP, Rosada RS, Santos RR, Jr Bonato VL, Sartori A, Silva CL.. 2006. Tissue distribution of a plasmid DNA encoding Hsp65 gene is dependent on the dose administered through intramuscular delivery. *Genet Vaccines Ther*. 4: 1.

Cologna R, Armstrong PM, Rico-Hesse R. 2005. Selection for virulent dengue viruses occurs in humans and mosquitoes. *J Virol.* 79(2):853 - 859.

Colombage G, Hall R, Pavy M, Lobigs M. 1998. DNA-based and alphavirus-vectored immunisation with prM and E proteins elicits long-lived and protective immunity against the flavivirus Murray Valley encephalitis virus. *Virology.* 250(1): 151-163.

Cui T, Sugrue RJ, Xu Q, Lee AK, Chan YC, Fu J. 1998. Recombinant dengue virus type 1 NS3 protein exhibits specific viral RNA binding and NTPase activity regulated by the NS5 protein. *Virology* 246(2): 409-417.

Davis BS, Chang GJ, Cropp B, Roehrig JT, Martin DA, Mitchell CJ, Bowen R, Bunning ML. 2001. West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. *J Virol.* 75(9): 4040-4047.

Davis HL, Whalen RG. 1995. DNA-based immunization. *Mol Cell Biol Hum Dis Ser.* 5: 368-387.

Davis HL, McCluskie MJ. 1999. DNA vaccines for viral diseases. *Microbes Infect.* 1(1): 7-21.

Dertzbaugh MT. 1998. Genetically engineered vaccines: an overview. *Plasmid*. 39(2): 100-113.

Deubel V, Kinney RM, Trent DW. 1988. Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus, Jamaica genotype: comparative analysis of the full-length genome. *Virology*. 165(1): 234-244.

Dobaño C, Rogers WO, Gowda K, Doolan DL. 2007. Targeting antigen to MHC Class I and Class II antigen presentation pathways for malaria DNA vaccines. *Immunol Lett.* 111(2): 92-102

Donnelly JJ, Wahren, B, Liu MA. 2005. DNA vaccines: progress and challenges. *J Immunol* 175(2): 633-639.

Drape RJ, Macklin MD, Barr LJ, Jones S, Haynes JR, Dean HJ. 2006. Epidermal DNA vaccine for influenza is immunogenic in humans. *Vaccine*. 24(21): 4475-481.

Duarte dos Santos CN, Frenkiel MP, Courageot MP, Rocha CF, Vazeille-Falcoz MC, Wien MW, Rey FA, Deubel V, Desprès P. 2000. Determinants in the envelope E protein and viral RNA helicase NS3 that influence the induction of apoptosis in response to infection with dengue type 1 virus. *Virology*. 274(2): 292-308.

Dupuis M, Denis-Mize K, Woo C, Goldbeck C, Selby MJ, Chen M, Otten GR, Ulmer JB, Donnelly JJ, Ott G, McDonald DM. 2000. Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice. *J Immunol*. 165(5): 2850-2858.

Edelman R. 2005. Dengue and dengue vaccines. *J Infect Dis*. 191(5): 650-653.

Edelman R. 2007. Dengue vaccines approach the finish line. *Clin Infect Dis*. 45 Suppl 1: S56-60.

Encke J, zu Putlitz J, Geissler M, Wands JR. Genetic immunization generates cellular and humoral immune responses against the nonstructural proteins of the hepatitis C virus in a murine model. *J Immunol*. 1998 161(9): 4917-4923.

Falconar AK. 1997. The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. *Arch Virol*. 142(5): 897-916.

Falconar AK. 1999. Identification of an epitope on the dengue virus membrane (M) protein defined by cross-protective monoclonal antibodies: design of an improved epitope sequence based on common determinants present in both envelope (E and M) proteins. *Arch Virol*. 144(12): 2313-2330.

Falconar AK. 2007. Antibody responses are generated to immunodominant ELK/KLE-type motifs on the nonstructural-1 glycoprotein during live dengue virus infections in mice and humans: implications for diagnosis, pathogenesis, and vaccine design. *Clin Vaccine Immunol*. 14(5): 493-504.

Falgout B, Chanock R, Lai CJ. 1989. Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2A. *J Virol*. 63(5): 1852-1860.

Falgout B, Bray M, Schlesinger JJ, Lai CJ. 1990. Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. *J Virol*. 64(9): 4356-4363.

Figueiredo LTM. 1996. Dengue in Brazil I: history, epidemiology and research. *Virus Rev Research* 01 (1-2): 9-16.

Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V. 1999. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. *J Virol*. 73(7): 6104-6110.

Freire MS, Marchevsky RS, Almeida LF, Yamamura AM, Caride EC, Brindeiro PA, Motta MC, Nogueira RM, Kubelka CF, Bonaldo MC, Galler R. 2007. Wild dengue virus types 1, 2 and 3 viremia in rhesus monkeys. *Mem Inst Oswaldo Cruz*. 102(2): 203-208.

Fu TM, Ulmer JB, Caulfield MJ, Deck RR, Friedman A, Wang S, Liu X, Donnelly JJ, Liu MA. 1997. Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol Med*. Jun;3(6): 362-371.

Fuller DH, Loudon P, Schmaljohn C. 2006. Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. *Methods.* 40(1): 86-97.

Gagnon SJ, Ennis FA, Rothman AL. 1999. Bystander target cell lysis and cytokine production by dengue virus-specific human CD4(+) cytotoxic T-lymphocyte clones. J Virol. 73(5): 3623-3629.

Galler R, Marchevsky RS, Caride E, Almeida LF, Yamamura AM, Jabor AV, Motta MC, Bonaldo MC, Coutinho ES, Freire MS. 2005. Attenuation and immunogenicity of recombinant yellow fever 17D-dengue type 2 virus for rhesus monkeys. *Braz J Med Biol Res*. 38(12): 1835-1846

Gould EA, Buckley A, Barrett AD, Cammack N. 1986. Neutralizing (54K) and non-neutralizing (54K and 48K) monoclonal antibodies against structural and non-structural yellow fever virus proteins confer immunity in mice. *J Gen Virol*. 67 (Pt 3): 591-595.

Green S, Rothman A. 2006. Immunopathological mechanisms in dengue and dengue hemorrhagic fever. *Curr Opin Infect Dis*. 19(5):429-436.

Gubler .J. 1998. Dengue and dengue hemorrhagic fever. *Clin Microbiol Rev*. 11(3): 480–496.

Gubler DJ. 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol. 10(2): 100–103.

Gurunathan S, Wu CY, Freidag BL, Seder RA. 2000. DNA vaccines: a key for inducing long-term cellular immunity. *Curr Opin Immunol*. (4): 442-447.

Guy B, Almond JW. 2008. Towards a dengue vaccine: Progress to date and remaining challenges. *Comp Immunol Microbiol Infect Dis*. 31(2-3): 239-252.

Guzmán MG, Kouri GP, Bravo J, Soler M, Vazquez S, Morier L. 1990. Dengue hemorrhagic fever in Cuba 1981: a retrospective seroepidemiologic study. *Am J Trop Med Hyg*. 42(2): 179-184.

Guzmán MG, Kourí G. 2002. Dengue: an update. *The Lancet Infection Disease* 2: 33-42.

Guzmán MG. 2005. Global voices of science. Deciphering dengue: the Cuban experience. *Science*. 309(5740): 1495-1497.

Halstead SB. 1970. Observations related to pathogensis of dengue hemorrhagic fever. VI. Hypotheses and discussion. *Yale J Biol Med*. 42(5): 350-362.

Halstead SB, O'Rourke EJ. 1977. Antibody-enhanced dengue virus infection in primate leukocytes. *Nature* 265(5596): 739-741.

Halstead SB. 1979. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J Infect Dis*. 140(4): 527-533.

Halstead SB. 2007. Dengue. *Lancet* 370: 1644–1652.

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature.* 408(6813):740-745.

Henchal EA, Henchal LS, Schlesinger JJ. 1988. Synergistic interaction of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challemge with dengue 2 virus. *J. Gen. Virol*. 69: 2101-2107.

Holmes EC, Twiddy SS. 2003. The origin, emergence and evolutionary genetics of dengue virus. *Infect Genet Evol*. 3(1): 19-28.

Hombach J, Barrett AD, Cardosa MJ, Deubel V, Guzman M, Kurane I, Roehrig JT, Sabchareon A, Kieny MP. 2005. Review on flavivirus vaccine development. Proceedings of a meeting jointly organised by the World Health Organization and the Thai Ministry of Public Health, 26-27 April 2004, Bangkok, Thailand. *Vaccine.* 23(21): 2689-2695.

Huang JH, Wey JJ, Sun YC, Chin C, Chien LJ, Wu,YC. 1999. Antibody responses to an immunodominant nonstructural 1 synthetic peptide in patients with dengue fever and dengue hemorrhagic fever. *J Med Virol.* 57(1): 1-8.

Huang KJ, Yang YC, Lin YS, Huang JH, Liu HS, Yeh TM, Chen SH, Liu CC, Lei HY. 2006. The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. *J Immunol.* 176(5): 2825–2832.

Inchauspe G, Vitvitski L, Major ME, Jung G, Spengler U, Maisonnas M, Trepo C. 1997. Plasmid DNA expressing a secreted or a nonsecreted form of hepatitis C virus nucleocapsid: comparative studies of antibody and T-helper responses following genetic immunization. *DNA Cell Biol.* 16(2): 185-195.

Ishikawa T, Takasaki T, Kurane I, Nukuzuma S, Kondo T, Konishi E. 2007. Co-immunization with West Nile DNA and inactivated vaccines provides synergistic increases in their immunogenicities in mice. *Microbes Infect* 9(9): 1089-1095.

Istúriz RE, Gubler DJ, Brea del Castillo J. 2000. Dengue and dengue hemorrhagic fever in Latin America and the Caribbean. *Infect Dis Clin North Am.* 14(1):121-140.

Jacobs SC, Stephenson JR, Wilkinson GW. 1992. High-level expression of the tick-borne encephalitis virus NS1 protein by using an adenovirus-based vector: protection elicited in a murine model. *J Virol.* 66(4): 2086-2095.

Jacobs SC, Stephenson JR, Wilkinson GW. 1994. Protection elicited by a replication-defective adenovirus vector expressing the tick-borne encephalitis virus non-structural glycoprotein NS1. *J Gen Virol.* 75 (Pt 9): 2399-2402.

Jacobs MG, Robinson PJ, Bletchly C, Mackenzie JM, Young PR. 2000. Dengue virus nonstructural protein 1 is expressed in a glycosyl-phosphatidylinositol-linked form that is capable of signal transduction. *FASEB J.* 14(11): 1603-1610.

Kang KK, Choi SM, Choi JH, Lee DS, Kim CY, Ahn BO, Kim BM, Kim WB. 2003. Safety evaluation of GX-12, a new HIV therapeutic vaccine: investigation of integration into the host genome and expression in the reproductive organs. *Intervirology*. 46(5): 270-276.

Kaufman BM, Summers PL, Dubois DR, Eckels KH. 1987. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. *Am J Trop Med Hyg*. 36(2): 427-434.

Kaur R, Rauthan M, Vrati S. 2004. Immunogenicity in mice of a cationic microparticle-adsorbed plasmid DNA encoding Japanese encephalitis virus envelope protein. *Vaccine.* 22(21-22): 2776-2782.

Kinney RM, Huang CY. 2001. Development of new vaccines against dengue fever and Japanese encephalitis. *Intervirology*. 44(2-3): 176-197.

Kochel T, Wu SJ, Raviprakash K, Hobart P, Hoffman S, Porter K, Hayes C. 1997. Inoculation of plasmids expressing the dengue-2 envelope gene elicit neutralizing antibodies in mice. *Vaccine* 15(5): 547-552.

Kochel TJ, Raviprakash K, Hayes CG, Watts DM, Russell KL, Gozalo AS, Phillips IA, Ewing DF, Murphy GS, Porter KR. 2000. A dengue virus serotype-1 DNA vaccine induces virus neutralizing antibodies and provides protection from viral challenge in Aotus monkeys. *Vaccine*. 18(27): 3166-3173.

Konishi E, Yamaoka M, Kurane I, Mason PW. 2000. Japanese encephalitis DNA vaccine candidates expressing premembrane and envelope genes induce virus-specific memory B cells and long-lasting antibodies in swine. *Virology.* 268(1): 49-55

Konishi E, Kosugi S, Imoto J. 2006. Dengue tetravalent DNA vaccine inducing neutralizing antibody and anamnestic responses to four serotypes in mice. *Vaccine.* 24(12): 2200-2207.

Kou Z, Quinn M, Chen H, Rodrigo WWSI, Rose RC, Schlesinger JJ, Jin X. 2008. Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among humoral peripheral blood mononuclear cells. *J. Med. Virol.* 80(1): 134-146.

Kozlowski PA, Cu-Uvin S, Neutra MR, Flanigan TP. 1997. Comparison of the oral rectal and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women. *Infect Immun*. 65(4): 1387-1394.

Krieg AM, Davis HL. 2001. Enhancing vaccines with immune stimulatory CpG DNA. *Curr Opin Mol Ther*. 3(1): 15-24.

Krieg AM. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol.* 20: 709-760.

Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, Jones CT, Mukhopadhyay S, Chipman PR, Strauss EG, Baker TS, Strauss JH. 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell*. 108(5): 717-725.

Kurane I, Zeng L, Brinton MA, Ennis FA. 1998. Definition of na epitope on NS3 recognized by human CD4+ cytotoxic T lynphocyte clones cross-reactive for dengue virus type 2,3 and 4. *Virology* 240: 169-174.

Kurane I. 2007. Dengue hemorrhagic fever with special emphasis on immunopathogenesis. *Comp Immunol Microbiol Infect Dis.* 30(5-6): 329-340.

Lai CJ, Bray M, Men R, Cahour A, Chen W, Kawano H, Tadano M, Hiramatsu K, Tokimatsu I, Pletnev A, Arakai S, Shameem G, Rinaudo M. 1998. Evaluation of molecular strategies to develop a live dengue vaccine. *Clin Diagn Virol*. 10(2-3): 173-179.

Lazo L, Hermida L, Zulueta A, Sánchez J, López C, Silva R, Guillén G, Guzmán MG. 2007. A recombinant capsid protein from Dengue-2 induces protection in mice against homologous virus. *Vaccine* 25(6): 1064-1070.

Leitner WW, Seguin MC, Ballou WR, Seitz JP, Schultz AM, Sheehy MJ, Lyon JA. 1997. Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from Plasmodium berghei malaria parasites. *J Immunol.* 159(12): 6112-6119.

Lemieux P. 2002. Technological advances to increase immunogenicity of DNA vaccines. *Expert Rev Vaccines*. 1(1):85-93.

Li H, Clum S, You S, Ebner KE, Padmanabhan R. 1999. The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. *J Virol.* 73(4):3108-3116.

Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S, Green S, Vaughn DW, Nisalak A, Ennis FA, Rothman AL. 2002. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. *J Infect Dis*. 186(8): 1165-1168.

Lin CF, Lei HY, Shiau AL, Liu HS, Yeh TM, Chen SH, Liu CC, Chiu SC, Lin YS. 2002. Endothelial cell apoptosis induced by antibodies against dengue virus nonstructural protein 1 via production of nitric oxide. *J Immunol.* 169(2): 657-664.

Lin CW, Liu KT, Huang HD, Chen WJ. 2008 Protective immunity of E. coli-synthesized NS1 protein of Japanese encephalitis virus. *Biotechnol Lett*. 30(2): 205-214.

Lin YL, Chen LK, Liao CL, Yeh CT, Ma SH, Chen JL, Huang YL, Chen SS, Chiang HY. 1998. DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. *J Virol*. 72(1): 191-200.

Lindenbach BD, Rice CM. 1997. trans-Complementation of yellow fever virus NS1 reveals a role in early RNA replication. *J Virol*. 71(12): 9608-9617.

Lindenbach BD, Rice CM. 1999. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. *J Virol*. 73(6): 4611-4621.

Lindenbach BD, Rice CM. 2001. Flaviviridae: the viruses and their replication. In Knipe KM, Howley PM (eds) *Fields Virology*. Lippincott-Wilkins, Philadelphia, PA pp. 991 – 1041.

Ling LM, Wilder-Smith A, Leo YS. 2007. Fulminant hepatitis in dengue haemorrhagic fever. *J Clin Virol*. 38(3): 265-268.

Liu MA. 2003. DNA vaccines: a review. *J Intern Med*. 253(4): 402-410.

Liu MA, Ulmer JB. 2005. Human clinical trials of plasmid DNA vaccines. *Adv Genet.* 55: 25-40.

Lozach PY, Burleigh L, Staropoli I, Navarro-Sanchez E, Harriague J, Virelizier JL, Rey FA, Desprès P, Arenzana-Seisdedos F, Amara A. 2005. Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. *J Biol Chem*. 280(25): 23698-23708.

Luckay A, Sidhu MK, Kjeken R., Megati S, Chong SY, Roopchand V, Garcia-Hand D, Abdullah R, Braun R, Montefiori DC, Rosati M, Felber BK, Pavlakis GN, Mathiesen I, Israel ZR, Eldridge JH, Egan MA. 2007. Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. *J Virol*. 81(10): 5257-5269.

Mackenzie JM, Jones MK, Young PR. 1996. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. *Virology*. 220(1): 232-240.

Mackenzie JS, Gubler DJ, Petersen LR. 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat. Med.* 10 (12 Suppl): S98-109.

Manam S, Ledwith BJ, Barnum AB, Troilo PJ, Pauley CJ, Harper LB, Griffiths TG, 2nd Niu Z, Denisova L, Follmer TT, Pacchione SJ, Wang Z, Beare CM, Bagdon WJ, Nichols WW. 2000. Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. *Intervirology* 43(4-6): 273-281.

Mangada MM, Rothman AL. 2005. Altered cytokine responses of dengue-specific CD4+ T cells to heterologous serotypes. *J Immunol*. 175(4): 2676-2683.

Markoff L, Chang A, Falgout B. 1994. Processing of flavivirus structural glycoproteins: stable membrane insertion of premembrane requires the envelope signal peptide. *Virology* 204(2): 526-540.

Martin JE, Pierson TC, Hubka S, Rucker S, Gordon IJ, Enama ME, Andrews CA, Xu Q, Davis BS, Nason M, Fay M, Koup RA, Roederer M, Bailer RT, Gomez PL, Mascola JR, Chang GJ, Nabel GJ, Graham BS. 2007. A West Nile virus DNA vaccine induces neutralizing antibody in healthy adults during a phase 1 clinical trial. *J Infect Dis. Dec* 15;196(12):1732-1740

Mateu GP, Marchevsky RS, Liprandi F, Bonaldo MC, Coutinho ES, Dieudonné M, Caride E, Jabor AV, Freire MS, Galler R. 2007. Construction and biological properties of yellow fever 17D/dengue type 1 recombinant virus. *Trans R Soc Trop Med Hyg*. 101(3): 289-298.

Mathew A, Kurane I, Rothman AL, Zeng LL, Brinton MA, Ennis FA. 1996. Dominant recognition by human CD8+ cytotoxic T lymphocytes of dengue virus nonstructural proteins NS3 and NS1.2a. *J Clin Invest.* 98(7):1684-91.

Mathew A, Kurane I, Green S, Stephens HA, Vaughn DW, Kalayanarooj S, Suntayakorn S, Chandanayingyong D, Ennis FA, Rothman AL. 1998. Predominance of HLA-restricted cytotoxic T-lymphocyte responses to serotype-cross-reactive epitopes on nonstructural proteins following natural secondary dengue virus infection. *J Virol*. 72(5):3999-4004.

McCluskie MJ, Davis HL. 1999. Mucosal immunization with DNA vaccines. *Microbes Infect.* 1(9):685-98.

McConkey SJ, Reece WH, Moorthy VS, Webster D, Dunachie S, Butcher G, Vuola JM, Blanchard TJ, Gothard P, Watkins K, Hannan CM, Everaere S, Brown K, Kester KE, Cummings J, Williams J, Heppner DG, Pathan A, Flanagan K, Arulanantham N, Roberts MT, Roy M, Smith GL, Schneider J, Peto T, Sinden RE, Gilbert SC, Hill AV. 2003. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat Med*. 9(6): 729-735.

zur Megede J, Chen MC, Doe B, Schaefer M, Greer CE, Selby M, Otten GR, Barnett SW. 2000. Increased expression and immunogenicity of sequence-modified human immunodeficiency virus type 1 gag gene. *J Virol*. 74(6): 2628-2635.

Mellado-Sánchez G, García-Cordero J, Luria-Pérez R, Lázaro-Olan L, Santos-Argumedo L, Gutiérrez-Castañeda B, Estrada-García I, Cedillo-Barrón L. 2005. DNA priming E and NS1 constructs--homologous proteins boosting immunization strategy to improve immune response against dengue in mice. *Viral Immunol* 18(4): 709-721.

Miller S, Sparacio S, bartenschlarger R. 2006. Subcellular localization and membrane topology of the Dengue virus type 2 Non-structural protein 4B. *J Biol Chem*. 281(13): 8854-63.

Ministério da Saúde. Fundação Nacional da Saúde. 2002. Dengue: aspectos epidemiológicos diagnósticos e tratamento. Ministério da Saúde. Fundação Nacional da Saúde.Brasília – Fundação Nacional da Saúde.

Ministério da Saúde http://www.saude.to.gov.br/pagina_adm/download/boletim_dengue_semana18.pdf.

Ministério da saúde http://portal.saude.gov.br/portal/arquivos/pdf/boletim_dengue_2803.pdf

Modis Y, Ogata S, Clements D, Harrison SC. 2004. Structure of the dengue virus envelope protein after membrane fusion. *Nature* 427(6972):313 – 319.

Modlin RL. 2000. Immunology. A Toll for DNA vaccines. Nature. 408(6813): 659-660.

Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, Sawasdivorn S, Duangchinda T, Dong T, Rowland-Jones S, Yenchitsomanus PT, McMichael A, Malasit P, Screaton G. 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat Med.* 9(7): 921-927.

Monath TP. 1994. Dengue: the risk to developed and developing countries. *Proc Natl Acad Sci USA* 91(7): 2395-2400.

Monath TP. 2007. Dengue and yellow fever--challenges for the development and use of vaccines. *N Engl J Med*. 357(22): 2222-2225.

Morozova OV, Maksimova TG, Bakhvalova VN. 1999. Tick-borne encephalitis virus NS3 gene expression does not protect mice from homologous viral challenge. *Viral Immunol*. 12(4): 277-280.

Mota J, Acosta M, Argotte R, Figueroa R, Méndez A, Ramos C. 2005. Induction of protective antibodies against dengue virus by tetravalent DNA immunization of mice with domain III of the envelope protein. *Vaccine* 23(26): 3469-3476.

Mukhopadhyay S, Kuhn RJ, Rossmann MG. 2005. A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol*. 3(1):13-22.

Murthy HM, Clum S, Padmanabhan R. 1999. Dengue virus NS3 serine protease. Crystal structure and insights into interaction of the active site with substrates by molecular modeling and structural analysis of mutational effects. *J Biol Chem*. 274(9): 5573-5580.

Muylaert IR, Galler R, Rice CM. 1997. Genetic analysis of the yellow fever virus NS1 protein: identification of a temperature-sensitive mutation which blocks RNA accumulation. *J Virol* 71(1): 291-298.

Niyomrattanakit P, Winoyanuwattikun P, Chanprapaph S, Angsuthanasombat C, Panyim S, Katzenmeier G. 2004. Identification of residues in the dengue virus type 2 NS2B cofactor that are critical for NS3 protease activation. *J Virol.* 78(24): 13708-13716.

Nogueira RM, Miagostovich MP, Lampe E, Souza RW, Zagne SM, Schatzmayr HG. 1993. Dengue epidemic in the stage of Rio de Janeiro, Brazil, 1990-1: co-circulation of dengue 1 and dengue 2 serotypes. *Epidemiol Infect.* 111(1): 163-170.

Nogueira RM, de Araújo JM, Schatzmayr HG. 2007. Dengue viruses in Brazil, 1986-2006. *Rev Panam Salud Publica* 22(5): 358-363.

OMS http://www.paho.org/english/ad/dpc/cd/dengue-cases-2007.htm

OMS http://www.who.int/csr/disease/dengue/en/index.html

Orozco J. 2007. Defeating dengue: a difficult task ahead. *Bull World Health Organ.* 85(10): 737-738.

Osanai CH, Travassos da Rosa AP, Tang AT, do Amaral RS, Passos AD Tauil PL. 1983. Dengue outbreak in Boa Vista, Roraima. Preliminary report. *Rev Inst Med Trop* 25(1): 53-54.

Pelizon AC, Martins DR, Zorzella SF, Trombone AP, Lorenzi JC, Carvalho RF, Brandão IT, Coelho-Castelo AA, Silva CL, Sartori A. 2007. Genetic vaccine for tuberculosis (pVAXhsp65) primes neonate mice for a strong immune response at the adult stage. *Genet Vaccines Ther* 5: 12.

Petersen LR, Roehrig JT. 2007. Flavivirus DNA vaccines--good science, uncertain future. *J Infect Dis.* 196(12): 1721-1723.

Phillpotts RJ, Venugopal K, Brooks T. 1996. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. *Arch Virol.*141(3-4): 743-749

Porgador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN. 1998. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J Exp Med.* 188(6): 1075-1082.

Porter KR, Kochel TJ, Wu SJ, Raviprakash K, Phillips I, Hayes CG. 1998. Protective efficacy of a dengue 2 DNA vaccine in mice and the effect of CpG immuno-stimulatory motifs on antibody responses. *Arch Virol.* 143(5): 997-1003.

Pryor MJ, Wright PJ. 1993. The effects of site-directed mutagenesis on the dimerization and secretion of the NS1 protein specified by dengue virus. *Virology* 194(2): 769-780.

Pryor MJ, Wright PJ. 1994. Glycosylation mutants of dengue virus NS1 protein. *J Gen Virol.* 75 (Pt 5): 1183-1187.

Purdue University http://physorg.com/news 125846262.html

Putnak R, Fuller J, VanderZanden L, Innis BL, Vaughn DW. 2003. Vaccination of rhesus macaques against dengue-2 virus with a plasmid DNA vaccine encoding the viral pre-membrane and envelope genes. *Am J Trop Med Hyg*. 68(4): 469-476.

Putnak JR, Kanesa TN, Innis,BL. 1997. A putative cellular receptor for dengue viruses. *Nat Med*. 3(8):828 – 829.

Putnak R, Fuller J, VanderZanden L, Innis BL, Vaughn DW. 2003. Vaccination of rhesus macaques against dengue-2 virus with a plasmid DNA vaccine encoding the viral pre-membrane and envelope genes. *Am J Trop Med Hyg*. 68(4):469-476.

Qi R, Zhang L, Chi CW. 2008. Biological characteristics of dengue virus and potential targets for drug design. *Acta Biochim Biophys Sin.* 40(2): 91–101.

Qu X, Chen W, Maguire T, Austin F. 1993. Immunoreactivity and protective effects in mice of a recombinant dengue 2 Tonga virus NS1 protein produced in a baculovirus expression system. *J Gen Virol*. 74 (Pt 1): 89-97.

Raviprakash K, Apt D, Brinkman A, Skinner C, Yang S, Dawes G, Ewing D, Wu SJ, Bass S, Punnonen J, Porter K. 2006. A chimeric tetravalent dengue DNA vaccine elicits neutralizing antibody to all four virus serotypes in rhesus macaques. *Virology*. 353(1): 166-173.

Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. *Am J Hyg* 27: 493-497.

Reyes-Del Valle J, Cháves-Salinas S, Medina F, Del Angel RM. 2005. Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. *J Virol.* 79(8): 4557-4567.

Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, Boshell J, de Mesa MT, Nogueira RM, da Rosa AT. 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. *Virology* 230(2): 244-251.

Robinson HL. 1997. Nucleic acids vaccines: an overview. *Vaccine* 15(8): 785-787

Robinson HL. 2003. Prime boost vaccines power up in people. *Nat Med.* 9(6): 642-643.

Robinson HL, Torres CA. 1997. DNA vaccines. *Semin Immunol.* 9(5): 271-283.

Rodriguez F, Zhang J, Whitton JL. 1997. DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. *J Virol.* 71(11): 8497-503.

Rodriguez F, Whitton JL. 2000. Enhancing DNA immunization.*Virology*. 268(2): 233-238.

Roehrig JT, Bolin RA, Kelly RG. 1998. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. *Virology* 246(2): 317-328.

Rothman AL. 2004. Dengue: defining protective versus pathologic immunity. *J Clin Invest.*113(7): 946-951.

Rottinghaus ST, Poland GA, Jacobson RM, Barr LJ, Roy MJ. 2003. Hepatitis B DNA vaccine induces protective antibody responses in human non-responders to conventional vaccination. *Vaccine.* 21(31): 4604-4608.

Sambrook J, Fritsch EF, Maniatis T. 1989. In *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Cold Spring Harbor New York N.Y.

Sampath A, Xu T, Chao A, Luo D, Lescar J, Vasudevan SG. 2006. Structure-based mutational analysis of the NS3 helicase from dengue virus. *J Virol.* 80(13): 6686-6690.

Sanger F, Nicklen S, Coulson AR. 1992. DNA sequencing with chain-terminating inhibitors. 1977. *Biotechnology*. 24: 104-108.

Sasaki S, Sumino K, Hamajima K, Fukushima J, Ishii N, Kawamoto S, Mohri H, Kensil CR, Okuda K. 1998. Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes. J Virol. 72(6): 4931-4939.

Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, Silverman GJ, Lotz M, Carson DA, Raz E. 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science*. 273(5273): 352-354.

Schatzmayr HG, Nogueira RM, Travassos da Rosa AP. 1986. An outbreak of dengue virus at Rio de Janeiro--1986. *Mem Inst Oswaldo Cruz.* 81(2):245 - 246.

Schlesinger JJ, Brandriss MW, Walsh EE. 1985. Protection against 17D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp48 and by active immunization with gp48. *J Immunol*. 135(4): 2805-2809.

Schlesinger JJ, Brandriss MW, Cropp CB, Monath TP. 1986. Protection against yellow fever in monkeys by immunization with yellow fever virus nonstructural protein NS1. *J Virol*. 60(3): 1153-1155.

Schlesinger JJ, Brandriss MW, Walsh EE. 1987. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. *J Gen Virol*. 68 (Pt 3): 853-857.

Schlesinger JJ, Foltzer M, Chapman S. 1993. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. *Virology*. 192(1): 132-141.

Schlesinger JJ. 2006. Flavivirus nonstructural protein NS1: complementary surprises. *Proc Natl Acad Sci U S A*. 103(50): 18879-18880.

Seneviratne SL, Malavige GN, de Silva HJ. 2006. Pathogenesis of liver involvement during dengue viral infections. *Trans R Soc Trop Med Hyg.* 100(7): 608-614.

Shafee N, AbuBakar S. 2003. Dengue virus type 2 NS3 protease and NS2B-NS3 protease precursor induce apoptosis. *J Gen Virol.* 84(Pt 8): 2191-2195.

Shedlock DJ, Weiner DB. 2000. DNA vaccination: antigen presentation and the induction of immunity. *J Leukoc Biol.* 68(6): 793-806.

Sheets RL, Stein J, Manetz TS, Duffy C, Nason M, Andrews C, Kong WP, Nabel GJ, Gomez PL. 2006. Biodistribution of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile virus is similar, without integration, despite differing plasmid backbones or gene inserts. *Toxicol Sci.* 91(2): 610-619.

Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, Zhang ZQ, Simon AJ, Trigona WL, Dubey SA, Huang L, Harris VA, Long RS, Liang X, Handt L, Schleif WA, Zhu L, Freed DC, Persaud NV, Guan L, Punt KS, Tang A, Chen M, Wilson KA, Collins KB, Heidecker GJ, Fernandez VR, Perry HC, Joyce JG, Grimm KM, Cook JC, Keller PM, Kresock DS, Mach H, Troutman RD, Isopi LA, Williams DM, Xu Z, Bohannon KE, Volkin DB, Montefiori DC, Miura A, Krivulka GR, Lifton MA, Kuroda MJ, Schmitz JE, Letvin NL, Caulfield MJ, Bett AJ, Youil R, Kaslow DC, Emini EA. 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 415(6869): 331-335.

de Souza LJ, Nogueira RM, Soares LC, Soares CE, Ribas BF, Alves FP, Vieira FR, Pessanha FE. 2007. The impact of dengue on liver function as evaluated by aminotransferase levels. *Braz J Infect Dis.* 11(4): 407-410.

Stephenson JR. 2005 Understanding dengue pathogenesis: implications for vaccine design. *Bull World Health Organ.* 83(4): 308-314.

Srivastava IK, Liu MA. 2003. Gene vaccines. *Ann Intern Med.* 138(7): 550-559.

Stiasny K, Heinz FX. 2006. Flavivirus membrane fusion. *J Gen Virol.* 87(Pt 10): 2755-2766.

Suksanpaisan L, Cabrera-Hernandez A, Smith DR. 2007. Infection of human primary hepatocytes with dengue virus serotype 2. *J Med Virol*. 79(3): 300-307.

Tan CH, Yap EH, Singh M, Deubel V, Chan YC. 1990. Passive protection studies in mice with monoclonal antibodies directed against the non-structural protein NS3 of dengue 1 virus. *J Gen Virol*. 71 (Pt 3): 745-749.

Tang DC, DeVit M, Johnston SA. 1992. Genetic immunization is a simple method for eliciting an immune response. *Nature*. 356(6365): 152-154.

Teixeira M da G, Costa M, da C, Barreto ML, Mota E. 2005. Dengue and dengue hemorrhagic fever epidemics in Brazil: what research is needed based on trends, surveillance, and control experiences? *Cad Saude Publica*. 21(5): 1307-1315.

Teo KF, Wright PJ. 1997. Internal proteolysis of the NS3 protein specified by dengue virus 2. *J Gen Virol*. 78 (Pt 2): 337-341.

Timofeev AV, Butenko VM, Stephenson JR. 2004. Genetic vaccination of mice with plasmids encoding the NS1 non-structural protein from tick-borne encephalitis virus and dengue 2 virus.*Virus Genes*. 28(1): 85-97.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22(22): 4673-4680.

Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Deck RR, DeWitt CM, Friedman A *et al.* 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science.* 259(5102): 1745-1749.

Ulmer JB, Donelly JJ, Liu MA. 1996. DNA vaccines promising: a new approach to inducing protective immunity. *ASM News* 62: 476-479.

Umareddy I, Chao A, Sampath A, Gu F, Vasudevan SG. 2006. Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. *J Gen Virol.* 87(Pt 9): 2605-2614.

Umareddy I, Pluquet O, Wang QY, Vasudevan SG, Chevet E, Gu F. 2007. Dengue virus serotype infection specifies the activation of the unfolded protein response. *Virol J*. 4: 91.

Valle RP, Falgout B. 1998. Mutagenesis of the NS3 protease of dengue virus type 2. *J Virol*. 72(1): 624-632.

Verthelyi D, Klinman DM. 2003. Immunoregulatory activity of CpG oligonucleotides in humans and nonhuman primates. *Clin Immunol*. 109(1): 64-71.

Volpina OM, Volkova TD, Koroev DO, Ivanov VT, Ozherelkov SV, Khoretonenko MV, Vorovitch MF, Stephenson JR, Timofeev AV.. 2005. A synthetic peptide based on the NS1 non-structural protein of tick-borne encephalitis virus induces a protective immune response against fatal encephalitis in an experimental animal model. *Virus Res.* 112(1-2): 95-99.

Wang E, Haolin N, Renling X, Barrett ADT, Watowich SJ, Gubler DJ, Scott W. 2000. Evolutionary relationship of endemic/epidemic and sylvatic dengue viruses. J. Virol 74 (7): 3227-3234.

Wang S, Heilman D, Liu F, Giehl T, Joshi S, Huang X, Chou TH, Goguen J, Lu SA. 2004a. DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague.*Vaccine.* 22(25-26): 3348-3357.

Wang Z, Troilo PJ, Wang X, Griffiths TG, Pacchione SJ, Barnum AB, Harper LB, Pauley CJ, Niu Z, Denisova L, Follmer TT, Rizzuto G, Ciliberto G, Fattori E, Monica NL, Manam S, Ledwith BJ. 2004b. Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation.*Gene Ther.* 11(8): 711-721.

Weiner DB; Kennedy RC. 1999. Genetic vaccines. *Sci Am* 281 (1): 34-41.

Welsh RM, Rothman AL. 2003. Dengue immune response: low affinity, high febrility. *Nat Med.* 9(7): 820-822.

Whitehead SS, Blaney JE, Durbin AP, Murphy BR. 2007. Prospects for a dengue virus vaccine. *Nat Rev Microbiol*. 5(7): 518-528.

WHO. 2007. Defeating dengue: a difficult task ahead. *Bull World Health Organ* 85 (10): 737–738.

Widera G, Austin M, Rabussay D, Goldbeck C, Barnett SW, Chen M, Leung L, Otten GR, Thudium K, Selby MJ, Ulmer JB. 2000. Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. *J Immunol*. 164(9): 4635-4640.

Winkler G, Maxwell SE, Ruemmler C, Stollar V. 1989. Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. *Virology*. 171(1): 302-305

Winkler G, Randolph VB, Cleaves GR, Ryan TE, Stollar V. 1988. Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. *Virology*. 162(1): 187-196.

Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. 1990. Direct gene transfer into mouse muscle in vivo. *Science* 247**:**1465-1468.

Woodland DL. 2004. Jump-starting the immune system: prime-boosting comes of age. *Trends Immunol*. 25(2): 98-104.

Wu CJ, Li TL, Huang HW, Tao MH, Chan YL. 2006. Development of an effective Japanese encephalitis virus-specific DNA vaccine. *Microbes Infect*. 8(11): 2578-2586.

Wu SF, Liao CL, Lin Y L, Yeh CT, Chen LK, Huang YF, Chou HY, Huang JL, Shaio MF, Sytwu HK. 2003. Evaluation of protective efficacy and immune mechanisms of using a non-structural protein NS1 in DNA vaccine against dengue 2 virus in mice. *Vaccine*. 21(25-26): 3919-3929.

Wu SJ, Grouard-Vogel G, Sun W, Mascola JR, Brachtel E, Putvatana R, Louder MK, Filgueira L, Marovich MA, Wong HK, Blauvelt A, Murphy GS, Robb ML,

Innes BL, Birx DL, Hayes CG, Frankel SS. 2000. Human skin Langerhans cells are targets of dengue virus infection. *Nat Med.* 6(7): 816-820.

Xu T, Sampath A, Chao A, Wen D, Nanao M, Chene P, Vasudevan SG, Lescar J. 2005. Structure of the Dengue virus helicase/nucleoside triphosphatase catalytic domain at a resolution of 2.4 A. *J Virol.* 79(16): 10278-10288.

Young PR, Hilditch PA, Bletchly C, Halloran W. 2000. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. *J Clin Microbiol*. 38(3): 1053-1057.

Zhang Y, Corver J, Chipman PR, Zhang W, Pletnev SV, Sedlak D, Baker TS, Strauss JH , Kuhn RJ , Rossmann MG. 2003. Structures of immature flavivirus particles. EMBO J. 22(11): 2604-2613.

Zhou Y, Ray D, Zhao Y, Dong H, Ren S, Li Z, Guo Y, Bernard KA, Shi PY, Li H. 2007. Structure and function of flavivirus NS5 methyltransferase.*J Virol*. 81(8): 3891–3903.

Zivny J, Kurane I, Leporati AM, Ibe M, Takiguchi M, Zeng LL, Brinton M A, Ennis FA. 1995. A single nine-amino acid peptide induces virus-specific, CD8+ human cytotoxic T lymphocyte clones of heterogeneous serotype specificities. *J Exp Med*. 182(3): 853-863.

ANEXOS

ANEXO 1

Circulating human antibodies against dengue NS1 protein: potential of recombinant D2V-NS1 proteins in diagnostic tests

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Circulating human antibodies against dengue NS1 protein: potential of recombinant D2V-NS1 proteins in diagnostic tests

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Abstract

The dengue virus (DV) causes one of the most important arthropod-borne human viral diseases throughout the tropical and subtropical countries. However, the morbidity and mortality of DV infections could be reduced with an early hospitalization care and a rapid risk identification of developing the dengue haemorrhagic fever (DHF). The nonstructural glycoprotein 1 (NS1) has been pointed as a reagent for immune-assay diagnostic test optimization. To evaluate this potential, recombinant DV2-NS1 proteins (rNS1) were produced from *Escherichia coli* (NS1EC) and insect cells (NS1IC) expression. The tests were performed by analysis of a human serum panel reacted against different rNS1 forms. The results demonstrated high correspondence between the DV positive sera and the assay results using native or refolded forms of either NS1IC or NS1EC. Also, the IgG and IgM anti-rNS1 level profiles showed distinct distribution, depending on protein form and disease status. However, the IgM anti-rNS1 reactions did not show sensibility to detect the DV in primary infections. The data obtained from the paired serum samples reactivity comparison suggested a heterogeneous human immune response and absence of correspondence between the IgG and IgM profile levels. Moreover, a patient with negative reference test could be detected by specific IgG anti-rNS1 assays presented here. Therefore, these results sustain the usefulness of dengue nonstructural proteins, in particular the NS1, in diagnostic tests as a complementary reagent.

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Keywords: Dengue; *Flavivirus*; NS1; Recombinant NS1

1. Introduction

Dengue has been considered a serious public health threat in tropical and subtropical countries (Halstead, 1990; Monath, 1994). The infection is caused by dengue virus (DV), which belongs to the *Flavivirus* genus and comprises four antigenically related serotypes. The disease spectrum of clinical illness range from asymptomatic or mild febrile illness to classic dengue fever (DF), to the life threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Gluber, 1998; Halstead, 1989). Often, the disease severity has been associated with heterotypic secondary infections (Rothman and Ennis, 1999), and circulating DV antibody levels, early in illness, has been correlated with the risk of developing DHF (Vaughn et al., 2000).

The DV nonstructural glycoprotein NS1 is expressed in mammalian cells in both membrane-associated and secreted forms and may present different molecular mass depending on glycosylation and distinct monomeric associations that change the protein conformational status (Flamand et al., 1999). Efforts have been done to determine the role of this

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protein in the dengue pathogeny and its potential as DHF predictor. Some data, in the current literature, suggest conflicting results. In one hand, several reports suggest that the DV-NS1 protein might play an important role in the pathogenesis of DHF and DSS, due to cross-reaction of antibodies generated against NS1 with self proteins (Falconar, 1997; Valdés et al., 2000). In the other hand, studies indicated that raised levels of antibodies anti-NS1 may confer protection against DV (Henchal et al., 1988; Schlesinger et al., 1993). In fact, it has been frequently observed that circulating IgG and IgM antibodies against DV-NS1 could be found in patient sera with primary and secondary infections, in different concentrations (Huang et al., 2001; Kuno et al., 1991; Libraty et al., 2002; Young et al., 2000). These data pointed this glycoprotein as a potential reagent to optimize the specificity of DV immuneassay tests. However, as proposed above, different results could be observed depending on the protein characteristics. The present study aimed to evaluate the potential of different forms of recombinant D2V-NS1 proteins in the dengue infection diagnostic. The analysis was done detecting the antibodies anti-rNS1 in a Brazilian serum panel, from patients with both primary and secondary infections. For this purpose, recombinant D2V-NS1 protein was expressed in bacteria and insect cells, purified and used in its native, refolded or denatured forms. The analyses were performed by rNS1-specific ELISA tests. Results revealed significant differences in the specificity and sensibility of the assays.

2. Materials and methods

2.1. Serum samples characterization

The serum samples analysed in this study was obtained from the collection of the Laboratory of Flavivirus at Oswaldo Cruz Foundation, Fiocruz, Brazil. Sixty samples were chosen at random from the collection, including specimens of the acute and convalescent phases. These specimens were obtained from the first to fourth days and from the second to fourth weeks that came after the disease onset, respectively. The first day was defined as the day when the first symptoms began. Primary and secondary DV infections were determined by IgM/IgG ELISAs and hemagglutination inhibition assays. All the tests were performed with paired plasma specimens, according to previous established serologic criteria (Innis et al., 1989; Miagostovich et al., 1999; WHO, 1997). Complementary DV identification was done by the virus isolation into *Aedes albopictus* C6/36 cell line (Gubler et al., 1984; Igarashi, 1978), by reverse transcriptase polymerase chain reaction (RT-PCR) (Lanciotti et al., 1992) and by IgM capture enzyme-linked immunosorbent assay (MAC-ELISA) (Kuno et al., 1987). All positive DV infection specimens were obtained from patients with symptoms of classical DF. The laboratorial serum characteristics are listed in Table 1. The samples are classified in the following groups: A and B, that comprised the samples from primary and secondary infections, respectively, with specimens collected only from one of the disease phases; P and S, samples from primary and secondary infections respectively, including, specimens of each patient collected from both acute and convalescent phase; C, the control group, composed by hospitalized individual sera with DV negative test; D, the control group composed by samples from healthy yellow fever vaccinated (Yfv) individual sera.

2.2. Virus, cell lines, plasmid and proteins

D2V New Guinea C strain was propagated into *A. albopictus* clone C6/36 cell line with RPMI 1640 (Sigma, USA) medium containing 2% fetal calf serum (Sigma, USA).

The rNS1 expressed in insect cells (NS1IC) was purchased from Hawaii Biotechnology Group Inc. (USA). These proteins, used either in native or heat-denatured form (10 min at 100 °C), were named NS1ICn and NS1ICd, respectively. To produce the rNS1 expressed in bacteria, *Escherichia coli*, M15 strain, and the vector pET23b (Novagen, USA) was used. The D2V-NS1 gene was amplified by RT-PCR, using total RNA extracted from mosquito-infected cells using appropriated primers (available upon request), and cloned in pET23b. The recombinant plasmid, pETNS1, was sequenced and checked with data on GeneBank (M29095). *E. coli* strain BL21(DE3) was transformed with the pETNS1 plasmid and grown to obtain the recombinant protein. The NS1 expression was then induced with 0.25 mM isopropyl-D-thiogalactopyranoside for 4 h. Bacterial culture was harvested and stored at −70 °C until use. The pellet, resuspended in lysis buffer (10 mM Tris, pH 8; 0.1% Triton 100X; 0.5 mM PMSF; 1% lysozyme and 5 mM imidazole), was sonicated, in ice bath, throughout four cycles of 15 s at 200 W, with intervals of 15 s and centrifuged. The recombinant protein was then purified by Ni-NTA super flow column (Qiagen, Germany) according to manufactures instruction. The protein was eluted in 4 M urea salt buffer with 60 mM imidazole and dialyzed in saline phosphate buffer, pH 7.4. Part of such purified protein was denatured by heat and named as NS1ECd, the other part was submitted to a refolded process, as described by Huang et al. (2001), and was designated as NS1ECr.

2.3. Electrophoresis and Western blot analysis

To determine the concentration and purity, the proteins were analysed by reagent assay (Bio-Rad, USA) and SDS-PAGE stained with silver nitrate 10% (Morrisey, 1981). Electrophoresis for the NS1ICn and NS1ECr were done in nondenaturant conditions, omitting the SDS and the 2-mercaptoethanol in the gel and in the sample buffer. Each recombinant protein was reacted with ascitic fluid of anti-D2V New Guinea C (NIH-V575 701 562) and monoclonal antibodies (MAbs) against D2V-NS1 linear epitopes, 1H7.4, 1A12.3 and 1G5.3 (Falconar et al., 1994) kindly gifted by Dr. Falconar.

Table 1
Diagnostical characteristics of the DV infected patient serum panel

Patient group	IgM[a]	IgG[b]	Isolation[c]	PCR[d]	Sample time (days)[e]
A (primary) (n = 17)	+	T	DV1 or 2		2–17
B (secondary) (n = 13)	+	T	DV1 or 2		2–17
P101	–	<40	DV1	ND	3
	+	10240			20
P102	–	<40	DV1	ND	4
	+	160			15
P201	–	<40	DV2	ND	1
	+	10240			31
P202	–	<40	DV2	ND	4
	+	160			15
P301	–	<40	–	DV3	4
	+	10240			20
P302	–	<40	–	DV3	2
	+	640			9
S101	+	<40	DV1	ND	3
	+	163840			20
S102	–	<40	DV1	ND	1
	–	40960			15
S201	–	2560	DV2	ND	2
	+	163840			15
S202	+	160	DV2	ND	3
	+	163840			15
S203	–	2560	DV2	ND	3
	+	40460			20
S204	–	160	DV2	ND	2
	+	163840			17
S301	–	160	DV3	ND	2
	+	163840			14
S302	–	2560	DV3	ND	3
	+	163840			14
S303	–	160	DV3	ND	4
	+	163840			17
D = DV negatives (n = 20)	–	–	–	–	2–20
	–	–			
YFv (n = 20)	–	–	–	–	ND

[a] IgM titer obtained by MAC-ELISA (Kuno et al., 1987).
[b] Titer detected by IgG ELISA (PanBio, Australia).
[c] Isolation and identification of the viral serotype (Gubler et al., 1984).
[d] Amplification of viral sequence by PCR (Lanciotti et al., 1992).
[e] Days after of the disease symptoms onset, when the serum samples were collected. Patient groups: A, nonpaired serum samples from individuals with primary infection; B, nonpaired serum samples from individuals with secondary infection; P, paired serum samples from primary infection; S paired serum samples from secondary infection; C, control reference group with DV negative serum samples; YFv, sera from yellow fever vaccinated healthy individual. T, IgG or IgM titers in accordance to the disease status; ND: not determined.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Immunomicrotiter plates (Nunc Maxisorp, Denmark) were coated with 100 μL of an antigen suspension of each recombinant protein (200 ng/well) diluted in PBS for 1 h at 37 °C. Plates were blocked with 200 μL of PBS containing 1% of slim milk, incubated overnight at 4 °C, and then washed five times with PBST. Human sera (100 μL/well), diluted 1:100 in buffer (PBST, 1% bovine serum albumin, 0.01% normal goal serum), were added and the plates were incubated for 1 h at 37 °C, followed by washing as described above. Antibodies were detected by adding 100 μL/well of 1:2000 diluted HRP-conjugated goat anti-human IgM or anti-human IgG immunoglobulin (Sigma, USA). Plates were in-

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P101	–	<40	DV1	ND	3
	+	10240			20
P102	–	<40	DV1	ND	4
	+	160			15
P201	–	<40	DV2	ND	1
	+	10240			31
P202	–	<40	DV2	ND	4
	+	160			15
P301	–	<40	–	DV3	4
	+	10240			20
P302	–	<40	–	DV3	2
	+	640			9
S101	+	<40	DV1	ND	3
	+	163840			20
S102	–	<40	DV1	ND	1
	–	40960			15
S201	–	2560	DV2	ND	2
	+	163840			15
S202	+	160	DV2	ND	3
	+	163840			15
S203	–	2560	DV2	ND	3
	+	40460			20
S204	–	160	DV2	ND	2
	+	163840			17
S301	–	160	DV3	ND	2
	+	163840			14
S302	–	2560	DV3	ND	3
	+	163840			14
S303	–	160	DV3	ND	4
	+	163840			17
D = DV negatives (n = 20)	–	–	–	–	2–20
	–	–			
YFv (n = 20)	–	–	–	–	ND

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protein were obtained (Fig. 3). The IgG anti-rNS1 level profile was significantly different, depending on the recombinant protein used in the assays. High antibody levels were detected in the reaction against the NS1IC protein, mainly with the native form (Fig. 3A and B). Such differences were more remarkable in the reactions of secondary sera against NS1ICn, which presented the highest values (Fig. 3A). The assays using NS1EC, in general, showed values lower than the earlier. The tests that were performed with the protein expressed in bacteria could not distinguish the DV infections status, even with the refolded form (Fig. 3C and D). Few cross-reactions with both proteins, NS1IC and NS1EC, were observed within the control and the YFv samples. These later, did not show positive reactions against the NS1ICn (Fig. 3).

Analyses of the IgM anti-rNS1 levels were also investigated. The reactions against the NS1ICn and NS1ECr proteins presented high intensity in secondary sera and the data suggests the presence of two distinct groups: one composed by individuals that presented low reactivity and another composed by individuals that showed high reaction intensities (Fig. 4A and B). Besides, the reactions with NS1ECd, presented the lowest absorbance values, even in those sera considered positive by the IgG assays.

3.3. *Profiles of NS1-specific IgG and IgM in a paired serum panel*

Levels of IgG and IgM anti-rNS1 were compared in six primary and nine secondary DV paired serum samples (listed in Table 1). No significant correlation ($r = 0.49$) was observed, in primary panel, between the level distributions of such immunoglobulins (Fig. 5A and B). In this panel, only two patients, P201 and P202, presented raised IgG levels of anti-NS1ICn and anti-NS1ECr from the acute to convalescent phase (Fig. 5A). In contrast, secondary serum samples, except for one of these patients, showed significant increase of the anti-NS1ICn IgG levels from the acute to the convalescent phase (Fig. 5B). Although several samples from secondary convalescent phase had higher levels of IgG anti-NS1ECr when compared with the acute phase, the reactions against NS1EC were, in general, weaker than those against NS1IC.

The IgM anti-rNS1 levels were negative in almost all the primary samples, except from the convalescent specimen P102, that reacted against the NS1ECr, and the specimens P202 and P302, that reacted against NS1ICn (Fig. 5C). Again, the IgM levels obtained with the secondary sera were significantly higher than those obtained with primary ones (Fig. 5D).

The results revealed no correlations between the levels of IgM and IgG against any rNS1 forms, either in primary or secondary serum panels. Despite some specimens (P102, S101, S102 and S201) have revealed high intensity reactions in the IgM assays performed with NS1ECr, in the IgG assays, the strongest reactions were detected against another rNS1. The samples S301 and S303 presented weak or negative values of IgM anti-rNS1 levels but intense reactions of IgG against NS1ECr and NS1ICn (Fig. 5). Moreover, the sample P301, that presented, in the acute phase, low anti-DV titers and

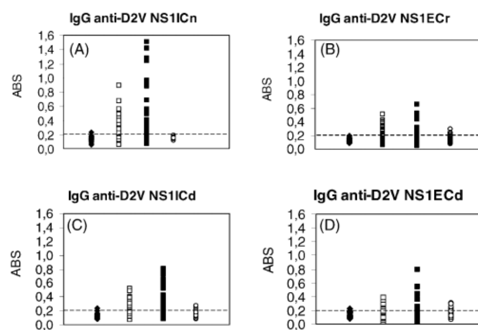

Fig. 3. Distribution of individual serum reaction values in the rNS1-specific IgG ELISA tests. A panel of primary (□) and secondary (■) sera from DV infected patients as well as negative control (♦) and YFv, yellow fever vaccinated healthy individuals (◯) serum samples. The cut off extinction (- - -) was determined as described in Fig. 2. Each spot represent the average from two individual experiments with duplicate for each serum sample ($P < 0.05$).

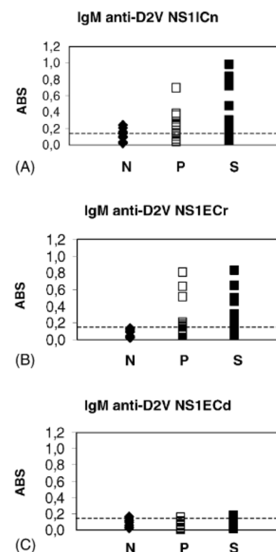

Fig. 4. Distribution of individual serum reaction values in the rNS1-specific IgG ELISA tests. A panel of primary (□) and secondary (■) sera from DV infected patients and negative control (♦) serum samples. The cut off extinction (- - -) was determined as described in Fig. 2. Each spot represent the average from two individual experiments with duplicate for each serum sample ($P < 0.05$).

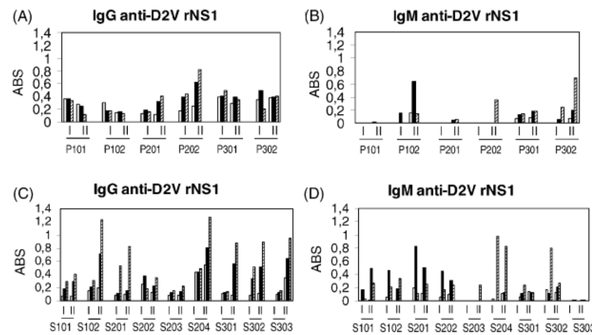

Fig. 5. Reactivity profile of paired serum samples to different rNS1 forms detected in IgG and IgM ELISA tests. Serum samples were collected from acute and convalescent phases of each patient. Diagnostic characteristics of primary (P) and secondary (S) samples are listed in Table 1. Open bars, NS1ECd; black bars, NS1ECr; hachured bars, NS1ICn. Data represent the mean of duplicate values ($P < 0.05$).

negative virus isolation, could be detected in the IgG anti-rNS1 assays (Table 1 and Fig. 5A).

4. Discussion

Several reports have detected high levels of either, antibodies anti-NS1 and the NS1 antigen in sera from patients infected with dengue virus (Alcon et al., 2002; Libraty et al., 2002; Young et al., 2000). Both of then have been suggested to be used for dengue diagnostic tests. However, the results obtained by distinct procedures could differ in levels of specificity and sensibility. To evaluate the potential use of the rNS1 proteins to optimize DV assays, a serum panel from Brazilian patients experiencing primary and secondary DF infections was analyzed by ELISA using four rNS1 forms. As expected, those proteins revealed differences on the electrophoretical patterns (Fig. 1). Such differences are related to the protein sources and their processing way, which interfered in their conformation and, consequently, in their electrophoretic mobility. It was interesting to note that all the rNS1 forms, including the NS1IC in its native conformation, reacted with the specific NS1 monoclonal with different intensities (data not shown). Considering that those MAbs were described as recognizing linear epitopes (Falconar et al., 1994), such data revealed the possibility of these proteins to expose epitopes able to react with those immunoglobulins independent on their conformation.

The reactions against the NS1ICn presented the highest correlation with the DV positive specimens, suggesting that antibodies anti-NS1 prevalent in the sera were those that recognized epitopes on the glycosylated NS1 form. However, our results also demonstrated that NS1ECr, a nonglycosylated refolded form studied by Huang et al. (2001), could be recognized by some serum specimens that did not react against the NS1ICn. These data points a heterogeneous pattern of human response to D2V-NS1 antigen. In fact, the present analysis showed a large diversity in the immune response elicited by each DV infected individual. Most of the sera contained antibodies that recognized the rNS1 proteins with different intensities.

When considered together, the results of the IgG assays performed by NS1ICn and NS1ECr proteins detected almost 90% of DV positive samples from the secondary panel (Fig. 1). However, the reaction intensities of the same patient differ significantly depending on the infection phase and on which rNS1 form was used. The samples that showed strong reactions against one of this proteins, did not present, necessarily, the same reaction level against the other (Fig. 5). Moreover, the reaction patterns showed variations for each immunoglobulin isotype with each different protein forms. The serum panel used in this study included several samples DV positive with IgM negative, mainly specimens from the acute infection phase. These results are in agreement with Kuno et al. (1998) that reported 5% of IgM negative specimens in positive dengue samples from individuals with both primary and secondary infections. Further, analysis to detect the appearance of the specific-DV IgM in human plasma found a delay of about 3 days after the symptoms onset (Alcon et al., 2002). The same authors reported the circulation of the antigen in samples at the first clinical symptoms, up to 9 days. Therefore, based on these data, it is clear that the infection stage is extremely relevant when collecting serum samples and this could have interfered in some of our results. However, several samples (S102, S201, S204 and S302), collected, respectively, at days 1 and 3 after the onset of illness, revealed specimens MAC negative with significant IgM anti-rNS1 levels. Moreover, the correspondence between specimens MAC positive, from primary infections, and the IgM anti-rNS1 assays, reinforce the previous reports, that pointed the NS1 presence in patient plasma early in the dengue infections as being presumably the cause of the early antibodies anti-NS1.

A remarkable absence of correspondence between the IgG and IgM levels in several serum samples was also observed. In the primary serum panel, the sample P202 showed specimens of the acute and convalescent phase with high levels of IgG against both proteins, NS1ICn and NS1ECr, but the IgM levels were undetectable in most of the reactions. Other samples (P102 and P302) that presented a later IgM response to one of the rNS1 sustain the evidence of the D2V-NS1 to elicit a large range of human immune response and this must be considered for future DV antibody assay optimization.

As expected, the prevalence of antibody isotype anti-rNS1, in samples from secondary infections, showed a significant shift from IgM to IgG. In addition, only among the secondary convalescent sera were observed a significant correlation between the IgG anti-NS1ICn levels and the disease status. These discrepancies could be analysed considering the interferences due to the protein source or to the patient inherent characteristics. About the protein, Se-Thoe et al. (1999) also detected different reaction patterns depending on cell source. About the human immune response, previous reports found significant differences in the DV-NS1 antibody levels in patient sera from different countries. This was associated with the local DHF incidence (Kuno et al., 1990; Kurane and Takasaki, 2001). Some authors suggested that these results could be associated with the population ethnical characteristics. Actually, Brazil shows low incidence of DHF/DSS, and its population is composed by a large ethnical miscegenation, which could explain the diversity of the immune response pattern observed in this study. Furthermore, it was possible that our results were underestimated since no immune–complex-dissociation procedure was used during the sample processing assays. Koraka et al. (2003) demonstrated the relevance of those methods to early diagnosis of DV infections. Indeed, our results, even under the present study conditions, demonstrated that a significant percentage of samples from the secondary serum panel changed the their immune response profile depending on protein form and/or disease phase. These results induce us to question the relevance of such tests as a prognostical assay for development of DHF. Are the circulating antibodies anti-NS1 definitively associated with the dengue morbidity, a cause or a consequence?

Several authors have proposed the DV-NS1 and the specific circulating anti-NS1 as factors associated with the ADE and the development of the DHF and DSS (Falconar, 1997; Shu et al., 2000; Valdés et al., 2000; Young et al., 2000). However, the literature presents conflicting data concerning to the role of this antigen and its correspondent antibodies in the severe clinical symptoms. Henchal et al. (1988) reported that NS1-raised antibodies might confer protection against DV. These proposal seems to have been confirmed by a prospective study that established a relationship of pre-existing DV neutralizing antibodies with lower viremia and milder disease (Endy et al., 2004). In spite of these reports, another works point different possibilities. In previous analysis, using monoclonals generated against D2V-NS1, it was suggested the potential of those antibodies for trigger the hemorrhagic phenomenon (Falconar, 1997). More recently, Libraty et al. (2002) demonstrated that free levels of secreted NS1 in plasma could be correlated with viremia levels and it was found a predictive value for the development of DHF. As pointed above, all our samples, from the secondary serum panel, that showed high levels of anti-rNS1 were from patients that presented symptoms of classical dengue with any hemorrhagic signals. Such observation might reflect differences in pathophysiological roles due to the presence of circulating anti-NS1. In fact, all samples tested in the present work, even those with high levels of anti-NS1ICn and anti-NS1ECr, contained low levels of anti-NS1ECd and presumably to linear epitopes. If we accept the possibilities proposed by Falconar (1997) that antibodies anti-NS1 that reacted with those epitopes that cross-react with human blood structures might contribute with the hemorrhagic symptoms, so its possible speculate that the absence of this kind of antibodies could prevent the severity of dengue infections. There are no evidences whether the patients, target of the present study that showed high anti-NS1 levels, also experienced an infection with high levels of serum circulating NS1 antigen or did not. In order to confirm this hypothesis, further analysis will be necessary amplifying the cohort with paired serum samples, including samples collected from individuals with DHF, the patient anamnesical data, genetical characteristics and previous medical history.

Although the correlation between NS1-specific antibodies, the presence of circulating NS1 protein and the severe disease symptoms is far from being definitely established, our results sustain evidences that reinforce the use of the antibodies anti-NS1 as a tool for the improvement of dengue diagnostic tests. The rNS1 proteins used in our study was isolated from D2V and showed similar reaction patterns with the sera collected from patients infected with other DV serotypes (1 and 3) in IgG assays. However, the number of sample size of DV positive patients infected with other serotypes was rather small and we are not able to perform a statistical analysis.

Finally, our observation emphasizes the usefulness of non-structural proteins, in particular the rNS1, in diagnostic tests as a complementary reagent. However, further knowledge advances are necessary concerning to the role of this protein in the establishment of the disease and the use of different recombinant NS1 forms to optimize the dengue diagnostic assays.

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References

Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M. Enzyme linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in blood during the acute phase of disease in patients experiencing primary and secondary infections. J Clin Microbiol 2002;40:376–81.

Endy TP, Nisalak A, Chunsuttitwat S, Vaughn DW, Green S, Ennis FA, et al. Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. J Infect Dis 2004;189:990–1000.

Falconar AKI. The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin, adhesin proteins and binds to human endothelial cells: potential implications in hemorrhagic fever pathogenesis. Arch Virol 1997;142:897–916.

Falconar AKI, Young PR, Miles MA. Precise localization of sequential dengue virus sub complex and complex B cell epitopes on the nonstructural-1 glycoprotein. Arch Virol 1994;137:315–26.

Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J Virol 1999;73:6104–10.

Gluber D. Dengue virus and dengue hemorrhagic fever. Clin Microbiol Rev 1998;11:480–96.

Gubler DJ, Kuno G, Sather E, Valez M, Olive A. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. Am J Trop Med Hyg 1984;33:158–65.

Halstead SB. Antibody, macrophages, dengue virus infection, shock and haemorrhage: a pathogenic cascade. Rev Infect Dis 1989;11:S830–9.

Halstead SB. Global epidemiology of dengue hemorrhagic fever. Southeast Asian Trop Med Publ Health 1990;21:636–41.

Henchal EA, Henchal LS, Schlensinger JJ. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol 1988;69:2101–7.

Huang JL, Huang JH, Shyu RH, Teng CW, Lin YL, Kuo MD, et al. High-levels expression of recombinant dengue viral NS1 protein and its potential use as a diagnostic antigen. J Med Virol 2001;65:553–60.

Igarashi A. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. J Gen Virol 1978;40:531–44.

Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, Suntayakorn S, et al. An enzyme-linked immunoassay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. Am J Trop Med Hyg 1989;40:418–27.

Koraka P, Burghoorn-Mass CP, Falconar A, Setiati TE, Djamiatun K, Groen J, et al. Detection of immune–complex-dissociated nonstructural-1 antigen in patients with acute dengue virus infections. J Clin Microbiol 2003;41:4154–9.

Kuno G, Gomez I, Gubler DJ. Detecting artificial anti-dengue IgM immune complexes using an enzyme-linked immunosorbent assay. Am J Trop Med Hyg 1987;36:153–9.

Kuno G, Vorndan AV, Gubler DJ, Gomes I. Study of anti-dengue NS1 antibody by Western blot. J Med Virol 1990;32:102–8.

Kuno G, Gomes I, Gubler DJ. An ELISA procedure for the diagnosis of dengue infections. J Virol Meth 1991;33:101–13.

Kuno G, Cropp CB, Wong-Lee J, Gubler DJ. Evaluation of an IgM immunoblot kit for dengue diagnosis. Am J Trop Med Hyg 1998;59:757–62.

Kurane L, Takasaki T. Dengue fever and dengue hemorrhagic fever: challenges of controlling an enemy still at large. Rev Med Virol 2001;11:301–11.

Lanciotti R, Calisher C, Gubler DJ, Chang G, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 1992;30:545–51.

Libraty DH, Young PR, Pickering D, Endy TP, Kalayanararooj S, Green S, et al. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlated with development of dengue hemorrhagic fever. J Infect Dis 2002;186:1165–8.

Miagostovich MPR, Nogueira M, Santos FB, Schatzmayr HG, Araujo ES, Vorndam V. Evaluation of an IgG enzyme-linked immunosorbent assay for dengue diagnosis. J Clin Virol 1999;14:183–9.

Monath TP. Dengue: the risk to developed and developing countries. Proc Natl Acad Sci USA 1994;91:2395–400.

Morrisey JH. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 1981;117:307–10.

Rothman A, Ennis FA. Immunopathology of dengue hemorrhagic fever. Virology 1999;257:1–6.

Schlesinger JJ, Foltzer M, Chapman S. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis. J Gen Virol 1993;76:217–20.

Se-Thoe S, Ng M, Ling A. Retrospective study of Western blot profiles in immune sera of natural dengue virus infections. J Med Virol 1999;57:322–30.

Shu P, Chen L, Chang S, Yueh Y, Chow L, Chien L, et al. Dengue NS1 antibody response: isotype distribution and serotyping in patients with dengue fever and dengue hemorrhagic fever. J Med Virol 2000;62:224–32.

Valdés K, Alvarez M, Pupo M, Vázquez S, Rodríguez R, Guzmán MG. Human dengue antibodies against structural and nonstructural proteins. Clin Diagn Lab Immunol 2000;7:856–7.

Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 2000;181:2–9.

World Health Organization (WHO). Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd ed. Genova: WHO; 1997.

Young PA, Paige A, Bletchly C, Halloran W. An antigen capture enzyme-linked immunosorbent assay reveals high levels of dengue virus protein NS1 in sera of infected patients. J Clin Microbiol 2000;38:1053–7.

ANEXO 2

Liver injury and viremia in mice infected with dengue-2 virus

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Liver injury and viremia in mice infected with dengue-2 virus

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Abstract

The goal of this study was to test the feasibility of BALB/c mice as an experimental model in the study of dengue disease. BALB/c mice were intraperitoneal infected with DENV-2 obtained from a human patient. Histopathological analysis of infected animals revealed liver injury with viral antigens detection. In initial stages, the most prominent lesions were vacuolization and diffuse steatosis in hepatocytes. Serum levels of ALT and AST increased progressively, reaching the highest values 7 days p.i. and decreasing at the 14th day. Since levels of circulating virus were very low, viremia was analyzed in C6/36 cells. Virus presence was detected by ultrastructural analysis, confirmed by RT-PCR assays. Period of viremia was analyzed by flow cytometry with cells incubated with mouse-infected sera collected in different days, revealing peak virus levels at the 7th day p.i. All such data correlate to the development of the disease described in humans.
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Keywords: DENV-2; Mouse; Liver; Viremia; Histopathology; Transaminases

Introduction

Dengue is an acute disease caused by the infection of dengue virus (DENV), which consists of four distinct antigenic types (DENV-1 to -4). The disease presents a wide range of clinical symptoms, including a mild self-limiting acute febrile illness (DENV fever) and hemorrhagic fever and/or shock syndrome (DHF/DSS) (Halstead, 1989). The clinical features of DHF include plasma leakage, a tendency to bleed, and liver involvement (Rothman and Ennis, 1999), which can progress to DSS and death (Henchal and Putnak, 1990). Although the liver is not a major target organ, the involvement of the liver in the pathogenesis of dengue virus infection, in particular concerning the development of DHS, was demonstrated by the abnormal liver function, tissular injury, presence of viral antigens, and RNA in human liver tissue (Bhamarapravati and Halstead, 1964; Bhamarapravati et al., 1967; Burke, 1968; Kalayanarooj et al., 1997; Kuo et al., 1992; Miagostovich et al., 1997; Nguyen et al., 1997; Rosen et al., 1989, 1999; Wang et al., 1990).

In general, most experimental studies dealt with suckling or young mice inoculated by the intracerebral route of a mouse-adapted DENV-2 (Nath et al., 1983; Raut et al., 1996). Animal responses to virus infection (clinical signs and/or degree of injury) varies according to mice strain, although these experimental models normally show that mice are susceptible for DENV infection. The full DHF/DSS manifestations, however, do not seem to occur in standard laboratory mice.

Some studies are based on other inoculation routes, such as intraperitoneal and intravenous, which resembles more to the natural infection in human population. Huang et al. (2000a, 2000b) showed that A/J mice inoculated intra-

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venously with a non-adapted DENV-2 presented a transient thrombocytopenia and anti-platelet antibodies. Such alterations were remarkable reduced when BALB/c or B6 mice were used (Huang et al., 2000a, 2000b). In nude mice, petechial and gastrointestinal hemorrhages have been observed (Raut et al., 1996). In the model of infection developed in SCID mice submitted to the transplantation of human hepatocarcinoma cell line (HepG2) foci of tissular hemorrhagy, hemoconcentration and fluid extravasation, thrombocytopenia, and prolonged partial thromboplastin time were achieved (An et al., 1999). This model takes advantage of the fact that HepG2 cells, a human hepatoma cell line, which conserved numerous characteristics of differentiated hepatocytes (Knowles et al., 1984), is permissive for DENV infection and replication. HepG2 cells transplanted into SCID mice are able to restock liver, and after viruses inoculation became the major target for their replication. Infected cells die by an apoptotic mechanism as a direct consequence of the viral infection (Marinneau et al., 1998). Moreover, these animals developed a hind-leg paralysis, liver injury, and renal dysfunction. Viremia in this model peaked at 8-day post-infection (p.i.) but viruses were already seen in 60% of mice at day 5. Viruses were detected in the hepatic tissue, brain, and blood (An et al., 1999).

The present study has been undertaken to characterize a mouse model of DENV-2 infection by intraperitoneal inoculation of a non-adapted virus isolated from a human patient. BALB/c mice developed an apparently mild infection, but with liver injury characterized by histopathological aspects and biochemical tests. Viral antigens were detected in focal areas of the damaged liver, confirming the presence DENV-2 in such tissue. Viremia was analyzed by flow cytometry with C6/36 cells incubated with mouse-infected sera collected in different times, which revealed peak virus levels at the 7th day p.i. in most animals. All such data correlate to what is observed in the human disease and therefore demonstrate that BALB/c mice strain is a susceptible animal to DENV-2 and can be used as an experimental model for the study of the pathogenesis of dengue disease.

Results

Mice infection

All mice inoculated with DENV-2 survived to the infection and did not present clinical signs, although some tissue pathological alterations could be detected. Liver of control mice, inoculated only with culture medium, did not exhibit any modification in their structure (Fig. 1a). However, hepatic injury was seen in all DENV-2-infected mice beginning from the 2nd until the 17th day p.i. (Figs. 1b–j). At the 2nd day p.i., hepatic plates maintained its architecture and were generally constituted by mononucleated hepatocytes, although some of them were binucleated. Some hepatocytes were slightly enlarged due to vacuolization while others were diminished. Hepatocyte vacuolization was observed all over the hepatic acini, but was more prominent in the zone II of the liver acini where cells were sparse. Slight variation in hepatocyte size could be observed (midzonal, Fig. 1b). At the 3rd day p.i., diffuse steatosis were noted in hepatocytes around the central vein area, mainly in sinusoidal side. Numerous hyperplastic Kupffer cells were detected in sinusoids capillary (Fig. 1c) and an increase of monocytes cells was observed in sinusoids (Fig. 1d). At the 7th day p.i., an intense area of edema was evident around the hepatocytes nucleus, causing a progressive increase of necrosis area in focal parenchyma. Sinusoid capillaries presented several monocytes and lymphocytes (Fig. 1e). Diffuse necrose of hepatocytes was also observed surrounding portal areas with increased monocyte infiltration (Fig. 1f). At the 13th day p.i., hepatocytes exhibited changes in their nuclear content and lipid-like nuclear inclusions were occasionally seen in vacuolated cells (Fig. 1g). Rare isolated or grouped inflammatory cells (mononuclear cells) were focally seen inside sinusoids at the 13th day p.i. Inflammation in hepatic lobuli was characterized by the presence of polymorphonuclear cells surrounding tumefacted hepatocytes with an intense cytoplasmic clarification (Fig. 1h). At the 17th day p.i., apoptotic cells were seen at the sinusoidal space as well as necrotic hepatocytes (Fig. 1i). Although at this time point hepatocytes seemed to be regenerated, focal rarefaction of sinusoidal cells and sinusoidal disappearance were occasionally seen at the 17th day p.i. (Fig. 1j).

The semiquantitative analysis demonstrated that in general at the 2nd day p.i., hepatocytes did not change their diameter in relation to control mice ($P > 0.05$). However, at the 13th day p.i., their diameter was significantly increased ($P < 0.05$) (Fig. 2a). Sinusoidal capillaries had a diminished diameter ($P < 0.05$) at the 2nd day p.i. and became significantly enlarged later on ($P < 0.05$) (Fig. 2b). Analysis of sinusoidal cells revealed a significantly decrease of the number such cells in all time points ($P < 0.05$) (Fig. 2c).

Detection of viral antigen in the liver of infected mice

In order to confirm the presence of DENV-2 in liver tissue, immunohistochemistry assays were performed using a monkey polyclonal anti-DENV-2 antibody. At the 2nd day p.i., viral antigens were detected in focal hepatocytes (Fig. 3c) and in the capillar endothelium of the central lobular vein (Fig. 3d). The antigens were also detected in the hepatocytes around the portal space (Fig. 3e). At the 13th day p.i., virus antigens were also detected in focal zones (Fig. 3g), although in a large extension as compared to the 2nd day p.i. Antigens were observed in plates of hepatocytes, in the same areas that exhibiting hepatic injury (Fig.

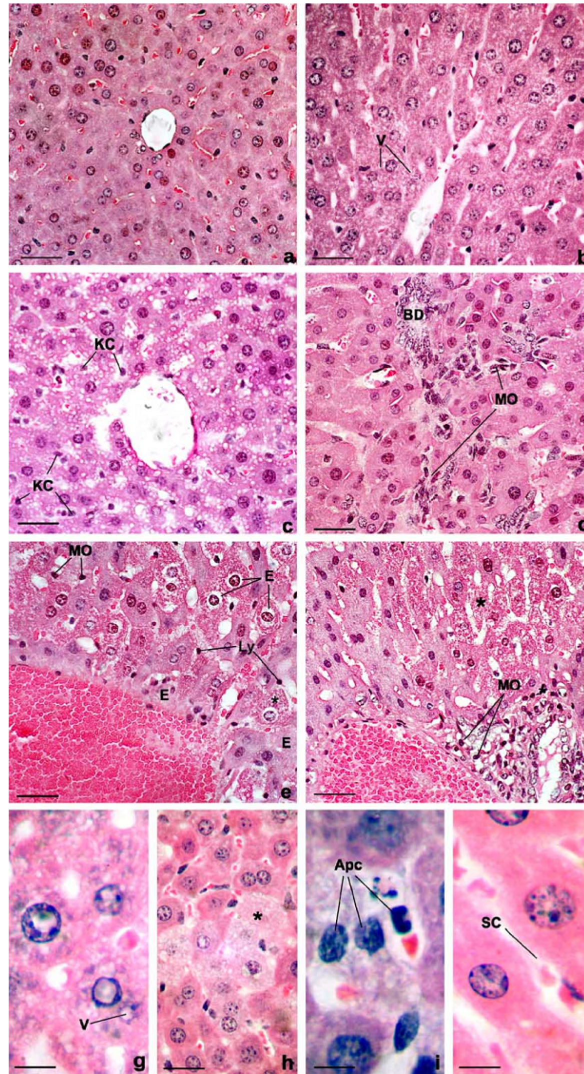


Fig. 1. Histological analysis of liver sections of control and DENV-2-infected mice, HE staining. (a) Liver section of a mouse inoculated with L-15 medium, presenting hepatocytes, sinusoid capillars, and a central vein without alterations (scale bar = 30 μm); (b) liver of a mouse at the 2nd day p.i., showing vacuolization (V) of hepatocytes and dilatation of sinusoid capillars around the central vein (scale bar = 20 μm); (c and d) liver of a mouse at the 3rd day p.i. with diffuse steatosis at the sinusoidal side of hepatocytes and increase of the number of Kupffer cells (KC), observed around the central vein (c), and microvesicular steatosis noted in the portal space and monocyte cells (MO) observed inside the sinusoids (d) (scale bars = 30 μm); (e and f) liver of a mouse at the 7th day p.i. with necrosis of hepatocytes and tissue edema (E) and an increase of the number of monocytes (MO) and lymphocytes (LY) inside the sinusoid capillaries (e), and hepatocyte necrosis (asterisk) with the presence of monocytes (MO) observed in portal areas (f) (scale bars = 30 μm); (g and h) liver section of a mouse at the 13th day p.i. in which lipid-like nuclear inclusions may be observed in vacuolated (V) and tumefacted (asterisk) hepatocytes (scale bar = 8 μm and 15 μm in panels g and h, respectively); (i and j) liver section of a mouse at the 17th day p.i. with apoptotic cells (Apc) occurring inside the sinusoidal capillar space (i) and the disappearance of sinusoidal cells (SC) (j) (scale bars = 6 μm).

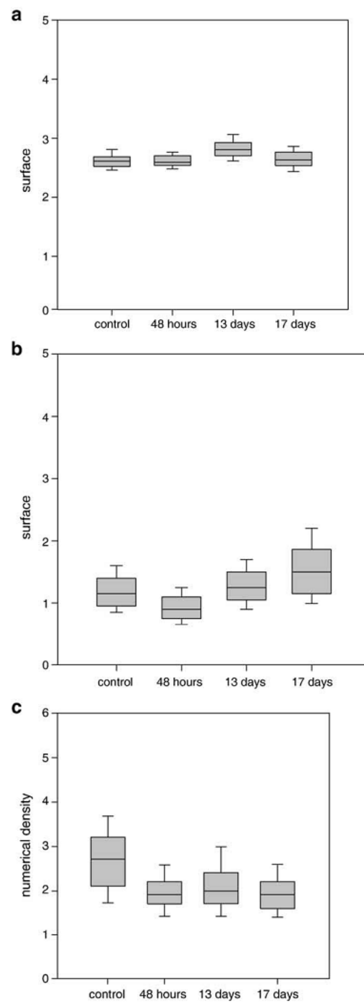


Fig. 2. Semiquantitative analysis of hepatocyte (a), sinusoidal capillars (b), and sinusoidal cells (c). (a) Hepatocytes in liver injury did not change their diameters in relation to control mice ($P > 0.05$), at the 2nd day p.i., while their diameters were significantly increased ($P < 0.05$) at the 13th day p.i.; (b) sinusoidal capillar had a diminished diameter ($P < 0.05$) at the 2nd day p.i. and became enlarged significantly ($P < 0.05$) in late infection; (c) the number of sinusoidal cells diminished at the 2nd day p.i. and increase in late infection ($P < 0.05$).

3g). As expected, negative control, constituted of liver of non-infected mice reacting with anti-DENV-2 antibodies, did not present any virus antigen (Fig. 3a). Furthermore, livers of infected mice at the 2nd and 13th days p.i. also did not present any reaction with the anti-human IgG conjugate (Figs. 3b and f, respectively).

Transaminase level quantifications in serum samples of DENV-2-infected mice

In order to investigate whether the hepatic injury detected in infected mice correlates to alterations in transaminase levels, semiquantitative analyses of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were performed. In general, at the 7th and 14th days p.i., levels of ALT and AST increased in most of the tested serum samples as compared to control sera (Fig. 4). Apparently, both transaminases presented peak level at the 7th day p.i. The statistical analysis of the data, however, showed no significance with the $P = 0.051$ at the 7th day p.i. The lack of significance was probably due to a considerable inter-individual variation.

Detection of DENV-2 in the C6/36 cell line inoculated with sera from infected BALB/c mice

Since levels of circulating virus in infected mice were very low (data not shown), for further analysis, C6/36 cells were used in order to amplify the amount of DENV-2 particles obtained from serum samples collected from these animals. Cells inoculated with mouse sera from the 2nd and the 11th days p.i., examined 15 days after incubation, presented viral particles inside the cisternae of the rough endoplasmic reticulum (Figs. 5c and d, respectively). Vacuolization was also observed in these cells, similar to those detected in positive control cells, infected with DENV-2 (Fig. 5b).

Detection of DENV-2 viral genome by RT-PCR amplification of the NS1 gene sequence

One DNA fragment of 1.1 kb, corresponding to the NS1 gene sequence, was detected in extracts of cells inoculated with mouse sera collected at the 2nd and the 11th days p.i. No amplification was observed in control samples cultivated with non-inoculated mouse sera (Fig. 6). These results confirmed the presence of DENV-2 in serum samples of infected mice and indicated that such virus is still able to infect other cells.

Detection of the period of viremia in serum of BALB/c mice infected with DENV-2 and inoculated in C6/36 cells (flow cytometry)

In an attempt to quantify the degree of infection in the present mouse model, flow cytometry techniques were applied. A DENV-2 hyperimmune mouse ascitic fluid was used for the quantification of the virus in the cultures treated with sera of infected animals. The serum was purchased in three different time periods after infection (3, 7, and 14

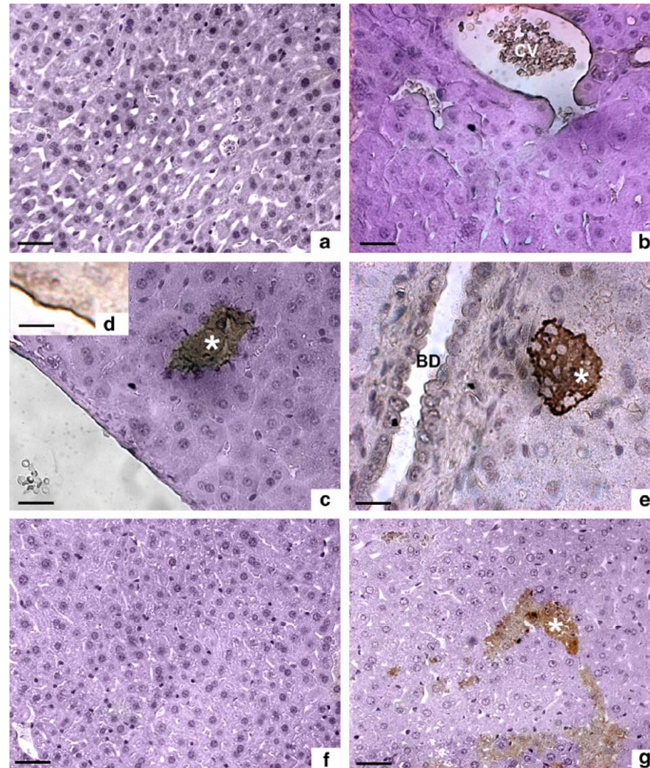


Fig. 3. Detection of DENV-2 antigens in liver of infected mice. (a) Negative control obtained with liver of a non-infected mouse (scale bar = 10 μm); (b) liver of an infected mouse at the 2nd day p.i., incubated only with anti-human IgG-horseradish peroxidase conjugate, central vein (scale bar = 10 μm); (c–e) liver of an infected mouse at the 2nd day p.i., incubated with anti-DENV-2 antibodies and anti-human IgG-horseradish peroxidase conjugate. Note the positive reaction in hepatocytes in focal midzonal (c) and portal space (e) areas (asterisk) (scale bar = 5 μm), and in endothelium capillar (d) (scale bar = 1 μm), biliar duct (BD); (f and g) liver of an infected mouse at the 13th day p.i., incubated only with the secondary antibody (f) or with primary and secondary antibodies (g) (scale bars = 10 μm). Note the positive reaction in several hepatocytes (asterisk).

days). Fig. 7a shows original histograms of one sample at the three different time periods illustrating the clear reaction of this antibody. Anti-DENV-2 was significantly positive in cells incubated with serum collected 7 and 14 days after infection, where a clear shift to the right side of the graph was observed. The peak of viremia was detected using the flow cytometer, revealing a ten-fold increase in the percentage of positive cells for the anti-DENV-2 antibodies 7 days after infection, followed by a subsequent decline of viremia at day 14th p.i. (Figs. 7a and b). A considerable individual variation, however, was determined. Six out of the 8 animals studied were positive to anti-DENV-2 (Figs. 7b and c). Four positive animals achieved the peak of viremia at day the 7th day p.i., whereas two animals behaved differently, one reaching its highest level of infection at the 14th day p.i. and the other at the 3rd day p.i. (Figs. 7b and c).

Discussion

Dengue is an intriguing disease and the mechanisms involved in the pathogenesis of DHF/DSS are yet poorly understood. In an attempt to understand the disease and/or to test new drugs or vaccines, several studies suggested the use of murine models (Atrasheuskaya et al., 2003; Huang et al., 2000a, 2000b; Shresta et al., 2004), although none of them reproduce the exactly symptoms observed in humans. In the present report, we evaluated the use of BALB/c as an experimental mouse model for DENV-2 infection. A

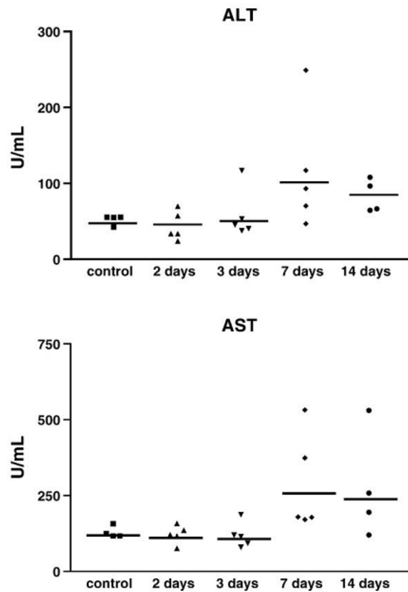


Fig. 4. This serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice experimentally infected with DENV-2, at the 2nd, 3rd, 7th, and 14th days p.i.

DENV-2 isolated from a serum sample obtained from one patient, followed by cultivation in mosquito cells, was directly inoculated in mice by the intraperitoneal route. Animals did not present clinical signs of the infection, significant hepatic alterations were observed. Histopathological analysis revealed focal injury in lobular parenchyma in hepatocytes. These results correlate to what is observed in humans, in which liver seems to be one target organ. The biology and clinical aspects of hepatitis is a well-known feature of human dengue infection (Innis, 1995; Rosen et al., 1989), although DENV can lead to a mild degree of hepatic involvement. However, it has been reported in recent years a severe involvement of the liver, including cases of fulminant hepatitis with high mortality (Lum et al., 1993).

The hepatic injury observed in the present mouse model began 2 days p.i., and at the 3rd day p.i. hepatocytes presented diffuse steatosis in midzonal areas. Necrosis of hepatocytes and a strong flux of edema was observed at 7 days p.i. The descriptions of liver histopathology in humans infected with DENV is yet scarce. The study of a dengue fatal case in human also showed diffuse hepatitis with midzonal necrosis and steatosis recruitment of inflammatory cells into hepatic parenchyma (Huerre et al., 2001). The data reported in this study correlated with our results. The observed hepatic injury was similar to that of early stages of yellow fever infection. An increase of plasma was reported when infection occurred through the intraperitoneal route. Simultaneously an increase in the transaminase levels, fatty changes in hepatocytes, Kupffer cell hyperplasia, and centrilobular and midzonal necrosis was observed (Innis, 1995; Kuo et al., 1992). The inflammatory infiltrate presented monocytes and lymphocytes in sinusoids of focal areas of the portal space. In accordance to this observations, in our model, mice infected with DENV-2 also presented numerous hyperplastic Kupffer and monocytes cells into sinusoids capillar and portal space, respectively. Some reports have shown that endothelial and Kupffer cells were susceptible for DENV replication (Hall et al., 1991; Innis, 1995) suffering, therefore, viral cytopathic effects, which may explain the hyperplasia observed. In fact, in the present work, viral antigens were detected in capillar endothelium of the central lobular vein confirming its susceptibility to virus replication, as well as in several hepatocytes. However, such antigens were not verified in Kupffer cells. In humans, antigens of DENV-2 have been detected in liver tissue, either in Kupffer's cells or in hepatocytes, spleen, lymph nodes, pulmonary alveolae, blood mononuclear cells, peripheral B cells, and thymic cortex (Couvelard et al., 1999; Hall et al., 1991; Huerre et al., 2001; Innis, 1995; Kuo et al., 1992; Monath, 1986; Nguyen et al., 1997; Rosen et al., 1999; Scott et al., 1980; Waterman et al., 1985). Further studies will be necessary in order to analyze or evaluate the extension of injury in other organs in mice, with the presence of viral antigens.

Apoptosis is a major feature of viral hepatitis (Galle et al., 1995) and also participates of the DENV pathological spectrum. In our work we could note the presence of apoptotic cells, mainly at the sinusoidal space, in later stage of infection, at the 17th day p.i. This apoptosis is probably related to focal rarefaction of sinusoidal cells and sinusoidal endothelial. In vitro, various cell lines susceptible for DENV, such as HepG2 and a mouse neuroblastoma cell line (Neuro2a), exhibited apoptotic cell death when infected with DENV (Desprès et al., 1996; Marianneau et al., 1997). In humans, apoptosis could also occur in endothelial and Kupffer cells, therefore leading to the focal sinusoidal denudation demonstrated herein. Probably the sinusoidal denudation seen in DENV-2 infection may cause the microvascular barrier derangement and may be pivotal in the development of parenchymal damage, contributing to the microvascular disruption caused by cytokines secreted by activated cells (Dhawan et al., 1990; Khanna et al., 1990).

The degree of hepatocytic viral injury induced in DENV infection could also be observed in the transient liver dysfunction depicted by enzyme levels. Serum levels of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) can be significantly higher in humans infected with any of the DENV serotypes. In general, DF is usually associated with mild to moderate elevations of such enzymes, while in patients with DHF/DSS ALT and AST

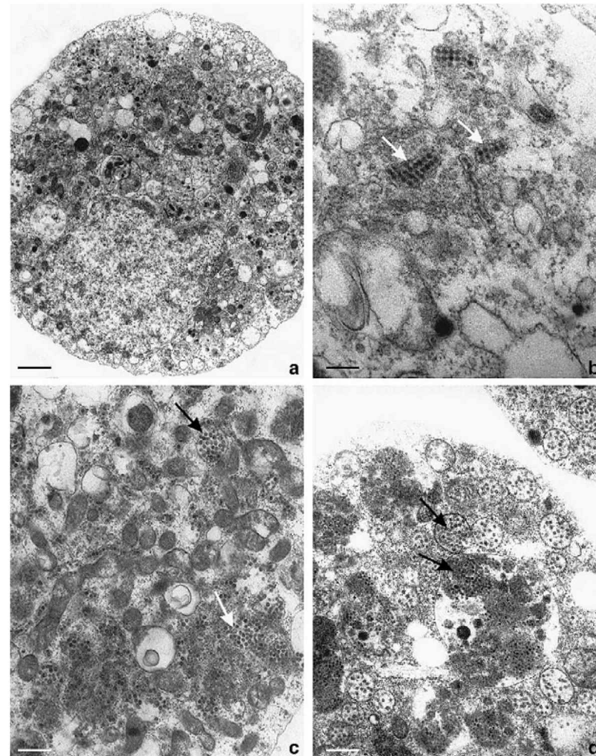


Fig. 5. Electron microscopy of C6/36 cells incubated with sera from DENV-2-infected mice. (a) Negative control of C6/36 cell (scale bar = 1 μm); (b) positive control of C6/36 cell infected with DENV-2. Note the virus particles (arrow) (scale bar = 0.2 μm); (c and d) C6/36 culture cells inoculated with sera from mice collected at the 2nd (c) and 11th days p.i. (d). Note the virus particles inside vesicles and vacuoles (arrows) (scale bar = 0.5 μm).

levels became remarkable higher (Kuo et al., 1992; Souza et al., 2004). Peak levels of the transaminases normally occurred at the 7th day p.i. and decreased in the following 2 weeks. Similar results were observed in the experimental mouse model here presented, in which levels of both hepatic transaminases (ALT and AST) increased in most of the tested serum samples, with peak levels at the 7th day p.i. Moreover, AST levels in mice were higher than ALT, which is also verified in humans (Nguyen et al., 1997).

Another parameter for evaluation of DENV infection is the viremia, which correlates to transaminase levels and other hepatic damage. In most experimental mouse models, viremia could not be characterized, probably due to the fact that circulating DENV is found in a very low level in such animals, which stands below the sensibility assays for normal virus titrage and/or isolation (Bhamarapravati and Halstead, 1964; Boonpucknavig et al., 1981; Nath et al., 1983; Shresta et al., 2004). In order to amplify the amount of DENV-2 particles obtained from mouse sera, we inoculated C6/36 cells with serum samples collected in different time points during the animal infection. Viral particles could be detected inside those cells by ultrastructure analysis and its presence was confirmed by PCR assays. Such results indicated that in fact DENV-2 particles were circulating in blood samples of infected mice and that such virus maintains its capacity to replicate in different cells.

In an attempt to detect the period of viremia in these animals, C6/36 cells were incubated with mouse serum samples purchased in different times and analyzed by flow cytometry. Peak of viremia was observed at the 7th day p.i. in most animals followed by a subsequent decline at the 14th day p.i. In humans, the peak of viremia is also verified around the 7th day p.i. with a decrease after 1 week (Kuo et al., 1992; Mohan et al., 2000). Huang et al. (2000a, 2000b) also detected viremia in blood samples of A/J mice by RT-PCR but not in BALB/c mice. However, virus could only be detected 2 days after inoculation by the intravenous route

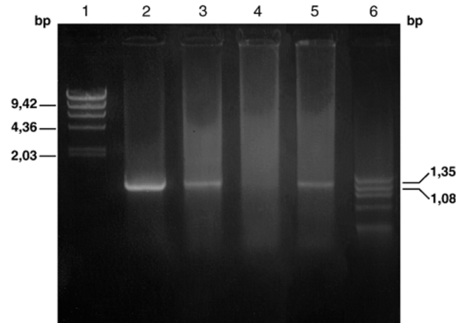


Fig. 6. The DENV-2 NS1 gene amplified by PCR and electrophoresed in 1% agarose gel stained with ethidium bromide. Serum samples collected from mice infected with DENV-2 were added to C6/36 cells and PCR reactions were performed using cDNAs made from extracts of such cells. Lanes: (1) standard marker of λ DNA digested with *Hind*III; (2) extract of cells cultivated with mouse sera collected 2 days following virus infection; (3) extract of cells cultivated with mouse sera collected 11 days following virus infection; (4) negative control with extract of cells cultivated with non-infected mouse sera; (5) positive control with extract of cells infected with DENV-2; and (6) standard marker of ϕ ×174 DNA digested with *Hae*III.

and it disappeared in the following days. Similar results were described by Atrasheuskaya et al. (2003), using young BALB/c mice inoculated with DENV-2 by the intraperitoneal route, in which viremia was detected by PCR after the 2nd day p.i. These experiments, nevertheless, were conducted with a previous mouse brain adapted. In our work, assays were performed with a DENV-2 isolated from a patient serum, cultivated in mosquito cells, and directly infected in mice by the intraperitoneal route.

In our mouse model we found: hepatic alterations, including liver histopathological injury with the presence of viral antigens, elevated levels of transaminases, mainly at the 7th day p.i., and a correlation between the peak of viremia, and ALT and AST levels. Our results revealed the ability of a DENV-2 circulating in a human population to infect BALB/c mice and are therefore a relevant physiological approach. Moreover, in conclusion, they demonstrated that this mouse model is feasible for the study of DENV disease. Furthermore, studies of experimental drugs or vaccines might be performed using the BALB/c mice.

Material and methods

Virus

DENV-2 was isolated from a patient serum during an outbreak of the virus in the state of Rio de Janeiro, in 1995 (Nogueira et al., 1995), kindly provided by the Flaviviruses Laboratory of the Department of Virology, Fiocruz, RJ. Virus had not undergone any passage in mouse brain. It was propagated in *Aedes albopictus* mosquito cell line monolayers (C6/36), in 10 ml tubes, with L-15 medium (Sigma, USA) supplemented with 1% non-essential amino acids, 10% tryptose phosphate broth and 10% fetal bovine serum, and maintained at 28 °C for 15 days.

Animals and experimental protocol

All 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. Adult male BALB/c mice (age of 2 months), weighing 25 g, obtained from the mouse colony maintained in the Department of Virology of the Instituto Oswaldo Cruz, were submitted to an intraperitoneal inoculation with DENV-2 (10^4 $TCID_{50}$/0.2 ml). For histopathological analysis and biochemical tests, mice, anesthesiated with 4% chloral hydrate (0.4 ml/25 g of animal), were sacrificed by total cardiac puncture, while for viremia detections blood samples were collected several times by partial cardiac puncture. Control animals consisted of non-infected or L-15 medium inoculated mice, sacrificed as described above.

Histological study of liver (light microscopy)

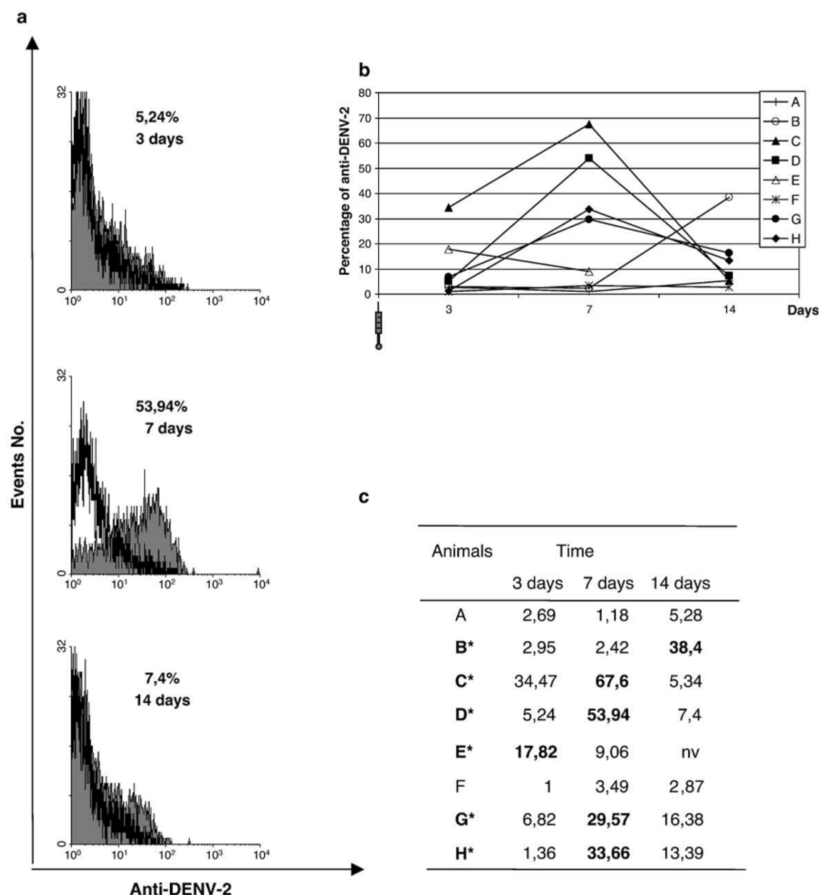
Five DENV-2 inoculated animals were sacrificed at the 2nd, 3rd, 7th, 13th, and 17th days p.i. Liver slices were fixed in Millonig's fixative, dehydrated in ethanol, and paraffin embedded. Sections (5 μm thickness) were stained with hematoxylin and eosin (HE).

Morphometrical analysis

Hepatocyte, sinusoidal density surface, and sinusoidal cells numerical density were obtained in HE stained slides, from animals at the 2nd, 13th, and 17th days p.i. A hundred and thirty images of liver parenchyma avoiding portal spaces were obtained in a NIKON Eclipse 104 light microscope (40× objective camera) and digitalized by a NIKON Coolpix 990 digital camera. From each images, diameters of 20 hepatocytes and 10 sinusoidal spaces, as well as sinusoidal cells counting, were achieved using a public software Scion (ScionCorp, USA). Data were submitted to statistical analysis using *t* test or Mann–Whitney rank sum test considering significance at $P < 0.05$.

Immunoperoxidase technique for the detection of viral antigen in mice liver

Paraffin-embedded sections of the liver of infected and control animals were deparaffinized with three washes in



Animals	Time		
	3 days	7 days	14 days
A	2,69	1,18	5,28
B*	2,95	2,42	**38,4**
C*	34,47	**67,6**	5,34
D*	5,24	**53,94**	7,4
E*	**17,82**	9,06	nv
F	1	3,49	2,87
G*	6,82	**29,57**	16,38
H*	1,36	**33,66**	13,39

Fig. 7. FACs analysis of C6/36 cells inoculated with sera collected from DENV-2-infected mice at different time points. (a) Original FACs histograms showing the time-dependent positivity for the anti-DENV-2 antibody in C6/36 cells treated with sera of one infected animal. Anti-DENV-2 = gray color/histogram, 2nd antibody = transparent overlaid histogram. Percentage of dengue virus and time of the analysis are shown at the right top of each histogram. (b) Graph showing the kinetic of infection for all animals studied as percentage of anti-DENV-2-positive cells. Each line represents one animal and each point represents the day of analysis. Day 1 of infection = [syringe symbol]. (c) Table containing the values for anti-DENV-2-positive cells for all infected animals in all studied days. Asterisk/bold* letters showing that in the majority of the animals studied the infection was detected using this experimental model (nv = no value).

xylol and serial washes in 100%, 90%, and 70% ethanol followed by a final wash in PBS. For detection of DENV antigens, samples of liver from 2 and 13 days p.i. were incubated over night at 4 °C with an anti-DENV-2 serum raised in monkey (gently provided by Dr. R. Galler, Laboratory of Molecular Biology of Flavivirus, Department of Biochemistry and Molecular Biology, Fiocruz, RJ). After washing with Tris–HCl (0.05 M pH 7.6), the sections were further incubated with rabbit anti-human IgG-horseradish peroxidase conjugate (Sigma) for 30 min at 37 °C. The slices were revelated with DAB (Sigma) and were counter-stained with Mayer's hematoxylin. Controls were stained with the secondary antibody only.

Biochemical analysis of serum hepatic enzyme

Five infected mice were bleed at the 2nd, 3rd, 7th, until the 14th days p.i. and serum samples were obtained after centrifugation at 400 rpm for 5 min and stored at −70 °C. Levels of alanine aminotransferase (ALT) and aspartate

aminotransferase (AST) were detected by the UV optimized (IFCC) method (Karmen, 1955), using commercial kits (Clm, Brazil).

Statistical analysis

Experimental results on biochemical tests were analyzed for their statistical significance by *t* Student and Kruskal–Wallis test $P = 0.051$.

Detection by transmission electron microscopy of DENV-2 in C6/36 cell line inoculated with sera from infected BALB/c mice

Serum samples (100 μl) collected from DENV-2-infected mice at the 2nd and 11th days p.i. were added to monolayers of C6/36 cells, grown as described above. Cells were daily observed for viral cytopathic effects for 15 days. Positive and negative controls consisted of DENV-2 (10^2 $TCID_{50}$/0.1 ml) infected and non-infected C6/36 monolayers, respectively. Cells were fixed in 1% buffered glutaraldehyde, dehydrated and embedded in epoxy resin for electron microscopy observations. Ultra-thin sections of 50–70 nm thickness were obtained using a diamond knife, double stained with uranyl acetate and lead citrate, and observed in a Zeiss EM-900 transmission electron microscope.

Detection of the NS1 DENV-2 gene by PCR amplification in C6/36 cell line inoculated with sera from infected BALB/c mice

C6/36 cells were used in order to increase virus titer. The cells were incubated with mice sera collected at the 2nd and 11th days p.i., as described above, and were used for the detection of the DENV-2 non-structural protein 1 (NS1). PCR reactions were performed using cDNAs made from extracts of such cells, and total RNAs were then extracted with Trizol (Invitrogen, USA), according to the manufacturer protocol. The RNA was used as template for the synthesis of a cDNA, carried out with the oligonucleotide antisense primer 5′-CAT AAG CTT ACA GAG GTT CCC CCA TG-3′, which hybridize between nucleotides 1422 and 1438 in the NS3 gene. The cDNA was then used for amplification of the NS1 gene sequence by Nested PCR. The first PCR reaction was made with two oligonucleotide primers (sense 5′-G GGG GAT ATC ATG CTG TCT GTG TCA CTA G-3′ and antisense 5′-G GGG CTC GAG TTA CCC TGT GAT CAA TG-3′), which anneals between nucleotides 1425 and 1441 and between nucleotides 125 and 141 in the E and NS2A protein genes, respectively. The oligonucleotide primers sense 5′-GGG GGA TAT CGA TAG TGG TTG CGT TG-3′ and antisense 5′-GGG GCT CGA GTT AGG CTG TGA CCA AG-3′ were then used for the amplification of the NS1 gene sequence in the second PCR reaction was. The two PCR reactions were programmed as follows: 94 °C for 2 min, 40 cycles of 92 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and an extension step at 72 °C for 5 min at the end of the cycle. The amplified products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed with Polaroid film.

Viremia detection by flow cytometry

Eight BALB/c mice were inoculated with DENV-2 as described above and serum samples of each animal were collected at the 3rd, 7th, and 14th days post-infection and stored at −70 °C. C6/36 cells were incubated with the serum and maintained for 10 days at 28 °C. Infected and non-infected cells were analyzed by a FACScalibur (Becton Dickinson). Performance control of the flow cytometer was accomplished on a regular basis using Calibrite Bead (Becton Dickinson). All analyses were performed with intracellular labeling techniques using a PBS/Saponin solution (0,05%) with preceding cell fixation (fixing reagents Paraformaldehyde 1%). Primary antibody used was a DENV-2 hyperimmune mouse ascitic fluid (ATCC, USA) and the second antibody was a fluorescein-conjugated goat anti-mouse IgG (Southern Biotechnology, USA). The standard incubation time with the first antibody was 1 h at 4 °C and samples underwent a supplementary 30-min incubation step with the second antibody.

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References

An, J., Kimura-Kuroda, J., Hirabayashi, Y., Yasui, K., 1999. Development of a novel mouse model for dengue virus infection. Virology 263, 70–77.

Atrasheuskaya, A., Petzelbauer, P., Fredeking, T.M., Ignatyev, G., 2003. Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. FEMS. Immunol. Med. Microbiol. 35, 33–42.

Bhamarapravati, N., Halstead, S.B., 1964. Studies on dengue virus infection I. Immunofluorescence localization of virus in mouse tissue. Arch. Pathol. 77, 538–543.

Bhamarapravati, N., Tuchinda, P., Boonpucknavik, V., 1967. Pathology of Thailand haemorrhagic fever: a study of 100 autopsy cases. Ann. Trop. Med. Parasitol. 61, 500–510.

Boonpucknavig, S., Vuttiviroj, O., Boonpucknavig, V., 1981. Infection of young adult mice with dengue virus type 2. Trans. R. Soc. Trop. Med. Hyg. 75, 647–653.

Burke, T., 1968. Dengue haemorrhagic fever: a pathological study. Trans. R. Soc. Trop. Med. Hyg. 62, 682–692.

Couvelard, A., Marinneau, P., Bedel, C., Drouet, M.T., Vachon, F., 1999. Report of a fatal case of dengue infection with hepatitis:- dengue antigens in hepatocytes and liver apoptosis. Hum. Pathol. 30, 1106–1110.
Desprès, P., Flamand, M., Ceccaldi, P.E., Deubel, V., 1996. Human isolates of dengue type 1 induce apoptosis in mouse neuroblastoma cells. J. Virol. 70, 4090–4096.
Dhawan, R., Khanna, M., Chatuverdi, U.C., Mathur, A., 1990. Effect of dengue virus-induced cytotoxin on capillary permeability. J. Exp. Pathol. 71, 83–88.
Galle, P.R., Hofman, W.J., Walczak, H., Schaller, H., Otto, G., Stremnel, W., Krammer, P.H., Runkel, L., 1995. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. J. Exp. Med. 182, 1223–1230.
Hall, W.C., Crowell, T.P., Watts, D.M., Barros, V.L.R., Kruger, H., Pinheiro, F., Peters, C.J., 1991. Demonstration of yellow fever and dengue antigens in formalin-fixed paraffin-embedded human liver by immunohistological analysis. Am. J. Trop. Med. Hyg. 45, 408–417.
Halstead, S.B., 1989. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. Rev. Infect. Dis. 11, 830–839.
Henchal, E.A., Putnak, R.J., 1990. The dengue viruses. Clin. Microbiol. Rev. 3, 376–396.
Huang, C.Y., Butrapet, S., Pierro, D.J., Chang, G.J., hunt, A.R., Bramarapravati, N., Gubler, D.J., Kinney, R.M., 2000a. Chimeric dengue type2 (vaccine strain PDK-53) dengue type 1 virus as a potential candidate dengue type 1 virus vaccine. J. Virol. 74, 3020–3028.
Huang, K.-J., Li, S.-Y.J., Chen, S.-C., Liu, H.-S., Lin, Y.-S., Yeh, T.-M., Liu, C.-C., Lei, H.-Y., 2000b. Manifestation of thrombocytopenia in dengue-2 virus infected mice. J. Gen. Virol. 81, 2177–2182.
Huerre, M.R., Lan, N.T., Mariameau, P., Hue, N.B., Khun, N.T., Khen, N.T., Drouet, M.T., Huong, V.T., et al., 2001. Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children. Virchows Arch. 438, 107–115.
Innis, B.L., 1995. Dengue and dengue haemorrhagic fever. In: Porterfield, J.S. (Ed.), Exotic Viral Infections. Chapman and Hall, London, pp. 103–146.
Kalayanarooj, S., Vaughn, D.W., Nimmannitya, S., Green, S., Suntayakorn, S., Kunentrasai, N., Viramitrachai, W., 1997. Early clinical and laboratory indicators of acute dengue illness. J. Infect. Dis. 176, 313–321.
Karmen, A.L., 1955. IFCC method for alanine aminotransferase. J. Clin. Invest. 34, 131.
Khanna, M., Chaturvedi, U.C., Sharma, M.C., Pandey, V.C., Mathur, A., 1990. Increased capillary permeability mediated by a dengue virus-induced lymphokine. Immunology 69, 449–453.
Knowles, B.B., Searls, D.B., Aden, D.P., 1984. Human hepatoma-derived cell lines. In: Chisari, F.V. (Ed.), Advances in Hepatitis Research. Massom, New York, pp. 196–202.
Kuo, C.H., Tai, D.L., Chang-Chien, C.S., Lan, C.K., Chiou, S.S., Liaw, Y.F., 1992. Liver biochemical tests and dengue fever. Am. J. Trop. Med. Hyg. 47, 265–270.
Lum, L.C.S., Lam, S.K., George, R., Devi, S., 1993. Fulminant hepatitis in dengue infection. South Asian J. Trop. Med. Public Health 24, 467–471.
Marianneau, P., Steffan, A.M., Royer, C., Drouet, M.T., Kirn, A., Deubel, V., 1997. Differing patterns of dengue and yellow fever viruses in a human hepatoma cell line. J. Infect. Dis. 178, 1270–1278.
Marinneau, P., Flamand, M., Deubel, V., Desprès, P., 1998. Apoptotic cell death in response to dengue virus infection: the pathogenesis of dengue hemorrhagic fever revised. Clin. Diagn. Virol. 10, 113–119.
Miagostovich, M.P., Ramos, R.G., Nicol, A.F., Nogueira, R.M.R., Cuzzi-Maya, T., Oliveira, A.V., Marchevsky, R.S., Mesquita, R.P., Schatzmayr, H.G., 1997. Retrospective study on dengue fatal cases. Clin. Neuropathol. 16, 204–208.
Mohan, B., Patwari, A.K., Anand, V.K., 2000. Hepatic dysfunction in childhood dengue infection. J. Trop. Pediatr. 46 (1), 40–43.
Monath, T.P., 1986. Pathobiology of the flaviviruses. In: Schlesinger, S., Schlesinger, M.J. (Eds.), The Togaviridae and Flaviviridae. Plenum, New York, pp. 375–440.
Nogueira, R.M.R., Miagostovich, M.P., Schatzmayr, H.C., Moraes, G.C., Cardoso, F.M.A., Ferreira, J., Cerqueira, V., Pereira, M., 1995. Dengue type 2 outbreak in the south of the state of Bahia, Brazil: laboratorial and epidemiological studies. Rev. Inst. Med. Trop. São Paulo 37, 507–510.
Nguyen, T.L., Nguyen, T.H., Tien, T.C., 1997. The impact of dengue hemorrhagic fever on liver function. Res. Virol. 148, 273–277.
Nath, P., Tandon, P., Gulati, L., Chaturvedi, U.C., 1983. Histological and ultrastructural study of spleen during dengue virus infection of mice. Indian J. Med. Res. 78, 83–90.
Raut, C.G., Deolankar, R.P., Kolhapure, R.M., Geverdhan, M.k., 1996. Susceptibility of laboratory bred rodents to the experimental infection with dengue virus type 2. Acta Virol. 40, 143–146.
Rosen, L., Khin, M.M., Tin, U., 1989. Recovery of virus from the liver of children with fatal dengue: reflections on the pathogenesis of the disease and its possible analogy with that of yellow fever. Res. Virol. 140, 351–360.
Rosen, L., Drouet, M.T., Deubel, V., 1999. Detection of dengue virus RNA by reverse transcription-polymerase chain reaction in the liver and lymphoid organs but not in the brain in fatal human infection. Am. J. Trop. Med. Hyg. 61, 720–724.
Rothman, A.L., Ennis, F.A., 1999. Immunopathogenesis of dengue hemorrhagic fever. Virology 257, 1–6.
Scott, R.M., Nisalak, A., Cheamudon, U., Seridhoranakul, S., Nimmannitya, S., 1980. Isolation of dengue virus from peripheral blood leukocytes of patients with hemorrhagic fever. J. Infect. Dis. 141, 1–6.
Shresta, S., Jennifer, L.K., Robert, B., Harris, E., 2004. Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice. Virology 319, 262–273.
Souza, L.J., Alves, J.G., Nogueira, R.M.R., Neto, C.G., Bastos, D.A., Siqueira, E.W.S., Filho, J.T.D.S., Cezário, T.A., Soares, C.E., Carneiro, R.C., 2004. Aminotransferase changes and acute hepatitis in patients with dengue fever: analysis of 1,585 cases. Braz. J. Infect. Dis. 8, 156–163.
Wang, L.Y., Chang, W.Y., Lu, S.N., Chen, T.P., 1990. Sequential changes of serum transaminase and abnormal sonography in patients with suspected dengue fever. Kao Hsiung Ihsueh Tsa Chih 6, 483–489.
Waterman, S.H., Kuno, G., Gubler, D.J., Sather, G.E., 1985. Low rates of antigen detection and virus isolation from the peripheral blood leukocytes of dengue fever patients. Am. J. Trop. Med. Hyg. 34, 380–384.

ANEXO 3

Histopathological aspects of Dengue-2 virus infected mice tissues and complementary virus isolation.

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Histopathological aspects of Dengue-2 virus infected mice tissues and complementary virus isolation

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SUMMARY - The difficulty in studying dengue virus (DENV) infection in humans and in developing a virus vaccine is the absence of a suitable animal model which develops the full spectra of the Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS). Despite the fact that viruses have been found in various animal tissues, we isolated DENV from tissues of adult BALB/c mice, inoculated with DENV serotype 2 (DENV-2) obtained from human serum. Viruses were ultrastructurally identified and immunolocalized by immunofluorescence techniques in C6/36 mosquito cell cultures, inoculated with tissues (liver, lung, kidney and cerebellum) macerate supernatant from mice, 48 h post-infection (p.i.). These organs, collected at the same stage of infection, were examined histologically. The histopathological analysis revealed focal alterations in all tissues examined. Liver contained focal ballooned hepatocytes, but without modifying the average diameter of the majority of hepatocytes. Sinusoidal lumen was significantly diminished at this stage but portal and centrolobular veins became congested. Lungs exhibited hemorrhagic foci in the alveolar space, vascular congestion and focal alveolitis. Cerebellar tissue showed rare foci of neuronal compactation (Purkinje cells) and perivascular oedema. In kidneys it was observed an increase in glomerular volume with augmented endocapillary and mesangial cellularity, with reactivity to anti-IgM in all glomeruli of infected mice. In conclusion, DENV-2 was found in all tissues examined early in the evolution of infection. Presence of viruses in tissues has mainly led to hemodynamic alterations with generalized vascular congestion and increased permeability, and mast cell recruitment in lungs. The latter could participate in the vascular modifications in tissues.

KEY WORDS *Dengue-2 virus - mouse - C6/36 cell line - tissue macerates - ultrastructure - histopathology*

INTRODUCTION

Dengue fever (DF) is a common disease in tropical countries. It is caused by four serotypes (dengue virus serotype 1 [DENV-1], DENV-2, DENV-3, DENV-4) of DENV (family *Flaviviridae*) transmitted to humans by some *Aedes* mosquitoes (Monath and Heinz, 1996). DENV-2 was first isolated in Brazil, during an outbreak in 1990 (Nogueira *et al.*, 1990). The World Health Organization estimates the number of annual dengue cases to be about 100 millions. DHF and DSS are the major complications that cause fatality in about 30,000 infected individuals per year (Knudsen, 1997). Considerable effort has been put to understand the pathogenetic mechanisms of dengue (DEN) infection and in vaccine development, but difficulties exist because of the lack of an appropriate small animal model (Bhamarapravati, 1993) which could mimic the DHF/DSS manifestations. In general mice are susceptible for DENV infection but inbred mice strains, in particular the C57BL/6 strain, was the most susceptible (Raut *et al.*, 1996). Despite the fact that signs of different severity were observed in these animals, viremia was not detected in these strains by routine method of virus titration, except in nude animals (Hotta *et al.*, 1981). However, presence of viral antigens has been demonstrated in several organs such as mesenteric lymph nodes, spleen, liver, and brain (Boonpucknavig *et al.*, 1981; Hotta *et al.*, 1981; Nath *et al.*, 1983). Until now the great majority of mice models of DEN infection has dealt with suckling or young mice infected by an intracerebral route of inoculation (Nath *et al.*, 1983;

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Raut *et al.*, 1996) of mouse-neuroadapted DENV (Desprès *et al.*, 1998; Atrasheuskaya *et al.*, 2003). Response of animals (clinical symptoms and/or degree of injury) varied according to the mice strain; however, the full DHF/DSS manifestations did not seem to occur in standard laboratory mice. In nude mice, petechial hemorrhages and gastrointestinal hemorrhage have been observed (Raut *et al.*, 1996) while a transient thrombocytopenia and anti-platelet antibodies were detected in A/J mice and in a mild extent, in BALB/c and B6 mice (Huang *et al.*, 2000). Moreover, foci of tissular hemorrhage, hemoconcentration and fluid extravasation, thrombocytopenia, and prolonged partial thromboplastin time were for the first time achieved in the model of infection developed in SCID mice, submitted to the transplantation of human hepatocarcinoma cell line (HepG2) (An *et al.*, 1999). HepG2 cells, a human hepatoma cell line, retain numerous characteristics of differentiated hepatocytes (Knowless *et al.*, 1984) and further, are permissive for DENV infection and replication. HepG2 cells transplanted into SCID mice are capable to repovoated liver, and after viruses inoculation became the major target for their replication. The infected cells, in turn, die by an apoptotic mechanism as a direct consequence of the viral infection (Marianneau *et al.*, 1998). Moreover, these animals developed a hind-leg paralysis, liver injury, and renal dysfunction. Viremia, determined by plaque-assay on a Vero cell monolayer, in this model peaked at 8 day post-infection, viruses were already seen in 60% of the mice at day 5. Viruses were only detected in the hepatic tissue, brain, and blood (An *et al.*, 1999). In the majority of animal models in which viremia was successfully characterized, viremia was obtained from RT-PCR of blood samples (Huang *et al.*, 2000; Atrasheuskaya *et al.*, 2003). These experiments were characterized by the use of either a mouse-adapted DENV strain in inbred young BALB/c mice (Atrasheuskaya *et al.*, 2003) or local virus isolate (DENV-2) in A/J mice strain (Huang *et al.*, 2000).

The present study was conceived to characterize the initial injury caused by DENV in liver, lung, kidney and cerebellum of BALB/c mice infected with DENV-2, and viruses presence at 48 h p.i. Differing from the majority of mice models, DENV-2 was obtained from a patient serum, propagated in *Aedes albopictus* mosquito cell line (C6/36) and was inoculated by a peripheral route (intraperitoneally). Presence of DENV antigen and virus particles, in monolayers of C6/36 cells inoculated with macerated tissue supernatants, was investigated by using light and electron transmission microscopy.

MATERIALS AND METHODS

Virus

The viruses used in our experiments were isolated from a patient serum during an epidemic of DENV-2 in the state of Rio de Janeiro in 1995 and propagated in the *Aedes albopictus* mosquito cell line (C6/36). The serum was tested by the indirect immunofluorescence technique using a type specific DENV-2 monoclonal (3H5, DENV-2) antibody. The virus had not undergone passage in mouse brain. The titer of the virus ($10^{6.3}$ $TCID_{50}$/0.1 mL) was calculated by the method of Reed and Muench (1938). The mice were inoculated with doses of 10,000 $TCID_{50}$/0.2 mL.

Cells

The isolation of the DENV was in virus-sensitive clone C6/36 from Singh's *Aedes albopictus* cells (Singh, 1967).

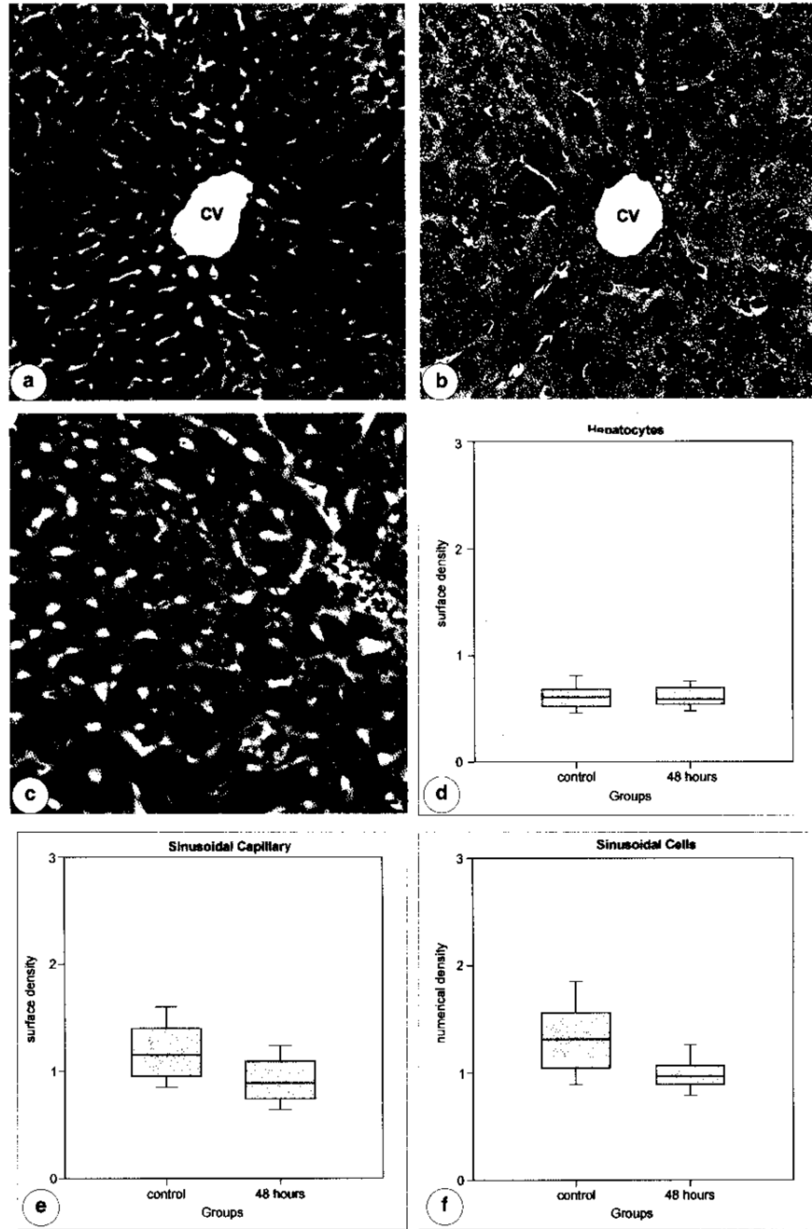
Animals

Adult male BALB/c mice, aged 2 months and weighing 25 g, were obtained from the mouse colony maintained in the Department of Virology of the Instituto Oswaldo Cruz, FIOCRUZ. Mice were peritoneally inoculated with DENV-2 (dose of 10,000 $TCID_{50}$/0.2 mL) and sacrificed after 48 h. Non-infected mice and mice inoculated with L-15 medium were used as controls and sacrificed at the same time.

Morphology of the tissues

The animals were peritoneally anaesthetized with 4% chloral hydrate (0.4 mL/25 g of animal) and tissue (liver, lung, kidney and cerebellum) fragments were collected from

FIGURE 1*a-f* (*a*) Normal liver of BALB/c mice. Central medzonal area. Note centrolobular vein (CV) with hepatocytes (H) around, sinusoidal capillary (SC), endothelial cell (EC). Bar = 50 µm. (*b*) Normal liver of BALB/c mice inoculated with L-15 medium. Central medzonal area. Note centrolobular vein (CV) with hepatocytes (H) around, sinusoidal capillary (SC), endothelial cell (EC). Bar = 0.03 µm. (*c*) Liver of BALB/c mice infected with DENV-2, 48 h p.i. Central medzonal area. Note swollen (star), vacuolization (asterisk) and division (•) of hepatocyte cells (H) and hyperplasia of Kupffer cells (KC). Sinusoidal capillary (SC), endothelial cells (EC). Bar = 0.03 µm. (*d*) Analysis of surface density of hepatocytes, 48 h p.i. Hepatocytes in liver did not change in their diameters ($p > 0.05$) in relation to liver control. (*e*) Analysis of surface density of sinusoidal capillary, 48 h p.i. Surface density of sinusoidal capillary in liver significantly decreased ($p < 0.05$) in relation to liver control. (*f*) Analysis of numerical density of sinusoidal cells, 48 h p.i. The number of sinusoidal cells in liver significantly decreased ($p < 0.05$) in relation to liver control.



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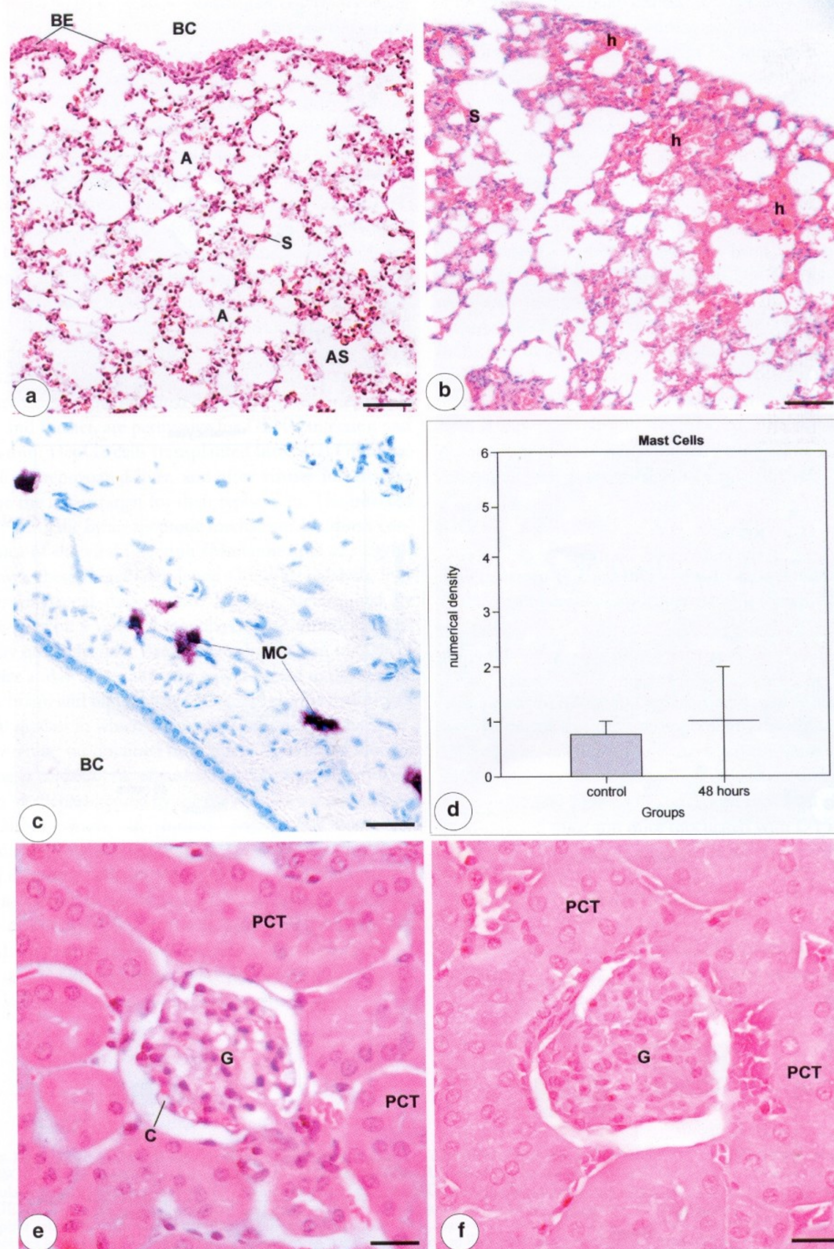
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5
4
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2
1
0
control
48 hours
Groups
d
PCT
G
C
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f

infected (48 h p.i.) and non-infected mice. Samples were fixed in Millonig's fixative, dehydrated in ethanol and paraffin-embedded. Sections (5 µm thickness) were stained with haematoxylin and eosin and toluidine Perez blue (only lung tissues).

Morphometrical analysis

Liver - The surface density of hepatocytes, and sinusoidal capillaries and cells numerical density were obtained from histological sections stained with haematoxilyn and eosin. A hundred and thirty images of the non-infected animals and a hundred and thirty images of the infected animals of liver parenchyma, avoiding portal spaces, were obtained in a 40× objective camera. From each image, 20 hepatocytes and 10 sinusoidal spaces were measured using a public software Scion (ScionCorp., USA), and all sinusoidal cells on each image were quantified.

Lung - Numerical density of mast cells were obtained from histological sections stained with toluidine Perez blue lung fragments. Thirty four images of non-infected animals and thirty four images of infected animals of pulmonary parenchyma, were obtained in 20× objective camera and all mast cells on each image were quantified.

Spleen - Numerical density of glomerular cells were obtained in histological sections stained with haematoxilyn and eosin. Seventy images of non-infected animals and seventy images of infected animals of kidney were obtained in 20× objective camera and all glomerular cells on each image surface were quantified.
All images were obtained with a NIKON Eclipse 104 light microscope and digitalized using a NIKON Coolpix 990 camera. Data were submitted to statistical analysis using t-test or Mann Whitney rank sum test considering $p < 0.05$.

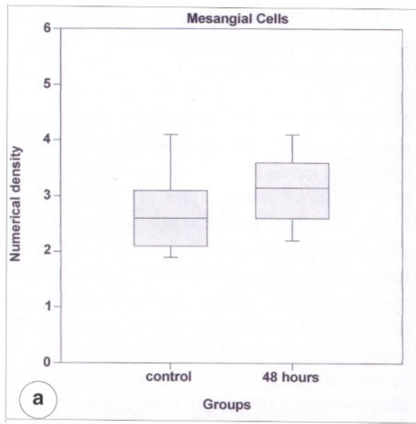
Direct immunofluorescence for immunoglobulins - Frozen sections of kidney of infected, L-15 medium inoculated and normal mice were tested for the presence of immunoglobulins using anti-mouse IgM conjugated with FITC (Caltag, USA). In brief, 5 µm sections were fixed with iced acetone and incubated with antibodies (dilution: 1:50) and observed in a confocal laser microscope.

Isolation of DENV-2 in C6/36 cell line inoculated with supernatant of tissue macerates from infected BALB/c mice - Tissue (liver, lung, kidney and cerebellum) fragments were washed in phosphate saline buffer (PBS, pH 7.2) and macerated in Leibovitz medium (L-15), supplemented with antibiotics. The suspension was incubated for one hour for antibiotic action, centrifuged at 1400 g for five minutes in a refrigerate centrifuge and the supernatant was collected. The cell monolayers were inoculated with 100 µl of supernatant and incubated for 1 h at 28 °C for virus adsorption. Subsequently, monolayers were grown in L-15 medium supplemented with 1% non-essential aminoacids, 10% tryptose phosphate broth and 10% fetal bovine serum. The tubes were kept at 28 °C and observed daily for viral cytopathic effects for fifteen days, when cultures were collected for morphological analyses. As negative control, were used C6/36 normal monolayers, while positive control consisted of monolayers inoculated with DENV-2 at the same concentration as used to infect mice. Both monolayer controls were kept in culture for the same period as done for test monolayers.

Indirect immunofluorescence - C6/36 monolayers were tested for presence of the viral antigen using type-specific serotype 2 monoclonal (3H5, DENV-2) antibody.

Transmission electron microscopy - C6/36 monolayers were fixed in 1% glutaraldehyde in cacodylate buffer (0.2 M, pH 7.2), dehydrated in acetone, post-fixed with 1% buffered osmium tetroxide, embedded in epoxy resin and polymerized at 60°C during three days. Ultrathin sections of 50-70 nm thickness were obtained using a diamond knife (Diatome) adapted to a Reichert-Jung Ultracut E microtome. The sections were picked up onto copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1993) and observed in a Zeiss EM-900 transmission electron microscope.

FIGURE 2*a-f* (*a*) Normal lung of BALB/c mice. Note a bronchiole (BC) with alveolar spaces (A) around. Alveolar septa (S) and alveolar sac (AS). Bronchiolar epithelium (BE). Bar = 50 µm. (*b*) Lung of BALB/c mice infected with DENV-2, 48 h p.i. Note swollen alveolar septa (S) and hemorrhagic foci (h). Bar = 100 µm. (*c*) Lung of BALB/c mice infected with DENV-2, 48 h p.i. Note mast cells (MC) in peribronchiolar space. Bronchiole (BC). Bar = 50 µm. (*d*) Analysis of numerical density of mast cells. The number of mast cells in lung, 48 h p.i. was significantly increased ($p < 0.05$) in relation to lung control. (*e*) Normal kidney of BALB/c mice. Cortical area. Note proximal convoluted tubules (PCT), glomeruli (G) with some capillaries (C). Bar = 0.02 µm. (*f*) Kidney of BALB/c mice infected with DENV-2, 48 h p.i. Cortical area. Note increase of glomerular cells. Glomeruli (G). Proximal convoluted tubules (PCT). Bar = 0.02 µm.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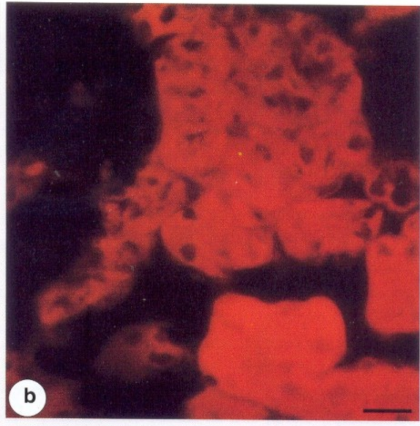
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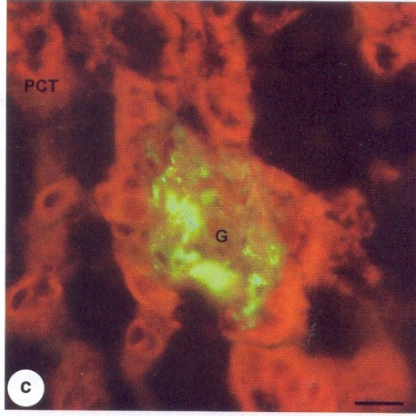
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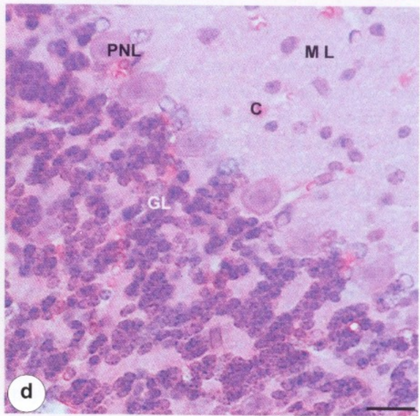
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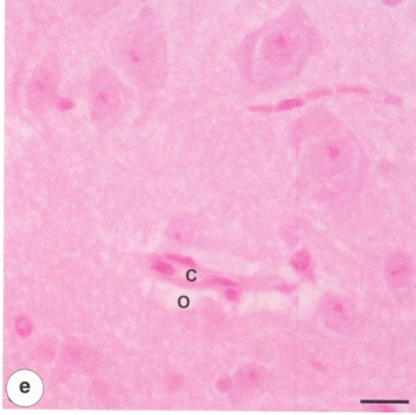
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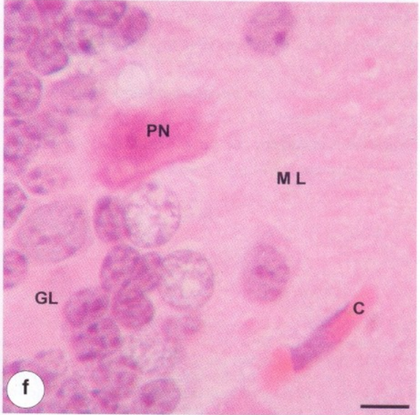
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RESULTS

Clinical signs

The infected BALB/c mice did not show either signs or increased mortality.

Histopathology and morphometry of tissues

At 48 h post-infection, tissues (liver, lung, kidney and cerebellum) were focally injured.
Hepatic tissue had, in general, a histological aspect similar to the controls (normal and L-15 medium-inoculated) (Fig. 1*a,b*) with only some focal mild alterations (Fig. 1*c*). Hepatic plates showed no significant modifications in relation to their surface density ($p > 0.05$) (Fig. 1*d*), contrasting with its increase at 13 days post-infection (data not shown). Scarse binucleated cells, or cells with enlarged nuclei were seen. Rare swollen cells or vacuolated hepatocytes were observed all over the liver parenchyma but more evident at the zone II of the liver acini (midzonal area) (Fig. 1*c*). Sinusoids, on the other hand, were significantly diminished ($p < 0.05$) in relation to normal liver as demonstrated by quantitative analysis of its surface density (Fig. 1*e*) as well as the numerical density of sinusoidal cells ($p < 0.05$) (Fig. 1*f*).
Lung from infected animals exhibited a slight thickening of alveolar septa due to the presence of engorged capillaries and a mononuclear inflammatory infiltrate (Fig. 2*b*), when compared to the control (Fig. 2*a*). In rare focal zones, parenchymal hemorrhage was observed (Fig. 2*b*), as also accumulation of alveolar macrophages. Mast cells were found in an increased amount ($p < 0.05$) (Fig. 2*d*) in peribronchiolar areas (Fig. 2*c*) in infected animals. In these areas it was verified the presence of a small amount of interstitial oedema.
Kidneys of infected animals exhibited only an increase in glomerular volume with augmented endocapillary (Fig. 2*f*) and mesangial cellularity ($p < 0.05$) (Fig. 3*a*) when compared to the control (Fig. 2*e*). Presence of immunoglobulins IgM in glomeruli (Fig. 3*c*) was observed in infected animals. Presence of immunoglobulins was not observed in glomeruli of animals control (Fig. 3*b*).
Cerebellar tissue was slightly damaged. A pericapillary oedema (Fig. 3*e*) was present, and sometimes was associated to an interstitial oedema with rare neuronal retraction (Purkinje neuron) (Fig. 3*f*), and cytoplasmic condensation, when compared to the control (Fig. 3*d*).

Isolation of DEN-2 virus in the C6/36 cell line

The syncytial cytopathic effect started to be visible around the 13th day p.i. in monolayers of the C6/36 cells of positive control and in the cultures inoculated with the supernatant of tissues.
At 15th day p.i. monolayer cells of the negative control showed no morphological alterations, exhibited neither DENV-2 antigen (Fig. 4*a*) nor virus particles (Fig. 4*g*).
In cell cultures of the positive controls and in monolayer cultures inoculated with the supernatant of the tissues, the DENV-2 antigen (Fig. 4*b-f*) was observed by indirect immunofluorescence technique. Presence of syncytial cytopathic effect was observed in all monolayers.
Ultrastructural observations of cell cultures of the positive controls and in monolayer cultures inoculated with the supernatant of the tissues, showed virus particles inside cysterns of the rough endoplasmic reticulum (Fig. 4*h-l*). In some monolayers, virus particles presented abnormal features, frequently lacking nucleocapsids (Fig. 4*l*).

DISCUSSION

The knowledge about DEN infection is mainly derived from the findings found in humans in autopsy material and blood samples. Therefore, pathogenesis of DEN remains difficult to study because of the lack of an adequate animal model. The great majority of models use suckling or young mice inoculated with a neuroadapted mouse DENV strain (Nath *et al.*, 1983; Raut *et al.*, 1996). Response of animals (clinical signs and/or degree of injury) varied according to the mice strain but in general these experimental models showed that mice is susceptible for DENV infection (Meiklejohn *et al.*, 1952; Lin *et al.*, 1998; Johnson and Roehrig, 1999). However, the full DHF/DSS manifestations did not seem to occur in standard laboratory mice (Huang *et al.*, 2000). The susceptibility of BALB/c mice inoculated with neuroadapted mouse DENV strain, by intraperitoneal route has been demonstrated (Atrasheuskaya *et al.*, 2003). In our studies, male BALB/c mice, demonstrated to be permis-

FIGURE 3*a-f* (*a*) Analysis of numerical density of mesangial cells, 48 h p.i. The number of mesangial cells in kidney was significantly increased ($p < 0.05$) in relation to kidney control. (*b*) Normal kidney of BALB/c mice. Cortical area. Presence of immunoglobulin was not observed. Bar = 0.02 μm. (*c*) Kidney of BALB/c mice infected with DENV-2, 48 h p.i. Cortical area. Note presence of immunoglobulin IgM in glomeruli (G). Proximal convoluted tubules (PCT). Bar = 0.02 μm. (*d*) Normal cerebellum of BALB/c mice. Note molecular layer (ML), Purkinje neuron layer (PNL) and granular layer (GL). Capillary (C). Bar = 0.02 μm. (*e,f*) Cerebellum of BALB/c mice infected with DENV-2, 48 h p.i. Note pericapillary oedema (o) and retraction of Purkinje neuron (PN). Molecular layer (ML), granular layer (GL), capillary (C). Bar = 1.6 μm.

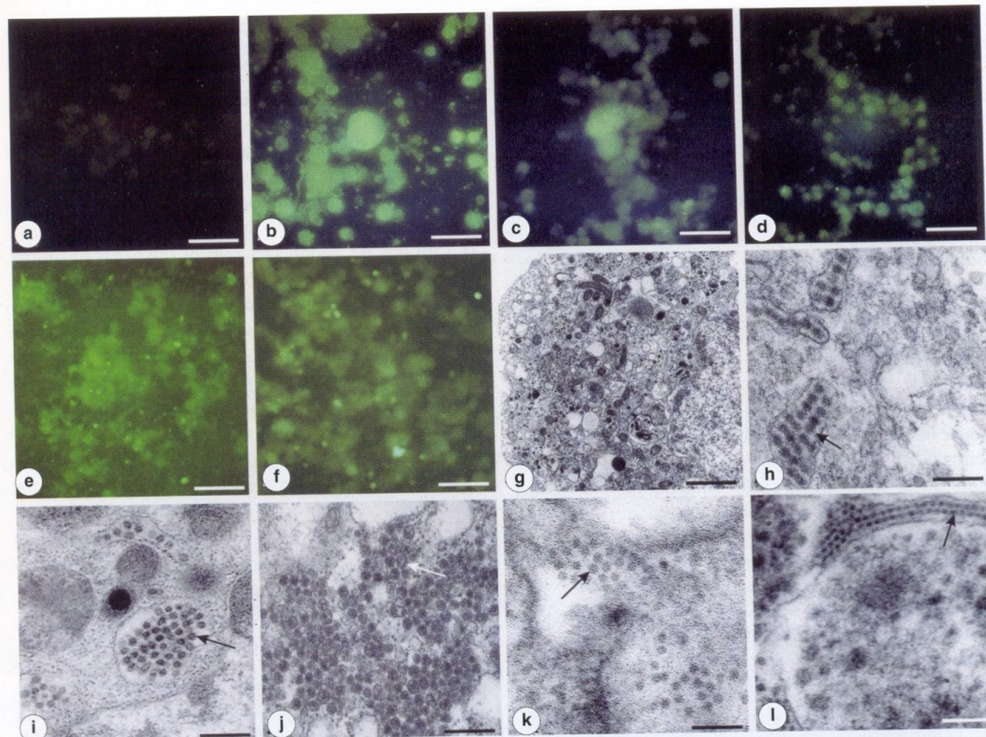

FIGURE 4*a-l* (*a-f*) Detection of DENV-2 antigen in monolayers of C6/36 by immunofluorescence technique. (*a*) Negative control. (*b*) Positive control. (*c*) Monolayer infected with macerate of hepatic tissue supernatant. (*d*) Monolayer infected with macerate of lung supernatant. (*e*) Monolayer infected with macerate of kidney supernatant.(*f*) Monolayer infected with macerate of cerebellum supernatant. Bar = 50 µm. (*g-l*) Detection of DENV-2 particles in monolayers of C6/36 by electron microscopy technique. (*g*) Negative control. Bar = 1. 25 µm. (*h*) Positive control. Bar = 0.23 µm. (*i*) Monolayer infected with macerate of hepatic tissue supernatant. Bar = 0.35 µm. (*j*) Monolayer infected with macerate of lung supernatant. Bar = 0.26 µm. (*k*) Monolayer infected with macerate of kidney supernatant. Bar = 0.3 µm. (*l*) Monolayer infected with macerate of cerebellum supernatant. Bar = 0.09 µm. Virus particles (arrows).

sive hosts for DENV-2 (virus non-adapted in mouse brain), when infected by intraperitoneal route.

Histopathological observations of lung, liver and cerebellum of animals 48 h p.i., exhibited mild modifications of their structure. Despite the fact that a widespread mild injury was detected in these animals, no signs of disease were observed, probably mimicking what occurs in the majority of human cases (Burke *et al.*, 1988; WHO, 1999). The histopathological alterations observed in our animals are very mild when compared with observations of other animal models (Raut *et al.*, 1996). In these animal models the mice were inoculated by intravenous route with a neuroadapted mouse DENV strain.

In hepatic tissues few binucleated cells or with enlarged nuclei, scarce thickened or vacuolized hepatocytes observed. The sinusoidal capillary was compressed when compared with hepatic tissues of non-infected mice. The alterations observed in liver of mice in our experiments were less severe than observed in other animal models and in human cases. Virus particles and antigen of DENV were detected in monolayers of C6/36 cells inoculated with supernatant of liver macerate of infected animals. Studies with BALB/c mice infected by intraperitoneal route, dilatation of sinusoidal capillary, hyperplasia of Kupffer cells, infiltrate of inflammatory cells, focal disorganization of the endothelial lining, vacuolization of hepatocytes and alteration of levels of aspartate (AST) and alanine (ALT) transaminase enzymes were observed (Paes *et al.*, 2002). Presence of DENV antigen in Kupffer cells was observed in studies with atymic nude BALB/c mice (Hotta *et al.*, 1981*b*). Severe damage in hepatic tissues has been observed in studies with BALB/c mice infected with neuroadapted mouse DENV strain (Atrasheuskaya

et al., 2003). The histopathological alterations observed in these animals were similar at DHF and DSS. The DENV has been isolated of the liver of fatal cases of DHF and the DENV antigen has been detected in Kupffer cells and hepatocytes (Fresh *et al.*, 1969; Rosen *et al.*, 1989; Innis, 1995; Bhamarapravati, 1997; Couverland *et al.*, 1999; Rosen *et al.*, 1999). Researchers postulate that hepatocytes and Kupffer cells can be target cells in DENV replication (Burke, 1968; Huerre *et al.*, 2001). This fact suggest that the liver can be target organ in the DF, DHF and DSS and that virus can replicate in liver cells causing hepatic injury.

Multifocal interstitial pneumonia with rare areas of hemorrhage was observed in lung tissue of our animals. Virus particles and antigen of DENV were detected in monolayers of C6/36 cells inoculated with supernatant of lung macerate of infected animals. Our results are similar to those shown in other groups of mice and in human necropsy studies (Burke, 1968; Hotta *et al.*, 1981*a*; Bhamarapravati, 1989). Increase of mast cells in peribronchiolar space and in the interalveolar septa in lung of mice of our experiments, probably contributes to the permeability changes seen in this organ. Tissue mast cells probably are also involved in DEN injury, since they are usually found in normal and abnormal alveolar wall (Fox *et al.*, 1981*a*). They produce a series of vasoactive mediators, including histamine and histamine-like products. Histamine causes increased capillary permeability by opening the intercellular junctions (Khanna *et al.*, 1990). Russel (1971) suggested that the histamine can be the mediator of the vascular permeability in DHF and in DSS. In studies with cases of DHF and DSS, increase of levels of histamine in urine was observed (Tuchinda *et al.*, 1977). Studies with mice inoculated with cytotoxic factor obtained from spleen of mice infected by DENV showed alterations in blood-brain barrier, leading to oedema, which was mediated via liberation of histamine (Chaturvedi *et al.*, 1991).

Histopathological alterations in kidney of mice infected by DENV have been poorly described. In our experiments was observed increased volume of glomeruli that could be related to the increased cellularity in glomeruli as well as due to the vascular congestion and immunoglobulins (IgM) presence in glomerular vessels. Virus particles and antigen of DENV were detected in monolayers of C6/36 cells inoculated with supernatant of kidney macerate of infected animals. According to Boonpucknavig *et al.* (1981), in studies with Swiss albino mice inoculated intraperitoneally with a mouse-adapted strain of DENV, mononuclear cell infiltration was seen at 14 days p.i. DENV antigen antibody complement complexes were located in the glomeruli, but without significant effect on glomerular function. In human cases of DF, increase of mesangial cells was observed (Bhamarapravati, 1997). Ours findings differ from studies with BALB/c mice infected with neuroadapted mouse DENV strain, where any histopathological alteration was observed in kidney (Atrasheuskaya *et al.*, 2003).

In our studies, cerebellum tissue showed focal alterations in the Purkinje neuron layer and oedema in the gray matter. Virus particles and antigen of DENV were detected in monolayers of C6/36 cells inoculated with supernatant of cerebellum macerate of infected animals. DENV has been shown to lack the ability to invade the central nervous system from the periphery (Nathanson and Cole, 1970), but there are reports of definitive development of cerebral oedema in cases of DF/DHF/DSS (Halstead, 1981) and the antigen of DENV has been detected in brain of human fatal cases of DEN (Miagostovich *et al.*, 1997; Nogueira *et al.*, 2002). During DENV-2 infection of mice, the blood-brain barrier is damaged, resulting in leakage of protein-bound Evans blue dye and radiolabeled mouse red blood cells into the brain substance (Chaturvedi *et al.*, 1991). In studies with atymic mice and BALB/c mice, virus particles and antigen of DENV were detected in neuron cytoplasm (Hotta *et al.*, 1981). Viral RNA has been demonstrated in cytoplasm of glial cells and in macrophages of brain of ICR mice, infected by intracerebral route (Lucia and Kangwanpong, 1994). Ours findings differ from studies with BALB/c mice infected with neuroadapted mouse DENV strain, where any histopathological alteration was observed in cerebellum (Atrasheuskaya *et al.*, 2003).

In our studies the presence of DENV antigen and viral particles in C6/36 cell cultures inoculated with supernatant of the tissues (liver, lung, kidney and cerebellum), macerates of BALB/c mice, 48 h post-infection, by intraperitoneal route, is an indirect proof of the DENV-2 infection, and confirms that BALB/c mice tissues are susceptible for DENV-2.

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REFERENCES

AN J., KIMURA-KURODA J., HIRABAYASHI Y. and YASIU K., 1999. Development of a novel mouse model for dengue virus infection. *Virology*, **263**, 70-77.

ATRASHEUSKAYA A., PETZELBAUER P., FREDEKING T.M. and IGNATYEV G., 2003. Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. *FEMS Immunol. Med. Microbiol.*, **35**, 33-42.

BHAMARAPRAVATI N., 1989. Homostatic defects in dengue hemorrhagic fever. *Rev. Infect. Dis.* (*Suppl.*), 4, S826-S829.

BHAMARAPRAVATI N., 1993. Pathology of dengue haemorrhagic fever. In: 'Monograph on Dengue/Dengue Haemorrhagic Fever: WHO, Regional Office for South-East Asia'. Thongcharoen P. ed., New Delhi, pp. 72-79.

BHAMARAPRAVATI N., 1997. Pathology of dengue infections. In: 'Dengue and Dengue Haemorrhagic Fever'. Gubler D.J. and Kuno G. eds., Cab International, Wallingford, pp. 115-132.

BOONPUCKNAVIG S., VUTTIVIJOJ O. and BOONPUCKNAVIG V., 1981. Infection of young adult mice with dengue virus type 2. *Trans. R. Soc. Trop. Med. Hyg.*, 75 (5), 647-653.

BURKE D.S., NISALAK A., JOHNSON D.E. and SCOTT R.M., 1988. A prospective study of dengue infections in Bangkok. *Am. J. Trop. Med. Hyg.*, 38, 172-180.

BURKE T., 1968. Dengue hemorrhagic fever, a pathological study. *Trans. R. Soc. Trop. Med. Hyg.*, 62, 682-691.

CHATURVEDI U.C., DHAWAN R., KHANNA M. and MATHUR A., 1991. Breakdown of the blood-brain barrier during dengue virus infection of mice. *J. Gen. Virol.*, 72, 859-866.

COUVERLAND A., MARIANNEAU P., BEDEL C., DROUET M.T., VACHON F., HENIN D. and DEUBEL V., 1999. Report of a fatal case of dengue infection with hepatitis. Demonstration of dengue antigens in hepatocytes and liver apoptosis. *Hum. Pathol.*, 30, 1106-1110.

DESPRÈS P., FRENKIEL M-P., CECCALDI P-E., DOS SANTOS C.D. and DEUBEL V., 1998. Apoptosis in the mouse central nervous system in response to infection with mouse-neurovirulent dengue viruses. *J. Virol.*, 72 (1), 823-829.

FOX B., BULL T.B. and GUZ A., 1981. Mast cells in human alveolar wall: an electron microscopic study. *J. Clin. Pathol.*, 34, 1333-1342.

FRESH J.W., REYES V., CLARKE E.J. and UYLANGCO C.V., 1969. Philippine hemorrhagic fever: a clinical, laboratory, and necropsy study. *J. Lab. Clin. Med.*, 73, 451-458.

HALSTEAD S.B., 1981. Immune enhancement of dengue infection-research question. In: 'Proceedings of the First ICMR Seminar on Dengue Haemorrhagic Fever 1981, International Center for Medical Research'. Hotta S. ed., Kobe University School of Medicine, Kobe, Japan, pp. 115-128.

HOTTA H., MURAKAMI I., MIYASAKI K., TAKEDA Y., SHIRANE H. and HOTTA S., 1981*a*. Experimental dengue virus infection using athymic nude mice. In: 'Proceedings of the First ICMR Seminar on Dengue Haemorrhagic Fever 1981, International Center for Medical Research'. Hotta S. ed., Kobe University School of Medicine, Kobe, Japan, pp. 151-158.

HOTTA H., MURAKAMI I., MIYASAKI K., TAKEDA Y., SHIRANE H. and HOTTA S., 1981*b*. Inoculation of dengue virus into nude mice. *J. Gen. Virol.*, 52, 71-76.

HUANG K-J., LI S-Y., CHEN S-C., LIU H-S., LIN Y-S., YEH T-M, LIU C-C. and LEI H-Y., 2000. Manifestation of thrombocytopenia in dengue-2-virus-infected mice. *J. Gen. Virol.*, 81, 2177-2182.

HUERRE M.R., LAN N.T., MARIANNEAU P., HUE N.B., KHUN H., HUNG N.T., KHEN N.T., DROUET M.T., HUONG V.T.Q., HA D.Q., BUISSON Y. and DEUBEL V., 2001. Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamense children. *Virchows Arch.*, 438, 107-115.

INNIS B., 1995. Dengue and dengue hemorrhagic fever. In: 'Kass Handbook of Infectious Disease; Exotic Viral Infections'. Porterfield J.S. ed., Chapman Hall, London, pp. 103-146.

JOHNSON A.J. and ROEHRIG J.T., 1999. New mouse model for degue virus vaccine testing. *J. Virol.*, 73 (1), 783-786.

KHANNA M., CHATURVEDI U.C., SHARMA M.C., PANDEY V.C. and MATHUR A., 1990. Increased capillary permeability mediated by dengue virus-induced lymphokine. *Immunology*, 69, 449-453.

KNOWLESS B.B., SEARLS D.B. and ADEN D.P., 1984. Human hepatoma-derived cell lines. In: 'Advances in Hepatitis Research'. Chisari F.V. ed., New York, Masson, pp. 196-202.

KNUDSEN A.B., 1997. Global strategy for the prevention and control of dengue and dengue haemorrhagic fever. In: 'Factors in the Emergence of Arbovirus Diseases'. Saluzzo J.F. and Dodet B. eds., Paris, Elsevier, pp. 129-140.

LIN Y., LIAO C.L., CHEN L.K., YEH C.T., LIU C.I., MA S.H., HUANG Y.Y., HUANG Y.L., KAO C.L. and KING C.C., 1998. Study of dengue virus infection in SCID mice engrafted with human K562 cells. *J. Virol.*, 72, 9729-9737.

LUCIA H.L. and KANGWANPONG D., 1994. Identification of dengue virus-infected cells in paraffin-embedded tissue using in situ polymerase chain reaction and DNA hybridization. *J. Virol. Methods*, 48, 1-8.

MARIANNEAU P., FLAMAND M., DEUBEL V. and DESPRÈS P., 1998. Apoptotic cell death in response to dengue virus infection: the pathogenesis of dengue hemorrhagic fever. *Clin. Diagn. Virol.*, 10, 113-119.

MEIKLEJOHN G., ENGLAND B. and LENNETTE E.H., 1952. Adaptation of dengue virus strains in unweaned mice. *Am. J. Trop. Med. Hyg.*, 1, 51-58.

MIAGOSTOVICH M.P., RAMOS R.G., NICOL A.F., NOGUEIRA R.M.R., CUZZI-MAYA T., OLIVEIRA A.V., MARCHEVSKY R.S., MESQUITA R.P. and SCHATZMAYR H.G., 1997. Retrospective study on dengue fatal cases. *Clin. Neuropathol.*, 6 (4), 204-208.

MONATH T.P. and HEINZ F.X., 1996. Flaviviruses. In: 'Fields Virology'. Fields B.N., Knipe D.M. and Howley P.M., Philadelphia, PA, Lippincott-Raven Press, pp. 961-1034.

NATHANSON N. and COLE G.A., 1970. Immunosuppression and experimental virus infection of the nervous system. *Adv. Virus Res.*, 16, 397-428.

NATH P., TANDON P., GULATI L. and CHATURVEDI U.C., 1983. Histological and ultrastructural study of spleen during dengue virus infection of mice. *Indian J. Med. Res.*, 78, 83-90.

NOGUEIRA R.M.R., FILIPIS A.M., COELHO J.M., SEQUEIRA P.C., SCHATZMAYR H.G., PAIVA F.G., RAMOS A.M. and MIAGOSTOVICH M.P., 2002. Dengue virus infection in central nervous system (CNS): case report from Brazil. *Southeast Asian J. Trop. Med. Public Health*, 33 (1), 68-71.

NOGUEIRA R.M.R., MIAGOSTOVICH M.P., LAMPE E. and SCHATZMAYR H.G., 1990. Isolation of dengue virus type 2 in Rio de Janeiro. *Mem. Inst. Oswaldo Cruz*, 85, 253.

PAES M.V., BARRETO D.F., PINHÃO A.T., TAKIYA C.M., CÔRTES L.M.C., MAJEROWICZ S. and BARTH O.M., 2002. Ultrastructural aspects of mouse liver infected with dengue-2 virus. *Virus Rev. Res.*, 7 (2), 38-46.

RAUT C.G., DEOLANKAR R.P., KOLHAPURE R.M. and GOVERDHAN M.K., 1996. Susceptibility of laboratory-bred rodents to the experimental infection with dengue virus type 2. *Acta Virol.*, 40, 143-146.

REED L.J. and MUENCH H., 1938. A simple method of stimating fifty percent endpoints. *Am. J. Hyg.*, 27, 493-497.

REYNOLDS E.S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.*, 17, 208-212.

ROSEN L., DROUET M.T. and DEUBEL V., 1999. Detection of dengue virus RNA by reverse transcription-polymerase chain reaction in the liver and lymphoid organs but not in the brain in fatal human infection. *Am. J. Trop. Med. Hyg.*, 61, 720-724.

ROSEN L., KHIN M.M. and TIN U., 1989. Recovery of virus from the liver of children with fatal dengue. Reflections on the pathogenesis of disease and its possible analogy with that of yellow fever. *Res. Virol.*, 140, 351-360.

RUSSEL P.K., 1971. Immunopathological mechanism in the dengue shock syndrome. *Prog. Immunol.*, 1, 831.

SINGH K.R.P., 1967. Cell cultures derived from larvae of *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.). *Curr. Sci.*, 36, 506-508.

TUCHINDA M., DHORREININTRA B. and TUCHINDA P., 1977. Histamine content in 24 h urine in patients with dengue haemorrhagic fever. *Southeast Asian J. Trop. Med. Public Health*, 8, 80.

WORLD HEALTH ORGANIZATION (WHO), 1999. Prevention and control of dengue and dengue haemorrhagic fever. Comprehensive guidelines. New Delhi, India: WHO Regional publication, SEARO, n. 29, pp. 134.