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EXPEDIENTE No. : 12069004
ASUNTO : SOLICITUD DE REGISTRO DE LA PATENTE DE
INVENCION **MÉTODO, PLASMIDEO Y**
COMPOSICIÓN PARA INDUCIR UNA RESPUESTA
INMUNE CONTRA VIRUS DE LA DENGUE BASADO
EN VACUNAS DE ADN Y VIRUS QUIMERICOS
SOLICITANTE : FUNDAÇÃO OSWALDO CRUZ.

JUAN CARLOS URIBE RESTREPO mayor de edad, domiciliado en la ciudad de Bogotá D.C, República de Colombia, abogado titulado e identificado como aparece al pie de mi firma, en mi calidad de apoderado de la sociedad **FUNDAÇÃO OSWALDO CRUZ**, existente y organizada conforme a las leyes de Brasil, con domicilio en la ciudad de Rio de Janeiro, Brasil, por medio del presente escrito doy respuesta al oficio No. 6982, notificado por fijación en lista de fecha 17 de junio de 2014, y conforme a la prórroga de términos legales presentada el 28 de agosto de 2014, me permito manifestar y anexar lo siguiente:

1. Manifiesto

• Respecto de la **UNIDAD DE INVENCION**

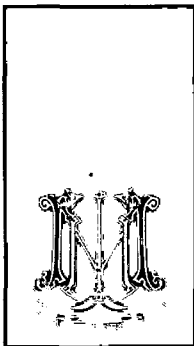
- 1.1 El examinador señala que la solicitud no cumple con el requisito de unidad de invención porque se refiere a 2 grupos inventivos. El señor examinador define que el concepto común inventivo es la vacuna de ADN contra el dengue, concepto divulgado por Imoto et al., 2006 (Vaccine, vol. 25, no. 6: 1076-1084, Mota et al., 2005 (Vaccine, Vol. 23: 2469-3476) y D1. Indica que los grupos inventivos están definidos por las siguientes características esenciales:

1° Reivindicaciones 3-6. Composición/kit comprendiendo una vacuna de ADN y virus quiméricos abarcando el virus vacunal de la fiebre amarilla contra el virus del dengue.

2° Reivindicaciones 7-10. Plásmido recombinante codificando la proteína del sobre viral E por cada serotipo del virus del dengue.

El señor examinador sugiere al solicitante restringir la materia a patentar en un sólo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica.

- 1.2 Ahora bien, para cumplir con los términos de Unidad de Invención conforme se indica en el artículo 28 de la Decisión 486 de la CAN, relacionados por el señor examinador en su oficio para subsanar la materia expuesta que se refieren a un plásmido recombinante que codifica el sobre viral de proteína (E) por cada serotipo del virus del dengue, nos permitimos **DIVIDIR LA PRESENTE SOLICITUD EN DOS SOLICITUDES INDEPENDIENTES** y con ello cumplir con este requisito solicitado por el señor examinador.



1.3 Para tal efecto anexamos al presente:

- i) Un nuevo capítulo reivindicatorio para conformar con el texto descriptivo de la presente y todo su cuerpo una primera solicitud que hace referencia a la Composición/kit comprendiendo una vacuna de ADN y virus quiméricos abarcando el virus vacunal de la fiebre amarilla contra el virus del dengue, con un pliego de 3 reivindicaciones.
- ii) Adicionalmente señalo que por separado se está radicando una solicitud divisional que hace referencia a Plásmido recombinante codificando la proteína del sobre viral E por cada serotipo del virus del dengue, con un pliego de 4 reivindicaciones.

Adicionalmente, cabe reiterar que los nuevos capítulos reivindicatorios contenidos en las dos solicitudes ahora independientes no implican una adición a la invención inicialmente presentada, sino que hace referencia a las sugerencias del señor examinador con el fin de precisar y aclarar la invención.

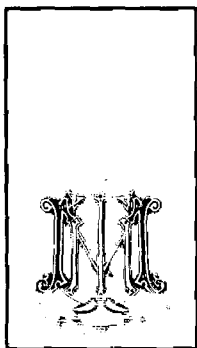
- Respecto a la **EXCEPCIÓN A LA PATENTABILIDAD**

- 1.4 En cuanto a los términos de patentabilidad, el señor examinador se refiere a que el objeto de las reivindicaciones 1 a 2 no es patentable de acuerdo con el Art 20 literal d) de la Decisión 486 de la CAN y agrega que las reivindicaciones mencionan un proceso para mejorar la eficacia de la protección y corresponden a un método de tratamiento que no es objeto de patentabilidad. Sobre este respecto, me permito informar a ese despacho que con la presente respuesta, anexamos un nuevo pliego reivindicatorio sobre el cual se ha modificado conforme lo indicado para reclamar el uso de la composición de las vacunas de ADN y 17D virus quiméricos. Para tal efecto se ha modificado la reivindicación 2 para incorporar el protocolo invertido con el principal virus quimérico e impulsar las vacunas de ADN, tal como se describe en la invención. El siguiente es el texto de la solicitud de la solicitud correspondiente a la PCT presentada:

“En el sistema de dosis y refuerzo de la presente invención, la primera inmunización (dosis) se realizó con vacunas de ADN, mientras que el refuerzo se hizo por la inmunización con virus quimérico. También es posible invertir este protocolo, con la primera inmunización con virus quiméricos y el refuerzo con vacunas de ADN, administradas en intervalos variados.”.

- **DE LA SOLICITUD DE PATENTE**

- 1.5 Respecto al capítulo reivindicatorio, el señor examinador se refiere a que la composición de las reivindicaciones 3-5 debe ser caracterizada a partir de los elementos que la conforman y las proporciones de los mismos. Agrega, que el virus quimérico y la vacuna deben ser definidas a partir de su estructura, es decir por la secuencia composición de vacuna de ADN y advierte que se menciona su forma de obtención pero no que comprende, ni las secuencias específicas de manera que el virus quimérico debe ser definido a partir de su estructura indicando la secuencia del virus modificado. Agrega también que el término virus quimérico 17D no es claro

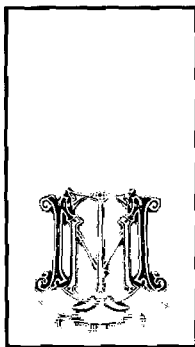


puesto que el virus debe ser definido a partir de su estructura, es decir a partir de su secuencia de nucleótidos. Para tal efecto, el nuevo pliego reivindicatorio ha modificado la reivindicación 3 e incorpora el objeto de las cláusulas 4 y 5 anteriores.

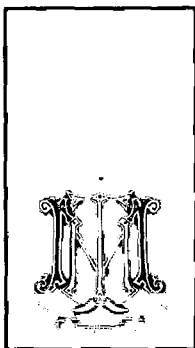
- 1.6 Seguido, el examinador se refiere a que el kit de la reivindicación 6 está conformado por un elemento que ya había sido reclamado en las reivindicaciones precedentes, de esta manera dicha reivindicación parece referirse al uso dado a dichos elementos y define que ello no es objeto de patentabilidad. En relación a este requerimiento, esta cláusula 6 original (nueva reivindicación 4) se ha modificado de manera que se ha destacado la diferencia entre el objeto de la reivindicación 6 (un equipo) del objeto de las reivindicaciones anteriores (composición).
- 1.7 Adelante también define que las reivindicaciones 7 y 9 no son concisas, porque presentan una excesiva complejidad, lo cual dificulta determinar la extensión del objeto que se busca proteger y define que las reivindicaciones 7 a 10 no son claras ni concisas y sugiere definir el plásmido por su secuencia específica y no por la secuencia codificada por el plásmido. Agrega también que las reivindicaciones 8 y 10 no son claras al no indicar el número de la secuencias (SEQ ID NO:) y sugiere eliminar la referencia al número de Genbank e incluir el número de secuencia. Respecto a tal requerimiento, la solicitante ha modificado las reivindicaciones donde las cláusulas originales 7 y 8, ahora las reivindicaciones 1 y 2 de la divisional, fueron modificados para incluir la región que corresponde a la ectodominio de la proteína (80%) y no la descripción de la forma en que se obtuvo.
- 1.8 La Solicitante, también agrega que las cláusulas 5 a 8 se han cancelado de este pliego reivindicatorio y se han presentado como caracterizaciones de la solicitud divisional modificado según lo sugerido por el examinador en el presente Oficio objeto de respuesta. Reclamaciones originales 9 y 10, ahora las reivindicaciones 3 y 4 presentadas como una solicitud divisional, de manera que se modificaron para incluir la región que corresponde al dominio III y no la descripción de la forma en que se obtuvo. En la nueva serie de caracterizaciones todos los términos que se consideran como definición abierta como "contiene" y "comprende" se modificaron.
- 1.9 Así las cosas, con base en el nuevo capítulo reivindicatorio anexo a la presente, la solicitante espera haber definido de manera clara y precisa la invención sobre la cual se busca protección, particularmente, respecto a lo que define el señor examinador en el escrito de Oficio objeto de la presente, agregando que tales correcciones no conforman una adición a la materia inicialmente presentada sino por el contrario corresponden a las aclaraciones solicitadas por ese despacho.

- **EXAMEN DE PATENTABILIDAD.**

- 1.10 Ese despacho concluye que en materia de patentabilidad de la presente invención, los documentos **D1: Raviprakash et al., 2009. Human Vaccines 5:8, 520-528;** y, **D2: Guirakhoo et al., 2004. Journal Of Virology, p. 4761-4775,** afectan la patentabilidad de las cláusulas 3 a 6. Para tal efecto, presenta una tabla comparando la materia reivindicada.
- 1.11 El documento D1 - Raviprakash et al, 2009 Vacunas Humanos. 5: 8, 520 a 528, se refiere a avances en el desarrollo de vacuna contra el dengue.

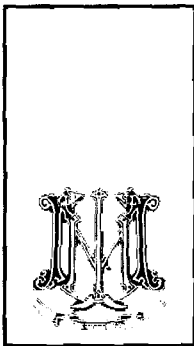


- 1.12 El documento D2, - Guirakhoo et al, 2004, Journal of Virology, P. 4.761-4.775, se refiere a la seguridad y eficacia de las formulaciones de vacunas quiméricas dengue-amarillo tetravalente del virus en primates no humanos.
- 1.13 El examinador menciona que la solicitud de patente en examen carece de inventiva. Sin embargo, la combinación de vacuna de ADN basado en el sobre viral de proteína del virus del dengue y la fiebre amarilla 17D/dengue virus quimérico no fue sugerido, usado o probado por otros grupos de investigación anteriores a la presente o incluso hoy en día.
- 1.14 El examinador cita el artículo de revisión sobre las vacunas contra el dengue de Raviprakash et al., 2009 (D1). De hecho, este artículo menciona otros estudios con vacunas de ADN contra el dengue basado en el módulo de expresión con el prM y de longitud completa E. Sin embargo, el enfoque mencionado en D1 es diferente de nuestro enfoque con la vacuna de ADN y plásmidos pE1 y pE2. En ambos casos, no utilizamos el gen que codifica la proteína prM. Los documentos citados en D1, así como otros del estado de la técnica, sugirieron que la proteína E del virus del dengue no podía ser expresada adecuadamente en la ausencia de la proteína prM que actúa como una chaperonina. La Solicitante revela estudios que han demostrado lo contrario de lo expuesto en el documento D1. De hecho, la presencia de proteína prM a la expresión correcta del ectodominio de la proteína E (80% de dominios de codificación I, II y III de la proteína E, y sin el resto de la terminal hidrofóbica C) no era necesaria para la inducción de la respuesta inmune protectora. Por lo tanto, basado en las enseñanzas de D1 no era obvio que la vacuna de ADN pE1 sería eficaz para mediar la expresión correcta del ectodominio de la proteína E y, como consecuencia, para generar una respuesta inmune protectora contra el Dengue.
- 1.15 D1 y D2 también mencionan que las vacunas quiméricas de la fiebre amarilla-dengue desarrollado por Monath, Guirakhoo (Guirakhoo es también el autor del documento D2 citado en el presente oficio) entre otros, destaca la seguridad y la inmunogenicidad de estas quimeras en ambos ensayos preclínicos y clínicos de fase 1. Los autores no sugieren en cualquier momento que el virus quimérico 17D/dengue se puede utilizar en combinación con vacunas de ADN. Por el contrario, los informes de D2 sugieren el uso de tales virus quiméricos suficientes para inducir una respuesta inmune eficaz y protectora contra el dengue. En ningún caso, los autores del documento D2 sugieren combinaciones con la quimera 17D/dengue para producir una vacuna más inmunogénica. Sin embargo, estudios más recientes (anexo 1, Sabchareon et al. 2012) demostraron que la vacuna tetravalente con el virus quimérico 17D/dengue no fue capaz de inducir protección frente al virus del dengue tipo 2 en niños en Tailandia, a pesar de que estos niños exhibieron niveles satisfactorios de anticuerpos neutralizantes contra el dengue tipo 2. Esto puede ser debido a la falta de respuesta inmune celular eficiente y protectora inducida por dicha vacuna (tetravalente). Por lo tanto, un experto en la técnica tras la lectura de los documentos D1 y D2 solos o en combinación, se habría desanimado para combinar el virus quimérico 17D con otras estrategias de vacunación.
- 1.16 Nuestros estudios han demostrado que la combinación con la vacuna de ADN con el virus quimérico 17D-D2, dio como resultado una respuesta inmunitaria más eficiente y protectora con la inducción de ambos brazos de la respuesta inmune (humoral y



celular). De hecho, cuando se combinaron las dos vacunas (vacuna de ADN y virus quimérico 17D), se observó un efecto sinérgico sobre la activación de la respuesta inmune humoral, con aumento de los niveles de anticuerpos neutralizantes contra el dengue, pero no en la respuesta inmune celular (Anexo 2, Azevedo et al. 2013). Una respuesta inmune celular robusta sólo se obtuvo cuando los animales también fueron inoculados con la vacuna de ADN. Este resultado fue sorprendente, porque desde el estado conocido de la técnica, la quimera-17D-D2 (que es un virus atenuado) también induce una fuerte respuesta inmune celular. Por lo tanto, de nuevo, estos estudios demuestran que la administración de la vacuna de ADN con el virus quimérico 17D virus quimérico no es obvia.

- 1.17 Por lo tanto, la presente invención tiene una actividad inventiva. La combinación de vacuna de ADN y el virus quimérico 17D/dengue fue capaz de inducir una protección completa en los animales (sin ningún signo de morbilidad). El examinador señaló que, de acuerdo con las figuras 3A y 3B de la invención, resulta pE1D2 aislado y pE1D2 + 17D-D2 similar. Sin embargo, este no es el caso ya que con sólo la pE1D2, así como con sólo el 17D-D2, aunque los animales sobrevivieron a la exposición al virus, presentaron signos de morbilidad. Por otro lado, con la combinación de los ratones pE1D2 + 17D-D2 no mostraron morbilidad. El examinador también señaló que con pE1D2 los animales sólo recibieron 2 dosis, mientras que con la pE1D2 + 17D-D2 en los ratones de régimen de inducción/potenciación recibieron 3 dosis. Esto es correcto. Sin embargo, se obtuvieron los mismos resultados cuando los animales fueron inmunizados con pE1D2 + 17D-D2 en el régimen de impulso primordial (3 dosis) o con inoculación simultánea (2 dosis), sin la morbilidad después de la exposición al virus, lo que revela el aumento de la protección inducida con la combinación de ambas vacunas. Además, la respuesta inmune inducida con los grupos (sólo inmunizados con la vacuna pE1D2, quimera pE1D2 + 17D-D2 o sólo 17D-D2) fue diferente (Anexo 2, Azevedo et al. 2013). Una vez más, la presente invención demuestra que las dos vacunas combinadas produjeron un efecto cooperativo, induciendo ambos brazos de la respuesta inmune (humoral y celular) de una manera eficiente. Esto fue posible porque, de acuerdo con el enfoque de la presente invención, la presentación del antígeno (proteína E) hacia el sistema inmune fue realizada de manera diferente y se configuró con inmunizaciones con vacunas aisladas. El enfoque y la estrategia son inesperados y no están contemplados por el estado de la técnica, ya que, como se mencionó anteriormente, se cree que la quimera 17D-D2 que es un virus atenuado, sería capaz de inducir eficazmente ambos brazos de la respuesta inmune.
- 1.18 En conclusión, no hay nada en los documentos referenciados por el señor examinador que sugiera los mismos elementos constitutivos de la presente invención. La técnica citada no reconoce el problema que se aborda en la presente invención, por lo que es posible afirmar que el capítulo reivindicatorio, debe considerarse novedoso y de altura inventiva. Por todo lo anterior, me permito en conclusión solicitar sea de nuevo revisada la presente solicitud y verificar las diferencias aquí expuestas y en consecuencia sea concedida la presente en los términos y el tenor de las nuevas cláusulas del capítulo reivindicatorio aquí modificadas.
- 1.19 Con lo anterior esperamos haber cumplido con los requerimientos exigidos por este despacho y solicitamos se practique un nuevo examen de fondo conforme el artículo



45 de la Decisión 486 de la Comisión de la Comunidad Andina y en consecuencia sea concedida la presente conforme el nuevo capítulo reivindicatorio anexo a la presente.

2. Anexo

2.1 Un nuevo capítulo reivindicatorio.

Al respecto reitero ante esa entidad que la invención radicada bajo el expediente de la referencia en adelante debe ser identificada bajo el título **COMPOSICIÓN/KIT COMPRENDIENDO UNA VACUNA DE ADN Y VIRUS QUIMÉRICOS ABARCANDO EL VIRUS VACUNAL DE LA FIEBRE AMARILLA CONTRA EL VIRUS DEL DENGUE.**

2.2 Estudio Sabchareon et al. 2012.

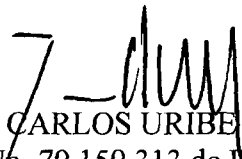
2.3 Estudio Azevedo et al. 2013.

Adicionalmente manifiesto a ésta entidad que por separado está siendo presentada la respectiva solicitud divisional, con el pago de la tasa oficial bajo el título **PLÁSMIDO RECOMBINANTE CODIFICANDO LA PROTEÍNA DEL SOBRE VIRAL E POR CADA SEROTIPO DEL VIRUS DEL DENGUE.**

4. Petición

De acuerdo con lo anterior, solicitamos revisar nuevamente la presente solicitud y verificar las diferencias aquí expuestas y en consecuencia sea concedida la presente invención en los términos y el tenor de las nuevas cláusulas del capítulo reivindicatorio aquí modificadas.

Del Señor Jefe,


JUAN CARLOS URIBE RESTREPO
C.C. No. 79.159.313 de Bogotá
T.P.A. 54.493 de Min. Justicia

ANEXO I.

- i) Un nuevo capítulo reivindicatorio.
- ii) Estudio Sabchareon et al. 2012.
- iii) Estudio Azevedo et al. 2013.

**COMPOSICIÓN Y KIT COMPRENDIENDO UNA VACUNA DE ADN Y
VIRUS QUIMÉRICOS ABARCANDO EL VIRUS VACUNAL DE LA
FIEBRE AMARILLA CONTRA EL VIRUS DEL DENGUE**

REIVINDICACIONES

1. Composición caracterizada por la combinación de vacunas de ADN y virus quiméricos 17D vacunal de fiebre amarilla en un sistema que consiste en:

- en el sistema de dosis (vacunas de ADN) y refuerzo (virus quiméricos), o en el protocolo invertido con la dosis (virus quimérico) y refuerzo (vacuna de ADN) o;
- sistema simultáneo de las dos estrategias vacunales (vacunas de ADN y virus quiméricos en una misma formulación).

2. Composición vacunal contra el virus dengue que consiste en:

(a) Vacunas de DNA contra los cuatro serotipos del virus dengue que consiste de distintos plásmidos recombinantes que codifican las proteínas E a partir de cada serotipo viral del virus dengue, o solamente los dominios III de estas proteínas;

(b) Virus quiméricos que consisten en el virus vacunal de fiebre amarilla 17D que codifican las proteínas PrM y E de los virus dengue de los distintos serotipos; y

(c) un vehículo farmacéuticamente aceptable: donde estas vacunas de ADN comprenden plásmidos pE1 o pE2, entre los cuales:

- el plásmido pE1 codifica el ectodominio del sobre de proteína E, que consiste en los dominios I, II y III de dicha proteína, a partir de los virus dengue, todas fusionadas a un péptido señal del activador de plasminogeno de tejido humano (t-PA),

- un plásmido pE2 codifica sólo el dominio III del sobre de proteína E del virus dengue, todas fusionadas a un péptido señal del t-PA.

3. Kit de vacunación contra el dengue caracterizado porque consiste de:

(a) un primer recipiente que contiene vacunas de ADN que codifican las proteínas E a partir de cada serotipo viral del virus dengue, fusionadas a un péptido señal del activador de plasminogeno de tejido humano (t-PA), o vacunas de ADN que codifican sólo los dominios III de las proteínas E de cada serotipo del virus dengue fusionado a un péptido señal de t-PA; y,

(b) un segundo recipiente que contiene virus quiméricos que consisten en el virus vacunal de fiebre amarilla 17D que codifican las proteínas prM y E de los virus dengue de los distintos serotipos.

-/-

Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial



Arunee Sabchareon, Derek Wallace, Chukiat Sirivichayakul, Kriengsak Limkittikul, Pornthep Chanthavanich, Saravudh Suvannadabba, Vithaya Jiwariyavej, Wut Dulyachai, Krisana Pengsaa, T Anh Wartel, Annick Moureau, Melanie Saville, Alain Bouckennooghe, Simonetta Viviani, Nadia G Tornieporth, Jean Lang

Summary

Background Roughly half the world's population live in dengue-endemic countries, but no vaccine is licensed. We investigated the efficacy of a recombinant, live, attenuated tetravalent dengue vaccine.

Methods In this observer-masked, randomised, controlled, monocentre, phase 2b, proof-of-concept trial, healthy Thai schoolchildren aged 4–11 years were randomly assigned (2:1) to receive three injections of dengue vaccine or control (rabies vaccine or placebo) at months 0, 6, and 12. Randomisation was by computer-generated permuted blocks of six and participants were assigned with an interactive response system. Participants were actively followed up until month 25. All acute febrile illnesses were investigated. Dengue viraemia was confirmed by serotype-specific RT-PCR and non-structural protein 1 ELISA. The primary objective was to assess protective efficacy against virologically confirmed, symptomatic dengue, irrespective of severity or serotype, occurring 1 month or longer after the third injection (per-protocol analysis). This trial is registered at ClinicalTrials.gov, NCT00842530.

Findings 4002 participants were assigned to vaccine (n=2669) or control (n=1333). 3673 were included in the primary analysis (2452 vaccine, 1221 control). 134 cases of virologically confirmed dengue occurred during the study. Efficacy was 30·2% (95% CI –13·4 to 56·6), and differed by serotype. Dengue vaccine was well tolerated, with no safety signals after 2 years of follow-up after the first dose.

Interpretation These data show for the first time that a safe vaccine against dengue is possible. Ongoing large-scale phase 3 studies in various epidemiological settings will provide pivotal data for the CYD dengue vaccine candidate.

Funding Sanofi Pasteur.

Introduction

Dengue has become one of the most important and widespread arthropod-borne viral diseases of human beings, with about half the world's population now at risk.¹ WHO estimates that 50–100 million dengue infections occur each year in more than 100 countries, and that half a million people develop severe dengue necessitating hospital admission, although the true figures could be higher.^{1–3} Symptomatic infection is classified as either dengue or severe dengue, depending on whether the patient recovers after the initial 3–7-day febrile phase, or develops complications as a result of a systemic vascular leakage syndrome.^{1,4} There is no specific treatment and, in absence of a vaccine, prevention relies on individual protection against mosquitoes and vector control strategies that, in view of the continuing expansion of dengue, have shown their limits as standalone measures.

The major challenges facing vaccine research and development include the existence of four pathogenic dengue virus serotypes (DENV1–4) that compete and interact at the immunological level, as well as more practical challenges, such as the lack of suitable animal models or a correlate of protection.^{5–7} In more than half a century of research, various vaccine approaches have

been attempted and several candidate vaccines are in early clinical or preclinical development.⁸

One candidate vaccine, CYD-TDV, is a recombinant, live, attenuated, tetravalent dengue vaccine based on the yellow fever 17D vaccine strain and produced in Vero cells.⁷ Phase 1 and 2 trials have been undertaken in southeast Asia and Latin America in cohorts of adults and children who were either immunologically naive against dengue and other flaviviruses before vaccination or who had some degree of pre-existing flaviviral immunity due to vaccination against yellow fever or Japanese encephalitis or natural exposure in endemic areas.^{7,9–13} These studies have shown that a three-dose regimen given over 12 months is well tolerated and elicits balanced neutralising antibody responses against the four serotypes in diverse epidemiological settings. We present the primary report of the first clinical trial of the protective efficacy of this investigational dengue vaccine. The trial was designed and undertaken in an endemic area according to WHO recommendations.¹⁴ The primary objective was to assess protective efficacy after three injections against virologically confirmed symptomatic dengue, irrespective of severity or serotype. Passive surveillance for admissions to hospital owing to fever due to dengue is ongoing.

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Methods

Study design and participants

We undertook an observer-blind, randomised, controlled, monocentre phase 2b trial of the efficacy of CYD-TDV against virologically confirmed symptomatic dengue. The study was done in the Muang district, Ratchaburi province, Thailand, which is about 100 km west of Bangkok. An epidemiological study to prepare this site for a dengue vaccine efficacy trial was done in 2006–09 and showed that the site was suitable for the trial in terms of disease incidence, the co-circulation of all four dengue virus serotypes (predominantly DENV1, then 2), adequate infrastructure for surveillance, and high community awareness of the disease.^{15–17} The study was done at Ratchaburi Regional Hospital (RRH), the province's principal medical care facility, and involved 35 schools in the district. We enrolled schoolchildren aged 4–11 years who were in good health based on medical history and physical examination, and whose parent or legal guardian had no plans to leave the study area. We excluded children with acute febrile illness at enrolment, those with congenital or acquired immunodeficiency or other disorders listed in the protocol, and those receiving immunosuppressive therapy or other treatments or vaccines prohibited by the protocol (appendix).

See Online for appendix

The ethical review committee for research in human subjects, Ministry of Public Health, Thailand, approved the protocol, amendments, consent, and assent forms. We obtained written informed consent from parents or legal guardians of all participants, and written assent from all participants aged 7 years or older. The trial was undertaken in accordance with the principles of the Declaration of Helsinki, and in compliance with good clinical practice guidelines.

An independent data monitoring committee (IDMC) oversaw the study. Safety data after the first vaccination of a first cohort of 150 children were reviewed by the IDMC and their recommendation to proceed with the trial was submitted to the ethical review committee for approval. The committee approved continuation and at this point required that the study design be changed from active (rabies vaccine) to placebo control. Placebo was therefore used for all subsequent control vaccinations.

Randomisation and masking

At vaccination centres set up at RRH and in 20 of the participating schools, children were randomly assigned (2:1) to receive three doses of dengue vaccine or a control product at months 0, 6, and 12. Investigators used an interactive web-based response system (IWRS) to sequentially allocate a unique inclusion number to each enrolled child. Subsequent steps occurred in a separate room away from the masked-observer investigator. Using the IWRS, designated unmasked trial personnel (who were not involved in data collection or trial assessments) were informed of a product code assigned to the participant's inclusion number, and administered the

product labelled with that code. The randomisation list that assigned a product code to each inclusion number was generated under the sponsor's responsibility by block randomisation with block sizes of six and stratification by vaccination centre.

Procedures

The recombinant, live, attenuated, tetravalent dengue vaccine (CYD-TDV) has been described elsewhere.⁷ It was presented as a powder and saline solvent (0.4% NaCl, containing human serum albumin) for reconstitution immediately before use. Reconstituted vaccine contained $5 \pm 1 \log_{10}$ median cell culture infectious dose of each of the four CYD vaccine viruses per 0.5 mL dose. The control product was inactivated rabies vaccine (Verorab, Sanofi Pasteur, Lyon France) for the first injection of the first 50 children randomly assigned to the control group, and 0.9% NaCl saline placebo for all other injections. All products were supplied by the sponsor and were injected subcutaneously in the upper arm.

We actively followed up all children to detect acute febrile illness based on daily surveillance of school registers during school terms for absenteeism, followed by phone calls or home visits to absentees, and on phone calls twice per week, mobile phone text-messages, or home visits throughout school holidays. In case of febrile illness at any time (defined as illness with two temperature readings of 37.5°C or higher at least 4 h apart), parents were asked to take their child to RRH for diagnosis and treatment. The surveillance system also captured spontaneous consultations at RRH. Consecutive febrile episodes separated by a symptom-free interval of 14 days or longer were regarded as separate episodes. Paired serum samples were collected at presentation (ie, acute sample, collected no later than 7 days after fever onset) and 7–14 days later (convalescent sample) and sent to the sponsor's Global Clinical Immunology (GCI) laboratory (Swiftwater, PA, USA) and to the Centre for Vaccine Development (Mahidol University, Bangkok, Thailand). We screened acute samples for presence of flavivirus using an initial PCR assay. Positive samples were tested for wild-type dengue virus with a serotype-specific quantitative RT-PCR, derived from a published method.¹⁸ In parallel, all acute samples were tested for the presence of dengue NS1 antigen with a commercial ELISA kit (Platelia, Bio-Rad Laboratories, Marnes-La-Coquette, France).¹⁹ An episode of virologically confirmed dengue was defined as a positive result in either one of the serotype-specific PCRs, or by NS1-antigen ELISA. A case was defined as a first episode of virologically confirmed dengue. Active surveillance was maintained until each participant had been followed up for at least 13 months after the third vaccination.

Severe dengue was defined as an episode that either fulfilled the 1997 WHO criteria for dengue haemorrhagic fever (DHF),²⁰ or was classified as severe by the IDMC on the basis of the WHO criteria and an additional list of symptoms such as visceral manifestations (appendix).

We documented and assessed all serious adverse events (SAEs) as defined in International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use guidance until the sixth month after the last injection, and thereafter any fatal SAE or vaccine-related SAE. The IDMC regularly reviewed all SAEs. We also assessed vaccine reactogenicity in the first 1050 enrolled children using conventional methods as described elsewhere for previous trials with this vaccine.^{11,12}

We assessed dengue immune responses in the first 300 enrolled children at RRH in sera collected at enrolment, and before and 28 days after each injection. Sera were sent to GCI for measurement of neutralising

antibody titres against each of the four the CYD parental dengue viruses with the plaque-reduction neutralisation test (PRNT₅₀), as described.^{21,22} The assay's quantitation limit was 10 (1/dil). Samples lower than this value were assigned the titre 5 and regarded as seronegative.

Statistical analysis

WHO guidelines for the clinical evaluation of dengue vaccine in endemic areas recommend that the primary analysis of vaccine efficacy (VE) consider fully vaccinated volunteers.¹⁴ Therefore our primary objective was to establish VE against cases (ie, participants with an episode) of symptomatic, virologically confirmed dengue occurring more than 28 days after the third vaccination according to

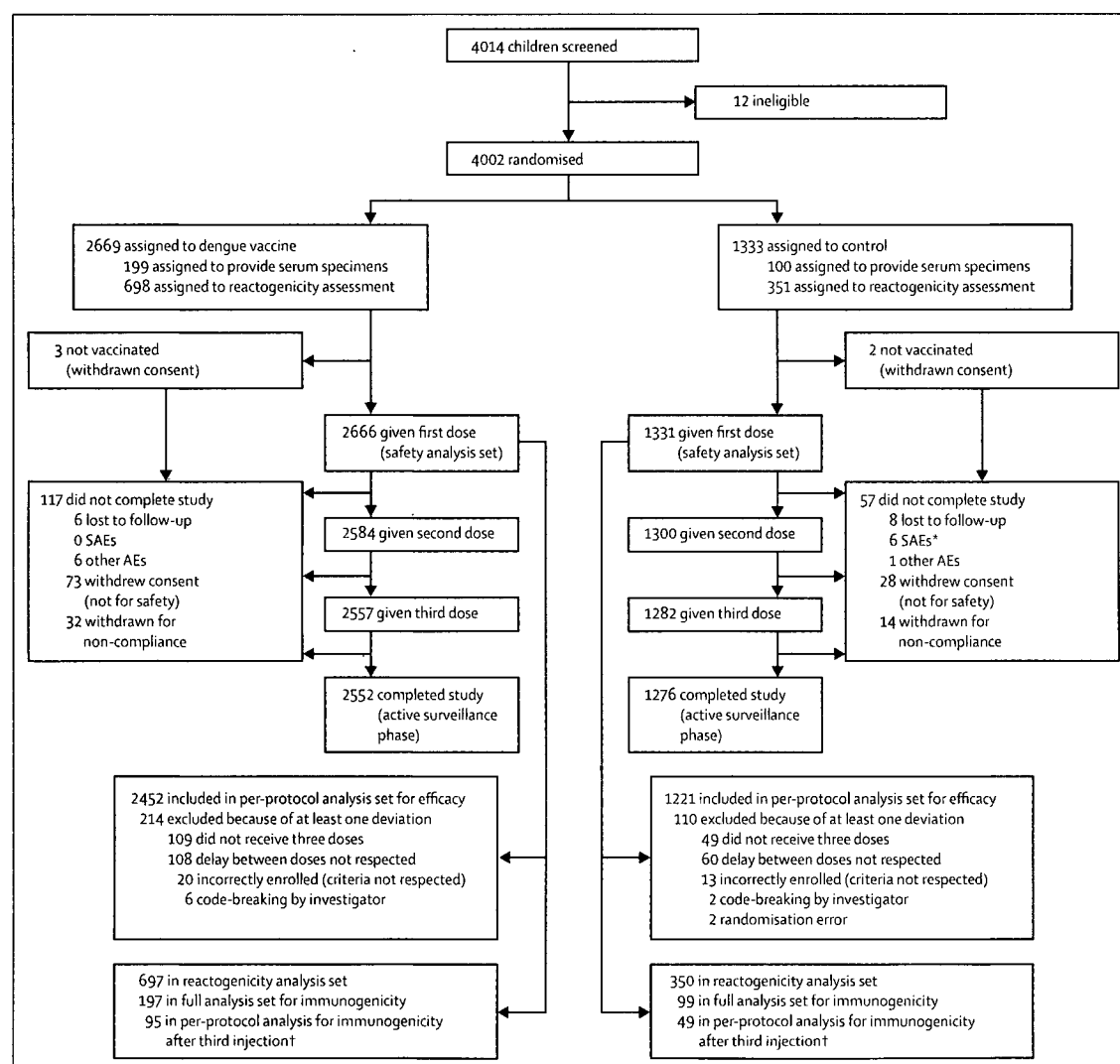


Figure: Trial profile

SAE=serious adverse event. AE=adverse event. *Six in the control group discontinued because of SAEs: four deaths, 1 acute idiopathic thrombocytopenic purpura, one acute febrile illness. †For the first cohort of 150 enrolled children, the ethics committee's approval to proceed with the trial was not received in time to perform the second injection within the time window allowed in the protocol (injections occurred at month 9), resulting in the exclusion of these children from the per-protocol analyses of efficacy and immunogenicity.

the equation: $VE = 100 \times (1 - ID_{CYD} / ID_{Control})$, where ID is the incidence density calculated as the number of cases divided by the total disease-free, person-time at risk in each group. With an assumed disease incidence of 1.3%, a true VE of 70%, a minimum follow-up of 1 year after the third vaccination, and a subject attrition rate of 7.5% per year, 4002 participants assigned with a 2:1 ratio to dengue vaccine or control were needed to show, with more than 80% power, and 95% confidence, that VE was not null. Analyses were based on the two-sided 95% CI of VE, calculated using the Exact method.²¹ A 95% CI that excludes zero shows a significant difference at a two-sided α level of 0.05. The primary analysis was done on the per-protocol population—ie, in those who satisfied the enrolment criteria, who had correctly received all three doses of the assigned vaccine at months 0, 6 (± 15 days), and 12 (± 30 days), and for whom group allocation had not been unmasked.

As secondary and observational objectives, we established efficacy against cases occurring more than 28 days after the second injection, irrespective of protocol compliance, and against all cases occurring after at least one injection (intention-to-treat analysis).

We also planned to assess efficacy against severe dengue, and against cases that were either virologically confirmed or serologically suspected. There were too few severe cases to make the first of these analyses meaningful. The analysis of virologically confirmed or serologically suspected cases will be reported separately. Other secondary and observational analyses that will be reported separately were the relation between neutralising antibody titres and the occurrence of dengue, and levels of viraemia.

Serotype-specific incidence was to be calculated as an observational objective. In an exploratory analysis,

defined in the statistical analysis plan, the serotype-specific relative risk was estimated, which is presented here as serotype-specific VE (ie, 1–relative risk). In an exploratory analysis, defined post-hoc, the serotype distribution in the two groups was tested for heterogeneity using Fisher's exact test and χ^2 test. Analyses for safety and immunogenicity endpoints were descriptive, using 95% CI, Student *t* test for normally distributed continuous variables, and χ^2 for the homogeneity between two categorical variables. $p < 0.05$ was regarded as significant.

The sample sizes of the immunogenicity and reactogenicity subsets were arbitrarily defined as no hypotheses were tested.

This trial is registered at ClinicalTrials.gov, NCT00842530.

Role of the funding source

The sponsor contributed to all study stages, including trial design, sample testing, statistical analysis, and the writing of this report. AS, DW, CS, KL, PC, VJ, WD, KP, TAW, AM, MS, AB, SV, NGT, and JL had complete access to the data. AS, DW, and JL had primary responsibility for the decision to submit for publication.

Results

Between Feb 5, 2009, and Feb 5, 2010, 4002 children were enrolled, of whom 3839 (96%) received three injections and 3673 (92%) were included in the per-protocol analysis set for efficacy (figure). Age and sex ratios were similar in both groups (table 1). At baseline, more than 90% of children sampled (immunogenicity subset) were seropositive against DENV or Japanese encephalitis virus (table 1). Active surveillance detected 2266 febrile episodes, 2263 (99.9%) of which were tested by both RT-PCR and NS1-antigen ELISA in acute blood samples. For 2256 (>99%) episodes, these samples were collected within 7 days of fever onset.

During the study, 134 children had virologically confirmed dengue, four of whom (all in the control group) had two episodes. Of these 134 cases, 77 occurred more than 28 days after the third injection in the per-protocol population and were included in the primary analysis: 45 occurred during 2522 person-years at risk in the vaccine group, whereas 32 cases occurred during 1251 person-years at risk in the control group. The corresponding vaccine efficacy was 30.2% (95% CI –13.4 to 56.6; table 2). Efficacy after at least one injection (intention-to-treat analysis) was 34.9% (95% CI 6.7–54.3; see appendix for Kaplan-Meier curves of virologically confirmed dengue, occurring after the first injection).

Non-primary analyses assessed serotype-specific relative risk and further post-hoc analyses revealed heterogeneity in the distribution of serotypes between groups, suggesting that efficacy differed between serotypes (table 2). Against DENV1, 3, and 4, efficacy after at least one injection was 61.2%, 81.9%, and 90.0%, respectively, and statistically superior to 0. Against DENV2, which accounted for 79 (59%) of 134 episodes, VE was

	Dengue vaccine (n=2669)	Control (n=1333)
Per-protocol analysis set for efficacy		
n	2452	1221
Age (years)	8.18 (2.04)	8.23 (2.06)
Boys	1187 (48%)	583 (48%)
Full analysis set for immunogenicity		
n	197	99
Age (years)	8.26 (1.74)	8.12 (1.74)
Boys	84 (43%)	46 (46%)
Body-mass index (kg/m ²)	16.4 (3.4)	16.8 (3.7)
Anti-DENV or anti-JEV prevalence*	179 (91%)	91 (92%)
Anti-JEV prevalence*	157 (80%)	77 (78%)
Anti-DENV prevalence (≥ 1 serotype)*	138 (70%)	68 (69%)

Data are n, mean (SD), or n (%). DENV=dengue virus. JEV=Japanese encephalitis virus. *Anti-DENV and anti-JEV seroprevalence defined as the percentage of participants with a plaque-reduction neutralisation test (PRNT₅₀) titre of 10 or higher.

Table 1: Baseline characteristics of participants

	Dengue vaccine		Control		Efficacy	
	Person-years at risk	Cases or episodes*	Person-years at risk	Cases or episodes*	% (95% CI)	Heterogeneity p value†
>28 days after three injections (per-protocol analysis)						
Cases	2522	45	1251	32	30.2% (-13.4 to 56.6)	0.0340
Serotype 1 episodes	2536	9	1251	10	55.6% (-21.6 to 84.0)	..
Serotype 2 episodes	2510	31	1250	17	9.2% (-75.0 to 51.3)	0.0309
Serotype 3 episodes	2541	1	1257	2	75.3% (-375.0 to 99.6)	..
Serotype 4 episodes	2542	0	1263	4	100.0% (24.8 to 100.0)	..
NS1-antigen positive only episodes	2542	4	1265	0	ND	..
>28 days after two injections						
Cases	3824	61	1905	47	35.3% (3.3 to 56.5)	0.0057
Serotype 1 episodes	3855	10	1921	16	68.8% (27.0 to 87.4)	..
Serotype 2 episodes	3824	44	1918	22	-0.3% (-75.8 to 41.1)	0.0009
Serotype 3 episodes	3860	2	1924	6	83.4% (7.1 to 98.4)	..
Serotype 4 episodes	3864	1	1934	4	87.5% (-26.5 to 99.7)	..
NS1-antigen positive only episodes	3863	4	1936	1	-100.5% (-9771.8 to 80.2)	..
After at least one injection (intention-to-treat analysis)						
Cases	5292	76	2630	58	34.9% (6.7 to 54.3)	0.0027
Serotype 1 episodes	5343	14	2666	18	61.2% (17.4 to 82.1)	..
Serotype 2 episodes	5312	52	2662	27	3.5% (-59.8 to 40.5)	0.0007
Serotype 3 episodes	5348	4	2667	11	81.9% (38.8 to 95.8)	..
Serotype 4 episodes	5353	1	2679	5	90.0% (10.6 to 99.8)	..
NS1-antigen positive only episodes	5351	5	2681	1	-150.5% (-11748.3 to 72.0)	..

ND=not determined. *A case was defined as a first episode of virologically confirmed dengue by either serotype-specific PCR, or NS1-antigen ELISA. Serotype-specific efficacy was calculated including all episodes of that serotype; four children with two virologically confirmed dengue episodes during the study were therefore included once in each of the two serotype-specific analyses concerned. †Fisher's exact test was used to test heterogeneity of serotype distribution between groups among the four serotypes and χ^2 was used to test the distribution between groups of serotype 2 versus the other three serotypes; NS1-antigen positive only cases (ie, RT-PCR negative cases) were excluded from heterogeneity testing.

Table 2: Serotype-specific and overall efficacy of CYD tetravalent dengue vaccine against virologically confirmed dengue disease

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	Dengue vaccine group			Control group					
	n	%	95% CI	First cohort*			Second cohort*		
				n	%	95% CI	n	%	95% CI
Safety analysis set									
n	2666	..	..	50	..	..	1281	..	..
SAEs at any time	315	11.8	10.6-13.1	8	16.0	7.2-29.1	168	13.1	11.3-15.1
Vaccine-related SAEs at any time	0	0.0	0.0-0.1	0	0.0	0.0-7.1	1	0.1	0.0-0.4
SAEs within 28 days after any injection	50	1.9	1.4-2.5	2	4.0	0.5-13.7	35	2.7	1.9-3.8
Vaccine-related SAEs within 28 days after any injection	0	0.0	0.0-0.1	0	0.0	0.0-7.1	1	0.1	0.0-0.4
Reactogenicity analysis set									
n	697	..	..	50	..	..	300	..	..
AE within 30 min of injection	0	0.0	0.0-3.6	0	0.0	0.0-7.1	0	0.0	0.0-1.2
Solicited injection site reaction within 7 days of injection	426	61.6	57.8-65.2	29	58.0	43.2-71.8	189	63.2	57.5-68.7
Solicited systemic reaction within 14 days of injection	538	77.7	74.5-80.8	38	76.0	61.8-85.9	223	74.3	69.0-79.2
Unsolicited AE within 28 days of injection	317	45.5	41.7-49.3	20	40.0	26.4-54.8	142	47.3	41.6-53.2
Vaccine-related unsolicited AE within 28 days of injection	10	1.4	0.7-2.6	0	0.0	0.0-7.1	1	0.3	0.0-1.8
AE leading to study discontinuation	0	0.0	0.0-0.5	0	0.0	0.0-7.1	0	0.0	0.0-1.2

Data are n, %, or 95% CI of participants with AEs at least once during the study. Safety analysis set=all vaccinated participants, analysed according to the vaccine received. AE=adverse event. SAE=serious adverse event. Injection site reaction=pain, erythema, or swelling. *The first cohort received one injection of rabies vaccine followed by two placebo injections; the second cohort received three placebo injections.

Table 3: Summary of all reported SAEs and of AEs reported in the reactogenicity subset after at least one injection

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low and not statistically superior to 0. In data from the control group, the incidence rate of virologically confirmed dengue was 1.73% in 2010 and 2.33% in 2011.

584 SAEs occurred during the study: 366 were reported for 315 (12%) of 2666 children in the vaccine group, and 218 were reported for 176 (13%) of 1331 children in the

	All episodes			Dengue virus serotype 2		
	Dengue vaccine group	Control group	p value*	Dengue vaccine group	Control group	p value*
Number of episodes	76	62	..	52	27	..
Duration of clinical syndrome (days)						
Mean	5.39 (2.34)	5.84 (2.68)	0.30	5.23 (2.39)	5.89 (2.21)	0.24
Median	5.0 (3.5-7.0)	5.5 (4.0-8.0)	..	5 (3-7)	6 (4-8)	..
Range	1-12	1-15	..	1-12	2-10	..
Duration of fever (days)						
Mean	4.13 (1.98)	4.40 (1.97)	0.42	4.08 (1.96)	4.22 (1.58)	0.74
Median	4.0 (2.5-5.0)	5 (3-6)	..	4.0 (2.5-5.0)	5 (3-5)	..
Range	1-10	1-9	..	1-10	1-7	..
Episodes leading to hospital admission	32 (42%)	30 (48.4)	0.46	20 (38.5)	15 (55.6)	0.14
Duration of hospital stay (days)						
Mean	4.91 (1.33)	5.17 (1.97)	0.55	5.05 (1.50)	4.80 (1.78)	..
Median	5 (4-6)	5 (4-6)	..	5 (4-6)	5 (3-6)	0.66
Range	3-8	2-11	..	3-8	2-9	..
Episodes with any haemorrhagic signs	28 (37%)	23 (37%)	0.98	16 (31%)	12 (44%)	0.22
Spontaneous bleeding	10 (13%)	10 (16%)	..	4 (8%)	5 (19%)	..
Bleeding with blood transfusion	0	0	..	0	0	..
Plasma leakage	4 (5%)	3 (5%)	..	2 (4%)	1 (4%)	..
Thrombocytopenia $\leq 50 \times 10^9/L$	6 (8%)	8 (13%)	..	4 (8%)	6 (22%)	..
Thrombocytopenia $\leq 100 \times 10^9/L$	16 (21%)	16 (26%)	..	10 (19%)	8 (30%)	..
Shock	0	2 (3%)	..	0	0	..
Organ impairment	1 (1%)	1 (2%)	..	1 (2%)	0	..

Data are n (%), mean (SD), median (IQR), or range. Episodes=all symptomatic, virologically confirmed dengue occurring during the study. *t test was used to compare means, χ^2 test was used to compare proportions between groups.

Table 4: Clinical characteristics of virologically confirmed dengue episodes

	Dengue vaccine (n=2669)			Control (n=1333)	
	12-year-old girl	6-year-old girl	6-year-old boy	12-year-old girl	10-year-old boy
Onset	182 days after dose 2	328 days after dose 3	344 days after dose 3	48 days after dose 3	170 days after dose 3
Presented with	Headache, anorexia, vomiting, abdominal pain, confusion	Rash, cough, anorexia, vomiting, abdominal pain	Headache, anorexia, abdominal pain	Headache, rash, abdominal pain	Headache, rash, vomiting, anorexia, abdominal pain
Additional clinical information	No clinical shock, CSF normal, CT brain normal, tonsillitis	No clinical shock, no other visceral manifestations	No clinical shock, no other visceral manifestations	Clinical shock (BP 80/50 mm Hg) after defervescence, hepatomegaly	No clinical shock, no other visceral manifestations
Duration of hospital stay (days)	7	6	3	11	10
Tourniquet test	Negative	Positive	Not done	Negative	Positive
Spontaneous bleeding	No	No	Haematemesis	Mucosal bleeding	Haematemesis
Minimum platelet count ($\times 10^9/L$)	48	6	25	18	51
Plasma leakage	No	>20% increase in haematocrit	>20% increase in haematocrit	Clinical pleural effusion, confirmed by chest radiograph, >20% increase in haematocrit	>20% increase in haematocrit
Dengue WT qRT-PCR					
Serotype	DENV2	DENV1	PCR negative	DENV1	DENV2
Virus titre ($\log_{10}$ GEq/mL)	9.89	5.46	NA	9.76	9.83
NS1 antigen ELISA	10.5	8.60	8.54	10.3	5.85
Diagnosis	Dengue fever with encephalopathy	DHF grade 1	DHF grade 2	DHF grade 3	DHF grade 2

BP=blood pressure. NA=not applicable. DHF=dengue haemorrhagic fever.

Table 5: Summary of dengue episodes classified as severe according to independent data monitoring committee or WHO 1997 classifications²⁰

control group (table 3). No vaccine-related SAEs were reported in the dengue group, but one occurred in the placebo control group. SAEs were medical disorders consistent with the age group and occurred at similar rates in each group when we considered the study period as a whole, as well as SAEs occurring within 28 days of an injection. In the reactogenicity analysis set, the proportion of each group with adverse events (AEs) was similar (table 3). Unsolicited AEs within 28 days of any vaccination were reported for 317 (45%) of 697 children in the vaccine group and 142 (47%) of 300 receiving placebo control. Vaccine-related unsolicited AEs within 28 days of any vaccination were reported for ten (1%) of 697 children in the vaccine group and one (<1%) of 300 receiving placebo control. Four children in the control group died during the study (drowning, traffic accident, T-cell lymphoma, head injury).

The clinical characteristics of dengue episodes were similar in the two groups when we considered all episodes, irrespective of serotype (table 4). These characteristics were also similar in the two groups when we considered serotype 2 episodes only (ie, the serotype against which the vaccine did not show efficacy). Five dengue episodes were classified as severe dengue according to IDMC or WHO 1997 definitions. Three occurred in the vaccine group, two in the control group (table 5). The incidence density of severe dengue after at least one injection was 0.058% (3 cases/5149 person-years) in the vaccine group, and 0.078% (2 cases/2578 person-years) in the control group. One episode of grade 3 DHF occurred in the control group; other episodes were grade 2 DHF or milder. All five children recovered without sequelae within 12 days.

In the immunogenicity subset, geometric mean titres (GMTs) increased after the first dengue vaccination compared with baseline (n=197) and were higher after the second and third injections compared with after injection 1 (table 6). 4 weeks after the third injection, GMTs were in the range 146 against DENV1 to 405 against DENV3, decreasing to values in the range 76.5–153 at 1 year after the third injection. In the control group (n=99), GMTs were in the range of 20–50 at each timepoint and were highest against DENV2 and DENV3.

Discussion

This phase 2b, proof-of-concept study was designed with the primary objective of establishing efficacy against virologically confirmed dengue of any serotype after three injections, under the assumption that the number of cases would not allow a meaningful estimation of serotype-specific efficacy. Our primary estimate of efficacy was lower than projected and was not significant. However, the number of observed cases was substantially higher than expected, allowing us to consider efficacy per serotype. Efficacy estimates against DENV1, 3, and 4 were in a range consistent with our assumed overall efficacy of 70% after three injections, and these estimates were significant after at least one vaccination, but not after the

Dengue vaccine group (n=197)				Control group (n=99)			
	m	GMT (95% CI)	Seropositive* (n, %)	m	GMT (95% CI)	Seropositive* (n, %)	
Baseline							
Serotype 1	197	42.8 (30.7–59.6)	108 (55%)	99	26.6 (17.6–40.2)	48 (48%)	
Serotype 2	197	56.8 (40.3–80.1)	115 (58%)	98	43.7 (27.8–68.7)	57 (58%)	
Serotype 3	197	31.5 (24.2–41.0)	119 (60%)	99	28.7 (19.3–42.6)	55 (56%)	
Serotype 4	197	28.1 (21.7–36.4)	111 (56%)	99	23.2 (15.6–34.6)	45 (45%)	
28 days after first injection							
Serotype 1	197	94.4 (66.4–134.3)	144 (73%)	98	27.7 (18.1–42.3)	47 (48%)	
Serotype 2	197	195 (143–266)	172 (87%)	99	42.9 (27.2–67.6)	56 (57%)	
Serotype 3	197	111.9 (85.8–145.9)	169 (86%)	99	27.0 (18.3–39.8)	52 (53%)	
Serotype 4	197	138 (106–178)	169 (86%)	99	24.2 (16.4–35.8)	46 (46%)	
28 days after second injection							
Serotype 1	94	120.7 (79.4–183.5)	84 (89%)	49	21.9 (12.4–38.6)	22 (45%)	
Serotype 2	94	326 (230–462)	93 (99%)	49	43.5 (23.3–81.1)	28 (57%)	
Serotype 3	94	195 (144–263)	93 (99%)	49	24.6 (14.4–42.1)	27 (55%)	
Serotype 4	94	159 (121–210)	91 (97%)	49	21.2 (12.3–36.4)	22 (45%)	
28 days after third injection							
Serotype 1	95	146.1 (98.5–216.7)	90 (95%)	49	23.9 (14.0–40.9)	27 (55%)	
Serotype 2	95	310 (224–431)	94 (99%)	49	52.2 (26.8–101.7)	29 (59%)	
Serotype 3	95	405 (307–534)	95 (100%)	49	48.9 (25.5–93.9)	29 (59%)	
Serotype 4	95	155 (123–196)	93 (98%)	49	19.4 (11.6–32.2)	21 (43%)	
1 year after third injection							
Serotype 1	95	76.5 (48.2–121.5)	73 (77%)	48	20.7 (12.0–35.8)	22 (46%)	
Serotype 2	95	122.5 (78.8–190.4)	81 (85%)	48	38.5 (20.6–72.1)	27 (56%)	
Serotype 3	95	94.8 (65.2–137.9)	85 (89%)	48	25.6 (14.5–45.3)	26 (54%)	
Serotype 4	95	153 (110–212)	89 (94%)	48	37.5 (20.1–69.2)	26 (54%)	

m=number of participants per protocol at that point in the study and for whom data are available for that endpoint. PRNT₅₀=plaque-reduction neutralisation test. GMT=geometric mean titre. *Titre 10 or higher.

Table 6: Geometric mean PRNT₅₀ antibody titre against vaccine parental dengue strains at baseline and after each injection (per-protocol immunogenicity analysis)

third possibly because of the lower number of cases. Conversely, efficacy was not shown against DENV2. This lack of efficacy against DENV2, and the fact that DENV2 was the prevalent serotype during the study, diminished the overall vaccine efficacy in this setting (panel).

More than 91% of enrollees completed the study per protocol, and the active surveillance system implemented for the study successfully collected acute blood samples for virological confirmation from more than 2200 febrile episodes within 7 days of fever onset. This high compliance probably reflects the importance of site preparation activities, and beyond that the importance of dengue for the study team and the population of Ratchaburi.^{15–17}

The vaccine's safety and reactogenicity profile was good and consistent with previous clinical trials using the recombinant CYD dengue vaccine technology.^{7,9–12} The absence of vaccine-related SAEs or any other safety signal after 2 years of active follow-up of more than 2600 vaccinated children is particularly noteworthy. Theoretical safety concerns associated with the potential increase in the rate or severity of dengue disease by an incomplete immune response against the four serotypes of dengue

Panel: Research in context**Systematic review**

A search of PubMed with the search terms “dengue”, “vaccine”, and “efficacy” did not show any previous clinical study of efficacy of a dengue vaccine. No date or language restrictions were applied.

Interpretation

Although the primary estimate of efficacy in our study was lower than projected and was not significant, the study's secondary findings have major implications for the continuing development of dengue vaccines. This candidate vaccine was immunogenic for all four serotypes and protected against three of the four serotypes (1, 3, and 4) at levels consistent with the initial hypothesis. Against the fourth serotype, however, no protection was seen in this setting despite satisfactory immunogenicity, and this factor affected the primary outcome. These findings challenge the vaccine development hypothesis that by inducing balanced levels of neutralising antibodies as measured by plaque-reduction neutralisation test, tetravalent vaccination would provide similar levels of protection against the four serotypes.²² Crucially, the antibody-dependent enhancement hypothesis that has hampered vaccine development to date was not borne out by this study.⁵⁷ There was no sign of enhanced disease in breakthrough cases after vaccination with this tetravalent YF17D-based recombinant dengue vaccine during the 2 years of study follow-up.

have hampered vaccine development.⁵⁷ In this context, the absence of any sign of disease enhancement after 2 years of follow-up after the first injection, in the presence of non-protective immune responses against the circulating DENV2 viruses, is an important and reassuring finding. Indeed, the duration of fever and the rate of admission to hospital were no higher in cases in the vaccine group compared with the control group, and there was no excess in severe cases or in the classic clinical signs of dengue such as bleeding, plasma leakage, or thrombocytopenia.

The observed lack of efficacy against DENV2 despite satisfactory immunogenicity is surprising and will need further investigation. It leads us to question the robustness of the assumption that to protect against dengue, vaccination must induce balanced immunogenicity against all four serotypes as assessed by PRNT₅₀, regarded as the most relevant assay to measure antibodies that protect epithelial cells from dengue virus infection.²¹ In vivo, however, Fc receptor-bearing cells are crucially important in supporting dengue infection. Testing the biological relevance of measuring DENV neutralisation in a system modelled on in-vivo target cells therefore deserves further study.

An antigenic mismatch between the CYD2 vaccine virus and the DENV2 virus or viruses that caused disease in our cohort is possible. The Asian 1 genotype of DENV2 circulating in southeast Asia consists of several lineages, one of which has mutations in domain 2 of the E protein (at E83, E226, and E228) that could have implications for viral fitness.²⁴ The aminoacid residues at these positions in the donor wild-type virus for the CYD2 vaccine (PUO-218) correspond to those of the Asian/American genotype.^{24,25} Antibody responses after vaccination against DENV2 were higher than those against DENV1

and DENV3, and were similar to responses noted in earlier studies in the region.^{10,12} A previous study in non-human primates showed that sera raised against CYD-TDV neutralise in an SN₅₀ assay a broad range of isolates across all the DENV serotypes, genotypes, geographical origins, and isolation years,²⁶ and this could still be the case for the DENV2 viruses circulating in our study. Whether immune responses to DENV non-structural proteins (which are not encoded in the CYD vaccine viruses) contribute to the overall protective response to DENV2 also needs further clarification. Also, the DENV2 antibody titres might not have been high enough to protect against this serotype or the particular lineage of viruses circulating in Ratchaburi during the study period. The monocentre design of this phase 2b study in a single area of Thailand and the predominance of this one serotype therefore constitutes its principal limitation.

The worldwide dynamics of dengue viruses are complex. The prevailing serotype and the distribution of viruses differ from region to region and evolve with time as a result of serotype and lineage replacement events.^{4,15,24,27–29} Intrinsic and extrinsic host factors can also play a part. Under different epidemiological circumstances, the vaccine as currently formulated might provide significant benefit. Further evaluation of vaccine efficacy in different epidemiological settings, against a wide range of dengue viruses of differing virulence and viral fitness, circulating in both epidemic and endemic contexts, is important. Ongoing phase 3 efficacy studies in more than 30000 volunteers in ten countries in Latin America and Asia will provide pivotal data for this candidate vaccine's efficacy (NCT01374516 and NCT01373281).

In conclusion, although the assumed high efficacy against all four serotypes of dengue virus was not shown, our study constitutes the first ever demonstration that a safe dengue vaccine is possible. In the context of WHO goals to reduce dengue mortality by at least 50% and the morbidity rate by at least 25% by 2020,³⁰ this study represents a major milestone. These findings have important implications for dengue vaccine development and support the continued evaluation of this dengue vaccine based on the recombinant CYD dengue vaccine technology.

Contributors

All authors participated in the design or implementation of the study, or the analysis and interpretation of findings. AS, TAW, AB, and JL were involved in all phases of the study. TAW, AB, NGT, MS, and JL contributed to the study design. AS was the principal investigator. AS, CS, KL, PC, SS, VJ, WD, and KP contributed to the implementation of the study and supervision at the site. DW was the sponsor's clinical study leader and responsible medical officer throughout the study. TAW was responsible for the safety follow-up during the study. AM designed and did the statistical analysis. AS, CS, DW, SV, NGT, and JL interpreted the efficacy results. AS, DW, CS, KL, PC, VJ, WD, KP, TAW, AM, MS, AB, SV, NGT, and JL had access to the study data, contributed to data interpretation, and reviewed and revised drafts of this report; all approved the final version. The report was written by Grenville Marsh (Sanofi Pasteur).

Conflicts of interest

DW, TAW, AM, MS, AB, SV, NGT, and JL are employees of Sanofi Pasteur and own shares or stock options in the company. AS, CS, KL, PC, SS, VJ, WD, and KP declare that they have no conflicts of interest.

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The Synergistic Effect of Combined Immunization with a DNA Vaccine and Chimeric Yellow Fever/Dengue Virus Leads to Strong Protection against Dengue

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Abstract

The dengue envelope glycoprotein (E) is the major component of virion surface and its ectodomain is composed of domains I, II and III. This protein is the main target for the development of a dengue vaccine with induction of neutralizing antibodies. In the present work, we tested two different vaccination strategies, with combined immunizations in a prime/booster regimen or simultaneous inoculation with a DNA vaccine (pE1D2) and a chimeric yellow fever/dengue 2 virus (YF17D-D2). The pE1D2 DNA vaccine encodes the ectodomain of the envelope DENV2 protein fused to t-PA signal peptide, while the YF17D-D2 was constructed by replacing the prM and E genes from the 17D yellow fever vaccine virus by those from DENV2. Balb/c mice were inoculated with these two vaccines by different prime/booster or simultaneous immunization protocols and most of them induced a synergistic effect on the elicited immune response, mainly in neutralizing antibody production. Furthermore, combined immunization remarkably increased protection against a lethal dose of DENV2, when compared to each vaccine administered alone. Results also revealed that immunization with the DNA vaccine, regardless of the combination with the chimeric virus, induced a robust cell immune response, with production of IFN- γ by CD8⁺ T lymphocytes.

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Introduction

Dengue is an important viral disease, consisting of a global public health problem in tropical and subtropical regions of the world including the Americas, where the main vector is the mosquito *Aedes aegypti*. It is estimated that over 2.5 billion people live in areas of dengue risk in which 50 to 100 million of infection occur annually and about 250 to 500 thousand patients develop the most severe symptoms of the disease, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), with more than 20,000 deaths [1], [2]. Despite several efforts, there is still neither an effective antiviral therapy nor a preventive vaccine against dengue commercially available [3].

There are four antigenically distinct dengue viruses (DENV1-4), which belong to the family Flaviviridae, genus Flavivirus [4]. The DENV genome is a positive single-stranded RNA, encoding a polyprotein which is processed 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 [5]. The E glycoprotein is the major component of virion surface and it is associated with several biological activities. It acts as a binding protein, interacting with receptors present on host cell surface and leading to endocytosis of

the virus particle. It also mediates fusion of envelope and host cell membranes, which culminates with the nucleocapsid disassembly and release of virus genome for polyprotein synthesis [5], [6]. The virus particle contains 90 homodimers of the E protein and its ectodomain is composed of the domain I, II and III [6].

This protein is the main target for the induction of neutralizing antibodies and therefore most vaccines being developed against DENV are based on the stimulation of immune responses towards the E glycoprotein [7], [8]. One of the main problems for developing a vaccine against dengue is the requirement for a protective immune response against all four serotypes, without the risk of inducing severe disease [9], [10]. This rationale is particularly attributed to epidemiological observations that most severe dengue cases occur in individuals experiencing a secondary DENV infection [11] and an inefficient immunization against one serotype may increase the risk of DHF/DSS development if the vaccinated host acquires an infection with such serotype.

Several vaccine approaches have been proposed to combat dengue disease, including the use of inactivated or live attenuated viruses, chimeric live viruses, subunit antigens and DNA immunizations [9], [12], [13]. Immunization with tetravalent formulations containing sets of live attenuated viruses lead to

unbalanced immune responses against the four serotypes, due to the interference of one virus in the replication of the others and/or the immunodominance of the response against some antigens upon others [9]. In fact, clinical studies with chimeric attenuated viruses by Sanofi-Pasteur, which is the most advanced tetravalent live attenuated dengue vaccine, revealed the necessity of three doses with several month intervals to reach seroconversion against all the four serotypes [14], [15]. On the other hand, the DNA vaccine is a gene-based strategy which seems not to cause interference upon combined immunization [16]. Actually, DNA vaccines have been shown to be significantly more effective when combined in other immunization approaches, such as in prime/boost regimen, leading to a synergistic effect of the immune response that can reduce the number of doses required for protection [17–19].

Therefore, in the present work we evaluated the combination of these two vaccine strategies for eliciting a robust immune response and protection against dengue. Balb/c mice were immunized with a DNA vaccine (pE1D2), which encodes the ectodomain of the envelope DENV2 protein, previously constructed by our group [20], combined to a chimeric yellow fever/dengue virus (YF17D-D2) [21]. Mice were inoculated with these vaccines by prime-boost or simultaneous immunization protocols and we observed a synergistic effect on the elicited immune response, increasing neutralizing antibody titers and conferring protection against a lethal challenge with DENV2. Results also revealed that immunization with the DNA vaccine, regardless of the combination with the chimeric virus, induced a robust cell immune response with the production of INF- γ by CD8+ T lymphocytes.

Materials and Methods

Viruses and Cell Lines

The DENV2, strain New Guinea C (NGC) (GenBank M29095), was used for construction of the pE1D2 recombinant plasmid. This strain was neuroadapted by passaging in newborn mouse brains and used in challenge assays. The DENV2 44/2 [21] was used for plaque reduction neutralization test. Both viruses were propagated in Vero cell monolayers (ATCC), cultivated in Medium 199 with Earle salts (E199, Sigma, USA), buffered with sodium bicarbonate, supplemented with 5% fetal bovine serum (FBS, Invitrogen, USA) and maintained at 37°C in 5% CO₂.

Vaccines

The DNA vaccine pE1D2. The DNA vaccine pE1D2 was previously described [20]. Briefly, this plasmid encodes the NGC DENV2 ectodomain (domains I, II and III) of E protein fused to a signal sequence derived from the human tissue plasminogen activator (t-PA). The pcTPA plasmid [22], without the E gene, was used as a control. Plasmids were isolated from transformed *Escherichia coli*, DH5- α strain, purified by Qiagen Endofree Plasmid Giga Kit (Qiagen, Germany) following manufacturer's instruction, suspended in endotoxin-free sterile water and stored at -20°C until use.

Chimeric virus YF17D-D2. The YF17D-D2 (P44/S) vaccine is a chimeric yellow fever (YF)/dengue 2 virus, constructed by replacing prM and E genes of the live attenuated virus vaccine YF17D by these genes from DENV2, as described previously [21]. The chimeric virus stock was amplified and tittered on Vero cells and stored at -70°C until use.

Mice Immunization

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 (approval ID: L-067/08). Groups of Balb/c mice (n = 10), male SPF with 4 to 6 weeks old, were inoculated by three different immunization protocols and challenged with DENV2, as summarized on table 1. The single type protocol was performed by immunization with the DNA vaccine or chimeric virus alone. Briefly, each animal was inoculated with the pE1D2 vaccine or the control plasmid (pcTPA) (100 μ g DNA/dose/animal) intramuscularly (i.m.), as described [20], or subcutaneously (s.c.) with the chimeric YF17D-D2 virus (10⁵ PFU/dose/animal). In both cases animals were immunized with one or two doses, given 2 weeks apart. In the prime-boost protocol (DNA/chimera), animals were inoculated, as described above, with one or two doses of plasmid DNA (prime) followed by a booster dose of YF17D-D2, given 10 days after the last DNA dose. Another group was immunized by an inverse prime-boost scheme (chimera/DNA), following the same doses and inoculation routes. Finally, in the simultaneous protocol, mice were inoculated with one or two doses of a mixture with the plasmid DNA and chimeric virus by the intramuscular route (Mix: DNA+chimera), or with two doses of each vaccine by different routes (DR: i.m. and s.c. for plasmid DNA and chimeric virus, respectively), given two weeks apart. Non immunized mice were also used as negative control. Animals were bled by retro-orbital puncture before inoculation (preimmune sera) and one day before challenge. Serum samples were collected and stored at -70°C until use.

Plaque Reduction Neutralization Test (PRNT50)

Neutralizing antibodies against DENV2 were measured using plaque reduction neutralization tests (PRNT) performed in Vero cells, with 96-well plates, as previously described [20]. Briefly, serum samples were serially diluted (from 1:5 to 1:640) in 50 μ L of E199 medium followed by the addition of approximately 30 PFU of DENV2, and incubated at 37°C for 1 h. Vero cell suspensions were added and plates were incubated at 37°C for 3 h. Cells were overlaid with 100 μ L of E199 medium with 3% carboxymethyl-cellulose and plates were incubated for 7 days at 37°C in 5% CO₂. Cells were then fixed with 10% formalin, stained with crystal violet and plaques were manually counted. Neutralizing antibody titers were expressed by 50% of plaque reduction (PRNT₅₀%). This assay is well established in the laboratory, with reproducible results, and it is used for testing mouse individual serum samples, whose volumes are small.

Interferon Gamma ELISPOT Assay

Spleen cells from groups of immunized mice (n = 5), isolated 10 days after the last immunization, were used in IFN- γ ELISPOT test. The assay was performed with a 9-mer peptide (SPCKIPFEI), corresponding to 331–339 amino acid residues of the DENV2 E protein, which is an immunodominant CD8+ T cell epitope in Balb/c mice specific for the serotype 2 [23]. Animals were immunized with the DNA vaccine and/or chimeric virus, as summarized in table 1. The IFN- γ ELISPOT mouse set (BD Biosciences) was used as previously described [24], in accordance with the manufacturer's instruction. Briefly, 96-well plates were coated overnight at 4°C with 5 μ g/mL IFN- γ capture monoclonal antibody (100 μ L in PBS/well), followed by washing and blocking with supplemented RPMI-1640 medium (Sigma), for 2 hours at room temperature. Splenocytes (2 $\times$ 10⁵ cells/well) were added in 100 μ L RPMI-1640 with the subsequent addition of 100 μ L of the E peptide (final concentration of 10 μ g/mL). Non-stimulated and concanavalin A (Con A, 5 μ g/mL) stimulated cells were used as negative and

Table 1. The different immunization protocols using the pE1D2 DNA vaccine and/or YF17D-D2 chimeric virus.

Groups	Vaccines			Challenge ^d	
	0	15	25	30	35
Plasmid 1d (i.m.) ^a	pE1D2	–	–	DENV2	–
Plasmid 2d (i.m.) ^a	pE1D2 or pcTPA	pE1D2 or pcTPA	–	DENV2	–
Chimera 1d (s.c.) ^a	–	–	YF17D-D2	–	DENV2
Chimera (s.c.) ^a	YF17D-D2	YF17D-D2	–	DENV2	–
Plasmid 1d (i.m.)+Chimera (s.c.) ^b	pE1D2	YF17D-D2	–	DENV2	–
Plasmid 2d (i.m.)+Chimera 1d (s.c.) ^b	pE1D2 or pcTPA	pE1D2 or pcTPA	YF17D-D2	–	DENV2
Chimera 1d (s.c.)+Plasmid 2d (i.m.) ^b	YF17D-D2	pE1D2	pE1D2	–	DENV2
Mix 1d (i.m.): Plasmid+Chimera ^c	pE1D2 or pcTPA+YF17D-D2	–	–	DENV2	–
Mix 2d (i.m.): Plasmid+Chimera ^c	pE1D2 or pcTPA+YF17D-D2	pE1D2 or pcTPA +YF17D-D2	–	DENV2	–
DR: Plasmid (i.m.)+Chimera(s.c.) ^c	pE1D2 or pcTPA+YF17D-D2	pE1D2 or pcTPA+YF17D-D2	–	DENV2	–

Plasmids: pE1D2 (DNA vaccine) or pcTPA (control).

Chimera: YF17D-D2 (Chimeric virus constructed by replacing the prM and E genes from attenuated yellow fever 17D virus by those from DENV2).

^aSingle type immunization;

^bCombined immunization with prime-booster regimen;

^cCombined immunization with simultaneous inoculations;

^dChallenge with DENV2 by the intracerebral route.

i.m.: intramuscular route; s.c.: subcutaneous route; Mix: plasmid DNA (pE1D2 or pcTPA)+chimeric virus (YF17D-D2); DR: different routes of inoculations for plasmids (i.m.) and chimeric virus (s.c.).

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positive controls, respectively. Splenocytes were cultured for 20 h at 37°C in 5% CO₂. Plates were washed once with water and then with 0.05% Tween 20-PBS (PBST), followed by incubation for 2 h at room temperature with 2 µg/mL of biotinylated IFN-γ detection antibody, diluted in 100 µL PBS with 10% FBS. Plates were washed again with PBST and incubated for 1 h at room temperature with streptavidinhorseradish peroxidase diluted 1:100. Spots were revealed with AEC substrate reagent set (BD Bioscience) at room temperature and counted with an Immunospot reader (Cellular Technology Ltd, Cleveland, OH) using the Immunospot Software Version 3. Results were expressed as the average from triplicate wells of spot-forming cells (SFC) per 2×10⁵ cells, after subtraction of background values detected in splenocytes incubated only with medium.

Lethal challenge with DENV2. Mice were challenged with a mouse brain adapted NGC DENV2, ten or fifteen days after the last immunization. Animals were anesthetized with a mixture of ketamine-xylazine [25] and intracerebrally (i.c.) inoculated with 30 µL of 4.32 log₁₀ PFU of DENV2, which corresponds to approximately 4 LD₅₀, diluted in E199 medium supplemented with 5% FBS. Animals were monitored for 21 days for mortality and morbidity. The evaluation of different morbidity degrees was performed using an arbitrary scale ranging from 0 to 3 in each animal group (0 = none, 1 = mild paralysis in one hind leg or alteration of the spinal column with a small hump, 2 = severe paralysis in one hind leg and alteration of the spinal column with a small hump or severe paralysis in both hind legs, 3 = two severe hind leg paralysis and deformed spinal column or death).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (La Jolla, USA), version 5.02. For the analysis of survival and morbidity rates, statistical significances were evaluated by chi-square test, while differences in the degree of morbidity, PRNT₅₀ titers and ELISPOT assay were analyzed by Mann-Whitney test. Values were considered significant at P<0.05.

Results

Induction of Neutralizing Antibodies against DENV2

The humoral immune response induced by the DNA vaccine (pE1D2) and/or the chimeric YF/DENV virus (YF17D-D2) was evaluated in Balb/c mice by the presence of neutralizing antibodies against DENV2. Serum samples from mice inoculated with the different immunization protocols (Table 1), as well as control animals, were collected 24 h before virus challenge and titrated for neutralizing antibodies (PRNT_{50%}), performed with a DENV2 isolate different from that used for vaccine constructions. Animals inoculated with the single type immunization protocols, only with the pE1D2 DNA vaccine or the YF17D-D2 chimeric virus, showed detectable neutralizing antibodies. Immunization with two doses of the DNA vaccine elicits a slight increase of neutralizing antibody levels when compared to inoculation with one dose (PRNT median of 67 and 40, respectively), while two doses of the chimeric virus induced significant higher PRNT comparing to what it was observed with one dose of this vaccine (median of 349 and 22, respectively) (Fig. 1). However, combined immunizations with the two vaccine strategies revealed an increase in the antibody response. Mice immunized with two doses of the pE1D2 DNA vaccine and boosted with one dose of YF17D-D2 virus presented high antibody titers (median = 560), in which 50% of these animals reached the maximal neutralization titer detected in our assay (≥640) (Fig. 1A). Furthermore, all mice inoculated with the reversed prime-booster protocol, with one dose of YF17D-D2 and two doses of pE1D2, presented the highest neutralizing antibody titer (≥640). On the other hand, animals inoculated with the prime-booster system using the control pcTPA plasmid [pcTPA (2d)+YF17D-D2 (1d)] did not reveal any increase in the humoral immune response (median = 20). In this group, the observed neutralizing antibody titers were, in fact, due to the immunization with the chimeric virus, as can be seen when we compare PRNT in mice receiving only one dose of the YF17D-D2 (median = 22) (Fig. 1A). The immunization scheme with one dose of each vaccine, pE1D2 (prime) and YF17D-D2 (booster), induced

significantly lower neutralizing antibody titers (median = 165) when compared to groups that received the former prime-boost protocol, with two doses of pE1D2 and one dose of YF17D-D2 (Fig. 1A). In addition, the simultaneous immunization protocols (Mix), with both vaccines combined in a single formulation, also elicited high levels of neutralizing antibodies, either with one or two doses. In fact, 40% and 90% of animals inoculated with one or two doses of the Mix, respectively, showed the highest antibody titers detected in our assay (≥ 640) (Fig. 1B). Thus, the scheme of two doses of mixed pE1D2 DNA vaccine and YF17D-D2 virus revealed to be as effective in generating neutralizing antibody levels as one dose of live YF17D-D2 virus followed by two booster doses of the DNA vaccine. In addition, simultaneous immunization with the two vaccines inoculated by different routes (DR) also elicited high neutralizing antibody levels, in which 60% of animals presented maximum PRNT (titers ≥ 640) (Fig. 1B). Pre-immune serum samples and sera collected from pcTPA-inoculated mice [pcTPA (2d)] did not present detectable neutralizing antibody titers against DENV2 (data not shown).

Production of IFN- γ Elicited by the Different Immunization Protocols

The IFN- γ ELISPOT assay was performed in order to investigate CD8 $^{+}$ T cell responses induced by the DNA vaccine and/or chimeric virus, with different immunization protocols (Table 1), using the DENV2 specific E_{331–339} immunodominant epitope, previously described in Balb/c mice [23]. Splenocytes from animals immunized with two doses of the live YF17D-D2 chimeric virus presented significantly higher SFC values when compared to cells collected from mice inoculated with only one dose of such vaccine (Fig. 2). However, mice administered with the pE1D2 DNA vaccine alone, regardless the number of dose (one or two doses), presented a remarkable increase of this immune response, with approximately seven fold more IFN- γ positive SFC numbers when compared to results observed with YF17D-D2-inoculated animal groups (Fig. 2). On the other hand, combined immunizations with these two vaccines, either with the prime-boost scheme or with simultaneous inoculation in a single formulation (Mix) or with different routes (DR), also elicited high numbers of SFC secreting IFN- γ (Fig. 2). Positive control, using Con A as stimulating antigen, confirmed the cell viability from all mouse groups (data not shown).

Protection against DENV2 Challenge

The protective efficacy of the different immunization protocols were evaluated in vaccinated animals challenged with a lethal dose of a mouse brain-adapted DENV2. Mice were monitored the following 21 days after challenge for the development of morbidity and mortality. The different morbidity degrees were evaluated using an arbitrary scale, regarding mainly hind leg paralysis, alterations in spinal column and death.

Results indicated that all animals immunized with a prime-boost protocol, with one or two doses of the pE1D2 (prime) followed by a booster dose with YF17D-D2 or in the reverse scheme (one dose of YF17D-D2 and two doses of pE1D2), survived virus challenge (Fig. 3A). Similar results were also observed in mice immunized with two doses of pE1D2 vaccine and all these groups showed statistically significant differences of survival rates ($p < 0.0001$) when compared to control groups (non-immunized and pcTPA-inoculated animals).

However, only animals vaccinated with the prime-boost protocol, with either two doses of pE1D2 and one dose of YF17D-D2 or with the reverse scheme, revealed a total absence of morbidity after virus challenge (Fig. 3B). Additionally, only one

mouse (1/10) in the group immunized with one dose of each vaccine in the prime-boost regimen showed clinical signs of infection (Fig. 3B). On the other hand, although 70% and 60% of animals submitted to a single type immunization, with one dose of the pE1D2 or YF17D-D2 vaccines, respectively, survived the DENV2 challenge (Fig. 3A), 50% and 80% of these mice presented morbidity, respectively (Fig. 3B). Furthermore, although most mice immunized with the prime-boost protocol using the pcTPA control plasmid [pcTPA (2d)+YF17D-D2 (1d)] also survived the virus challenge (80%) (Fig. 3B), the majority of them showed remarkable clinical signs of infection (Fig. 3B).

Protocols of combined immunizations using simultaneous inoculations also induced 100% survival, regardless of the number of doses (Fig. 4A). Additionally, animals immunized with two doses of these combined vaccines (Mix 2d), showed complete protection against DENV2, without any morbidity (Fig. 4B). On the other hand, 30% of mice immunized with two doses of the YF17D-D2 presented clinical signs of infection (Fig. 4B). The protective efficacy was also evaluated in animals inoculated simultaneously with the two vaccines administered by different routes (DR) and results revealed the same level of protection (100% survival, without any clinical signs of infection) (Fig. 4).

Discussion

In the present report, we investigated the immune response and protection induced with a combination of two different vaccine strategies against dengue in mice, using a DNA plasmid (pE1D2) and a yellow fever/dengue chimeric virus (YF17D-D2). Both vaccines contain sequences from the DENV2 envelope gene. The pE1D2 DNA vaccine encodes 80% of the DENV2 envelope protein sequence, corresponding to its ectodomain (domains I, II and III) without the C-terminal stem-anchor region, fused to the t-PA signal peptide [20]. Expression of the recombinant E protein was previously demonstrated *in vitro*, in BHK cells transfected with the pE1D2 plasmid, and results revealed that the t-PA signal sequence was able to mediate secretion of the ectodomain with expected molecular weight [20], which is an important characteristic for efficient humoral immune response activation by DNA vaccines [26–28]. The YF17D-D2, in its turn, is a live attenuated chimeric virus derived from the yellow fever 17D vaccine virus in which the pre-M and E genes were replaced by the corresponding sequences from DENV-2 [21], [29].

The envelope glycoprotein is present on the surface of virus particle and it contains several epitopes that elicit neutralizing antibodies against dengue [7], [30–32]. Such antibodies have an important role in dengue immunity, by blocking the binding of virions to cell receptors which culminates in the failure of virus infection and replication, and they have generally been used as a marker for vaccine effectiveness [16]. Most of dengue neutralizing antibodies is directed to the E protein and some studies suggested that the domain III is the major target for the induction of such antibodies [7], [33], since it is the binding domain for the attachment of DENV to cell receptors [6], [31]. However, we previously observed that a DNA vaccine based only on the domain III elicited a weak neutralizing antibody response in mice when compared to full-length ectodomain-based vaccine [20]. Besides, other reports have also suggested that domains I and II contain epitopes which seem to be important for the generation of neutralizing antibody against DENV [30], [34].

We observed that immunization with one or two doses of the pE1D2 DNA vaccine induces detectable levels of neutralizing antibodies against DENV2, ranging from 1:8 to 1:240. In a previous work with this DNA vaccine we observed a significant

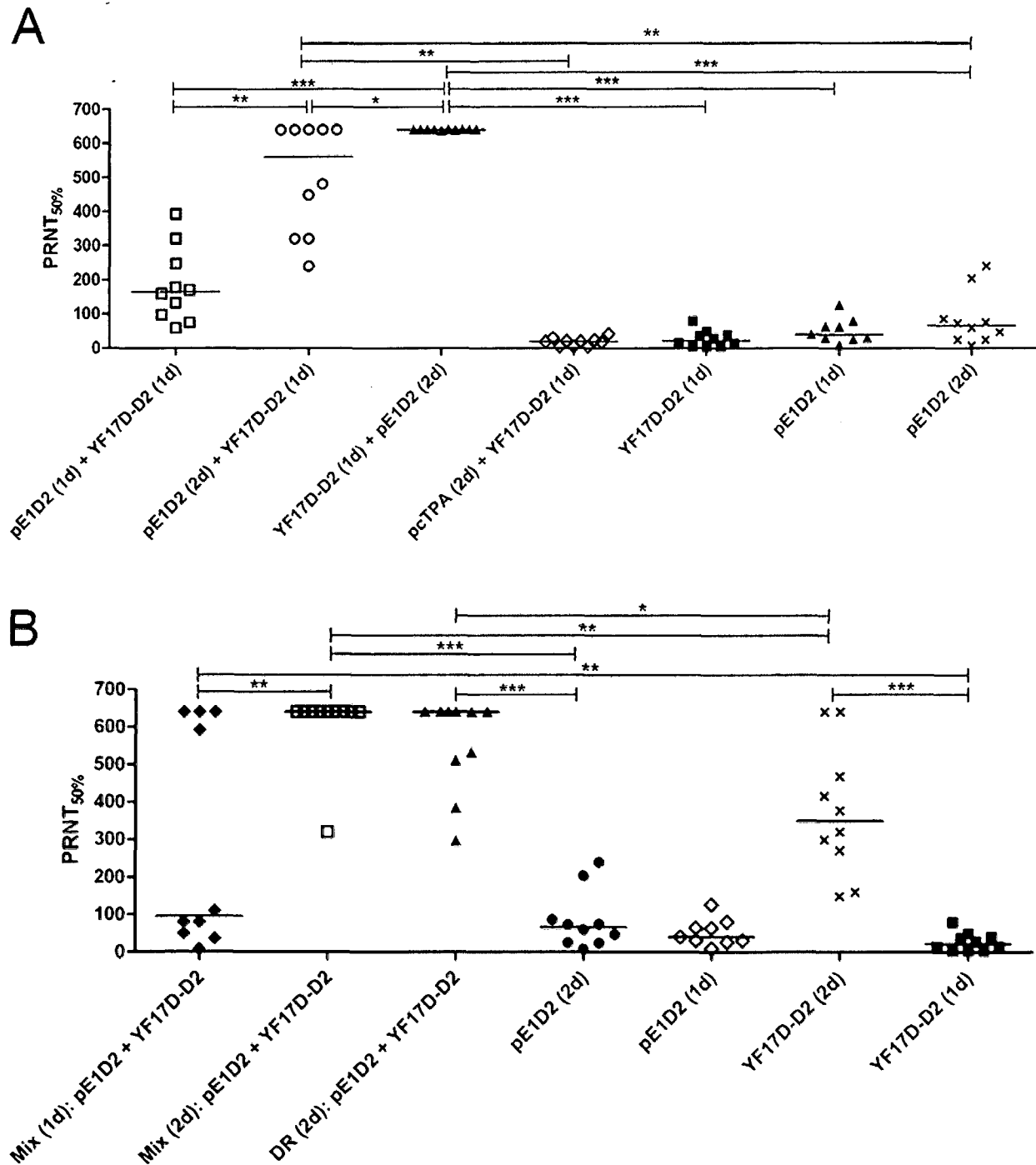


Figure 1. Neutralizing antibody responses against DENV2. Panels A and B refer to samples tested from animals immunized with prime-boost protocols or with simultaneous inoculations, respectively, and control groups. Individual serum samples were collected 24 h before virus challenge. Sera were serially diluted from 1:5 to 1:640 and the PRNT_{50%} was performed in 96-well plates. Asterisks indicate differences that are statistically significant using the Mann-Whitney test (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$). One dose = 1d; two doses = 2d. doi:10.1371/journal.pone.0058357.g001

increase of neutralizing antibody levels after challenge with DENV2 by the intracerebral route, indicating the activation of immunological memory with rapid and strong secondary humoral response, which may also contribute for protection [20]. Mice immunized with the YF17D-D2 chimeric virus also presented neutralizing antibodies, mainly those animals inoculated

with two virus doses, (PRNT median = 349). However, such humoral immune response increased remarkably when animals were inoculated with the combination of the two vaccine strategies, most of them reaching the maximal antibody level detected in our PRNT assay (titers ≥ 640). The synergistic effect on the immune response was observed even when experiments

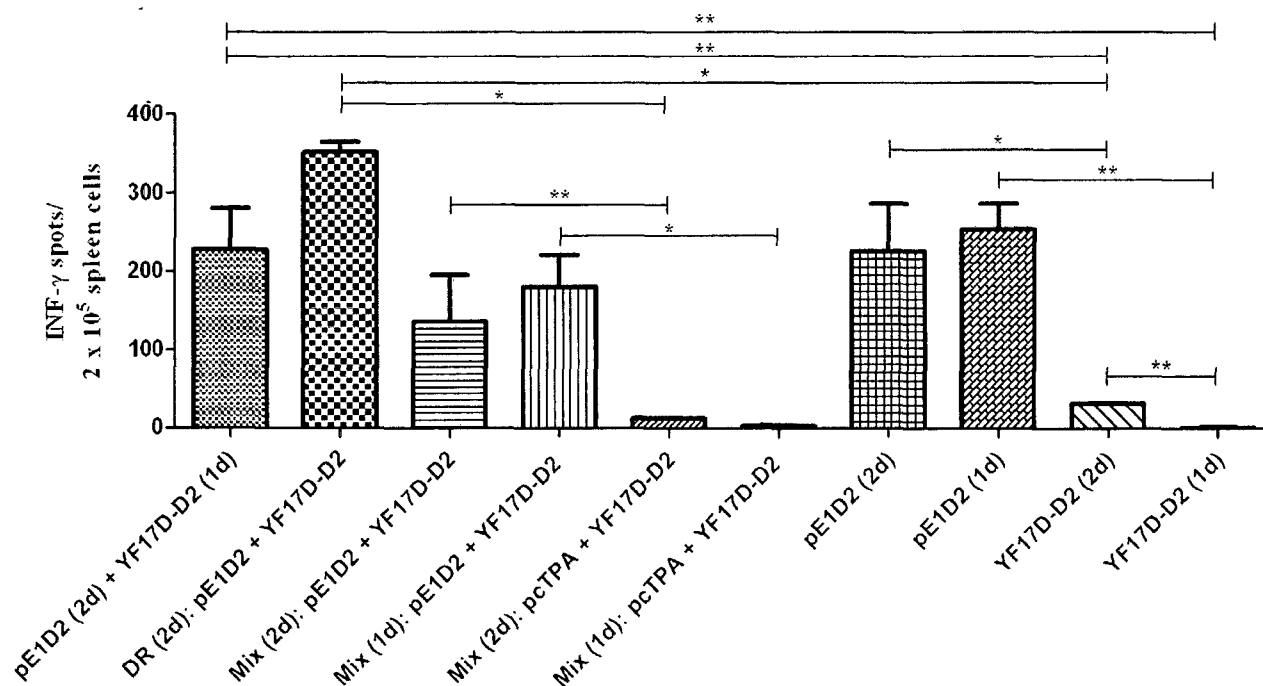


Figure 2. Production of IFN- γ by CD8 $^{+}$ T cells from mice immunized with different vaccination protocols. Splenocytes collected from the different animal groups ($n = 5$) were stimulated with a T-cell specific peptide and the number of spot-forming cells (SFC) were quantified in a 24 h ELISPOT assay. Asterisks indicate statistically significant differences using the Mann-Whitney test (* $p < 0.05$; ** $p < 0.01$). One dose = 1d; two doses = 2d. doi:10.1371/journal.pone.0058357.g002

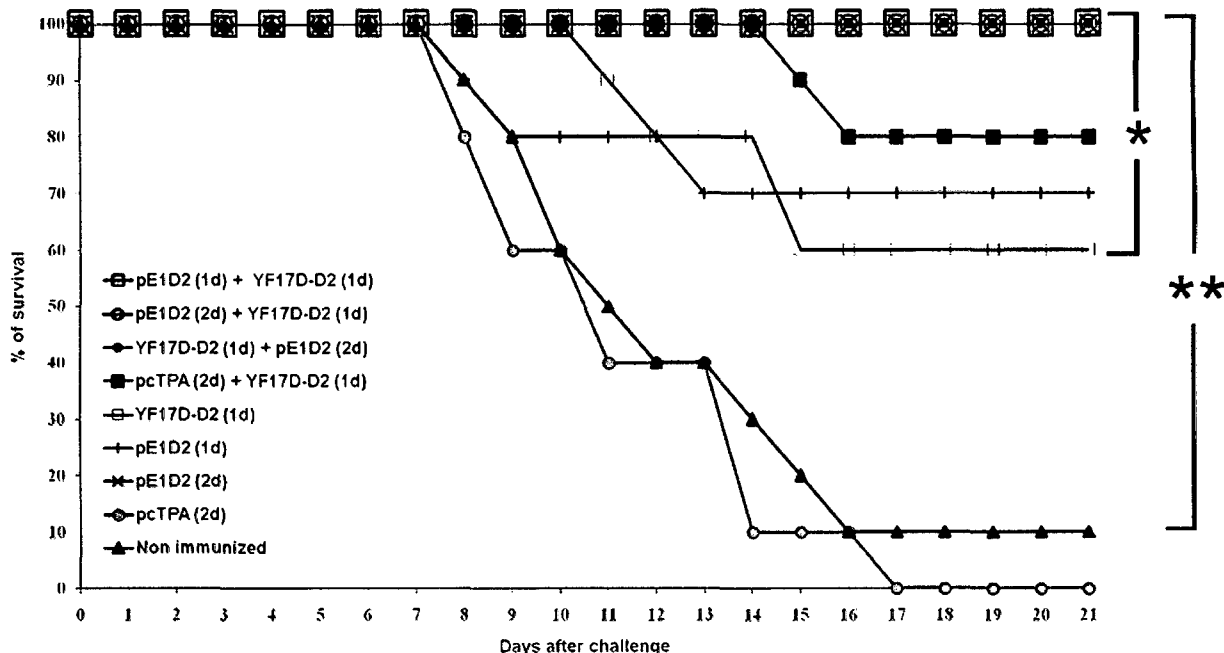
were performed with the prime/boost immunization protocol using only one dose of each vaccine. However, the highest antibody levels were detected in animals receiving two doses of the DNA plasmid followed by one dose of the chimeric virus. Moreover, the immunization with the reversed prime/boost regimen (chimeric virus/DNA vaccine) was also effective and induced an even more efficient neutralizing antibody response. Some reports also demonstrated that DNA vaccines combined with other immunization strategies induces higher humoral immune responses against different pathogens, including dengue viruses, when compared to each approach tested singly [17], [18], [35–40]. The prime/boost approach using DNA vaccine has been investigated with different combinations, including subunit vaccines, inactivated pathogens as well as the use of live viral vectors [17], [36], [38–41]. However, as far as we know, our work is the first report of using the combination of a DNA vaccine and a chimeric yellow fever/dengue virus for induction of an immune response against DENV. Besides, regarding dengue vaccines, none of the trial products tested in humans showed yet to be completely protective, thus indicating the need to pursue new strategies to reach a broader protection.

In addition, the simultaneous inoculation of the pE1D2 DNA vaccine with the YF17D-D2 chimeric virus also revealed a potent synergistic effect on the neutralizing antibody response in mice, regardless of the immunization route, with an unique formulation of both vaccines delivered by the i.m. route (Mix) or with inoculations by the i.m. and s.c. routes for the DNA vaccine and the chimeric virus, respectively (DR). Previous reports using DNA vaccine and other strategies, such as recombinant proteins or inactivated virus, also showed an increase in antibody response when both vaccines were administered simultaneously [37], [38]. Apparently, the use of two different immunization strategies, either by a prime/boost regimen or with simultaneous administrations,

lead the antigen to the mouse immune system in a distinct way, which may deliver it to different antigen presenting cells, thus, eliciting different immune responses either in its magnitude and/or its quality. This can be the reason why chimeric virus YF17D-D2 inoculations did not induce strong IFN- γ responses as we detected in animals immunized with the DNA vaccine pE1D2.

Although most of the studies concerning the immune response induced towards the E protein are based on antibody production, other reports suggests that the activation of T cells with IFN- γ expression may also contribute to protection against dengue [42–45]. In our work, we observed a robust production of IFN- γ by splenocytes collected from mice immunized with the pE1D2 DNA vaccine and stimulated with the peptide E_{331–339}, which was previously described as an immunodominant cytotoxic CD8 $^{+}$ T cell epitope in Balb/c mice specific for the serotype 2 [23]. Similar results were detected with cells obtained from animals immunized with the combination of the two vaccines (pE1D2 and YF17D-D2), either in the prime/boost system or with simultaneous inoculations. On the other hand, mice vaccinated only with the YF17D-D2 chimeric virus showed a significantly lower IFN- γ response. The induction of robust cellular immune responses in animals inoculated with DNA vaccines against different pathogens has been widely described [46], [47]. However, different from what we observed in the antibody production, we did not detected a synergistic effect in the IFN- γ -positive T cell response in mice immunized with the combination of the two vaccines. In fact, the number of SFC in the ELISPOT assay with splenocytes collected from these mice were similar to what we detected in animals immunized with only the pE1D2, suggesting that such response was induced predominantly by the DNA vaccine. The cellular immune response is a key aspect for the induction of a long-lasting immunologic memory [48–50]. Therefore, these results reinforce the idea that the combination of the two immunization strategies

A



B

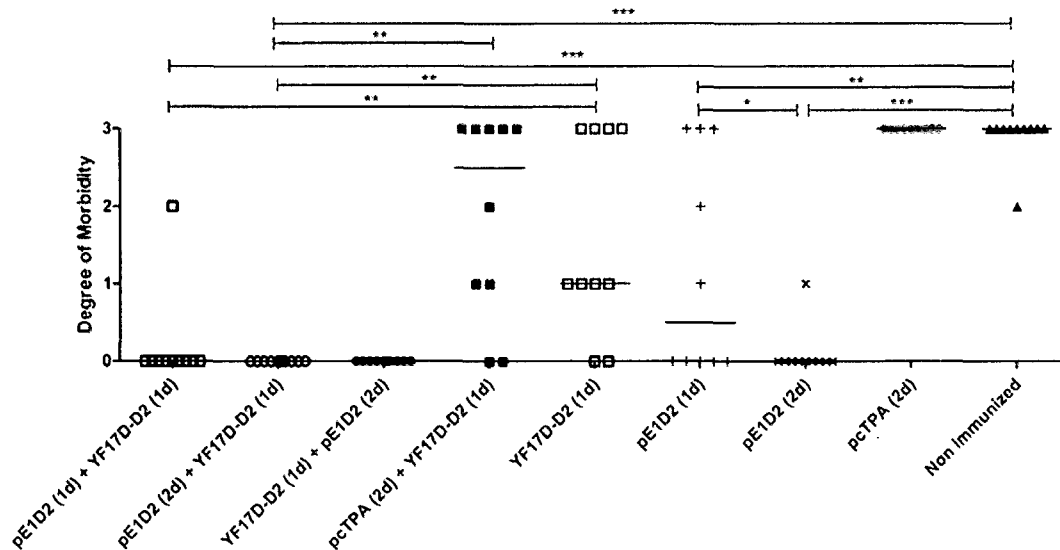


Figure 3. The protective efficacy of vaccination with pE1D2 and YF17D-D2 against DENV2 in prime-booster regimen. Groups of Balb/c mice (n = 10) were immunized with pE1D2 (i.m.) and/or YF17D-D2 (s.c.), administered singly or in a prime-booster regimen, and challenged (i.c.) with the NGC DENV2. Vaccinated and control mice were monitored for 21 days after challenge with the record of survival rates (A) and degree of morbidity (B). Asterisks indicate statistically significant differences using the chi-square test in (A) (*p<0.001; **p<0.0001) and the Mann-Whitney test in (B) (*p<0.05; **p<0.01; ***p<0.001). One dose = 1d; two doses = 2d.
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can lead to a more efficient vaccine. This is an important finding, since recently results were published concerning the protective efficacy of the tetravalent Sanofi-Pasteur vaccine, also based on chimeric yellow fever/dengue virus, in a phase 2 trial with children [51]. Authors showed that, although children presented high levels of dengue-2 neutralizing antibodies, they were not protected against this virus. Such result reveals that other branch of the

immune response should be investigated and that the induction of a robust cellular response with production of IFN- γ may be important for protection.

In accordance to the immune response detected in vaccinated mice and discussed above, the challenge experiments with a lethal dose of DENV2 also revealed in the present work that the combination of the two different immunization strategies were

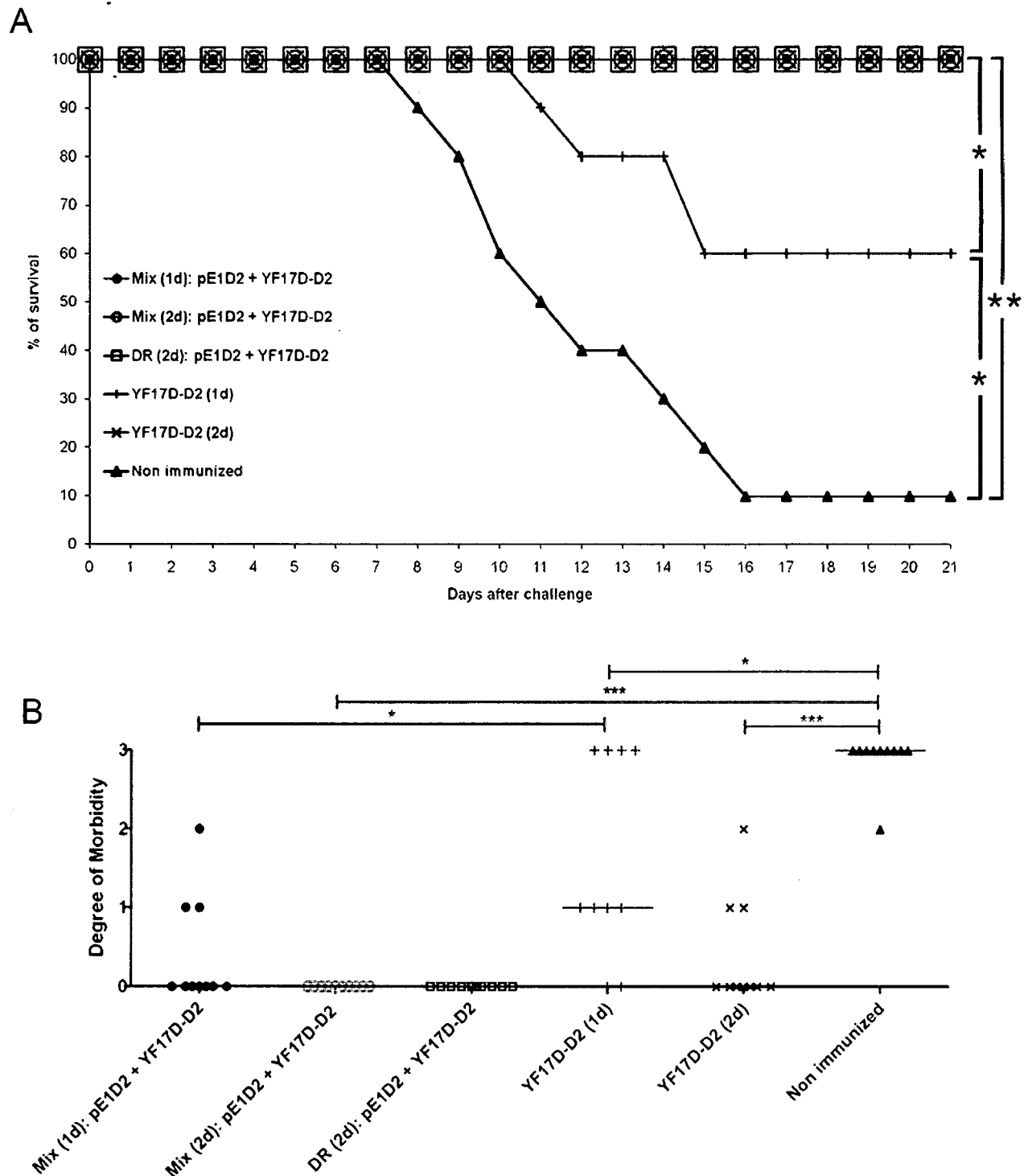


Figure 4. The protective efficacy of vaccination with pE1D2 and YF17D-D2 against DENV2 inoculated simultaneously. Groups of Balb/c mice (n = 10) were immunized by the i.m. route with a mixture of these two vaccines (Mix) or by the i.m. inoculation of the pE1D2 plasmid and s.c. administration of the YF17D-D2 chimeric virus simultaneously (different routes, DR). Animals were challenged (i.c.) with the NGC DENV2 and monitored for 21 days after challenge with the record of survival rates (A) and degree of morbidity (B). Asterisks indicate statistically significant differences using the chi-square test in (A) (*p<0.001; **p<0.0001) and the Mann-Whitney test in (B) (*p<0.05; **p<0.01, ***p<0.001). One dose = 1d; two doses = 2d.
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more protective than each vaccine administered singly, specially when two doses of the DNA vaccine pE1D2 were administered.

These animals were fully protected, without presenting any clinical sign of the dengue infection, either in mouse groups submitted to

prime/boost immunizations [pE1D2(2d)+YF17D-D2(1d) or YF17D-D2(1d)+pE1D2(2d)] or with simultaneous inoculations [Mix (2d): pE1D2+ YF17D-D2 or DR: pE1D2+ YF17D-D2].

Although symptoms manifested in mice inoculated with dengue virus by the intracerebral route are not exactly the same as observed in humans, it has been reported that infection with dengue can also lead to encephalitis in some fatal cases, with detection of virus antigens or the virus RNA in the brain [52–54]. Therefore, such evidences indicate the involvement of the central nervous system in the pathogenesis of dengue. Moreover, we choose this challenge model because it is the most widely used model for vaccines tests against dengue virus [12], [20–21], [24], [27], [55–56]. Hence, results presented in the present work can be compared to those described in several studies. Most animals were challenged 15 days after the last immunization because in older animals the effect of the dengue inoculation is reduced. However, another mouse group simultaneous inoculated with the two vaccine strategies was also challenged 30 days after the last immunization and all vaccinated mice survived virus infection, without the appearance of any clinical sign of infection (data not shown).

The YF17D-D2 chimeric virus used in our experiments was constructed in Brazil [21] by the exchange of the prM-E genes. The Sanofi-Pasteur also developed a dengue vaccine based on a tetravalent formulation of prM-E chimeric viruses and this vaccine has undergone extensive clinical testing [14], [15], [51], [57]. Such trials revealed the necessity of three doses with several month intervals to achieve a balanced antibody response against all four dengue serotypes [14], [15]. The administration of several doses for long periods may represent one problem for vaccination in dengue endemic regions, since it can leave the population more susceptible to the development of severe disease until it became immunologically covered and protected. Therefore, combined immunization of a chimeric yellow fever/dengue virus with a DNA vaccine, as proposed in the present work using DENV2 as a proof of principle, may circumvent such difficulty for induction of robust immune responses against all serotypes, reducing the number of doses and/or the vaccination intervals. Moreover, it is important to note that when the pE1D2 or the YF17D-D2 vaccines were tested alone [20–21] they do not induced fully protection against dengue, which was only achieved after immunization of both strategies in the same animal. Furthermore, the combination of the two different immunization strategies administered simultaneously (Mix or DR groups), instead of the prime/boost regimen, is an attractive approach for vaccination campaigns, since it can facilitate the logistic for inoculations using a single formulation. On the other hand, the reversed prime-boost

system also opens a perspective for the use of a DNA vaccine as a booster, particularly in individuals who do not achieve satisfactory neutralizing antibody levels against the four serotypes after immunization with the yellow fever/dengue chimeric virus. In this case, the booster DNA vaccine may be administered as monovalent or tetravalent formulations. However, further studies will be necessary for testing such hypothesis using tetravalent DNA vaccine and chimeric virus formulations.

Another problem concerning dengue tetravalent vaccines based on live attenuated viruses is the possibility of viral interference, leading to heterogeneous immune responses against each serotype [9]. On the other hand, DNA vaccine appears not to cause interference upon combined immunization and in fact it seems to be more effective when combined to other vaccine strategies [17–19]. Although virus interference may still occur when the two strategies are used, the DNA immunization may also help for balancing the immune response induced against each serotype, providing a booster response for one or some virus when necessary. However, further experiments will be conducted to test if the interference phenomenon may be overcome in a tetravalent vaccine consisting of chimeric virus YF17D-dengue and DNA vaccines.

Additionally, the Mix regimen used in the present work represents an interesting strategy because it can reduce the number of doses. We used the term “dose” as the number of immunization in each animal at a specific time point. Although mice received the double concentration of each vaccine in the Mix (2d), for example, they were given simultaneously, thus maintaining interval between doses (two weeks). Furthermore, in the case of Mix (1d), although three mice presented clinical signs of infection, this system was more protective when we compared to pE1D2 (1d) or YF17D-D2 (1d). Moreover, when we think about the immunization schedule, the Mix (1d) can be considered also more protective than the YF17D-D2 (2d), since it attained the same result (100% survival with 30% morbidity) as the second group but in a single vaccination time.

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Author Contributions

Conceived and designed the experiments: AMA ASA. Performed the experiments: ASA AJG MA. Analyzed the data: AMA ASA AJG RG. Contributed reagents/materials/analysis tools: AMA MSF RG. Wrote the paper: AMA ASA RG.

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