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Immunization with a live attenuated dengue-2-virus candidate vaccine (16681-PDK 53): clinical, immunological and biological responses in adult volunteers

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A live dengue-2 (DEN-2) candidate vaccine (strain 16681-PDK 53), attenuated by passage in primary dog kidney cells, was tested in ten adult volunteers for evaluation of the safety, infectivity and immunogenicity of a dose of 1.9-2.7 × 10^4 plaque-forming units. Five of the volunteers were nonimmune to either dengue or Japanese encephalitis (JE) viruses; the other five were nonimmune to dengue but immune to JE. After receiving 1.0 ml of the vaccine subcutaneously, all ten volunteers developed neutralizing antibodies to DEN-2 which were maintained for at least one and a half years. None of the subjects developed abnormal signs or symptoms and the results of clinical chemistry investigations were within normal range throughout the 21 days of observation after the immunization. Virus isolated from one viraemic volunteer retained the small-plaque and temperature-sensitive growth characteristics of the vaccine virus in vitro. *Further testing of this candidate vaccine in humans is indicated.*

Dengue haemorrhagic fever (DHF) is a serious manifestation of dengue virus infection and remains a major cause of morbidity and mortality among children in Thailand and neighbouring countries. Outbreaks have occurred in countries where the disease appeared to have waned, as in Malaysia in 1982 (*1*). Epidemics of DHF have also occurred in regions of the world not previously affected, e.g., in Cuba in 1981 (*2*). Vector control and other public health measures appear to be of limited value in most instances. Immunization against dengue infection remains an unexplored possibility. Since immunity against all four dengue virus types can be demonstrated in the majority of adults in dengue endemic areas, it is conceivable that immunization with appropriate dengue vaccines will elicit protective immunity in persons at risk. This vaccine for protecting against and controlling dengue haemorrhagic fever/dengue shock syndrome would have to be tetravalent in order to be effective against all four dengue serotypes and to minimize the risk of subsequent infection by a type not included in the vaccine. Ideally the vaccine should be given to children aged six months to one year as the principal target population, should induce a seroconversion rate of at least 95%, and should produce lifelong immunity. Based on the experience with other viral vaccines it should be a live attenuated vaccine.

Dengue-2 (DEN-2) virus has been passaged serially in primary dog kidney (PDK) cells and various biological markers, such as temperature of restricted replication, small plaque size, growth in human monocytes, extension of survival time in suckling mice, cytopathic effect (CPE) in LLC-MK2 cells and monkey virulence, have been used to evaluate changes in biological properties that may be suitable for empirical assessment (*3*). The present report describes a phase 1 trial of DEN-2 (strain 16681-PDK 53) candidate vaccine in ten human volunteers, using undiluted virus. This candidate vaccine fulfilled all the requirements for safety, including the monkey neurovirulence test, according to the U.S. Food and Drug Administration's (FDA) requirements for live viral vaccines (*4*).

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Table 1. Summary of biological properties of uncloned DEN-2 (16681) virus at different passage levels in PDK cells

Biological attribute	PGMK[a] parent	Number of passages in PDK cells			
		30	40	50	53
In vitro tests:					
LLC-MK_2 plaque size[b]	LP,MP,SP	SP	PP	PP	PP
LLC-MK_2 cytopathic effect	4+	1+	0	0	0
Temperature sensitivity (°C)	40.2	39.9	38.2	38.0	37.8
Growth in human peripheral blood leukocytes	+	+	+	+	+
In vivo tests:					
Suckling mouse neurovirulence (mean day of death)	13.4	14.8	18.6	17.8	21[c]
Monkey viraemia	4/4	ND[d]	0/1	ND	2/4
Monkey HI antibody response	4/4	ND	1/1	ND	4/4
Monkey PRNT antibody response	4/4	ND	1/1	ND	4/4

[a] PGMK = primary green monkey kidney cells.
[b] Plaque size: LP ⩾5 mm; MP = 2–4 mm; SP = 1 mm; PP < 1 mm.
[c] No deaths till day 21.
[d] ND, not done.

MATERIALS AND METHODS

The candidate vaccine

DEN-2 candidate vaccine was derived from a parental strain (16681) that had been isolated from a patient with DHF in Thailand and passaged in tissue culture before inoculation in *Toxorhynchites amboinensis* (*5*). The virus was then serially passaged in PDK cell cultures at 32 °C, without cloning or deliberate selection. Biological markers at passage 53 (Table 1) indicated a sensitivity at 37.8 °C, small plaque size (of 1 mm in diameter or less), moderate growth in monocytes (10–100 plaque-forming units (PFU)/ml), loss of neurovirulence for suckling mice, and low viraemia in monkeys compared with the parental strain (*5*). One litre each of the virus grown in PDK cells at passage 51, 52 and 53, representing the master seed, the production seed and the candidate vaccine, respectively, was prepared and preserved in 3.5% human albumin, final concentration. The candidate vaccine was centrifuged at 1050 *g* for one hour at 0–4 °C. The supernatant was filtered through 0.22-μm filters and dispensed in 1.0 ml vials which were stored at −80 °C. The titre of the virus in the candidate vaccine ranged from 2 to 5×10^4 PFU/ml in LLC-MK2 cells. Ampoules of 0.5 ml of candidate vaccine were lyophilized and stored at −80 °C for reference purposes.

Safety tests for the cell substrate, the production seed and the candidate vaccine were designed according to the United States FDA regulations for the production of live attenuated viral vaccines (*6*). The tests included microbial sterility, search for adventitious agents in primary green monkey kidney cells, primary rhesus monkey kidney cells, primary rabbit kidney cells, WI-38 and MRC-5 cells, and tests in adult and infant mice, guinea-pigs and rabbits. Haemadsorbing agents were sought in PDK-cell cultures used for the production seed and candidate vaccine preparations. DEN-2 infected PDK cells and fluids were also searched for adventitious agents by electron microscopy. All these tests were done in our laboratory in Bangkok. Further tests, including microbial sterility and cell culture study for adventitious agents were done on the control fluid, production seed and candidate vaccine in the Department of Tropical Medicine and Medical Microbiology at the University of Hawaii. Identity tests, using DEN-2 specific monoclonal antiserum (CDC Atlanta), were also done at the Hawaii laboratory.

For the monkey neurovirulence tests, ten rhesus monkeys were inoculated with control fluid (2 animals), parental virus (2 animals), and candidate vaccine (6 animals) into the brain and spinal cord, according to the U.S. FDA procedures (*4*). The monkeys were kept at the University of Hawaii laboratory and inspected daily by a veterinary pathologist (one of the authors, S.A.) for 17 days, at which time they were sacrificed and autopsied. Needled tracts were identified in the brain and spinal

cord if possible. Formalin-fixed brain and spinal cord tissues were sectioned in our laboratory in Bangkok. Histopathology slides were prepared and reviewed by S.A. first, and later by Lt.-Col. James Moe of the Walter Reed Army Institute of Research (Washington, DC, USA). These tests revealed no increase in the number or severity of the lesions observed in the vaccinated group of monkeys compared with the two control monkeys. It was concluded that neither parental DEN-2 nor DEN-2 PDK 53 exhibited significant viral neurovirulence.

An international Peer Review Committee established by the WHO Regional Office for South-East Asia reviewed all the findings, inspected the record books and visited the laboratories. Their approval for a clinical trial was first obtained before the protocol for the trial was submitted to the ethical and human experimentation committees of Mahidol University and the Ministry of Public Health.

Clinical trial

In order to minimize the risk of transmission of dengue virus from the volunteers after receiving the candidate vaccine, the clinical trial was conducted in the subdistrict of Pong Mae Lob in the province of Lampoon, about 700 km north of Bangkok and 60 km away from the nearest town. The site was surveyed twice, in 1983 and 1984; no *Aedes aegypti* was found but there was a low density of *A. albopictus*.

Ten adult males living in Pong Mae Lob were recruited on the basis of a seroepidemiological study carried out in 1984. Their ages ranged from 18 to 30 years. All the volunteers were able to read and after discussing the contents of the consent form with one of the authors (S.Y.), signed it without any reservation. They all appeared to be healthy by clinical, biochemical and haematological examinations, with the exception of intestinal parasitic infestation with mild to moderate eosinophilia in some cases. Five of the 10 volunteers showed no serological evidence of previous infection by dengue or Japanese encephalitis (JE) viruses, by plaque reduction neutralization tests (PRNT) and haemagglutination inhibition (HI). The remaining 5 cases showed PRNT responses to JE virus, indicating previous infection with this virus. DEN-2-infection enhancing antibodies were studied in 9 out of the 10 volunteers, using a microtest and the human monocyte cell line, U-937 (*7*). Five demonstrated antibody to JE virus and 4 had no detectable antibodies to any flavivirus. None of these sera had DEN-2 enhancing activity. On the day before the immunization, blood was taken from all the subjects and the sera were tested by PRNT against DEN-1, 2, 3, 4 and JE viruses. Two volunteers received the vaccine on 17 May 1984; the remaining eight received it 7 days later.

After inoculation with the candidate vaccine virus, the volunteers were kept in four mosquito-screened rooms at the subdistrict health centre. They were examined according to the clinical protocol by either the attending nurse or two physicians (authors S.Y. and T.C.) for abnormalities in clinical symptoms (Table 2) and signs (temperature, blood pressure, cold extremities, restlessness, lethargy, rash, nose

Table 2. Frequency of clinical symptoms in 10 volunteers after receiving DEN-2 (16681-PDK 53) candidate vaccine

Symptoms	Volunteer No.										Frequency
	1	2	3	4	5	6	7	8	9	10	
Fever	–	–	–	–	–	–	–	–	–	–	0/10
Headache	–	d11[a]	d9, 10	–	–	d17	–	d8, 11	–	d10, 12	5/10
Eye pain	–	–	d10	–	–	–	–	–	–	–	1/10
Muscle pain	d1	–	d10	–	–	–	–	–	–	d13, 14	3/10
Abdominal pain	–	–	d8, 9, 17	–	d15	–	–	d8	d9	d17	5/10
Abdominal tenderness	–	–	–	–	–	–	–	–	–	–	0/10
Cough	–	–	–	–	–	d17	d6	–	–	d6–12, 17	3/10
Nausea, vomiting	–	–	–	–	–	–	–	d8	–	d11–12	2/10
Diarrhoea	–	–	d19	–	–	–	–	–	d19	–	2/10
Anorexia	–	–	d6	–	–	–	–	–	d9	d11	3/10

[a] d11 = day 11, i.e., the symptom was manifest on day 11 in this volunteer.

bleeding, other bleeding, rhinitis, pharyngeal injection, lymph node enlargement, hepatomegaly, splenomegaly, and lesions at the inoculation site). The intention was to keep the volunteers in confinement for 21 days but since all of them felt no ill effect (or only minor discomfort) and were used to being active in the open mountain environment, this resulted in their going out periodically during the 21 days of observation. Most of them came back at meal times and when blood samples had to be taken, and they never entered any towns where the potential risk of *A. aegypti* existed.

The inoculating dose of the candidate vaccine was 1 ml subcutaneously. The material was prepared by carefully mixing two 1 ml vials and using half of this for the injection; the remaining 1 ml was kept on wet ice and flown to Bangkok on the same day for virus titration.

Laboratory studies

Blood samples were taken on days 0, 6, 10, 14, 18, 21, 30, 60, 180, 1 year, and 1½ years after inoculation; for isolation of the virus, the sera were separated at the Bangkok laboratory on the same day and inoculated into C6/36 and LLC-MK2 cells. Blood samples taken on the same days were also processed for serological analyses (HI and PRNT against DEN-1, 2, 3, 4, and JE virus) (*8*, *9*) and for clinical chemistry (blood urea nitrogen, creatinine, aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT)) and haematological studies (complete blood counts, total and differential white cell counts, total platelet counts, prothrombin time, partial thromboplastin time, total haemoglobin). Urinalysis and hepatitis B surface antigen (HBsAg) studies were also performed.

RESULTS

Signs and symptoms

In this study, none of the volunteers showed abnormal signs, such as elevated temperature, bleeding, hypotension or organ involvement. One volunteer showed some degree of restlessness on day 12 and another had rhinitis on day 8. No reactions at the inoculation site were observed except for a centrally blanched, circumscribed erythematous skin rash at a venepuncture site on day 8 in one case. Some mild symptomatic abnormalities were noted in some subjects, mostly after day 6; half the volunteers complained of headache (occurring mostly between days 8 and 17) and abdominal pain, the other symptoms being less frequent (Table 2).

Clinical chemistry and haematology

None of the volunteers showed abnormalities in the clinical chemistry investigations during the study. HBsAg was not detected in any of the volunteers. The only haematological abnormality was eosinophilia in 4 of the volunteers before the inoculation. During the study, a depression of total white blood cell counts occurred in all cases around day 10 and returned to near original values by day 14 or later (Fig. 1). There was no absolute leukopenia. At the same time there was lymphocytosis (by percentage), monocytosis and formation of atypical lymphocytes. Of interest was the presence of giant platelets in 6 of the 10 volunteers, even though thrombocytopenia was not observed.

Virological study

Titration of the inoculated vaccine showed that the volunteers received from 1.9 to 2.7×10^4 PFU/ml. Viraemia was detected in one of the ten volunteers on day 10, after amplification in LLC-MK2 and C6/36 cells (but not by direct plaque assay). This virus formed uniform small plaques and did not grow at 37.9 °C. It was identified to be DEN-2 by the breakthrough neutralization test.

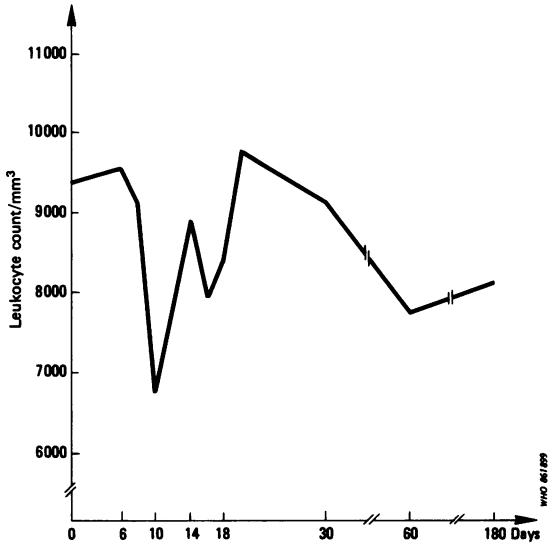

Fig. 1. Mean leukocyte counts in 10 volunteers after immunization with the DEN-2 (16681-PDK 53) candidate vaccine.

Table 3. Flavivirus antibody responses on day 0 and day 30 after DEN-2 immunization

Immune status and volunteer No.	Day	HI titres					PRNT titres				
		DEN-1	DEN-2	DEN-3	DEN-4	JE	DEN-1	DEN-2	DEN-3	DEN-4	JE
DEN(−) JE(−)											
1	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
	30	<10	10	<10	<10	<10	15	260	23	20	ND[a]
2	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
	30	<10	10	<20	10	<10	<10	59	<10	<10	ND
4	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	ND
	30	160	160	320	320	160	<20	600	20	<20	ND
5	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
	30	<10	<10	<10	<10	<10	<20	305	<20	<20	ND
10	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
	30	80	160	160	320	160	21	680	25	<20	ND
DEN(−) JE(+)											
3	0	<10	<10	<10	<10	10	<10	<10	<10	<10	15
	30	160	160	320	640	160	<20	620	<20	14	ND
6	0	<10	<10	<10	<10	10	<10	<10	<10	<10	280
	30	160	160	160	320	160	33	1200	32	<20	ND
7	0	<10	<10	<10	<10	10	<10	<10	<10	<10	100
	30	160	160	160	320	160	47	670	48	34	ND
8	0	<10	<10	<10	<10	10	<10	<10	<10	<10	130
	30	320	320	640	1280	320	94	1150	57	50	ND
9	0	<10	<10	<10	<10	<10	<10	<10	<10	<10	28
	30	80	80	80	160	80	38	400	50	22	ND

[a] ND, not done.

Antibody response

Table 3 shows the HI and PRNT titres in the ten volunteers on day 0 and day 30 after immunization. In the flavivirus nonimmune group, seroconversion was detected by HI in 4 of the 5 volunteers (not in No. 5), with homologous titres ranging from 0 to 160. However, seroconversion was evident in all five non-immune volunteers when assayed by PRNT. Three persons (No. 2, 4 and 5) in this group showed specific neutralizing antibodies to DEN-2, while the other two (No. 1 and 10) showed broad reactions to other dengue serotypes (DEN-1, 3 and 4). All the JE-immune volunteers developed an immune response to dengue antigens when analysed either by HI or PRNT. In this group, the antibodies were always broadly reacting to all 4 dengue serotypes, although homologous titres were higher than the heterologous ones.

In the flavivirus nonimmune group, neutralizing antibodies were detected in three individuals on day 14 and in all 5 by day 21, attaining peak PRNT titres of 40 to 680 (Table 4). In the JE-immune group, neutralizing antibodies started to be detected on day 14 in all of them, with peak titres of 400 to 1200. Peak neutralizing antibody titres in both groups were attained around days 21 to 30. Follow-up for a period of one and a half years showed persistence of neutralizing antibodies in all ten volunteers.

DISCUSSION

DEN-2 candidate vaccine (16681-PDK 53) was given to ten volunteers; five of them were flavivirus nonimmune while the other five were dengue non-immune but JE immune. All of them showed specific neutralizing antibody responses against DEN-2 virus, beginning on day 14, reaching a peak between days 21 and 30, and remaining high for at least one and a half years after the inoculation. All five of the first group of volunteers (flavivirus nonimmune) clearly showed a primary type of response specific to DEN-2, with low or no neutralizing antibody to DEN-1, 3 or 4. In the second group (dengue nonimmune but JE immune), there was a broad neutralizing reaction to all types of dengue but the response to DEN-2 was always much stronger. The HI responses to DEN-2 in both groups of volunteers appeared to be not as

Table 4. Follow-up of neutralizing antibody titres in volunteers after receiving the DEN-2 candidate vaccine

Immune status and volunteer No.	Antibody titre after immunization							
	0	14 days	21 days	30 days	60 days	180 days	1 year	1½ years
DEN(−) JE(−)								
1	<10	<10	80	260	163	93	81	63
2	<10	42	59	40	42	90	84	45
4	<10	25	620	600	430	180	82	110
5	<10	38	58	305	85	44	42	25
10	<10	<10	170	680	370	255	240	122
DEN(−) JE(+)								
3	<10	83	480	620	275	178	175	100
6	<10	26	1160	1200	739	139	120	58
7	<10	42	520	670	277	210	72	74
8	<10	88	930	1150	460	169	180	75
9	<10	15	200	400	89	75	110	37

striking as the neutralizing responses. One individual did not show any HI response at all, while his neutralization antibody titres went up like the others in this group. The capability of the DEN-2 candidate vaccine (16681-PDK 53) to provoke a consistent immune response in humans has been further confirmed in a related clinical trial involving 25 subjects, all of them dengue and JE nonimmunes (*12*).

Although there was only one virus isolation from one volunteer on day 10, another isolation was obtained in our second clinical trial on day 6, indicating that viraemia probably occurred around days 6 to 10. The first virus isolated displayed the biological characteristics of the original candidate vaccine, showing that no reversion had occurred after one human passage.

Previous studies with other DEN-2 candidate vaccines showed mild to moderate dengue-like illness in inoculated human volunteers (*10*, *11*). These self-limiting untoward reactions were characterized by low-grade temperature, headache, muscle pain and/or rash. Our group of volunteers showed no untoward reaction. Although there was no absolute leukopenia ($<4000/mm^3$), the total white blood cell count did fall, reaching the lowest level around day 10, which probably corresponded with the end of the viraemic phase.

DEN-2 (16681-PDK 53) is a very promising candidate vaccine in terms of immunogenicity and reactogenicity and deserves to be further developed. This has been confirmed by the recent successful conclusion of the second clinical trial involving 25 subjects to determine the minimum infectious doses. An important observation is that, in contrast to recent experiences with the DEN-2 (PR 159-S1) (*10*, *11*) and DEN-4 (H-241) (*13*) candidate vaccines in human volunteers, it is possible to obtain a high-titre specific immune response to dengue, without the recipients showing dengue-like illness.

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RÉSUMÉ

IMMUNISATION PAR UN VACCIN CANDIDAT VIVANT ATTÉNUÉ CONTRE LE VIRUS DE LA DENGUE TYPE 2 (16681-PDK 53): RÉPONSE CLINIQUE, IMMUNOLOGIQUE ET BIOLOGIQUE CHEZ LES VOLONTAIRES ADULTES

Une préparation susceptible d'être utilisée comme vaccin vivant contre la dengue type 2 (DEN-2) (souche 16681-PDK 53), atténuée par passage en culture primaire de cellules rénales de chien, a été expérimentée chez dix volontaires adultes en vue de l'évaluation de son innocuité, de son infectiosité et de son immunogénicité, à la dose de $1{,}9\text{-}2{,}7 \times 10^4$ unités formatrices de plages. Cinq de ces volontaires étaient dépourvus d'immunité vis-à-vis de la dengue ou de l'encéphalite japonaise; les cinq autres n'avaient aucune immunité vis-à-vis de la dengue mais avaient une immunité à l'égard de l'encéphalite japonaise. Après injection de 1,0 ml de cette préparation par voie sous-cutanée, les dix volontaires ont élaboré des anticorps neutralisants vis-à-vis du DEN-2, qui se sont maintenus pendant au moins un an et demi. Aucun sujet n'a présenté de symptômes anormaux et les résultats des examens de chimie clinique se situaient dans les limites normales pendant les 21 jours d'observation après injection. Le virus isolé chez un volontaire virémique conservait les caractéristiques de croissance (plages de petite taille et thermosensibilité) du virus vaccin *in vitro*. Il est donc indiqué de poursuivre l'experimentation de ce vaccin potentiel chez l'homme.

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