A SHORT HISTORY OF DENGUE AND MAHIDOL DENGUE VACCINE

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Abstract. In 1980, Mahidol University committed to develop a live-attenuated tetravalent DENV vaccine. The DENV vaccine development project was supported by a grant from the WHO Regional Office for South-East Asia (ICP RPD 002/DHF). DENV-1 and -2 obtained from DHF patients and DENV-4 obtained from DF patients were serially passaged in primary dog kidney (PDK) cells certified to be free from human and canine infectious agents. DENV-3 obtained from DHF patients was first passaged in primary green monkey kidney (PGMK) cells and then in certified Fetal Rhesus Lung (FRhL) cells. The degree of attenuation was empirically based on certain biological markers. Bulk seed productions were eventually prepared in pilot production facilities at Mahidol University's Centre for Vaccine Development at the Institute of Molecular Biosciences. They were subjected to general safety tests and monkey neurovirulence tests in accordance with the US FDA requirements. These pre-clinical tested candidate DENV viruses were approved for proceeding to the clinical evaluation phase by a WHO-appointed Scientific Steering Committee and by the Ethical Review Committee of the Thai Ministry of Public Health. The monovalent live-attenuated viruses – DENV-1 PDK-13, DENV-2 PDK-53, DENV-3 PGMK-30/FRhL- 3 and DENV-4 PDK-48 – were first tested in flavivirus non-immune adult subjects, followed by bivalent, trivalent, and tetravalent vaccine clinical trials. All vaccine recipients developed either a mild or no adverse reaction to the vaccine. The immunogenicity data were discussed. Due to viral interference of each DENV components in the combinations, 12 DENV formulations were evaluated for confirmation of safety and immunoginecity profiles in 155 Thai children aged 3-15 years. Preliminary data were analyzed and processed for further development.

In order to make productive use of this research, Mahidol University entered into a collaborative licensing agreement in DENV vaccine production in 1993 with France based Sanofi Pasteur, the vaccine division of Sanofi-Aventis Group and the largest company in the world devoted entirely to human vaccines. DENV vaccine based on this approach was prepared for production on an industrial scale in France using specific-pathogen-free (SPF) dog colony and FRhL cells. The vaccine is presented in a lyophilized (freeze-dried) form and reconstituted with water for injection in order to deliver a 0.5 ml specified dose. Multiple dose presentations were planned for a target population of children and adults living in or travelling to DEN-endemic areas. The current strategy of creating tetravalent DENV vaccine formulations can lead to an unbalanced immune response. This is attributed to viral interference that apparently comes into play when three monovalent vaccine viruses DENV-1, DENV-2 and DENV-4 are mixed with DENV-3 to create a tetravalent formulation. More research is needed on a priority basis to work out the viral interference factor in order to make the production of a tetravalent vaccine out of our attenuated DENV-3 candidate vaccine strain a success.

Keywords: live attenuated tetravalent dengue vaccine, WHO/SEARO, PDK cells

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INTRODUCTION

Dengue fever and dengue hemorrhagic fever (DF/DHF) are caused by dengue (DENV) viruses. There are four antigenically related, but distinct, DENV serotypes (DENV-1 through DENV-4).

Humans are the amplifying vertebrate hosts, and *Aedes* mosquitoes are the primary mosquito vectors as well as the reservoir of infection. DENV infections cause a spectrum of diseases, ranging from asymptomatic infections to infections that are complicated by hemorrhage, shock, and death. Infection with DENV of one serotype results in apparent life-long monotypic immunity against that serotype but not against any other serotype. Thus, separate infections with all four DENV serotypes are theoretically possible in a single host. It should be noted that in Thailand, all the four DENV serotypes co-circulate, thereby resulting in multiple exposures and the potential for re-infection with different serotypes.

HISTORICAL DEVELOPMENT

The name dengue was accepted for standard usage by the Royal College of Physicians of London in 1869. Bancroft in 1906 published the first evidence of the fact that *Aedes aegypti* mosquitoes are vectors of the disease. In 1931, Simmons proved that *Aedes albopictus* is also an efficient vector. The demonstration that dengue virus can produce inapparent infection in certain species of monkeys led to the accumulation of evidence indicating that certain monkeys may be infected in nature that the infection can be transmitted by mosquitoes from monkey to monkey as well as from monkey to man and that monkeys may constitute one of the links in the chain of events which perpetuate the virus in nature. During World War II, extensive studies resulted in the demonstration of multiple immunologic types of the virus, known later on as dengue 1-4 serotypes.

Dengue Hemorrhagic Fever (DHF) was recognized as a new disease first in Manila in 1954. The disease affected mainly children and was characterized by acute onset of high fever, petichial hemorrhagic and shock. The second large outbreak occurred in Manila again in 1956 affecting more than 1,200 cases with 6% case-fatality rate. In 1958, an outbreak of DHF occurred in Bangkok and nearby areas. Almost 2,500 cases with 10 % case-fatality rate were recorded.

Since then, DHF has become a serious public health problem, causing large scale of morbidity and mortality among children in South-East Asia and Western Pacific Regions of WHO. Well-established epidemics have been reported from Myanmar, China, Cambodia, Indonesia, Lao PDR, Malaysia, Philippines, Thailand and Vietnam.

In the WHO South-East Asia Region, DHF is a major public health problem in Indonesia, Myanmar and Thailand.

The first meeting of the South-East Regional Advisory Committee on Medical Research (SEA/ACMR) held in New Delhi, 5-9 January 1976, recommended to the Regional Director that research on DHF be considered to be of high priority. During the second session of the SEA/ACMR held in New Delhi, 23-27 August 1976, a review on the history of the spread of this disease through several countries of the Region, with an evaluation of the current state of knowledge on its epidemiology, virology, pathogenesis and the related problems of clinical management.

A meeting of the Research Study Group on DHF was held in New Delhi on 24-25 February 1977. Several measures with potential for the prevention and control of this disease were considered. After detailed discussions, the group made its recommendations, of which the two important ones were: (i) vaccine research; and (ii) control of *Aedes aegypti.* The first plan of vaccine research was developed, which, inter alia, proposed that virologists from the South-East Asia and the Western Pacific regions be trained in research and development of the vaccine at the School of Tropical Medicine and Medical Microbiology, University of Hawaii. On the completion of the training, the participants on return to their respective countries were impressed upon to get directly involved in the national DENV vaccine development program. The time frame needed for the development of the DENV vaccine program was proposed to be 3-5 years.

It was understood that most countries with DHF problem would like to participate in the field trials of DENV vaccine at a later stage when the vaccines would be ready.

In 1978, a research steering committee recommended to WHO to take positive steps towards DENV vaccine development by designating the then Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, now known as the Centre for Vaccine Development, Mahidol University at Salaya, Thailand, to undertake the research for the development of the vaccine.

Funding of this project began in April 1980. Three laboratories were equipped for DENV vaccine research and development. A virologist was recruited and sent to the University of Hawaii for the initial phase of research as well as for advanced training while equipping of the laboratory continued. The laboratory was ready for operation in early November 1980. Detailed and comprehensive standard operating procedures (SOPs) for vaccine development were prepared. Protocols were available. Tests were signed by operators and were checked and signed by the supervisor.

In 1987, the site for DENV vaccine development moved to the Centre for Vaccine Development of Mahidol University at Salaya, Nakhon Pathom, Thailand. Equipments for the Government. Another four additional buildings were constructed between 1988-1990, which included an experimental animal house and vaccine pilot plant buildings. The entire vaccine compound was designated for DENV vaccine development. Airlocks and a hepafiltered air supply were generated to control potential cross-contamination.

DENGUE VACCINE DEVELOPMENT

Rationale for dengue vaccine development

The scientific hypothesis behind the development of tetravalent DENV vaccine against DHF could be summarized as follows:

1. Adults develop a higher rate of seroconversion of antibody response against DENV viruses and appeared to be less susceptible to DHF. The naturally acquired immunity appeared to protect the individuals against the infection. The immunization of target populations could result towards development of protective antibody response in individuals and could help in protecting the disease.

2. It has also been shown that a mono- or bitypic antibody response could be a risk factor for DHF if sequential infection by other types of DENV viruses occurred. It was imperative that DENV vaccine should be able to confer the protective immunity against all four types of DENV infection and provide a life-long immunity. This called for the development of a live-attenuated tetravalent DENV vaccine.

3. The target population for immunization against DHF should be toddlers 1-3 years old.

Technical consideration on dengue vaccine development at Mahidol University

The objectives of this program were to select strains of DENV-1, -2, -3, and -4, which showed promise of being attenuated for human use and produced in cell substrates. All the four DENV virus serotypes being developed in Thailand were passaged serially in cell culture without specific selection.

Dengue virus strains selected for attenuation attempts

Dengue 1 (16007-TC-10 2/14/74). Isolated from a DHF patient in Thailand in 1964 had been passaged in tissue culture before inoculation into *Toxorhynchitis amboinensis*. The first intrathoracic passage is No. 167164 and the second in 167376 (received from Dr Robert E Tesh on June 17, 1980).

Dengue 2 (16681 LLC-1 1/22/73). Isolated from a DHF case in 1964 from Thailand had been passaged in tissue culture before inoculation into *Toxorhynchitis amboinensis*. The first intrathoracic passage was N° 167165 and the second passage is 167377 and was received on 17 June 1980. The parent culture strain was virulent for man, having produced typical DF in a laboratory worker who was exposed accidentally (unpublished observations, Dr SB Halstead).

Dengue 3 (16562 TC-7 1/31/72). Virus was isolated in 1964 from a DHF case in the Philippines. It had been passaged in cell culture before inoculation of *Toxorhynchites amboinensis*. The

first intrathoracic passage was N° 167166; the second passage was N° 167378 (received for PDK cell passage on 17 June 1980). The parent culture passaged virus was demonstrated to be virulent for man having produced DF in an accidentally infected laboratory worker (unpublished observations, Dr SB Halstead).

Dengue 4 (1036). Virus was isolated from a DF cases in Indonesia in 1976 using *Aedes aegypti* and kindly furnished by Dr Duane G Gubler. The fourth passage was used to initiate the vaccine studies.

Mosquito inoculation

At the University of Hawaii (Pacific Research Unit), five adult laboratory-reared *Toxorhynchitis amboinensis* were inoculated intrathoracically with strains of DENV-1 to -4. The inoculum was approximately 0.0003 ml. Mosquitoes were maintained on 10% sucrose solution at 28°C for 12 days. At the end of the incubation period, each group of insects was killed by freezing, their heads removed and triturated in 5.0 ml phosphate buffer saline, containing 0.5% gelatin, 30% heat-inactivated calf serum and penicillin and streptomycin. After centrifugation at 5°C for 30 minutes, each supernatent fluid was inoculated into another group of five *Toxorhynchitis amboinensis*. These insects were also held at 28°C for 13 days. At the end of the incubation period, freezing killed them.

Preparation of mosquito suspensions

The head was removed from the infected mosquitoes with a sterile surgical blade and placed in a mortar. Body parts were kept in a sterile vial and frozen at -70°C. The virus diluent, with 30% heat inactivated calf serum in phosphate-buffered saline, pH 7.5, penicillin/streptomycin, was added to ground mosquito heads, 2.5 ml/5 mosquitoes. After centrifugation at 10,000 rpm for 30 minutes at 5°C, the supernatant fluids were filtered through a 0.45 micron Millipore filter. Filtrates were used to inoculate primary dog kidney and Green Monkey Kidney (GMK) cells.

Preparation of primary dog kidney cells

The work was done in the laboratories of the Department of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine, and was supported, in part, by a Grant from the Rockefeller Foundation to Dr SB Halstead.

Each lot and sub-lot of dog kidney cells were subjected to safety tests to assure that the cells and supernatant fluid were free of infectious agents. Tests included for exclusion of bacterial, fungi, mycoplasma and cytopathic and hemadsorbing agents.

Development of attenuated strains of DENV 1-4 viruses

Mosquito suspension of the DENV-1 (16007), DENV-2 (16681), and DENV-4 (1036) were attenuated by serial passages in PDK cell culture ate 32°C without cloning or deliberate selection. The procedure relied on the spontaneous appearance of variants and selection for attenuated variants by the biological pressure of the abnormal host cell. This general method had been successful with several other live virus vaccines, *eg* rubella and mumps.

The DENV-3 (16562) virus was attenuated by serial passages in GMK cell; however, attempts to adapt it to PDK cells had failed.

DENV viruses were serially passaged in PDK cells (Fig 1).

At every fifth passage level, a moderate-sized virus seed was prepared. This virus was studied for plaque size morphology in LLC-MK2 cells, temperature sensitivity to of replication shut-off, suckling mouse neurovirulence and growth in human monocytes, viremia and antibody response in primates.

When the passaged virus presented a reduction in plaque size, temperature sensitivity for replication and absence of viremia and reduced antibody response in monkeys, a "Master seed", "Production seed" and "Candidate vaccine" were prepared. Safety tests on the Production seed and Candidate vaccine included the inoculation of neutralized virus into adult and suckling mice, guinea pigs, rabbits and several tissue culture systems. Furthermore it was also the assured that the candidate vaccine produced no neurovirulence

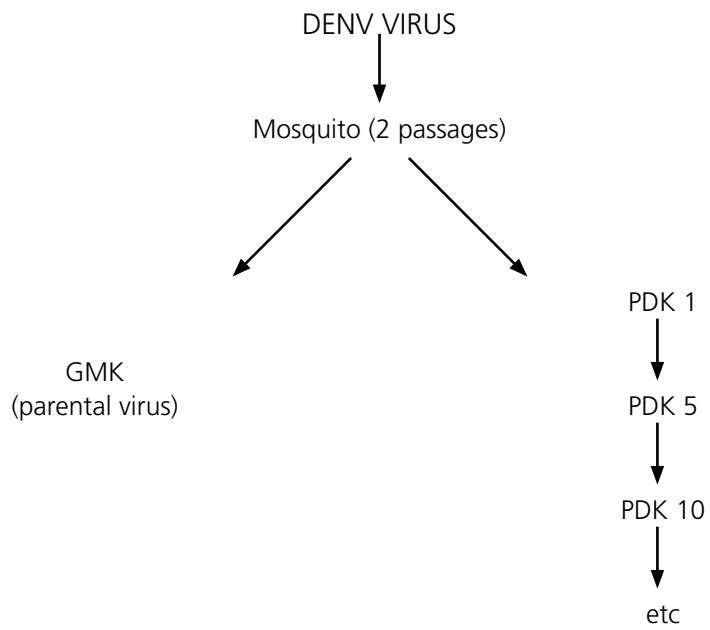

Fig 1–Schema for attempted attenuation of dengue viruses.

following intracerebral inoculation in monkeys. The attenuated strains this developed could help towards worldwide stock of candidate dengue vaccines.

What constitutes a satisfactory level of attenuation remains uncertain. Hypothetically, we would like to have a vaccine which is avirulent, that is, viruses which do not have the capability to cause direct cell injury, but the protective antigenic epitopes of these avirulent viruses should still be preserved and effectively presented to both the B and T lymphocytes of the vaccines to confer both humoral and cellular immunity. It is very difficult to define the attenuation of the dengue viruses by a specific series of the biological markers. These observations remained unsubstantiated due to the fact that there was, no known animal model for DHF. Man represented the only alternative testing model of vaccine efficacy.

Markers of attenuation

To define the level of attenuation of the viruses at the present, it could at best be empirical. The assessment was based on the findings of a combination of markers.

Evidence for attenuation was based on a comparison of the high passage viruses with the parent virus in several *in vivo* and *in vitro* tests: plaque size, temperature sensitivity, replication in human monocytes, and monkey viremia. These characteristics had been shown to be related to human virulence with other experimental dengue vaccines.

The DENV-1 PDK 43, DENV-2 PDK 53, DENV-3 GMK 33 and DENV-4 PDK 48 candidate viruses produced a uniform plaque size when assayed in LLC-MK2 cells. They revealed temperature sensitivity by the plaquing efficiency test. High PDK or GMK passages had significantly reduced virulence for suckling mice by the intracerebral route. All the DENV candidate viruses produced low or no ability to replicate in human monocytes *in vitro*. All of them showed low or no viremia after inoculation with 104-105 plaque forming unit (pfu) of each candidate viruses with moderate specific neutralizing antibody responses. Reduced neurovirulence for mice was observed with DENV-1, DENV-2 and DENV-3 candidate viruses. However, the DENV-4 PDK48 candidate viruses still revealed

modulate neurovirulence in suckling mice.

Safety test

Safety tests of the cell substrate, the candidate viruses and the candidate vaccines were designed according to the United States FDA regulations as applied to live attenuated vaccines produced in the United States. Tests included microbial sterility; and search for adventitious agents in PGMK cells, adult and infant mice, guinea pigs, and rabbits. Hemadsorption agents were sought in cell-culture experiments. A second tier of tests required for additional safety were performed at the virology laboratory of the Department of Tropical Medicine and Medical Microbiology, University of Hawaii.

The team could establish the capability to perform monkey neurovirulence test in Thailand and slides of monkey tissue were independently reviewed by an experienced neuropathologist.

Peer review of the vaccine development project

Candidate DENV vaccines considered to be sufficiently attenuated were submitted to an international panel of experts in DENV for review. This panel had met annually once a year in Bangkok for twelve times from 1983 to 1994 (Table 1). The function of the panel of experts was to review the scientific work, including visit to the site of vaccine development, in order to pursue and examine the facilities, and to audit the raw data. The record books were reviewed by two of the peer reviewers in detail for completeness and for the accuracy of summary data presented. The peer group gave recommendations to the Ministry of Public Health of Thailand and to WHO-SEARO based on their assessment whether the candidate vaccines were suitable for vaccine trials in human beings or not. This process was unique for WHO programs.

The DENV-1 (16007) PDK 43, DENV-2 (16681) PDK 53, DENV-3 (16562) GMK 30 FRhL 3 and DENV-4 (1036) PDK 48 met the US FDA requirements for microbial safety and monkey neurovirulence test for live attenuated viral vaccine conducted by laboratory at Mahidol University as well as by an independent laboratory at the Walter Reed Army Institute of Research, USA. They were approved by an international peer review group based on the examination of the result of safety test and by an on-site examination of the facilities, laboratory record and log books. Confirmatory histopathological examination was done at the ethical review conducted by a committee for human experimentation of the Mahidol University and by a similar committee of the Ministry of Public Health, which was satisfactory and these candidate vaccines were approved for clinical trials.

CLINICAL TRIALS OF DENGUE VACCINES

Clinical trials of monovalent dengue vaccines

The site for the small-scale experimental clinical trial was Lamphun and Loei Provinces, an area where there was low prevalence of *Ae. aegypti* mosquitoes. The trials were conducted during the cold season so as to minimize the risk of other arbovirus infections and possible transmission of vaccine viruses. The population chosen to conduct the trials consisted of flavivirus non-immune young male adults. The initial trial was conducted in two phases using first two and then eight volunteers to increase the safety factor. The protocol called

Table 1. Dengue Virus Development Program, WHO Peer Review Meeting, Bangkok, Thailand [a].

Serial No.	Date		
1st	1-5	August	1983
2nd	23-25	August	1984
3rd	31	July-2 Agust	1985
4th	20-22	August	1986
5th	27-30	July	1987
6th	1-5	August	1988
7th	7-11	August	1989
8th	29-30	September	1990
9th	26-28	August	1991
10th	24-26	August	1992
11th	23-25	August	1993
12th	29-31	August	1994

[a] Organized by WHO/SEARO, WHO Project: ICP RPD/002.

for close observation during the first 21 days.

DENV-1 (16007) candidate vaccines

The candidate DENV-1 (16007) PDK 43 vaccine was passed 43 times in PDK cell. The evolution of the biological markers tested was as follows: plaques in LLC-MK2 cells became of small size (=1 mm) after passages 10-15. Temperature sensitivity at 39°C was achieved at passage 30. Ability to grow in human peripheral blood lymphocytes (PBL) was lost at passage 20. Suckling mouse neurovirulence was reduced to minimal level at passage 15. After 43 passages, all monkeys that received the DENV-3 PDK 43 showed none or low viremia. Based on these results, passage 43 was selected for phase 1 trial.

Six flavivirus non-immune subjects were inoculated with a dose of 2.1 to 3.5 × 10^4 pfu. Clinical symptoms were mild in all volunteers and only one of them showed very minimal nose bleeding, without other hemorrhagic manifestations. In no case was absolute eukopenia observed. Clinical chemistry was normal in all volunteers. Immune response, as measured by plaque reduction neutralization test (PRNT), was detected in only 1 out of 6 flavivirus non-immune volunteers. Two of the seronegative subjects were challenged again with the same dose of DENV-1 PDK 43 at 3 months. Again, they failed to develop any neutralizing antibody. The conclusion was that DENV-1 PDK 43 had been over-attenuated and that it was necessary to try lower passage levels.

A candidate vaccine was then prepared from DENV-1 PDK 30, and after the usual safety tests, was infected into five adult male volunteers who were seronegative for both JE and DENV viruses. Two of the five seroconverted, but the responses were low. The exercise was thus repeated using DENV-1 PDK 20 but again the antibody responses were low and only three of the five seroconverted. The conclusion was thus reached that DENV-1, PDK 20, PDK 30 and PDK 43 were over-attenuated to be useful as candidate vaccines for DENV-1 in people that were seronegative for previous exposure to flaviviruses. The DENV-1 PDK 13 virus that was used in the next trial showed evidence of lesser attenuation than PDK 20, PDK 30 or PDK 43 in that it still replicated in human monocytes. When seven DENV and JE antibody negative male volunteers were injected with DENV-1 PDK13, five seroconverted to DENV-1 within 30 days. There was some evidence of rhinitis but this may have been coincidental and the significance of the observation could not be assessed. There was also a slight fall in leukocyte counts on day 10, but the virus was not isolated from blood at any stage.

DENV-2 (16681) candidate vaccine

The trial of the DENV-2 candidate vaccine, DENV-2 (16681) PDK 53, was carried out as phase 1a and 1b trial.

The initial phase 1a of DENV-2 PDK 53 candidate vaccine in ten 18-30-years-old male human subjects showed encouraging results. None of the 10 persons vaccinated were febrile or incapacitated; side-reactions possibly attributable to the candidate vaccine were limited to slight leukopenia, occasionally abnormally large platelets and a few transient complaints such as mild aches and pains.

All vaccinated persons developed DENV-2 neutralizing antibody. Those subjects with preexisting antibody to JE virus responded serologically more rapidly than those subjects without preexisting flavivirus antibody before vaccination.

Serological tests carried out one year after the vaccination showed that neutralizing antibodies were present in 100% of the volunteers.

The phase 1b trial of DENV-2 PDK 53 candidate vaccine involved sixteen 15-30-year-old male volunteers, 15 of whom were flavivirus non-immune. Four doses of varying virus dilutions were given to groups of four +volunteers each, and every person developed DENV-2 neutralizing antibodies, regardless of vaccine virus dilutions. Abnormal lymphocytes and a slight decrease in lymphocyte numbers were consistently observed between days 6 and 10. As in the phase 1a trial, no adverse reactions to the vaccine were observed.

Viremia was detected in one volunteer and virus isolated in C6/36 cells from day 6 serum.

The virus had growth characteristics similar to those of the candidate DENV vaccine virus. On the basis of 1a and 1b studies it was suggested that viremia occurred between days 6 and 10. It is unlikely to occur after day 14 because of the onset of neutralizing antibodies. It is possible that viremia may precede the time of the lowest white cell counts, which frequently occurred on day 6.

A dose response study in adults, based on 5-fold dilutions of the vaccine, showed an estimated 50% infectious dose of 5-7 pfu.

DENV-3 (16562) candidate vaccines

The DENV-3 (16562) parent virus did not grow in PDK cells and was passaged in GMK cells. At passage GMK 30, the virus was still able to replicate in PBL and produced plaques of varying sizes. After passage 34, plaques were uniformly small. Two GMK passages were selected for adaptation to FRhL cells: 30 and 35. In FRhL cells, DENV-3 attained titers one log higher than in GMK cells. With both passages (PGMK 30/F2) and GMK 35/F2), biological markers were considered to be satisfactory: plaques were of small size, no CPE in LLC-MK2 cells, temperature sensitive at 38.3°C, no growth in human PBL, and reduced neurovirulence for suckling mice.

No adventitious agents were found in GMK cells analysed by electron microscopy. Safety tests of GMK cells were being completed at the National Institute for Biological Standards and Control (London) and the National Biological Standards Laboratory (Canberra). The cells had been found to be free of mycoplasma, mycobacteria and other adventitious agents. Tests to detect simian retroviruses, SV5 and SV40, were negative.

Biological characteristics of DENV-3 candidate vaccine viruses

The DENV-3 (16562) GMK 30 passage virus had mixed plaque morphology (medium and small), a restrictive temperature of 40°C caused CPE in LLC-MK2 cells and grew in human PBL. DENV-3 GMK 30, FRhL-3 virus had small and pinpoint plaque morphology, restricted growth at 38°C, and did not cause CPE in LLC-MK2 cells. Considerable change, presumably selection, had occurred with FRhL passage. The virus recovered from a volunteer who received PGMK 30, FRhL-3 vaccine had biological markers (medium) plaque size, CPE in LLC-MK2 similar to earlier passage vaccine was either genetically prone to reversion or contained an undetected subpopulation of more virulent virus.

Three passage levels of DENV-3 (16562) were given to volunteers; GMK 33, PGMK 30-FRhL-2, and GMK 30 FRhL-3. The FRhL passaged viruses differed from the GMK 33 in being more temperature-sensitive, less able to produce CPE in LLC-MK2 cells, and having uniform small plaque morphology.

Four volunteers received the GMK 30 FRhL-3 virus at doses of 1×10^4 to 6.5×10^4 pfu. One of two volunteers seroconverted at the lower dose. The volunteer who failed to convert at the lower dose was revaccinated at the higher dose and seroconverted. Two volunteers seroconverted at a higher dose. Only minor symptoms and no fever were observed. Satisfactory primary immune responses were observed in three volunteers; the fourth, who was JE immune, had a secondary-type serological response. The virus isolated from the serum of one volunteer exhibited medium-sized plaque morphology and its characteristics of earlier passage virus.

In other trials, two volunteers received GMK 33 vaccine and four volunteers received GMK 30 FRhL-2 vaccine. Both of those vaccines contained both medium and small plaque sizes and were less temperature sensitive than the GMK 30 FRhL-3 vaccine. Both vaccines immunized satisfactorily at doses of 10^4; however, brief febrile responses and mild symptoms were observed.

The GMK-30, FRhL-3 vaccine appeared to be less reactogenic than the other two DENV-3 candidate vaccines and was immunogenic at a dose of 5×10^4.

DENV-4 (1036) PDK 48 candidate vaccine

DENV-4 (1036) virus was passaged in PDK cells to passage 48. Biological markers of PDK 48 included small plaque and no cytopathic effect in

LLC-MK2 cells, temperature replicative shutoff at 39°C, and average survival of 12 days in suckling mouse. PDK 48 virus replicated in peripheral blood mononuclear cells. Rhesus monkeys inoculated with PDK 48 virus did not develop viremia but seroconverted. On evaluation of the monkey neurovirulence tests, the panel of experts concluded that there was no significant difference between the parental DENV-4 virus and DENV-4 PDK 48 candidate vaccine, and it was thus acceptable to proceed with phase 1a clinical trial. An additional four rhesus monkeys had been tested with PDK 48 virus; enhanced neurovirluence was not found. The monkey neurovirulence test result was considered satisfactory, and it appeared feasible to proceed with PDK 48 as a candidate vaccine.

The phase 1a clinical trial was the inoculation of five flavivirus seronegative volunteers with 1-2×10^4 pfu of DENV-4 PDK 48. All volunteers developed specific neutralizing antibodies, which first appeared from days 13-16, and peaked in titer at day 30 post-inoculation. Clinical signs were unremarkable in all volunteers, and no volunteer developed fever. Clinical symptoms were generally absent, although two volunteers reported eye pain and headache. In one of these volunteers the headache re-occurred for a period of about 2 weeks. All volunteers showed normal blood chemistry profiles. Hematological studies revealed a transient increase in atypical lymphocytes in three volunteers. All volunteers showed a temporary depression in total white blood cell counts; however, there was no absolute leukopenia. Virus was recovered only from plasma, and the viremia appeared to be low in titer, since no virus could be detected by direct plaque assay. The recovered virus strains shared all biological markers with the vaccine candidate, except that one strain showed an extended mean day to death in suckling mice of 20 days.

The phase 1b trial was designed to determine the minimum infective dose of the DENV-4 vaccine candidate. The 1b trial was temporarily divided into two phases with groups of seven and five volunteers. The vaccine was diluted from 1:5 (3,700 pfu) to 1:1,000 (12-15 pfu) and each dilution was inoculated into 1-3 volunteers. None of the volunteers showed fever or rash, and clinical signs and symptoms were mild, although eight of the twelve volunteers reported transient headache and eye pain. Blood chemistries were normal, and hematological findings were similar to those seen in the phase 1a trial.

All groups of volunteers inoculated with a dilution of 7 × 10^2 pfu or greater developed specific neutralizing antibody. Two of the two volunteers at 7 × 10^2 pfu seroconverted, one of the two at 1.5 × 10^2 pfu seroconverted, and none of the three volunteers at 63-77 pfu seroconverted. In total, combining the phase 1a and phase 1b results, 10 of the 10 volunteers inoculated with vaccine doses of 7 × 10^2 pfu or greater seroconverted.

Polyvalent vaccine clinical trials

Bivalent vaccine DENV-2 (16681) PDK 53 and DENV-4 (1036) PDK 48 clinical trial. The aim of the DENV vaccine development program was to develop and administer a vaccine containing a mixture of multiple DENV serotypes. The rationale was based on the provision of providing protection to all serotypes that would minimize any chances of future DENV infection enhancement and adverse host reaction. This trial was designed to conduct in humans in support of the hypothetical concept that multiple simultaneous infections with candidate vaccines were possible, safe, and effective. Eleven male flavivirus non-immune subjects aged 16 to 31 years received bivalent DENV-2 and DENV-4 candidate vaccines.

The neutralizing antibody responses to DENV-2 at 6 months ranged from 1:52 to 1:440 and that of DENV-4 ranged from 1:44 to 1:310. A low titer of neutralizing antibodies to DENV-1, DENV-3 and JE viruses were detected early, but this disappeared by 6 months.

The bivalent DENV-2–DENV-4 candidate vaccine was both immunogenic and without unacceptable reactions. Moreover, the dose of DENV-2 and DENV-4 viruses was acceptable and formed to be the basis for future trials.

Bivalent vaccine DENV-1 (16007) PDK 13 and DENV-4 (1036) PDK 48 clinical trial. The bivalent DENV-1 and DENV-4 vaccine's human clinical trial was conducted in Loei Province, Thailand. Seven male flavivirus nonimmune subjects, aged 16 to 30 years, received bivalent DENV-1 and DENV-4 candidate vaccine.

All seven subjects seroconverted to both DENV-1 and DENV-4 since the presence of neutralization antibodies were detected by PRNT to both DENV-1 and DENV-4. There was no difference in response between those receiving candidate vaccines in separate arms and among those receiving mixed vaccine in another arm.

The bivalent DENV-1 and DENV-4 candidate vaccine induced specific response to both DENV-1 and DENV-4 but low titers of heterologous neutralizing antibody were found and the vaccine, was without any adverse reactions, among the recipients.

Bivalent vaccine DENV-1 (16007) PDK 13 and DENV-2 (16681) PDK 53 clinical trial. Seven male subjects aged between 17 and 32 years received bivalent DENV-1 and DENV-2 candidate vaccine.

All seven subjects seroconverted to both DENV-1 and DENV-4 since the presence of neutralization antibodies were detected by PRNT to both DENV-1 and DENV-4. There was no difference in response between those receiving candidate vaccines in separate arms and among those receiving mixed vaccine in another arm.

The bivalent DENV-1 and DENV-4 candidate vaccine induced specific response to both DENV-1 and DENV-4 but low titers of heterologous neutralizing antibody were found and the vaccine was without any adverse reactions among the recipients.

Bivalent vaccine DENV-1 (16007) PDK 13 and DENV-2 (16681) PDK 53 clinical trial. Seven male subjects aged between 17 and 32 years received bivalent DENV-1 and DENV-2 candidate vaccine.

All subjects seroconverted to DENV-1 and DENV-2 by 30 days. Titers of DENV-1 neutralizing antibody ranged between 1:27 and 1:70 in the six subjects who were non-immune before vaccination. Titers of DENV-2 ranged from 1:26 to 1:120 and at 30 days no cross-reaction with other flaviviruses was observed.

The bivalent DENV-1 and DENV-2 candidate vaccine was both immunogenic and without any adverse reactions.

Trivalent vaccine DENV-1 (16007) PDK 13, DENV-2 (16681) PDK 53 and DENV-4 (1036) PDK 48 clinical trial. The human clinical trial comprised of a mixture of three monovalent DENV vaccines (DENV-1, -2 and -4), which was inoculated into each of 12 male adults. The study was performed in a subdistrict of Loei Province in Northeast Thailand, where the prevalence of *Ae. aegypti* or *Ae. albopictus* mosquitoes was low. The objective of this study was to determine the safety and feasibility.

Of the 12 recipients, nine were flavivirus non-immune; they all developed serum neutralizing antibodies to all the three DENV viruses.

The results of this study showed that it was possible to infect humans safely with three attenuated DENV viruses. The median dose of DENV-1 virus was close to optimum whereas the dose of DENV-4 was too low. The successful administration of a trivalent DENV vaccine indicated that Mahidol University had achieved another important milestone on the road to the development of a tetravalent dengue vaccine.

Trials of tetravalent vaccine in children aged 5-12 years. The tetravalent DENV vaccine candidate appeared to be safe when administered to children aged 5-12 years. Children became just febrile, and this usually did not last for more than a day. One volunteer had a rash that persisted for three days.

Two trends of serological response to the tetravalent vaccine were observed among the volunteers. First, the infectious dose that was calculated for adults was not equivalent for the children in the age groups studied. A trend of an

increasing rate of seroconversion among children was noted with a decreasing vaccine dosage; however, an optimum dose for children 5-12-years-old still had to be determined.

Second, children with preexisting antibodies to either DENV or JEV appeared to respond better to the tetravalent vaccine than did children who were completely non-immune. Even so, not all volunteers with preexisting flavivirus antibodies responded to all four DENV serotypes.

Collaboration with manufacturer

The Mahidol vaccine was licensed to Pasteur Mérieux (PMsv, now Sanofi Pasteur) in France for large-scale production under Good Manufacturing Practice (GMP) conditions.

The master seed, the production seed and the candidate DENV vaccines were sent to Pasteur Merieux in February 1993, shortly after the agreement was signed in January 1993. Three technical meetings at Pasteur Merieux were held in Lyon, France, between 1994-1996. Industrial production of the four monovalent vaccines was achieved by 1995. All biological studies, including monkey neurovirulence studies were repeated.

The first clinical trial carried out using the Mahidol/PMsv tetravalent vaccine in US volunteers suggested that the combination of four attenuated strains appeared to result in increased reactogenicity and diminished tolerability. Antibody responses were predominantly directed against DENV-3 with low or undetectable titers against the remaining three serotypes. This outcome appeared to be the result of preferential replication of DENV-3 in the tetravalent vaccine. The mechanism of such viral interference was not known. But it had been suggested that the ratio of the four attenuated viruses in the tetravalent formulation may be an important factor. A subsequent clinical study in Thailand showed that varying and reducing the concentrations of the DENV-3 strain resulted in an improved clinical safety profile of the tetravalent vaccine. About 71% seroconversion (against all 4 serotypes) was observed after a two-dose vaccination schedule in this study. Several different reformulations of the tetravalent vaccine were being evaluated in order to provide a more balanced immune response to each serotype.

Second generation recombinant vaccines

A Cooperative Research and Development Agreement (CRADA) was entered into with the Division of Vector-Borne Infectious Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado, USA, to develop the second-generation recombinant vaccines using complementary DNA (cDNA) technology. As per the memorandum of understanding (MoU), the first shipment of candidate DENV vaccines (DENV-1 and DENV-2) was sent to CDC in August 1994. The DENV-3 and DENV-4 vaccines were sent shortly thereafter. The agreement called for Mahidol University to provide support for one locally trained technician and for CDC to provide support to one Thai investigator engaged in research and capacity-building activities at Fort Collins. Vaccine development studies were realized at CDC while biological marker testing was partially done in Thailand.

Conclusion on present status of PDK-based live-attenuated dengue vaccine

The importance of DENV vaccine development was imperative in order to improve public health throughout the world and was highly desirable for WHO to provide financial support for this program. The peer group summarized the progress as follows:

(1) Monovalent candidate vaccines

(a) DENV-1: A usable candidate vaccine.
(b) DENV-2: A near-perfect candidate vaccine.
(c) DENV-3: The most recently developed candidate vaccine, somewhat more reactogenic than the other candidate vaccines. A search for a better vaccine should proceed.
(d) DENV-4: A very good product.

(2) Bivalent and trivalent combinations using DENV-1 PDK 13, DENV-2 PDK 53 and DENV-4 PDK 48 had undergone phase 1 trials in adults with satisfactory results.

(3) Tetravalent vaccine was acceptably safe. Interference was noticed after mixing of the DENV-3 GMK-30/F3 in the combination.

LESSONS LEARNED

There was a general consensus that vaccination can be one of the most cost-effective ways to prevent DF and DHF. The aim of this project was to develop a safe and immunogenic vaccine against the four DENV serotypes. Each of the four monovalent vaccines as well as the bivalent and the trivalent vaccines were developed and tested step by step in the laboratory and in human volunteers. By 1992, the attenuated, tetravalent vaccine was being tested for immunogenicity and safety in human volunteers. Formal phase 1 and phase 2 clinical trials had proven the vaccines to be both safe and immunogenic in humans. Human trials of the tetravalent vaccine were successfully concluded.

In November 1992, WHO headquarters and WHO/SEARO announced the attainment of the objective of the dengue vaccine development project at Mahidol as follows: "Vaccine for Dengue Hemorrhagic Fever". From this study, it was proved that PDK cells could be used successfully for attenuation attempts. The DENV-2 PDK 53, which was one of the important outcomes of this study, has been further used as a backbone to construct live molecular DENV vaccines in the USA.

Research as well as relevant capability building activities at Mahidol University were established with the advice of the international peer group which met annually. However, the initial expectation in 1985 that DENV vaccine development would be completed within three years proved too optimistic.

Considerable research capacity building took place as part of the research project support during that decade. The various technologies required for vaccine development and laboratory-scale production were transferred. They included continuous tissue culture, development of PDK and other cell lines, monkey tests for neurovirulene, etc. The annual meetings of the peer group itself provided valuable scientific advice to the project. In addition, Mahidol University scientists were supported for visits and contacts with various scientists and institutions in other countries.

Meanwhile, Mahidol University expanded the physical and other infrastructure required for vaccine development and pilot scale up. A vaccine development center building, and a laboratory animal center were completed at the new Salaya campus. Equipment for up scaling was received as donation from the Italian Government.

The DENV vaccine development project was acknowledged to be a worthy scientific achievement in the area of health. Such achievements could occur due to the long-term commitment of scientists in Thailand, the continuous support of the Government of Thailand, and the initial impetus and sustained commitment and support provided by WHO/SEARO. The Government of Thailand and Mahidol University provided the major resources. WHO provided about USD 2.5 million during a period of 15 years. Other donors contributed substantial amounts at various stages of the project for specific components of the program. Success was due to scientific correctness of the research, the outstanding leadership of the late Prof N Bhamarapravati of Mahidol University, sound research management by several parties, and the sustained commitment and technical support through the years of the WHO Regional Office for South-East Asia.

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