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PHASE 1 STUDIES OF WALTER REED ARMY INSTITUTE OF RESEARCH CANDIDATE ATTENUATED DENGUE VACCINES: SELECTION OF SAFE AND IMMUNOGENIC MONOVALENT VACCINES

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Abstract. We describe the results of initial safety testing of 10 live-attenuated dengue virus (DENV) vaccine candidates modified by serial passage in primary dog kidney (PDK) cells at the Walter Reed Army Institute of Research. The Phase 1 studies, conducted in 65 volunteers, were designed to select an attenuated vaccine candidate for each DENV serotype. No recipient of the DENV candidate vaccines sustained serious injury or required treatment. Three vaccine candidates were associated with transient idiosyncratic reactions in one volunteer each, resulting in their withdrawal from further clinical development. Increasing PDK cell passage of DENV-1, DENV-2, and DENV-3 candidate vaccines increased attenuation for volunteers, yet also decreased infectivity and immunogenicity. This effect was less clear for DENV-4 candidate vaccines following 15 and 20 PDK cell passages. Only one passage level each of the tested DENV-2, -3, and -4 vaccine candidates was judged acceptably reactogenic and suitable for expanded clinical study. Subsequent studies with more recipients will further establish safety and immunogenicity of the four selected vaccine candidates: DENV-1 45AZ5 PDK 20, DENV-2 S16803 PDK 50, DENV-3 CH53489 PDK 20, and DENV-4 341750 PDK 20.

INTRODUCTION

The Walter Reed Army Institute of Research (WRAIR) and Mahidol University in Bangkok, Thailand have developed live-attenuated dengue virus (DENV) vaccine candidates.[1,2] These DENV vaccine candidates are attractive because they are capable of infection and replication in recipients, can induce both humoral and cellular immune responses following a single dose, and may be produced at relatively low cost.[3] In the absence of proven animal models, the principal means of determining the safety and immunogenicity of a vaccine candidate is clinical evaluation in volunteers.[4] Over the past 40 years, many DENV vaccine candidate strains were found to be unsuitable for humans because of over-attenuation that rendered them insufficiently immunogenic, or because they were under-attenuated, resulting in dengue fever.[5]

More recently, DENV vaccine candidates have been prepared after adaptation to growth in primary dog kidney (PDK) cells, and selection for modified viral phenotypes.[6] Preliminary clinical studies demonstrated that DENV strains could be attenuated for humans after passage in PDK cells.[7,8] Subsequently, a concerted investigation was undertaken at WRAIR to select an attenuated vaccine candidate for each DENV serotype. Experimental DENV vaccine candidates were prepared as described,[9] and their safety and immunogenicity were then tested in volunteers at one or more passage levels. The purpose of these clinical investigations was to select the least reactogenic and most immunogenic attenuated DENV serotype vaccine candidates for expanded clinical development and future possible combination into a multivalent vaccine candidate.

We describe the results of this program of safety testing and selection of WRAIR attenuated DENV-1, -2, -3, and -4 vaccine candidates for expanded clinical testing. The selection of the DENV-1 vaccine candidate has already been described in detail elsewhere.[10] Selected data from this study has been included herein for continuity and comparison with the other WRAIR attenuated serotype vaccine candidates.

MATERIALS AND METHODS

Selection of volunteers. Healthy male and female volunteers 18–45 years of age were examined and screened by a panel of tests, including blood chemistries, hematology, prothrombin time, partial thromboplastin time, urinalysis, and serology for hepatitis B surface antigen and antibodies to syphilis and to human immunodeficiency virus. Volunteers were excluded on the basis of persistent significant abnormalities or positive test results. Female volunteers were eligible to participate if they had a negative pregnancy test result within 48 hours of vaccination and stated in the consent form that they would avoid conception by using effective contraception for three months following vaccination. In addition, volunteers were excluded if they had a history of allergy to neomycin, streptomycin, or gentamicin compounds used to manufacture the vaccine candidates, or evidence of flavivirus immunity, which may affect responses to DENV vaccine candidates.[11] Flavivirus immunity was determined by the presence of hemagglutination-inhibition (HAI) antibodies against DENV types 1-4, Japanese encephalitis, or yellow fever viruses detectable at a serum dilution $\geq$ 1:10, a history of yellow fever or Japanese encephalitis vaccination, or documented flavivirus infection.

Volunteers were also required to score $\geq$ 70% on a written examination designed to test knowledge of all aspects of the clinical trial. Informed consent was subsequently obtained from each volunteer in compliance with U.S. 21 CFR Part 50-Protection of Human Subjects. The clinical protocol conformed to all relevant regulatory requirements, including the Declaration of Helsinki (Protocol), and Army Regulations 70-25-Use of Volunteers as Subjects of Research, and 40-7-Use of Investigational Drugs in Humans and the Use of Schedule I Controlled Substances. The studies were reviewed

and approved by the Human Subject Research Review Board, Office of the Surgeon General, U.S. Army, the WRAIR Human Use Research Committee, and the Institutional Review Board, University of Maryland at Baltimore.

Study vaccine candidates. Production and preclinical testing of DENV vaccine candidates are described in this supplement.[9] Before trial in volunteers, each vaccine candidate was confirmed to elicit substantially reduced viremia compared with its wild-type parent virus when administered to rhesus monkeys. Adequate modification measured by infection of rhesus monkeys indicated that the DENV vaccine candidate strains were appropriate for human testing. The selected study vaccine candidates are listed in Table 1.

Immediately before immunization, each vial of lyophilized vaccine candidate was reconstituted with sterile water for injection (United States Pharmacopeia). After immunization, unused portions of rehydrated vaccine candidate were maintained on ice and titrated within four hours in LLC-MK_2 cell monolayers.[12] Each volunteer received between 0.3×10^5 and 6.8×10^6 plaque forming units (PFU) of virus, depending on the injected candidate vaccine candidate (Table 1).

Study design. A standard randomized, single-blind, inpatient clinical protocol was used for all studies. The majority of the studies were conducted from 1991 to 1993 at the Center for Vaccine Development, University of Maryland (Baltimore MD). The pilot studies of DENV-2 S16803 PDK 40 vaccine candidate and DENV-4 341750 PDK 20 vaccine candidate were performed in 1996 and 1988, respectively, at the Medical Division, United States Army Medical Research Institute of Infectious Diseases (USAMRIID) (Fort Detrick, Frederick, MD).

These pilot clinical studies evaluated the safety of different vaccine candidates. Thus, the highest available passage for a particular strain, presumed to be the most attenuated, was tested first in three volunteers. Symptoms were monitored closely for three weeks, and if the volunteers remained well, the next available lower passage virus was tested. If one or more of the volunteers became ill, testing of lower passages of the vaccine candidate strain was not performed because it was presumed that lower passages were likely to be less attenuated. After testing of all acceptable passage levels in at least three volunteers, the lowest level that caused no illness was selected for testing in additional volunteers.

To allow careful observation, prevent exposure to other infectious diseases, and to prevent the possible infection of vector mosquitoes, volunteers were confined to the research ward from three days prior to inoculation until 20 days after immunization. All adverse experiences occurring within this period following administration of each vaccine candidate were recorded, regardless of severity or whether they were considered vaccination related. Acceptable safety of a vaccine candidate was defined by the protocol as the absence of the following serious adverse events: any severe clinical illness not explained by a diagnosis unrelated to the vaccination; persistent fever (oral temperature $\geq$ 38.5°C for four determinations over a 24-hour period, a maximum daily oral temperature $\geq$ 38.5°C on three successive days, or a temperature exceeding 40°C on any individual determination); thrombocytopenia (< 100,000 platelets/mm^3) or leukopenia (absolute neutrophil count < 1,000/mm^3) on two consecutive determinations; or a serum alanine aminotransferase (ALT) level of more than four times normal on three or more successive days that was otherwise unexplained. In addition, any experience that would suggest a significant adverse event potentially associated with the use of the vaccine candidate was documented as a serious adverse event.

Clinical evaluation. Volunteers were inoculated subcutaneously with 0.5 mL of undiluted vaccine candidate on study day 0. After immunization, vital signs were recorded every six hours. The injection site was examined and the maximum diameter of erythema and induration was measured and recorded daily. Clinical signs (fever > 37.8°C, rash, vomiting, petechiae, and liver and spleen enlargement) and symptoms (malaise, headache, myalgia, arthralgia, nausea, and eye pain or photophobia) were assessed daily for 21 days after vaccination. Symptoms were graded as none, mild (noticed symptom but continued ward activity), moderate (symptom interfered with normal ward activity), or severe (forced to bed by symptom). If requested by the volunteer, painful symptoms were treated with propoxyphene hydrochloride; antipyretics other than acetaminophen were not used. Observations were recorded on a standard checklist of symptoms and physical findings. Volunteers were discharged from the study ward on study day 21, and requested to return for serologic studies 1, 6, 12, and 24 months after vaccination.

Two healthy yellow fever–immune volunteers were immunized at USAMRIID in 1986 with a DENV-3 CH53489 (cl 24/28) PDK-0 vaccine candidate.[13] Medical records from the

TABLE 1

Walter Reed Army Institute of Research live-attenuted dengue virus (DENV) vaccine candidates*

Vaccine candidate	PDK passage level	Year	Study site	No. of volunteers	Dose ($\times 10^5$ PFU)	Reference
DENV-1	0	1984	USAMRIID	2	0.3	20
45AZ5	10	1991, 1992	CVD	9	2.8–3.5	10
	20	1991, 1992	CVD	10	7.7–38	
	27	1991	CVD	10	4.4–45	
DENV-2	30	1991, 1992	CVD	10	5.6–10	
S16803	40	1996	USAMRIID	3	5.0	
	50	1991	CVD	3	6.8	
DENV-3	0	1986	USAMRIID	2	1.8	13
CH53489	10	1992	CVD	3	3.8	
	20	1992	CVD	6	1.0–1.4	
DENV-4	15	1991	CVD	3	4.8	
341750	20	1988	USAMRIID	8	1.0	14

* PDK-primary dog kidney; PFU = plaque-forming units; USAMRIID = United States Army Medical Research Institute of Infectious Diseases, Fort Detrick (Frederick, MD); CVD = Center for Vaccine Development, University of Maryland (Baltimore, MD).

study were reviewed for presence or absence of the following signs and symptoms: fever, rash, malaise, headache, myalgia, arthralgia, and eye pain or photophobia. Viremia was measured daily. In contrast to the present trials, symptoms were not systematically recorded, and the intensity of symptoms was not graded. In addition, clinical experience with the DENV-4 341750 Carib PDK 20 vaccine candidate, given to eight volunteers at USAMRIID in 1988 and previously reported,[14] was summarized for comparison with that of the DENV-4 PDK 15 vaccine candidate.

Laboratory evaluation. Blood was collected from volunteers every other day through day 20 and on day 31 for testing of hemoglobin and hematocrit, white blood cell count with differential count, platelet count, and aspartate aminotransferase and ALT levels. In addition, blood was collected every other day through day 20 for virus isolation and antibody studies. Blood (20 mL) was allowed to clot at 4°C for ≤ 2 hours, and serum was decanted into 1-mL aliquots, frozen, and stored at -70°C until studied.

For determination of dengue viremia, serum was thawed and inoculated onto C6/36 *Aedes albopictus* mosquito cell monolayers and incubated at 28°C for 14 days. Supernatant culture fluid harvests were assayed for virus by plaque assay on LLC-MK_2 cells.[15] To quantitate the amount of virus in serum, a plaque assay was performed on C6/36 mosquito cells.[14] Cell culture flasks were inoculated with dilutions of plasma and adsorbed at 35°C for 1–2 hours. An overlay medium consisting of Hanks' balanced salt Solution and 0.75% agarose, 5% lactalbumin hydrolysate, 0.12M $NaHCO_3$, and antibiotics was added and all flasks were incubated at 35°C. After seven days, the flasks were stained with 5% liquid neutral red for 3–5 hours. Excess stain was removed and the plaques were read after 18 hours.

Tests for antibodies to DENV included an enzyme-linked immunosorbent assay (ELISA) and the HAI assay, as well as plaque reduction neutralization tests (PRNTs) performed using a DENV of the same serotype as the strain in the vaccine candidate being tested. Detection of IgM antibodies to DENV was performed by modification of an ELISA, where values > 0.10 optical density units were considered positive.[16] The HAI test was performed by the standard technique modified to microvolumes using 4–8 units of individual antigens and serum extracted with acetone to remove inhibitors.[17] The PRNT assays were performed in Vero cells by a modification of the method described by Russell and others,[18] in which the input viruses were low-passage parent vaccine candidate viruses.[19] The PRNT end points were defined as the lowest dilution that gave a 50% reduction in plaques ($PRNT_{50}$); a titer ≥ 1:10 was considered positive.

Definition of infection by the vaccine candidate. Infection by vaccine candidate was defined as replication of DENV in the volunteer, indirectly detected by the appearance of serum type-specific neutralizing antibody or IgM antibody to DENV antibody after immunization.[10] Viremia was not included as necessary for diagnosis of infection because it was never detected in the absence of an antibody response. A vaccine candidate failure was defined as an unacceptable adverse clinical response or failure to develop convalescent IgM or IgM or PRNT antibodies to DENV.

Statistical analysis. The relationship between passage level and the frequency and intensity of reactogenicity was analyzed, for DENV-2 vaccine candidate S16803 (PDK 30, 40, and 50) and for DENV-3 vaccine candidate CH53489 (PDK 10 and 20), using the Cochran-Armitage test for trend and Spearman's correlations, respectively. The symptoms and signs independently analyzed included the presence or absence and the number of days experiencing eye symptoms, headache, malaise, myalgia, arthralgia, rash, and fever (temperature > 37.8°C). The null hypothesis of equal reactogenicity at lower or higher PDK passages was evaluated at a probability of 5%.

By inspection of the data, the optimal passage level for each DENV vaccine candidate was determined based on the clinical and immunologic responses of each volunteer. The vaccine candidate which was well tolerated but which immunized approximately 80% of the volunteers was selected for further development.

RESULTS

Serious adverse events. None of the 65 recipients of WRAIR attenuated DENV vaccine candidates developed serious adverse experiences that were fatal, life-threatening, or resulted in hospitalization or prolongation of their ward stay. Conversely, three volunteers experienced idiosyncratic reactions that were considered severe (i.e., they substantially disrupted the subject's ability to carry out normal activities) following receipt of DENV-2 PDK 40, DENV-3 PDK 10, or DENV-4 PDK 15 vaccine candidates. These transient and self-limited responses are described in detail later in this report. On the basis of the illnesses experienced by these volunteers, these vaccine candidates were considered unsafe for continued administration in volunteers. No volunteers developed signs of hepatomegaly, splenomegaly, petechiae, or hemorrhage. No local reactogenicity (erythema, induration, warmth) was observed at injection sites following vaccination.

Clinical responses to attenuated DENV vaccine candidates. *DENV-1 45AZ5 vaccine candidates.* Vaccine candidates were prepared from three levels of PDK cell-passaged virus (PDK-10, PDK-20, and PDK-27) and injected into 9–10 volunteers.[10] There was a significant progressive decrease in reactogenicity and in hematologic changes from low to high PDK passage level, indicating progressive attenuation of the vaccine candidate strain for humans.

DENV-2 S16803 vaccine candidates. The initial clinical experience with the WRAIR DENV-2 S16803 vaccine candidates is summarized in Figure 1.

PDK-50 vaccine candidate. The DENV-2 PDK-50 vaccine candidate was tested in three volunteers. None developed oral temperatures ≥ 38.0°C, and two volunteers had only transient mild symptoms of malaise, headache, and eye symptoms (eye pain or photophobia). Laboratory findings included transient ALT elevations (< 3 times normal) in two of three, and mild leukopenia in one of three volunteers. Because of the acceptable safety profile of the PDK-50 vaccine candidate, the next lower available passage, PDK-30, was selected for clinical evaluation.

PDK-30 vaccine candidate. The PDK-30 vaccine candidate, which was tested in 10 subjects, produced symptoms compatible with mild-to-moderate dengue fever and was considered under-attenuated. Four volunteers (40%) developed low-grade fever (T_{max} = 38.5°C), over days 9–14 post-vaccination (median day 12) and 80% developed a rash. The majority of volunteers experienced eye symptoms (10 of 10), headaches

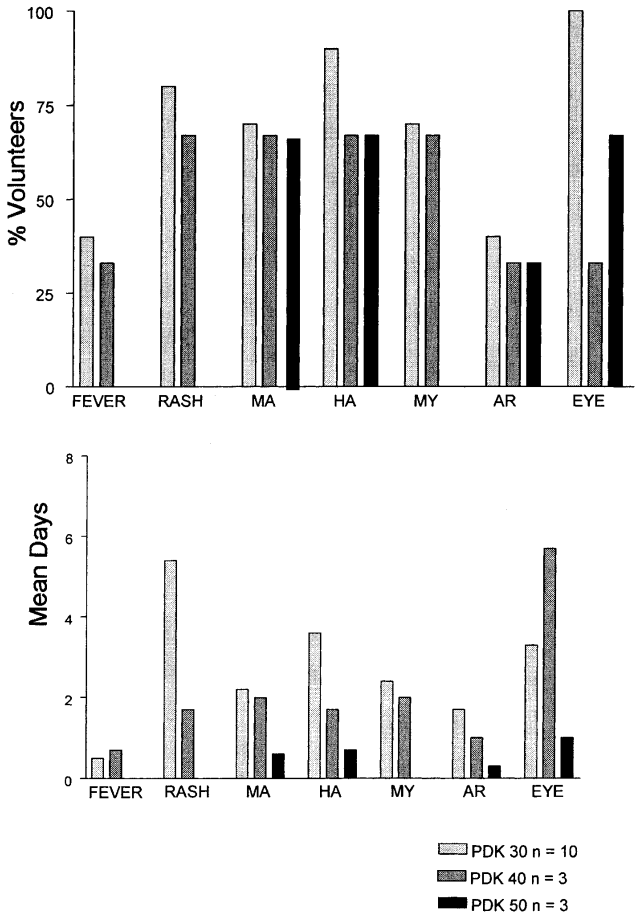

FIGURE 1. Clinical responses in recipients of dengue-2 S16803 virus vaccines. MA = malaise; HA = headache; MY = myalgia; AR = arthralgia; EYE = eye pain or photophobia; PDK = primary dog kidney.

(9 of 10), and malaise (9 of 10), while 70% had ≥ one or more severe symptom of headache, eye pain and photophobia, malaise, or myalgia. Three volunteers had mild elevation of their ALT levels.

PDK-40 vaccine candidate. Subsequently, the PDK-40 vaccine candidate became available and was tested in three volunteers. Two volunteers developed low-grade fever (< 38.1°C) with mild myalgias, headache, and rash 9–10 days after vaccination. Symptoms resolved spontaneously over a 2–3-day period without disability or requirement for medication. Accompanying symptoms were an unanticipated increase in serum liver enzyme levels to a maximum ALT level of 199 IU/mL in one (four-fold increase above normal) and a maximum ALT level of 77 IU/mL in the other (1.5-fold increase above normal). The third volunteer remained asymptomatic but also developed two-fold increases in the ALT level (to a maximum of 102 IU/mL). All laboratory abnormalities resolved within days without intervention, and all volunteers were in good health 21 days after receipt of the vaccine candidate. Because of the uniform occurrence of increase ALT levels in recipients of the PDK-40 vaccine candidate, consistent with mild injury to hepatocytes or other cells, no further development is planned for the product.

As shown in Figure 1, there was a trend towards decreased frequency of signs of fever and rash for the DENV-2 vaccine candidate between PDK-30 and PDK-50 passage levels. Furthermore, there was a decrease in oral temperature from a T_{max} of 38.5°C towards normal with increasing passage, but no change in duration of fever beyond one day. For the DENV-2 vaccine candidates, the frequency of eye symptoms, rash, headache, malaise and myalgia, and the duration of eye symptoms, rash, malaise, and fever were significantly associated with passage level.

DENV-3 CH53489 vaccine candidates.

PDK-0 vaccine candidate. The DENV-3 CH53489 (cl 24/28) PDK-0 vaccine candidate developed at WRAIR was administered to two healthy yellow fever–immune male volunteers in 1984 as a 0.5-mL subcutaneous inoculation of 2×10^4 PFU of virus.[13] The immediate post-immunization course was uneventful. By day 6, both volunteers were ill with dengue fever characterized by high fever, chills, myalgias, headache, malaise, and a diffuse erythematous rash. Both volunteers developed thrombocytopenia and leucopenia, but there were no signs of hemorrhagic fever. After a febrile period lasting five days, both men rapidly recovered and were well by day 21. Because of the severe illnesses experienced by both subjects, no further testing of this passage level was undertaken. Subsequently, PDK-10 and PDK-20 vaccine candidates were prepared from serial passage of the DENV-3 parent virus.

PDK-20 vaccine candidate. The PDK-20 vaccine candidate was given to six volunteers and resulted in mild reactogenicity. One subject experienced an early febrile illness on day 3 with transient fever (T_{max} = 38.2°C), pharyngitis, and cervical lymphadenopathy. No DENV was isolated from the volunteer's serum. This subject was felt to have had an intercurrent illness with fever unrelated to vaccination. Four of six volunteers developed short-lived symptoms of arthralgia, eye pain, and headache without rash. However, one volunteer had moderate symptoms of headache, malaise, and eye pain for three days (days 10–12) without fever. He also developed leukopenia and a sustained increase in the ALT level (maximum = 222 IU/mL); these laboratory abnormalities had resolved by follow-up at day 20. Another volunteer had a mild and reversible increase in the ALT level to less than twice the normal level. Because the DENV-3 PDK 20 vaccine candi date was safe with acceptable reactogenicity, the next lowest available passage vaccine candidate virus (PDK-10) was tested.

PDK-10 vaccine candidate. The PDK-10 vaccine candidate was unacceptably reactogenic. One of three volunteers developed low-grade fever on days 10 and 11 (T_{max} = 38.3°C), and a florid rash for 13 days. Another volunteer developed persistent pruritus associated with waxing and waning hives on days 6–9 post-vaccination, and tender cervical and axillary lymph nodes. He subsequently developed a maculopapular rash with malaise, headache, and myalgia on days 10–12, but without fever. This volunteer may have had an idiosyncratic allergic reaction to the vaccine candidate. These two volunteers also had leukopenia and increases in ALT levels to more than three times the normal level that resolved on follow-up on day 32.

The response to DENV-3 CH53489 vaccine candidates is summarized in Figure 2. Although there was a trend for less frequent and shorter duration signs and symptoms with increasing passage, none reached statistical significance in either analysis.

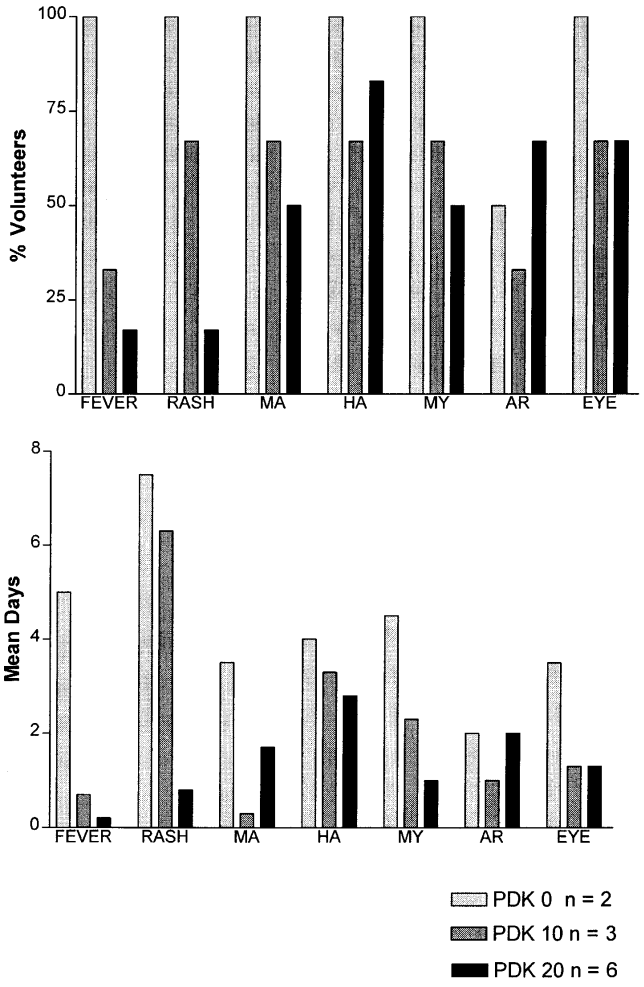

FIGURE 2. Clinical responses in recipients of dengue-3 CH53489 virus vaccines. MA = malaise; HA = headache; MY = myalgia; AR = arthralgia; EYE = eye pain or photophobia; PDK = primary dog kidney.

DENV-4 341750 vaccine candidates.

PDK-20 vaccine candidate. Eight volunteers received 10^5 PFU of PDK-20 vaccine candidate.[14] Five volunteers developed a scarcely noticeable macular, blanching rash and a minimal temperature increase (T_{max} = 38.1°C).

PDK-15 vaccine candidate. Three volunteers received this vaccine candidate and two experienced minimal symptoms. The third volunteer became acutely ill on day 8 with fever, edematous swelling of face and extremities, severe lassitude, rash, eye pain, photophobia, and arthralgias. Over the next three days, fever persisted with a T_{max} = 39.6°C, but signs and symptoms resolved spontaneously. Because of this severe adverse response to vaccination, further use of this passage level was terminated.

Viremia and immune responses to attenuated DENV vaccine candidates. Viremia and immune responses in volunteers receiving the WRAIR DENV-2, -3, and -4 vaccine candidates are shown in Table 2. The infectivity of the individual vaccine candidates, including the DENV-1 vaccine candidates, is summarized in this section.

DENV-1 45AZ5 vaccine candidates. The presence and mean duration of viremia decreased with progressive PDK cell passage from 100% incidence with low passages (PDK-0 and -10) to no detectable viremia after vaccination with PDK-27 virus.[10,20] In contrast, DENV-1 vaccine candidate immunogenicity, assessed as the frequency of neutralizing antibody seroconversion, was similar for candidates prepared at passage levels 0, 10, and 20 (80–100%), but appeared to decrease approximately two-fold to 40% for the vaccine candidate prepared at passage level 27. Interestingly, no neutralizing antibody was detected in three subjects in whom vaccine candidate virus infections were documented by detection of viremia or appearance of specific IgM antibody.[10]

DENV-2 S16803 vaccine candidates. No recipients of the PDK-50 vaccine candidate developed viremia; however, two of three developed low-titer neutralizing antibody by day 60. These findings suggested that the vaccine candidate virus was diminished in infectivity for humans. In contrast, two of three DENV-2 PDK-40 vaccine candidates had demonstrable viremia, and all three subjects developed high-titer neutralizing antibody after vaccination. As expected, infectivity of the DENV-2 PDK-30 vaccine candidate was highest: viremia was detected in all 10 volunteers, and all subjects seroconverted by day 60 with neutralizing antibody titers > 1:60 by day 60.

DENV-3 CH53489 vaccine candidates. DENV-3 retaining temperature sensitivity and small plaque phenotype of the vaccine candidate virus was recovered for six and seven days in the two yellow fever–immune recipients of the DENV-3 CH53489 (cl 24/28) PDK-0 vaccine candidate.[13] Subsequently, high-titered $PRNT_{50}$ and HAI antibody with a sec-

TABLE 2
Viremia and immune responses to Walter Reed Army Institute of Research dengue virus (DENV)-2, -3, and -4 vaccine candidates*

Vaccine candidate-PDK passage level	Viremia†			Seroconversion‡			GMT§	
	Virus isolation	Days detected (midpoint)	Virus titer (PFU/mL)	IgM	HAI	$PRNT_{50}$	Day 31	Day 60
DENV-2-30	10/10	6–12 (10)	3–1200	6/9	4/9	9/9	343	208
DENV-2-40	2/3	6–10 (8)	NA	3/3	2/3	3/3	640	561
DENV-2-50	0/3	–	–	1/3	0/3	2/3	50	20
DENV-3-0	2/2	3–10 (6)	NA	2/2	2/2	2/2	2,818	1,995
DENV-3-10	2/3	6–10 (8)	84–6,600	3/3	3/3	3/3	710	153
DENV-3-20	2/6	8–12 (10)	12–138	2/6	1/6	3/6	485	75
DENV-4-15	1/3	8–10 (9)	3–15	3/3	3/3	3/3	36	140
DENV-4-20	5/8	8–14 (10)	10–1,200	5/8	5/8	5/8	196	35

* GMT = geometric mean titer; PDK = primary dog kidney; PFU = plaque-forming units; HAI = hemagglutination inhibition; $PRNT_{50}$ = 50% plaque reduction neutralization test.
† Defined as positive virus isolation, with days of virus isolation and range of virus titer (in PFU/mL) detected after dengue vaccination.
‡ Defined as an IgM antibody value > 0.10 optical density units, an HAI antibody titer ≥ 1:10, or a neutralizing antibody titer ≥ 1:10 ($PRNT_{50}$).
§ Neutralizing antibody titer present 31 and 60 days following dengue vaccination.

ondary-infection-like cross reactivity was measured in serum collected on days 30 and 60 from both volunteers. Infectivity was similar in subjects who received the DENV-3 PDK-10 vaccine candidate: two of three developed viremia and vaccination induced neutralizing antibodies in all. In contrast, two of six DENV-3 PDK-20 vaccine candidates had detectable viremia, and only three volunteers subsequently seroconverted, suggesting diminished infectivity.

DENV-4 341750 vaccine candidates. Eight volunteers received 10^5 PFU of the PDK-20 vaccine candidate, and viremia and antibody response developed in five (63%).[14] The vaccine candidate prepared from a lower passage of this candidate, PDK-15, appeared to have been more infective. Virus was isolated from a single volunteer on days 8 and 10 following vaccination with a maximum titer of 15 PFU/mL. This volunteer developed a neutralizing antibody titer of 120 with an apparent secondary HAI response, and evaluation after the study showed that he had been previously exposed to St. Louis encephalitis virus (PRNT titer = 1:20 before vaccination). The two volunteers without detectable viremia developed neutralizing titers of 1:10 and 1:40 by day 31 after vaccination.

Selection of WRAIR DENV vaccine candidates. The results of the extended program of safety testing of the WRAIR PDK-attenuated DENV vaccine candidates are shown in Table 3, which lists the salient features of the vaccine candidates for each serotype. Increasing PDK cell passage resulted in decreasing mean illness score, which assessed duration and number of symptoms per volunteer. Increased PDK cell passage was also associated with decreased mean days of viremia, with the exception of DENV-4 vaccine candidates. The percentage of recipients infected decreased with increasing PDK cell passage level, as did the observed proportion seroconverting, defined as the percentage of recipients with neutralizing antibody titer $\geq$ 1:10. However, decreased immunogenicity of the higher passage vaccine candidate candidates for any serotype strain set is uncertain due to broad, overlapping confidence intervals. Of the tested DENV-2, -3, and -4 vaccine candidates, only one passage level each was judged acceptably reactogenic and suitable for expanded clinical study: DENV-2 PDK-50, DENV-3 PDK-20, and DENV-4 PDK-20.

DISCUSSION

Both the WRAIR and Mahidol DENV vaccine programs have developed several live vaccine candidates by attenuation through serial passage in PDK cells. The results of the pilot testing in small numbers of volunteers established that the WRAIR DENV vaccine candidates were not unacceptably reactogenic: no volunteers among 65 recipients developed responses that met or exceeded the defined stopping criteria for the study. Experimental infection following DENV vaccination, while occasionally uncomfortable, was tolerable and never required treatment of sustained serious injury. Three volunteers had transient idiosyncratic adverse reactions associated with DENV vaccination, resulting in withdrawal of the vaccine candidates they received from further clinical development.

The clinical experience demonstrated that increasing PDK cell passage of vaccine candidate viruses increased attenuation for volunteers. This effect is best seen with DENV-1 and DENV-3 viruses, in which PDK-0 viruses resulted in clinical dengue fever, while 20 PDK cell passages resulted in acceptable reactogenicity for these virus strains. However, increasing PDK cell passage decreased infectivity of vaccine candidate viruses, resulting in diminished immunogenicity. Furthermore, diminished viremia in humans appears to correlate with the experience in rhesus monkeys, with the exception of DENV-4 PDK-15.[9] These findings suggest that infectivity of an attenuated DENV vaccine candidate in volunteers was closely related to its immunogenicity.

The relationship between passage level and reactogenicity should be interpreted with caution because subjects who experienced one symptom were likely to experience several symptoms. Since our analytic methods assume independence of these symptoms, interpretations based on independent P values can be tenuous. However, rash showed a strong association with passage level (independent $P = 0.009$ for presence, $P = 0.01$ for duration). This is bolstered by a lack of significant correlation between rash and other symptoms for either DENV-2 or DENV-3 vaccine candidates (Spearman's tests).

Only vaccine candidates with acceptable reactogenicity profiles were selected for expanded clinical testing: DENV-1 45AZ5 PDK-20, DENV-2 S16803 PDK-50, DENV-3 CH53489 PDK-20, and DENV-4 341750 PDK-20. The reactogenicity of these vaccine candidates is similar to that observed in American volunteers receiving attenuated Aventis Pasteur/Mahidol vaccine candidate viruses.[21] Because of the broad confidence intervals in seroconversion due to small numbers of volunteers, subsequent studies will increase the

TABLE 3

Results of initial safety and immunogenicity trials of Walter Reed Army Institute of Research dengue virus (DENV) vaccine candidates*

Vaccine candidates	PDK passage level	Mean duration of viremia (days)	Mean illness score	Reactogenicity	No. infected†/ no. vaccinated	No. seroconverted‡	% seroconversion (95% confidence limit)
DENV-1	10	5.0	3.0	Acceptable	7/9	7	78 (40–97)
45AZ5	20	1.0	3.6	Acceptable	10/10	10	100 (69–100)
	27	0.0	1.7	Acceptable	7/10	4	40 (12–74)
DENV-2	30	2.2	19.1	Not acceptable	10/10	10	100 (69–100)
S16803	40	1.7	14.7	Not acceptable	3/3	3	100 (29–100)
	50	0.0	5.0	Acceptable	2/3	2	67 (9–99)
DENV-3	10	2.3	15.3	Not acceptable	3/3	3	100 (29–100)
CH53489	20	0.6	11.0	Acceptable	3/6	3	50 (12–88)
DENV-4	15	0.6	20.7	Not acceptable	3/3	3	100 (29–100)
341750	20	3.8	6.6	Acceptable	5/8	5	63 (24–91)

* PDK-primary dog kidney.
† Defined as positive for IgM antibody to dengue virus or 50% plaque reduction neutralization test ($PRNT_{50}$) seroconversion.
‡ Defined as a neutralizing antibody titer $\geq$ 1:10 ($PRNT_{50}$).

number of recipients of each of the four selected vaccine candidates. In addition, further tests will seek to determine whether immunogenicity of these attenuated vaccine candidates can be boosted through administration of two doses instead of the single dose used for these studies.

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