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Safety and immunogenicity of attenuated dengue virus vaccines (Aventis Pasteur) in human volunteers

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Abstract

A randomized, controlled, double-blinded study was conducted to determine safety and immunogenicity of five live attenuated dengue vaccines produced by Aventis Pasteur (AvP). The study was completed with 40 flavivirus non-immune volunteers: five recipients of each monovalent (dengue-1, dengue-2, dengue-3, or dengue-4) vaccine, ten recipients of tetravalent (dengue-1, dengue-2, dengue-3, and dengue-4) vaccine, and ten recipients of vaccine vehicle alone. All vaccines were administered in a single subcutaneous dose (range, 3.6–4.4 $\log_{10}$ plaque forming units). No serious adverse reactions occurred in volunteers followed for 6 months after vaccination. Five vaccine recipients developed fever ($T \geq 38.0$°C), including four tetravalent vaccinees between days 8 and 10 after vaccination. Dengue-1, dengue-2, dengue-3, or dengue-4 vaccine recipients reported similar frequency of mild symptoms of headache, malaise, and eye pain. Tetravalent vaccinees noted more moderate symptoms with onset from study days 8–11 and developed maculopapular rashes distributed over trunk and extremities. Transient neutropenia (white blood cells $<4000/mm^3$) was noted after vaccination but not thrombocytopenia (platelets $<100000/mm^3$). All dengue-3, dengue-4, and tetravalent vaccine recipients were viremic between days 7 and 12 but viremia was rarely detected in dengue-1 or dengue-2 vaccinees. All dengue-2, dengue-3, and dengue-4, and 60% of dengue-1 vaccine recipients developed neutralizing and/or immunoglobulin M antibodies. All tetravalent vaccine recipients were viremic with dengue-3 virus and developed neutralizing antibodies to dengue-3 virus. Seven volunteers also had multivalent antibody responses, yet the highest antibody titers were against dengue-3 virus. The AvP live attenuated dengue virus vaccines are safe and tolerable in humans. The live attenuated tetravalent dengue vaccine was most reactogenic, and preferential replication of dengue-3 virus may have affected its infectivity and immunogenicity. Published by Elsevier Science Ltd.

Keywords: Dengue; Vaccine; Tetravalent; Virus

1. Introduction

Dengue is the leading arboviral infection of humans [1]. Four serotypes of dengue virus are transmitted by *Aedes* spp. mosquitoes, and may result in incapacitating acute febrile illness in non-immune adults [2]. Occasionally, dengue may be complicated by hemorrhagic fever, which is fatal in about 0.5% of cases [3]. Following the explosive growth of the principal mosquito vector, endemic and epidemic dengue has spread throughout

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Central and South America, Southeast Asia, India, Africa, and the Caribbean and Pacific regions [4]. Vector control and other preventive measures have been limited in effect, often expensive and difficult to enforce. No specific therapy is available to prevent or ameliorate illness.

A safe and accessible dengue virus vaccine is needed to protect children and non-immune adults in regions with endemic or epidemic dengue. Ideally, vaccination should confer protection against all four dengue virus serotypes. Dengue vaccines using attenuated viruses have recently been shown to be protective against infection in animals [5]. Attenuated vaccines have several advantages over other vaccines: (1) as replicating agents, they induce both humoral and cellular immune responses; (2) they may immunize with a single dose; and (3) they may be produced at relatively low cost.

The Aventis Pasteur (AvP) attenuated tetravalent dengue virus vaccine was created from attenuated candidate vaccine viruses developed and studied in Thailand. These viruses were studied individually and in combination in clinical studies conducted by the Center for Vaccine Development, Mahidol University (Institute of Sciences and Technology for Development, Virus Production, Mahidol University Salaya campus, Nakhon Chaisri, Nakhon Pathom, Thailand) [6]. Working virus seeds and vaccine batches were produced in good manufacturing practice facilities at AvP, and a phase I study was initiated in the United States to determine the safety and immunogenicity of these attenuated dengue viruses and of a tetravalent vaccine formulation composed of all four viruses.

2. Materials and methods

2.1. Study design

This study was a randomized, controlled, double blind trial of four AvP attenuated dengue viruses (type 1, 2, 3 and 4 dengue viruses) and a tetravalent dengue vaccine formulated from them. Eligible volunteers were randomly allocated to one of the four attenuated dengue viruses, attenuated tetravalent dengue virus vaccine, or placebo (Table 1). Placebo recipients were inoculated with cell culture fluid and stabilizer without dengue virus. Safety of the vaccines was evaluated by monitoring signs, symptoms, and laboratory parameters, while immunogenicity was evaluated by measurement of dengue antibody responses. Each volunteer was followed for 6 months following vaccination.

Forty healthy male or female adult volunteers, 18–50 years old, were enrolled in the study: five for each of the monovalent vaccines, ten tetravalent vaccine recipients, and ten placebo recipients. Volunteers were excluded for any existing acute or chronic medical conditions, pre-existing flavivirus antibody before vaccination, evidence of an abnormality in a clinical or laboratory finding, or pregnancy. Each volunteer provided written informed consent after passing a manda-

Table 1
Baseline characteristics

	Vaccine					
	Type 1	Type 2	Type 3	Type 4	Tetravalent	Placebo
Number of volunteers/group	5	5	5	5	10	10
Number of males	3	4	3	5	9	5
Race						
Asian	0	0	0	0	1	0
Black	4	3	5	3	2	8
White	1	2	0	2	6	1
Other	0	0	0	0	1	1
Age (years)						
18–20	0	0	0	0	1	0
21–30	1	1	3	1	4	4
31–40	2	3	1	4	3	3
41–50	2	1	1	0	2	3
Laboratory values						
White blood cells (thousands/mm^3)	7.1 (1.8)[a]	6.3 (1.1)	7.2 (1.4)	6.9 (1.4)	6.1 (1.0)	8.6 (2.8)
Hematocrit (mg/dl)	43 (4)	42 (3)	42 (3)	43 (2)	43 (2)	41 (4)
Platelets (thousands/mm^3)	250 (62)	235 (38)	271 (82)	230 (48)	221 (46)	252 (60)
ALT (U/L)	27 (8)	23 (11)	34 (21)	38 (17)	39 (12)	24 (7)
AST (U/L)	28 (8)	22 (6)	24 (7)	31 (11)	28 (8)	20 (3)

[a] Data presented as mean value (S.D.).

tory test describing the purpose, risks, benefits, and basic comprehension of the study. This protocol was approved by the Human Subjects Research Review Board of the Office of the Surgeon General, US Army and conducted in accordance with Army Regulation 70-25. Random allocation of eligible volunteers to each study group was carried out using computer-generated lists. Eight eligible volunteers were discharged from the study before vaccination: six for abnormal laboratory results, one for acute illness, and another left for personal reasons. Their vaccine was assigned to the next eligible volunteer.

2.2. Study vaccines

The vaccines were furnished by AvP as single dose lyophilized vials to be reconstituted with 0.5 ml diluent. Each dose was formulated to contain the following virus titer: dengue-1 16007, 3.73 $\log_{10}$ plaque forming units (pfu); dengue-2 16681, 4.37 $\log_{10}$ pfu; dengue-3 16562, 3.70 $\log_{10}$ pfu; and dengue-4 1036, 3.63 $\log_{10}$ pfu. The lyophilized tetravalent vaccine contained 3.47 $\log_{10}$ pfu dengue-1, 3.90 $\log_{10}$ pfu dengue-2, 3.70 $\log_{10}$ pfu dengue-3, and 3.80 $\log_{10}$ pfu dengue-4 viruses, and was obtained by mixture of the four monovalent bulk vaccines. Each dengue virus in the tetravalent vaccine was titered by indirect fluorescent focus assay in Vero cells cultivated on 96-well plates. Detection of dengue virus dose infecting 50% of Vero cells (cell culture infective $dose_{50}$) after 7 days in culture was performed using dengue type-specific monoclonal antibodies (provided by the Centers for Disease Control and Prevention).

Vaccine residuals contained < 10 mg/ml neomycin, < 3.9 ng/ml bovine serum albumin, stabilizer (sugars and amino acids without animal proteins), and no preservatives. The placebo consisted of vaccine vehicle alone (stabilizer and media without virus). Vaccine vials were labeled with lot number and for investigational use only.

The lyophilized vials were reconstituted on the day of administration (study day 0) by a designated unblinded vaccine preparer. The individual was furnished with a sealed envelope for each volunteer, containing the assigned vaccine on a randomization/vaccine administration sheet. One vial of the assigned vaccine was prepared for each volunteer. Vaccine from a single vial (0.5 ml) was drawn into a single 1 ml syringe with a 25 G needle and administered subcutaneously to the volunteer. In addition, two vials of each test article to be received by volunteers were selected for determination of residual virus titer and prepared identically to vials used for vaccine administration. After all volunteers received their assigned vaccines, contents of the two syringes for titration were immediately placed in culture for determination of titer without freezing.

2.3. Clinical evaluation

All vaccine recipients underwent regular history and physical examination for signs or symptoms of dengue fever. Twelve examinations were performed in the month following vaccination, including daily evaluation on the research ward during anticipated period of viremia and symptoms (days 7–12). In addition, volunteers were asked to monitor temperature twice daily with digital thermometers (Graham-Field Inc., Hauppauge, NY); study staff reviewed and recorded temperature logs at each visit. Subsequently, they were asked to return for serologic studies 2, 4, and 6 months after vaccination.

At each clinic evaluation, study investigators interviewed volunteers for the presence of local and systemic symptoms. Severity of symptoms was measured on a scale of mild (requiring no change in activity nor medication), moderate (requiring change in activity and/or medication), and severe (requiring bed rest or loss of work). Study investigators examined the injection site for local erythema, warmth, tenderness, or edema. Arm pain was graded as none, mild (present with full use of arm), moderate (limited use of arm), or severe (inability to use arm). Physical examination included determination of enlargement or tenderness of axillary lymph nodes, liver or spleen. Appearance, location, and intensity of any rashes or petechiae were noted.

Dengue fever was defined in the protocol as a sustained febrile illness characterized by prominent headache and myalgias, which is variably accompanied by either one or more minor manifestations: retro-orbital pain, arthralgia, chills, malaise, fatigue, anorexia, nausea, abdominal pain, back pain, or a diffuse erythematous rash. The illness may last 5–6 days or more. Either leukopenia (total white blood cell count less than $4000/mm^3$), thrombocytopenia (< 150000 platelets/mm^3), or transient low elevations of liver enzymes must also be present. All signs and symptoms return to normal by day 21. The diagnosis of dengue fever is confirmed by isolation of virus from blood obtained from the volunteer during the early stages of illness [7].

2.4. Laboratory assays

Routine clinical laboratory assays were performed at each study visit for white blood cell (WBC), platelet, and hematocrit, as well as serum creatinine, alanine aminotransferase (AST), and aspartate aminotransferase (ALT) levels. Sera were also obtained for virus culture and identification, and for investigation of dengue-specific antibodies. Confluent LLC-MK2 monkey kidney cells were used to amplify viruses from sera collected on days 2–21 after vaccination [8]. Culture fluid harvests were subsequently assayed for the presence of virus by plaque assay on confluent Vero cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assays were performed to identify viruses by type from sera collected on days 2–21 following vaccination [9]. The enzyme-linked immunosorbent assay for detection of anti-dengue immunoglobulin (Ig)M and IgG antibodies was performed as described by Innis et al. [10]. The plaque reduction neutralization ($PRNT_{50}$) assay was performed by the method described by Russell et al., with exogenous complement added to the virus diluent [11]. Antibody assays were run using all four dengue virus serotypes.

2.5. Statistical analyses

Data for each volunteer were recorded on attached data forms and kept in the volunteer's study chart. Serological and virological results were recorded from WRAIR laboratory notebooks onto the appropriate forms and forwarded to the volunteer's study chart. In addition, data for vaccine recipients were entered into a computer database constructed using a standardized database entry program (Microsoft Access, version 7.0; Microsoft Corporation, Redmond, WA). WRAIR staff provided and maintained the central database and software to create it. To preserve confidentiality, biographical information was maintained only in the volunteer's study workbook. All other forms and computer entries were indexed by study number, volunteer initials, and date. Data were analyzed using SAS (version 6.12; SAS Institute, Cary NC).

Seroconversion rates were compared between the tetravalent vaccine group and placebo control group using Fisher's exact tests. This trial, with ten volunteers per group, had 80% power for detecting a difference in seroconversion rates of 55% if the seroconversion rate in the placebo group is 5% or less, with a Type I error of 5% (one-sided). Because of the small sample size, the study had low power for detecting smaller differences between groups. Analysis of variance was used to compare mean values of variables calculated for each group of tetravalent, monovalent, and placebo volunteers.

2.6. Quality control

Clinical evaluations were conducted in accordance with good clinical practice. Clinical laboratory assays were performed according to Clinical Laboratory Improvement Amendments in a laboratory certified by the College of American Pathologists. Placebo recipients were also included as negative controls for clinical and laboratory evaluations by investigators blinded to vaccine assignment. Bar-coded specimens were furnished to participating laboratories; whenever possible, and serial specimens from the same volunteer were assayed in parallel.

3. Results

3.1. Study population

Table 1 summarizes the baseline characteristics of the 40 men and women in the study group. Twenty-nine (73%) of the volunteers were male, and 25 (62%) were black. Most of the volunteers (75%) were between the ages of 21 and 40 years. At baseline, the placebo study group had the highest mean white blood count, absolute neutrophil count (ANC), platelet count, and the lowest hematocrit and serum AST. The tetravalent group had the highest mean ALT and AST. The study groups were similar with respect to their vital signs and the results of the physical examinations.

3.2. Clinical responses

There were no severe nor serious adverse events following immunization, including anaphylaxis, severe clinical illness, or unremitting fevers due to the vaccine, pronounced thrombocytopenia, or serum ALT levels of more than tenfold above baseline. One dengue-3 vaccine recipient was hospitalized from days 41 to 70 for a prior medical condition unrelated to immunization. A placebo recipient was lost to follow-up, defined as failure to attend three successive scheduled visits. A tetravalent vaccine recipient required an unscheduled visit on day 19 for symptoms of moderate headache, right eye pain, photophobia, and conjunctivitis. This volunteer continued to have persistent vascular headaches, which had decreased by day 21. Because of previous history of migraine headache, he was referred for neurological evaluation, where he was found to have a normal neurologic examination and cranial magnetic resonance imaging. His symptoms of headache and eye pain resolved on low-dose nortriptyline.

No volunteer in the placebo group or in any of the monovalent groups showed evidence of dengue fever. Ten days after vaccination, two volunteers in the tetravalent group had elevated temperature, moderate headaches, myalgia, macular and papular rashes, and low white blood count; these clusters of symptoms satisfy the protocol definition of dengue fever. However, in neither volunteer was the magnitude or duration of febrile illness sufficient to meet the protocol definition of severe dengue fever (≥ 39.4°C and ≥ 72 h, respectively). No volunteer had dengue hemorrhagic fever.

Five volunteers in the study became febrile ($T \geq 38.0$°C) following immunization. One volunteer developed fever ($T_{max} = 38.4$°C) and a flu-like syndrome 3 days after receiving dengue-2 vaccine. These symptoms resolved completely by day 5, and dengue virus was not isolated from sera collected during his febrile illness.

Table 2
Responses to vaccines

	Vaccines					
	Type 1	Type 2	Type 3	Type 4	Tetravalent	Placebo
Symptoms						
Malaise	3	2	2	4	7[a]	1
Headache	3	4	3	3	10[a]	1
Myalgia	2	2	3	2	7	2
Eye sx	2	1	4	4	6[a]	0
Pruritis	1	2	3	2	9[a]	2
Signs						
$T > 38°C$	0	1	0	0	4	0
Arm pain	1	0	2	1	0	0
Local signs	1	0	3	0	2	1
Adenopathy	1	0	2	0	0	0
Rash	1	1	3	5	10[a,b]	1
Laboratory values						
WBC $< 4000/mm^3$	1	2	3	1	10[a,b]	0
ANC $< 1000/mm^3$	0	0	1	0	0	0
Platelets $< 150\,000/mm^3$	0	0	1	1	2	0
ALT ≥ 50 U/l	2	2	2	5	7	3
AST ≥ 42 U/l	2	2	2	4	6	3

[a] $P < 0.05$, tetravalent versus placebo.
[b] $P < 0.05$, tetravalent vesrus monovalent.

Four tetravalent vaccine recipients developed brief fevers: one with $T_{max} = 38.2°C$ on day 10, another with $T_{max} = 38.3°C$ on day 9, and a third with temperatures to 38.2°C on days 9 and 10. The fourth volunteer experienced steady fever from days 8–10 with temperatures ranging from 38.4 to 39.2°C.

Table 2 shows the frequency of common symptoms reported by vaccinees: malaise, headache, myalgia, and eye symptoms (combining eye pain, photophobia, and conjunctivitis). Symptoms of feverishness or chills, anorexia, nausea, vomiting, or abdominal pain were rare, transient, and always mild. The frequency of symptoms was not different among the dengue-1, dengue-2, dengue-3, and dengue-4 vaccine recipients. To simplify further analysis, responses to dengue-1, dengue-2, dengue-3, and dengue-4 vaccines are combined into a single monovalent vaccine group (total, 20 volunteers).

The most commonly reported symptoms in monovalent vaccine recipients was headache (13 of 20 volunteers, or 65%), malaise (55%), eye pain (50%), myalgia (45%), and pruritis (40%). The most commonly reported symptoms in tetravalent vaccine recipients were headache (ten of ten volunteers, or 100%), pruritis (90%), myalgia (70%), malaise (70%), and eye pain (50%). In contrast, placebo recipients reported fewer symptoms of myalgia (two of ten volunteers, or 20%), pruritis (20%), headache (10%), and malaise (10%). Frequency of malaise, headache, eye pain, and rash was significantly greater ($P < 0.05$) in tetravalent or monovalent vaccine recipients than in placebo recipients. Moreover, pruritis was also significantly more common in tetravalent vaccine recipients than in monovalent vaccine or placebo recipients ($P = 0.003$).

The majority of volunteers immunized with monovalent vaccines reported transient mild symptoms; a few vaccinees experienced moderate symptoms, often accompanied by rash and pruritis. One volunteer who received dengue-3 vaccine developed severe symptoms, with headache on day 16 that resolved by the next day. Tetravalent vaccine recipients reported more moderate symptoms of malaise, headache, and myalgia, with onset from days 8 to 11. These symptoms were short-lived and resolved spontaneously or with acetaminophen. Two tetravalent vaccinees also described moderate pruritis, which resolved with one dose of antihistamine. No tetravalent vaccine recipient reported severe symptoms.

Local signs at the injection site were infrequent (Table 2). Transient arm pain without limitation of arm motion occurred in four monovalent vaccinees. Six volunteers had erythema: three dengue-3, two tetravalent, and a single placebo vaccinee. Two volunteers reported local tenderness: one dengue-3 vaccinee on day 14, and a dengue-1 vaccinee on days 2, 10, and 11. No volunteer had induration, warmth, or edema at the injection site. Axillary lymphadenopathy was noted in the injected arm in one dengue-1 vaccine recipient and two dengue-3 vaccine recipients; the dengue-1 vaccinee also noted ipsilateral axillary tenderness on day 14. Liver or splenic enlargement was absent.

Twenty of 30 immunized volunteers had maculopapular and often pruritic rashes, which appeared on days 8–12 and resolved by day 16 (Table 2). In ten monovalent vaccinees, these rashes were subtle maculopapular rashes distributed over the face, trunk, and sometimes extremities. More overt rashes, typically blanching maculopapular rashes distributed over trunk and extremities, were noted in all tetravalent vaccine recipients. The frequency of rash was significantly greater ($P < 0.05$) in tetravalent vaccinees than in monovalent vaccine or placebo recipients. No petechial or purpuric rash was observed.

3.3. Laboratory evaluation

The frequency of neutropenia (WBC $< 4000/mm^3$ or absolute neutrophil count $< 1000/mm^3$), thrombocytopenia (platelet count $< 150000/mm^3$), or liver injury (elevation in serum alanine aminotransferase greater than five times normal) after immunization is shown in Table 2. Neutropenia was noted in 17 of 30 immunized volunteers: all tetravalent vaccinees, three dengue-3 vaccinees, two dengue-2 vaccinees, one dengue-1 vaccinee, and one type 4 vaccinee. Decreases in circulating white blood cell occurred predictably after day 9, with a nadir on day 11, with duration of 1–3 days (Fig. 1). In all cases, WBC became normal by day 14 after immunization. A dengue-3 vaccine recipient had two consecutive absolute neutrophil counts below $1000/mm^3$, one of $990/mm^3$ on day 10 and an ANC of $925/mm^3$ on the following day. The volunteer experienced no ill effects from this potential serious adverse event, required no treatment for the transient decrease in ANC and, by day 14, spontaneously regained his normal total white blood cell count and ANC.

Three volunteers developed thrombocytopenia: one dengue-3 vaccinee, one dengue-4 vaccinee, and two tetravalent vaccine recipients. Depression of platelet counts generally coincided with the onset of neutropenia, with duration of 2 days; all counts returned to normal by day 16 (Fig. 1). No volunteer had a platelet count below 100000 platelets/mm^3. Monovalent and tetravalent vaccines had a statistically significant effect on the mean WBC ($P < 0.001$), absolute neutrophil count ($P = 0.001$), and platelet count ($P = 0.005$) compared with placebo. These effects were strongest in tetravalent vaccine recipients. There were no statistically significant differences in mean hematocrit among the three groups.

Elevation of serum ALT above normal values was observed in 18 of 30 immunized volunteers and three placebo recipients (Table 2) ($P = 0.08$ using a Fisher's exact test). The largest observed elevations were 208 U/l in a placebo recipient and 126 U/l in a tetravalent vaccinee. An analysis of variance comparing mean values of ALT during days 9–14 did not show a significant difference between the placebo and vaccine recipients ($P = 0.62$). Serum AST elevations paralleled rises in ALT; the maximum levels were 178 U/l in a placebo recipient and 108 U/l in a type 2 vaccinee. Peak rises in these enzymes typically occurred on day 14. No abnormality was found in serum creatinine among study volunteers.

Twenty-two volunteers tested positive for virus isolation following immunization with attenuated dengue vaccines (Fig. 2). All dengue-3, dengue-4, and tetravalent vaccinees developed viremia, while virus isolation was rare in volunteers who received dengue-1 or dengue-2 vaccines. Viremia typically occurred from days 7 to 12 after vaccination, but was also occasionally present by day 5 in dengue-3, dengue-4, or tetravalent dengue vaccinees. Only a dengue-4 vaccine recipient had viremia after day 12, on day 14. No viremia was detected in the third week following immunization.

The RT-PCR assay also identified a similar peak of viremia in vaccinees, but with significantly fewer positives than obtained with virus isolation (Fig. 2). The mean number of days with a positive response for the

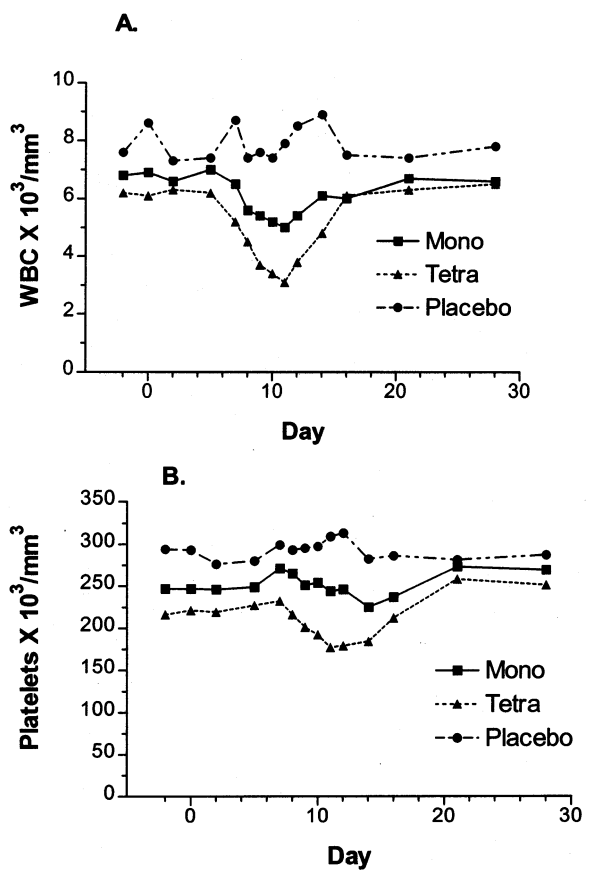

Fig. 1. Hematologic changes in recipients of AvP attenuated dengue virus vaccines. Anticoagulated blood specimens were obtained from volunteers following vaccination. Data shows the average white blood cell counts (A) and platelet counts (B) in recipients of monovalent vaccines (indicated by squares), tetravalent vaccine (triangles), and placebo (circles).

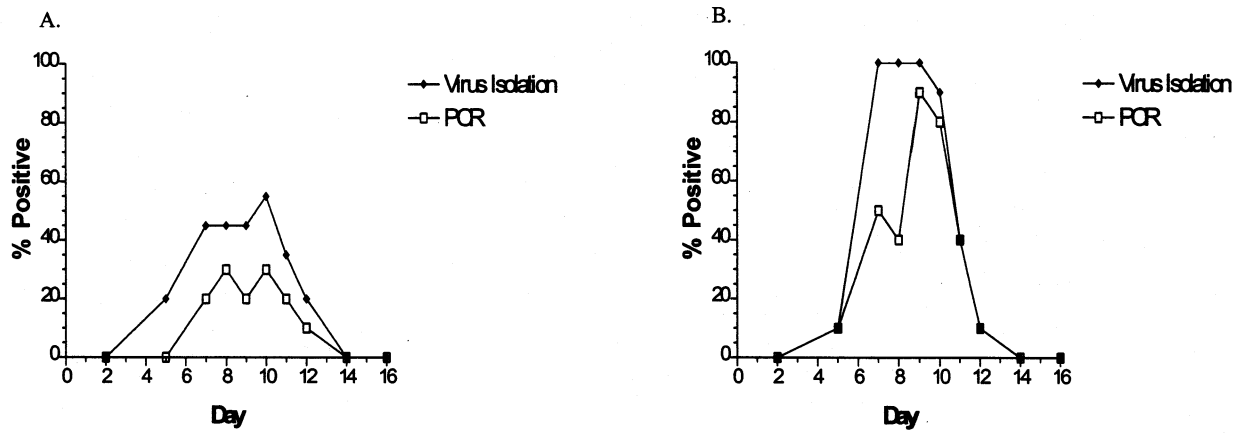

Fig. 2. Viremia in recipients of AvP attenuated dengue virus vaccines. Sera were collected from volunteers for 21 days following vaccination. Data show the percentage of vaccine recipients positive for virus by isolation (◆) and percentage of vaccine recipients positive for circulating dengue viral genome by RT-PCR (□). (A) Results for monovalent vaccines, (B) results for tetravalent vaccines. No placebo recipient was viremic by either assay, and no volunteers were viremic from 14 to 21 days after vaccination.

virus isolation assay (3.4 ± 0.4) was significantly higher than for the RT-PCR assay (1.9 ± 0.4), $t = 4.68$ (29 degrees of freedom) ($P = 0.0001$). However, the RT-PCR assay confirmed the type virus with which the volunteer was inoculated and never identified heterologous viral genomes. Viremia was detected in 20% of dengue-1 vaccinees, 20% of dengue-2 vaccinees, 80% of dengue-3 vaccinees, and 40% of dengue-4 vaccinees. Surprisingly, only dengue-3 virus was identified in all tetravalent vaccine recipients; one volunteer had concurrent dengue-3 and dengue-4 viremia on day 11.

All tetravalent, dengue-3, and dengue-4 vaccinees volunteers seroconverted by IgM assay, defined as IgM > 20 U on day 21; tetravalent vaccinees had the highest median IgM values (Fig. 2). Dengue-1 and dengue-2 vaccinees showed low rates of seroconversion (2/5 and 3/5, respectively), with lower titer IgM antibody. No placebo recipients developed IgM antibodies. IgG titers in volunteers were typically very low; seroconversion, defined as IgG > 20 U on day 28, was detected in five monovalent vaccinees. Three of these volunteers (a dengue-1, a dengue-2 and a dengue-4 vaccinee) demonstrated significantly more elevated IgG antibody titers compared with those observed in other vaccinees. There were no similar findings among tetravalent vaccinees.

Monovalent vaccine recipients experienced an overall seroconversion rate of 90% by the PRNT assay, defined as a $PRNT_{50}$ titer greater than 1:10. Antibody titers in monovalent vaccine recipients were highest against the type of dengue vaccine that was administered (homologous antibody). Geometric mean titers of homologous antibody declined with time after monovalent vaccination but remained positive to day 180 in 94% (17/18) of responders (Fig. 4). None of the placebo recipients developed detectable neutralizing antibodies. The three volunteers with elevated IgG responses also developed lesser neutralizing antibody responses against other type dengue viruses, suggesting exposure to flaviviruses prior to vaccination. Another dengue-4 recipient had transient antibodies to dengue-2 or dengue-3 virus after vaccination. Excluding the three possibly exposed volunteers from the analysis does not substantially alter the results.

The overall seroconversion rate in tetravalent dengue vaccine recipients was 100%, and seropositivity was maintained in all until day 180. All tetravalent vaccinees seroconverted, with the highest antibody titers, to dengue-3 virus (Fig. 3). Seven tetravalent vaccine recipients had antibody against more than one dengue virus, usually low titer antibody responses to dengue-1 and dengue-2 viruses. Antibody against dengue-4 virus at PRNT titer 220 was detected in only one tetravalent vaccine recipient, the sole volunteer with antibody against all four dengue viruses; another vaccinee developed trivalent responses (against dengue-1, dengue-2, and dengue-3 viruses) by day 180.

4. Discussion

The AvP monovalent and tetravalent attenuated dengue vaccines were safe and tolerable in volunteers. No severe adverse events or hospitalizations attributable to vaccination occurred in the Phase I trial. One dengue-3 vaccine recipient had clinically insignificant depression of absolute neutrophil count below $1000/mm^3$ for less than 48 h. No vaccine caused dengue fever as defined in the protocol, but one tetravalent vaccine recipient developed a dengue-like syndrome of 3 days duration.

The reactogenicity observed following receipt of the AvP dengue vaccines is consistent with the range of responses seen in recipients of other attenuated dengue

vaccines [12]. Monovalent vaccines resulted in transient mild symptoms of headache and myalgias with few detectable signs; the dengue-3 and dengue-4 vaccines were most reactogenic. The dengue-2 vaccine produced the least reported symptoms, consistent with the experience with Mahidol dengue-2 vaccine in Thai and American volunteers [13,14]. Similar but stronger responses were present in most recipients of tetravalent vaccine, who reported mild to moderate symptoms with fever and rash. Furthermore, there were detectable changes in WBC and platelet count following tetravalent vaccination. The combination of four attenuated strains, although never severe or unsafe, appeared to result in increased reactogenicity, consequently tolerability of these vaccines was diminished compared with monovalent vaccines.

Monovalent attenuated dengue vaccines induced virus serotype-specific antibody responses. All recipients of dengue-2, dengue-3, and dengue-4 vaccines seroconverted with homologous antibody, but frequency and magnitude of antibody responses were lower in dengue-1 vaccinees. The magnitude and pattern of these responses corresponded with the experience with the Mahidol vaccines [15]. In addition, the findings establish the correlation of vaccine viremia with induction of dengue antibody responses observed with other attenuated dengue vaccines [12,16].

After observing good immune responses following monovalent vaccination, neutralizing antibody responses after tetravalent dengue vaccination were predominantly directed towards dengue-3 virus. Tetravalent vaccination resulted in near complete obliteration of antibody responses to dengue-4 and dengue-2 viruses that were strong immunogens individually. Conversely, the majority of tetravalent vaccinees had antibody responses to dengue-1 virus, which was a relatively poor immunogen as a monovalent vaccine.

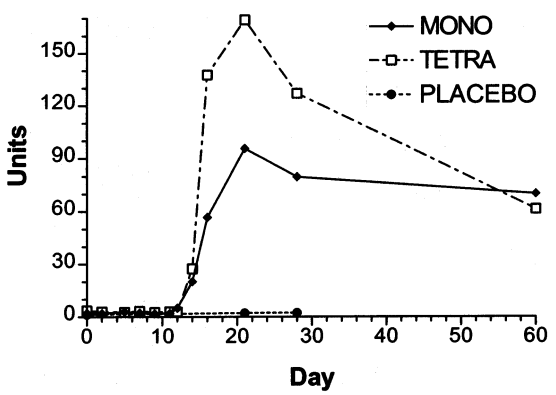

Fig. 3. IgM antibodies to dengue virus in recipients of AvP attenuated dengue virus vaccines. Sera were collected from volunteers for 60 days following vaccination. Data show the median IgM antibody values in recipients of monovalent vaccines (■), tetravalent vaccines (□), and placebo (○). As placebo recipients were uniformly negative (IgM < 20 U), no specimens were tested after study day 28.

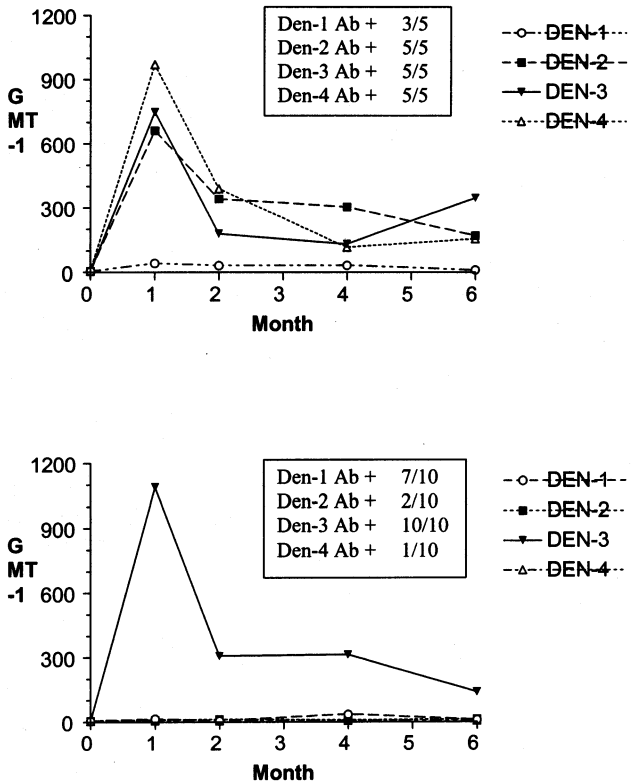

Fig. 4. Seroconversion ($PRNT_{50} \geq 10$) and geometric mean titer of dengue virus type-specific neutralizing antibodies in recipients of AvP attenuated dengue virus vaccines. Data in the upper panel show seroconversion rates (inset) and the inverse geometric mean titers of neutralizing antibodies in recipients of the attenuated monovalent dengue vaccines. Titers were calculated from $PRNT_{50}$ values. Responses were predominately monotypic with the exception of three vaccinees (see text). Data in the lower panel show seroconversion rates (inset) and the inverse geometric mean titers of neutralizing antibodies in recipients of the attenuated tetravalent dengue vaccine. Seroconversion rates and PRNT50 antibody titers to dengue-1, dengue-2, and dengue-4 viruses were uniformly lower than the dominant antibody responses to dengue-3 virus.

These restricted antibody responses after tetravalent vaccination were unexpected and different from those observed following tetravalent vaccination in Thailand [17].

The RT-PCR assays confirm universal viremia with dengue-3 virus among tetravalent vaccinees. The failure to detect other virus genomes, despite adequate viral recovery in monovalent vaccinees, suggests preferential replication of dengue-3 virus in tetravalent vaccinees. Apparent absence of replication of other type viruses in tetravalent vaccinees suggests the possibility that competitive interference exists amongst the AvP attenuated virus strains in the virus titers administered.

The mechanisms for this apparent viral interference are unknown. Inoculation of the four strains in Vero cells and in monkeys failed to demonstrate viral interference (J.-F. Saluzzo, unpublished data, 1994). Since the tropism of these attenuated viruses is presumably

identical, early entry and replication of dengue-3 virus may have resulted in induction of defective interfering virus particles and/or interferons from host cells, which prohibited entry or replication of other dengue viruses [18]. Relative doses of the four attenuated dengue viruses may be important in viral interference, as observed with other combination virus vaccines [19]. A factorial study design may be considered to test alterations in the formulation of the tetravalent vaccine that may result in changes in type-specific seroconversion.

Presence of broadly cross-reactive antibody responses following monovalent vaccination in three volunteers is puzzling. Despite extensive negative evidence from screening (immunization and travel review, serology for antibodies to dengue and other flaviviruses) before vaccination, these three volunteers had apparent secondary responses compared with other monovalent recipients. It is known that yellow fever immunity can enhance antibody responses to dengue vaccine [20]. These findings suggest that prior exposure to flavivirus may have been important in modifying the responses to dengue vaccines.

While tetravalent vaccination resulted in incomplete antibody responses, evaluation of T-cell responses from tetravalent vaccinees showed DV-specific proliferative and cytotoxic T lymphocyte responses to dengue-1, dengue-2, and dengue-3 viruses (Rothman et al., manuscript submitted). Monovalent vaccine recipients were not tested in this study, but the experience with tetravalent vaccines is consistent with that observed in recipients of attenuated monovalent dengue vaccines [21]. These findings suggest induction of broad type 1 $CD4^+$ and $CD8^+$ responses in these individuals, and support the hypothesis that live attenuated tetravalent dengue virus vaccines may immunize with a single dose. However, complete protection from enhanced dengue disease such as hemorrhagic fever probably requires induction of equivalent trivalent or tetravalent antibody and/or T cell responses [5].

The AvP attenuated dengue-1, dengue-2, dengue-3, and dengue-4 vaccines proved safe, tolerable, and immunogenic in volunteers. Neutralizing antibodies persisted to 6 months after a single dose in 89% of the responders. Ten recipients of the AvP tetravalent dengue vaccine noted more symptoms and signs, including fever and rash, yet none developed dengue fever. Universal dengue-3 viremia and subsequent induction of dominant antibody responses to dengue-3 virus suggests a pattern of viral interference in vivo. Changes in formulation of the four component attenuated dengue viruses, and/or addition of booster doses, may increase the immunogenicity of the tetravalent vaccine. The tetravalent vaccine formulation used in Thailand, based on the MID_{50} and titer of similar candidate vaccines, included 79- and 16-fold less dengue-2 and dengue-4 vaccine virus pfu per dose, respectively, than the tetravalent vaccine used in this study [6]. In this case, significant differences in proportion among candidate vaccine viruses may have helped induce more complete tetravalent antibody responses.

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