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A Phase 2 study of a purified, inactivated virus vaccine to prevent Japanese encephalitis☆

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Abstract

Japanese encephalitis (JE) is a serious disease caused by the JE virus. New generation JE vaccines are needed to prevent this disease. We conducted this Phase 2 randomized, open label, unblinded, single center study of a new, cell-culture derived, purified inactivated virus (JE-PIV) vaccine. The JE-PIV vaccine was administered in either two or three intramuscular (IM) doses (6.0 or 12.0 mcg each) with observation over 8 weeks. All volunteers completed the protocol without serious adverse reactions. Headache and transient tenderness at the injection site were the most common complaints. There were no laboratory abnormalities believed to be related to vaccine during the study. JE-PIV was well tolerated, resulted in high seroconversion rates [Day 56 (primary endpoint); 95–100%] and induced enduring immune responses up to 2 years after vaccination. Expanded Phase 3 trials are planned.

Keywords: Japanese encephalitis; Vaccine; Inactivated; Virus

1. Introduction

Japanese encephalitis (JE) is a disease caused by the Japanese encephalitis virus (JEV), a member of the Japanese encephalitis serological group of flaviviruses. It is transmitted by culicine mosquitoes (primarily *Culex tritaeniorhynchus*), with pigs and birds as amplifying hosts [1,2]. The geographic range of JEV extends from eastern, southern and southeastern Asia, to Papua New Guinea and the Torres Strait of northern Australia. Approximately 3 billion people (roughly 60% of the world's population) live in this region [3], with residents of rural agricultural areas being at particular risk. The geographical range of the virus may be spreading; cases of JE have been reported from as far as the Marianas Islands in the east, Pakistan in the west [4], Nepal in the north and Sri Lanka in the southwest [5]. There was a recent outbreak of JE in the Bihar states of northeastern India, and Nepal [6], and an ongoing outbreak in the eastern Uttar Pradesh, with 292 dead since April, 2006 [7]. JE, therefore, remains a significant public health problem in many Asian countries and as a result, poses risks to US military personnel stationed or deployed to endemic areas [8,9] and to travelers in Asia [10].

Japanese encephalitis is the most important cause of viral encephalitis in eastern and southern Asia, with 30,000–50,000 cases [11,12] reported annually (probably

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an underestimate due to underreporting [13]). The majority of infections are asymptomatic, with overt encephalitis occurring in only 1 out of every 50–1000 persons infected [14], but 25–30% of encephalitis cases are fatal, with as many as 30% of survivors left with neurological sequelae [10,15]. As vector control efforts have been largely ineffective and there are no antiviral agents effective against JEV, immunization is the principal countermeasure against this disease.

Worldwide, there are three types of JE vaccine in use; however, only the inactivated JE vaccine produced in mouse brain was distributed commercially and widely available internationally. A live, attenuated vaccine using the SA_{14}-14-2 viral strain has been used in millions of children in the Peoples Republic of China (PRC), used widely in Nepal, and recently, Korea. It has been recently introduced in India. An inactivated, PHK cell-derived vaccine using the P3 JEV strain has also been in use in the PRC for several years.

A formalin-inactivated, mouse brain-derived vaccine (JE-VAX®) was manufactured by the Foundation for Microbial Diseases of Osaka University in Japan (BIKEN) and was licensed in the US in 1992 [3]. Previous clinical trials have shown an efficacy rate of 91% [16,17]. Unfortunately, the use of this vaccine has been troubled by safety issues. Serious side effects such as anaphylaxis occurring typically 1–3 days (sometimes 2 weeks) after vaccination have been noted, with an incidence among US citizens of 15–62 per 10,000 [18]. In addition, the neural tissue substrate of the vaccine has raised concerns about the possibility of vaccine-related neurological side effects [19]. These concerns led to the suspension of routine vaccination with the mouse brain-derived inactivated JE vaccine in Japan in May 2005 due to possible cases of acute disseminated encephalomyelitis following JE vaccination. Surveillance of JE vaccine-related complications in Japan during the years 1965–1978 found neurological events (principally encephalitis, encephalopathy, seizures and peripheral neuropathy) occurring at a rate of 1–2.3 per million vaccinees [20]. This vaccine may also have been a contributing factor in the death of a US serviceman who received a first dose of JE-VAX® 60 h earlier [18]. Recently, manufacture of this vaccine was discontinued, leaving a significant deficit in the preventive armamentarium against JE for US travelers and the military.

To address a number of these issues, the World Health Organization proposed the development of a new generation of JE vaccines [21]. A new, purified, Vero cell-derived, inactivated JE virus vaccine (JE-PIV) was developed at the Walter Reed Army Institute of Research (WRAIR), Forest Glen, MD, USA, following a similar production methodology used for a dengue-2 PIV [23,24]. In pre-clinical studies, the potency of the JE-PIV was shown to be equivalent to JE-VAX® [23]. In a Phase 1 study, the JE-PIV was shown to be safe in subjects and stimulated immune responses in approximately 50% of vaccinees, presumably due to the low (0.5 and 2.0 mcg) doses given in the study (N. Kanesa-thasan, personal communication). This paper reports on the results of a Phase 2 study that was conducted from 2001 to 2003 using a new production lot of JE-PIV vaccine.

2. Methods

2.1. Subjects

A total of 94 eligible subjects took part in the study. Subjects were recruited at the WRAIR Clinical Trials Department from the military and civilian populations in the Washington, DC metropolitan area. Each subject provided informed consent. Inclusion criteria included age between 18 and 49 years, no significant health problems as established by medical history and laboratory evaluation, and, if female, not to be pregnant. Female subjects were required to have a negative urine pregnancy test prior to vaccination, and to agree to avoid pregnancy during the study and for 30 days after the last dose of vaccine. Exclusion criteria included the use of concomitant medications or vaccinations, history of seasonal allergies, allergy to any vaccine component, asthma, immunosuppressive disorders, neurological disorders and to have had no exposures (illness or vaccination) to dengue, JE, yellow fever (YF) or tick borne encephalitis viruses in the past. Subjects in the JE-VAX® group were required to have no prior history of allergies. Subjects who gave informed consent underwent a medical history and physical examination. Screening laboratory tests including complete blood count (CBC), alanine aminotransferase (ALT), creatinine (Cr), urinalysis, Hepatitis B surface Ag (HBsAg), Hepatitis C, HIV and flaviviral (JEV, dengue, YF) serology were done. Any identified abnormalities in medical history/physical examination or screening labs considered clinically significant in the opinion of the investigators lead to disqualification of the subject.

2.2. Vaccine

The study vaccine was a JE purified, inactivated virus (JE-PIV) vaccine, lot 0737, developed and manufactured by the Pilot Bioproduction Facility, WRAIR, Forest Glen, MD 20910, USA. The attenuated SA_{14}-14-2 vaccine strain, adapted to primary canine kidney cells [22], was further passaged in Vero cells. Vero cells used for production were a derivative of a certified cell line that has been used to produce more than 1 billion doses of licensed polio and rabies vaccines [25]. We chose to produce the inactivated vaccine using the attenuated JE strain because of manufacturing concerns. Other recently developed inactivated Hepatitis A vaccines [27] have also been produced using attenuated virus strains. After adaptation to Vero cells, a master seed (Vero-4) and production seed (Vero-5) were prepared and banked at −80 °C. A vaccine lot was prepared at Vero passage 6 by inoculation of Vero cells grown in 850 cm^2 roller bottles. After inoculation, virus was harvested on Days 3, 5, 7 and 9. A total of 10 L of harvested virus was pooled and clari-

Table 1
Comparison of JE-VAX® and JE-PIV

	JE-VAX®	JE-PIV
Parent virus seed	Nakayama (Virulent)	SA_{14}-14-2 (Attenuated)
Virus growth substrate	Mouse Brains	Mammalian cells (Vero)
Inactivation	Formalin	Formalin
Stabilizers	Porcine Gelatin	None
Adjuvant	None	Alum
Preservative	Thimerosal	None

fied by centrifugation followed by filtration and ultrafiltration (100,000 MWCO). To remove Vero cell DNA, the concentrated virus was treated with protamine sulfate, clarified and purified by zonal centrifugation in sucrose gradients. Gradient fractions were assayed for antigen activity associated with viral particles. Antigen positive fractions were pooled, diluted and formalin added at a concentration of 0.05% (v/v). After 10 days inactivation at 22 °C, formalin was neutralized with sodium bisulfite and the bulk vaccine stored at 4 °C. Pre-clinical testing consisted of tests for adventitious microbial agents, mycoplasma, cellular protein and DNA contaminants, endotoxin, reverse transcriptase (PERT assay), mouse immunogenicity and efficacy, and viral-specific protein and antigen. All test results were satisfactory and met specifications. Vero-specific cellular DNA was measured at 2 pcg/mL. For the final container vaccine lot no. 0737, the bulk vaccine was adsorbed to alum (Rehydragel), filled in 0.7 mL vials and stored at 2–8 °C. Final container vaccine was tested for sterility, pH, aluminum content, residual bisulfite, pyrogen and identity. All test results were satisfactory and met specifications. For dosing, each dose was formulated to contain either 6.0 or 12.0 mcg per dose. The immunogenic potency of JE-PIV was evaluated in vivo after adsorption to alum by administration of graded dilutions in mice. Following immunization at 0 and 4 weeks, antibody responses at 6 weeks were evaluated by a quantitative neutralizing antibody assay in comparison to a reference standard (JE-VAX®). Vaccine lot 0737 met the in vivo potency criteria (ED_{50} $\leq$5 ng) after vaccination. A comparison of the WRAIR JE-PIV and JE-VAX® vaccines is shown in Table 1.

2.3. Study design

This study was a randomized, open label, unblinded, single center trial. A total of 94 eligible subjects were allocated into one of 4 groups and vaccinated sequentially. Group 1 (24 subjects) received JE-PIV (6 mcg/dose) given IM on Days 0 and 28 (12 mcg total dose); Group 2 (24 subjects) received JE-PIV (6 mcg/dose) given IM on Days 0, 14 and 28 (18 mcg total dose); Group 3 (25 subjects) received JE-PIV (12 mcg/dose) given IM on Days 0 and 28 (24 mcg total dose); and Group 4 (21 subjects) received JE-VAX® given subcutaneously (SQ) according to the recommended schedule on Days 0, 7 and 28. Group 2 started at least 1 week after the first dose of Group 1 was given; Group 3 started at least 1 week after the second dose of Groups 1 and 2 was administered, and Group 4 started at least 1 week after the second dose of Groups 1, 2 and 3 was administered. The 6 and 12 mcg doses of JE-PIV were selected because they best approximated the amount of protein in JE-VAX® (approximately 6 mcg per dose), and because the 0.5 and 2.0 mcg doses did not stimulate an adequate immune response in the Phase 1 study. Each JE-PIV dose was given at least 2 weeks apart, because a 1-week interval between doses was deemed inappropriate for a vaccine containing alum.

On initial vaccination (Day 0), subjects were interviewed and examined for evidence of acute illness prior to inoculation. After vaccination, each subject was assessed clinically to identify local and systemic reactogenicity. From Day 0 to Day 56, subjects underwent 12 post-vaccination assessments including regular history and physical examinations, including measurement of vital signs and adverse events. In addition, each subject was to keep a diary of solicited symptoms and signs for 7 days after each vaccination. Solicited systemic symptoms included fever, headache, myalgias and other symptoms the subject may have experienced, while solicited local symptoms included arm pain, redness or swelling at the injection site. Intensity of symptoms following vaccination were graded as none (absent), mild (no interference with normal daily activities), moderate (limits normal daily activities) and severe (unable to perform normal daily activities). Relationship to study vaccine was categorized as not related, unlikely, suspected and probable. Adverse events (AE) and serious adverse events (SAE) were collected from Day 0 to Day 56.

Blood samples were obtained routinely from subjects on Day 0 and on Days 28 and 56 after the first dose for the following clinical laboratory measurements: complete blood count (CBC), serum alanine aminotransferase (ALT) and creatinine (Cr). JEV serology was performed on all subjects on Day 0 and on Days 28 and 56 after the first dose using the plaque reduction neutralization test ($PRNT_{50}$). Follow-up venipuncture for JEV serologic studies was optional for the subjects at 6, 12, 18 and 24 months after the first dose.

2.4. Specimen collection and analysis

Blood specimens were obtained at scheduled study visits by standard phlebotomy techniques. Blood tubes were processed and sent for routine clinical laboratory tests (CBC, ALT, Cr) to the Department of Clinical Pathology, Walter Reed Army Medical Center (WRAMC) and Department of Virus Diseases, WRAIR (JEV serology). Urine human beta-chorionic gonadotropin (β-HCG) was performed by a trained technician in the Department of Clinical Trials, WRAIR. Urinalysis was performed by the Department of Clinical Pathology, WRAMC. The handling of all specimens was done in class 2 laminar flow hoods to protect both the personnel and integrity of the samples.

2.5. JEV neutralization assay

This test was performed by the Clinical Testing Laboratory, Department of Virus Diseases, Walter Reed Army Institute of Research, Silver Spring, MD 20910, USA. The JE neutralization test was performed as described [26]. A 50% reduction of plaques in the PRNT at serum dilution of 1:10 was used as the lower cut-off for seroconversion. Serial four-fold dilutions (1:10, 1:40, 1:160 and 1:640) of serum were made. An equal volume of JE virus strain SA_{14}-14-2, diluted to contain 250–500 pfu/mL, was added to each serum dilution tube. Following incubation at 35 °C for 30 min, 0.2 mL was removed from each tube and inoculated onto triplicate six-well plates of confluent Vero cells. Flasks were incubated at 35 °C for 1 h and the monolayers overlaid with 5 mL of 0.6% agarose/medium 199 mixture. After incubation at 35 °C for 6 days, plaques were stained by addition of a second overlay containing neutral red stain in 0.6% agarose. Flasks were incubated overnight at 35 °C, the plaques were counted, and the PRNT50 determined by probit analysis using SPSS software (SPSS Inc., Chicago, IL).

2.6. Study cohorts

2.6.1. Intent-to-treat cohort

The intent-to-treat cohort included all subjects enrolled in the study for which data were available. For the intent-to-treat analysis of safety, this included all subjects for whom safety data were available. For the intent-to-treat analysis of immunogenicity, this included all subjects with available serological data.

2.6.2. Protocol-defined (PP) cohort for analysis of safety

The cohort included in the analysis of safety involved all subjects who received at least one dose of study vaccine, and who had not received a vaccine forbidden in the protocol.

2.6.3. Protocol-defined (PP) cohort for analysis of immunogenicity

The cohort included in the analysis of vaccine immunogenicity involved all evaluable subjects (i.e., those meeting all eligibility criteria and complying with the procedures defined in the protocol) for whom data concerning immunogenicity endpoint measures were available.

2.7. Determination of sample size

The purpose of the study was to demonstrate that the JE-PIV vaccine is immunogenic in the dosages and schedules tested. This study was the first evaluation of the proposed 6 and 12 mcg dosages of the JE-PIV vaccine and was, therefore, limited in size, largely by safety and practical considerations. The JE-VAX® vaccine was included as an active comparator because it is known to be immunogenic. The number of subjects enrolled was to be 25 per group.

The JE-VAX® vaccine was expected to result in a seroconversion rate (SCR) of >95%. A group size of 25 would permit detection of a difference between 95 and 68% seroconversion rates with 80% power by the χ-square test with a 0.05 one-sided significance level. This limit of detection was sufficient for the purpose of this protocol. The power to distinguish between groups based on GMTs was felt to be even greater. The drop out rate was expected to be from none to two per group. Due to the practical limitations of recruiting and following study subjects, the sample size was not to be enlarged in anticipation of possible dropouts.

2.8. Analyses

2.8.1. Analysis of demographics

The demographic characteristics (age in years, sex and race) of the study cohort is tabulated. The mean age of the enrolled subjects, as a whole and per group, was calculated.

2.8.2. Analysis of safety

For each group, the incidence of each solicited symptom over the 7-day follow-up period was reported. The relationship of solicited general symptoms to vaccination was determined. Serious adverse events reported during the study period, if any, were listed for each group.

2.8.3. Analysis of immunogenicity

Two analyses were planned: the first was an intent-to-treat analysis (ITT) including all subjects who received the study vaccine. The second analysis was an according-to-protocol analysis (ATP)/per protocol (PP) including only the subjects who fulfilled the criteria defined in the protocol. The according-to-protocol analysis was considered as being of primary interest for the efficacy analysis.

Seroconversion rates (SCR) and geometric mean titers (GMT) of anti-JEV antibodies were calculated with 95% confidence intervals for all time points for which blood samples were taken. Seroconversion was defined as the appearance of antibodies at $\geq$1:10 titer after vaccination in a subject who was previously seronegative. A seronegative subject was defined as a subject whose titer is <1:10. A neutralizing antibody titer of $\geq$1:10 generally is accepted as evidence of protection and post vaccination seroconversion [28–30]. The GMT was calculated using the log-transformation of measured titers for all specimens, whether above or below 1:10 titer, and taking the anti-log of the mean of these transformed values.

2.8.4. Statistical analysis

P values were calculated using the Fisher's exact test (GraphPad Software, © 2005) for Day 56 SCR, 2-sided, compared to licensed JE-VAX®, and the Independent *T*-test (GraphPad Software, © 2005) for Day 56 GMT, 2-sided, compared to licensed JE-VAX®. Nonparametric tests (Wilcoxson-Mann–Whitney) were done as described previously [31].

Table 2
Baseline demographics (all subjects)

	Group 1 (JE-PIV 12 mcg)	Group 2 (JE-PIV18 mcg)	Group 3 (JE-PIV 24 mcg)	Group 4 (JE-VAX®)
Gender				
Male	18	9	17	10
Female	6	15	8	11
Race				
African American	8	18	11	11
Caucasian	15	4	11	8
Native American	0	0	0	1
Hispanic	1	1	3	0
Asian	0	0	0	1
Other	0	1	0	0
Mean age	30.6	36.9	33.8	30.0

3. Results

Eighty-seven subjects completed the study; seven did not. In Group 1 (12 mcg total dose JE-PIV), one subject moved away from the study area and another withdrew consent prior to vaccination. In each of Groups 2 (18 mcg JE-PIV) and 3 (24 mcg JE-PIV), one subject was lost to follow-up after completing the vaccination series. Another subject in Group 3 was noted to have a diastolic blood pressure between 100 and 116 mmHg prior to receiving the final dose, and so was medically disqualified. In Group 4 (JE-VAX®), one subject was lost to follow up after a move out of the area, and one subject did not complete the vaccination series due to development of a rash after dose #2.

3.1. Demographics

Table 2 outlines the baseline demographics of all study subjects. Distribution of subjects by sex and age in the four groups was by chance. Group 2 had more females than Group 1 (15 versus 6, respectively, $P < 0.05$) and Group 3 (15 versus 8, respectively, $P < 0.05$); more African-Americans than Group 1 (18 versus 8, respectively, $P < 0.05$); and older mean age than Group 1 (36.9 versus 30.6, respectively, $P < 0.05$) and Group 4 (36.9 versus 30.0, respectively, $P < 0.05$).

3.2. Immunogenicity

Table 3 shows the SCR and GMT and 95% confidence intervals (95% CI) on Days 28 and 56. Table 4 shows the SCR and GMT with 95% CI for Days 180, 365, 540 and 720. On Day 28, 17/22 subjects (77%) in Group 1 (JE-PIV 6 mcg × 2) seroconverted with a GMT of 61. On Day 56, the primary endpoint, 21/22 subjects (95%) seroconverted with a GMT of 327. The one volunteer who did not seroconvert was found after completion of the Day 56 follow up to have been involved in another trial involving Neupogen™ administration and leukophoresis. Twenty-two of 23 (96%) of subjects seroconverted in both Groups 2 and 3 with the GMTs shown at Day 28 (Table 3). Twenty-three of 23 volunteers (100%) and 23/23 volunteers (100%) in Groups 2 and 3, respectively (PP cohort), seroconverted with the GMTs at Day 56 shown in Table 3.

In comparison, on Day 28, 16/19 (84%) JE-VAX® subjects in the PP cohort seroconverted. By Day 56, 14/19 (74%) of JE-VAX® subjects in the PP cohort remained seroconverted. This SCR was significantly less ($P < 0.05$) than the SCR in Groups 2 and 3 by Fisher's exact test (Table 3).

Table 3 shows that the GMT at Day 56 for Group 1 was 327. The GMT for Groups 2 and 3 were similar in range, though highest in Group 3 (24 mcg total dose) at Day 56. Interestingly, the peak GMT for Group 2 (6 mcg × 3) occurred at Day 28. For the JE-VAX® group, the GMT at Day 56 was 128, and was significantly lower than the corresponding GMTs in Groups 1 ($P < 0.005$) and 3 ($P < 0.001$). However, the lower number of volunteers entered in the JE-VAX® group precludes drawing a firm conclusion on the lower seroconversion rate on Day 56 of this group of 19 evaluable volunteers compared to the 68 evaluable JE-PIV recipients.

The persistence of neutralizing antibody in the serum in JE-PIV recipients is illustrated by the long-term follow up seroconversion and GMT results (Table 4). While in the JE-PIV groups the percentage of seroconverters varied between 83 and 100%, only half of the JE-VAX® subjects demonstrated maintenance of serum neutralization antibody over the long term. Table 4 also shows GMTs for Days 365, 540 and 720 in a small number of volunteers vaccinated with JE-PIV with still adequate seroconversion rates and titers, illustrating the persistence of serum antibody after vaccination with JE-PIV.

3.3. Safety

The majority of 73 study subjects immunized with the WRAIR JE-PIV reported no or few minor symptoms after the

Table 3
Seroconversion rates (SCR) and geometric mean titers (GMTs) for Day 28 and 56 after Dose #1 (primary endpoint), per protocol population

Group	Vaccine	Total dose	Day 28		Day 56	*P*-value*	Day 56	*P*-value**
			SCR (%)	GMT (95%CI)	SCR (%)		GMT (95%CI)	
1	JE-PIV	12 mcg	17/22 (77.3)	61.2 (37.1/101.0)	21/22 (95)	0.09	327.2 (253.3/422.8)	<0.005
2	JE-PIV	18 mcg	22/23 (95.6)	328.3 (189.1/569.8)	23/23 (100)	<0.05	186.1 (124.8/227.5)	0.56
3	JE-PIV	24 mcg	22/23 (95.6)	117.5 (76.0/181.8)	23/23 (100)	<0.05	516.3 (393.7/677.1)	<0.001
4	JE-VAX®	3 doses	16/19 (84.2)	131.7 (77.5/223.6)	14/19 (74)	–	128.3 (76.3/215.8)	–

* Fisher's exact test (GraphPad Software, © 2005) for Day 56 SCR, 2-sided, compared to licensed JE-VAX®.
** Independent *T*-test (GraphPad Software, © 2005) for Day 56 GMT, 2-sided, compared to licensed JE-VAX®.

Table 4
Long-term seroconversion rates (SCR) and geometric mean titers (GMTs) for Day 180, 365, 540 and 720 after Dose #1, per protocol population

Group	Vaccine	Total dose	Day 180		Day 365		Day 540		Day 720	
			SCR (%)	GMT (95%CI)	SCR (%)	GMT (95%CI)	SCR (%)	GMT (95% CI)	SCR (%)	GMT (95%CI)
1	JE-PIV	12 mcg	17/17 (100.0)	119.3 (82.7, 171.9)	11/11 (100.0)	51.6 (28.1, 94.7)	9/10 (90.0)	37.3 (25.1, 55.5)	7/8 (87.5)	89.1 (56.3/141.0)
2	JE-PIV	18 mcg	17/18 (94.4)	112.3 (71.2, 177.0)	16/16 (100.0)	90.0 (56.3,143.8)	6/6 (100.0)	66.3 (20.2,217.8)	5/6 (83.3)	110.0 (68.2/177.5)
3	JE-PIV	24 mcg	15/17 (88.2)	33.2 (21.5, 51.4)	11/11 (100.0)	69.7 (43.2,112.5)	10/11 (90.9)	46.5 (27.0, 80.2)	2/2 (100.0)	79.5 (0.9/6880.7)
4	JE-VAX®	3 doses	7/13 (53.8)	49.5 (23.3, 105.2)	6/11 (54.6)	30.3 (17.3, 53.3)	NA	NA	4/6 (66.7)	27.9 (12.7/61.2)

Table 5
Number of subjects with systemic adverse events per group, regardless of relationship to vaccine

	Group 1 ($n=24$)	Group 2 ($n=24$)	Group 3 ($n=25$)	Group 4 ($n=21$)
Fever				
Absent	19	20	22	20
98.5 to 99.5 °F	0	1	0	0
99.6 to <103 °F	5	3	3	1
Headache				
Absent	11	9	13	9
Mild	8	9	11	10
Moderate	5	6	1	1
Severe	0	0	0	1
Myalgia				
Absent	14	10	16	12
Mild	7	11	7	8
Moderate	3	3	2	1
Severe	0	0	0	0

Headache: mild (no interference with daily activities); moderate (limits normal activities); severe (unable to perform normal activities). Myalgia: mild (no interference with daily activities); moderate (limits normal activities); severe (unable to perform normal activities).

cumulative administration of 164 total injections. No subject in the JE-PIV groups developed severe symptoms requiring medication, bed rest, hospitalization or medical intervention. Systemic and local adverse reactions for each of the treatment groups irrespective of relationship to vaccine are listed in Tables 5 and 6, respectively.

The most frequently reported systemic adverse event in the JE-PIV groups was headache in 40/73 (55%) of subjects (Table 5), 28/40 (70%) of these considered suspected or probably related to JE-PIV vaccination, and these were equally distributed between the 6 and 12 mcg treatment groups. These

Table 6
Number of subjects with local reactions per group, regardless of relationship to vaccination

	Group 1 ($n=24$)	Group 2 ($n=24$)	Group 3 ($n=25$)	Group 4 ($n=21$)
Arm pain				
Absent	4	4	4	9
Mild	12	13	13	6
Moderate	8	7	8	6
Severe	0	0	0	0
Redness				
Absent	20	20	22	15
Mild	1	4	3	2
Moderate	3	0	0	3
Severe	0	0	0	1
Swelling				
Absent	22	20	20	14
Mild	1	2	3	3
Moderate	1	2	2	3
Severe	0	0	0	1

Pain: mild (mild discomfort when touched); moderate (spontaneously painful or pain on movement); severe (limits use of limb). Redness: mild (≤1 mm); moderate (>1 to <50 mm); severe (≥50 mm). Swelling: mild (≤2 mm); moderate (>2 to <50 mm); severe (≥50 mm).

headaches were of short duration, lasting at most half a day. Myalgia was suspected or probably related to JE-PIV vaccination in 28/33 (85%) of reports. In comparison, 10/12 (83%) reported instances of headaches and 8/9 (89%) reported instances of myalgias in Group 4 who received JE-VAX® were considered suspected or probably related to vaccination. The probable relationship with vaccination of these events was higher in the JE-VAX® group compared with the JE-PIV groups, but as this study was not blinded, observer bias cannot be excluded.

There were no oral temperatures in excess of 103.0 °F in any of the treatment groups. More (12/73, 16%) subjects in the JE-PIV groups had fever than in the JE-VAX® group (1/21, 5%). Among the JE-PIV subjects, 5 (21%), 3 (12%) and 3 (12%) subjects in Groups 1, 2 and 3, respectively, had oral temperatures 99.6 to <103 °F, while 1 (4%) had oral temperature 98.5–99.5 °F. Six of these 12 subjects (9% of the total treatment group) experienced fever considered related (suspected or probable) to JE-PIV vaccination. Vital sign values remained within normal limits for all treatment groups during the study.

The number of treatment-related local adverse events, suspected or probable, was equally distributed between the two vaccines. One subject was medically disqualified after receiving one dose of JE-VAX® due to rash. Two subjects in the JE-VAX® group experienced severe local reactions; one developed severe redness and swelling at the injection site of 20 mm × 40 mm size; another subject developed arm swelling of 30 mm × 25 mm size. No subjects in the JE-VAX® group required hospitalization, however, the subject noted above who developed severe redness and swelling after vaccination required antihistamines. As expected, the most commonly reported local sign following an IM injection with JE-PIV was arm pain, which occurred with equal frequency in the two- and three-dose groups. The number of reports of post-vaccination arm pain, redness, and swelling among the JE-PIV and JE-VAX® groups did not differ. There were no serious laboratory abnormalities (defined as Cr >1.4, AST or ALT >100 IU, Hct <25, WBC <3.0 or platelets <100 K) felt to be treatment-related. There were no changes from baseline hematology and chemistry values felt to be treatment-related.

4. Discussion

Effective vaccination strategies are a very important way to control the spread of communicable disease, especially those caused by viruses, which have no specific treatment. The use of JE vaccines has resulted in the control of this disease in areas and within populations where the vaccines are available and widely used such as the very young and the elderly. An effective vaccine is important not only for JE-endemic areas, but also for travelers and the military, two highly mobile populations which frequently require rapid induction of protective immunity before completing a full dosing schedule.

The current JE vaccine is a US FDA-licensed mouse-brain vaccine (JE-VAX®) manufactured in Japan and approved for protection of travelers and military personnel. JE-VAX® is efficacious, and immunity has been shown to persist for up to 3 years in a military population in a non-endemic area [32]. The current ACIP recommendation calls for a booster after 2 years [18]. However, the requirement for three doses in the primary immunization series, an interval of 4–5 weeks for induction of protective immunity, periodic boosters and high cost (approximately US$ 200 for the primary three-dose series) have limited the use of JE-VAX®. The vaccine has also been associated with serious systemic allergic (urticaria and/or angioedema) adverse events and neurological reactions at a rate of 6.3 per 100,000 doses [33]. The removal of this vaccine from the US market poses a threat to effective measures to protect against JE.

A new inactivated JE virus vaccine manufactured in an acceptable cell culture substrate in lieu of mouse brain tissue and having a low incidence of adverse events would represent an improvement over the previous licensed JE vaccine. A new vaccine capable of inducing rapid onset of protective immunity after two doses or less, and providing long lasting immunity without the need for booster doses would be an ideal replacement JE vaccine. This JE-PIV candidate is intended to meet these needs.

The purpose of this study was to show that JE-PIV is sufficiently immunogenic and well tolerated in the doses and schedules tested. In general, all dose regimens with JE-PIV resulted in higher seroconversion rates compared to JE-VAX®. High rates of seroconversion at Day 56, the primary immunogenicity endpoint, were observed for all three JE-PIV treatment groups in this study, with total doses ranging from 12 to 24 mcg. The lowest total dose of JE-PIV administered, 12 mcg, resulted in 95% seroconversion (21/22 subjects) on Day 56, while the higher total doses of JE-PIV tested (18 and 24 mcg) resulted in 100% seroconversion. The 18 and 24 mcg total doses of JE-PIV resulted in 96% seroconversion by Day 28. In contrast, only 16/19 JE-VAX® subjects (84%, Day 28), and 14/19 (74%, Day 56) seroconverted. However, two subjects in this group who seroconverted by Day 28 were excluded from the per-protocol analysis because they had not received the third vaccination. The lower number of 19 evaluable subjects entered in the JE-VAX® group precludes drawing a firm conclusion on the lower seroconversion rate on Day 56 compared to the 68 evaluable JE-PIV recipients.

JE-PIV recipients continued to exhibit high seroconversion rates up through Day 720; thus, it appears that the immune response stimulated by JE-PIV, at least in this small number of subjects, is durable for at least 2 years. In contrast, only half of the JE-VAX® recipients retained serum neutralizing antibody at titers ≥1:10 on Day 180 and Day 365. One volunteer in Group 1 (JE-PIV 6 mcg; Day 0, 28) received Neupogen™ (Filgrastim, G-CSF, Amgen) followed by leukophoresis during a separate clinical trial. This was made known to the investigators only after Day 56. This subject was considered a protocol violator, however, the subject

was included in the PP analysis, since the interaction of Neupogen with B-lymphocytes and their subsequent ability to produce specific antibodies is unclear. On the other hand, leukophoresis could also have resulted in this subject's failure to seroconvert.

In general, GMTs for all JE-PIV treatment groups were higher than in the JE-VAX® group. Only two time points show lower GMT following JE-PIV vaccination compared to JE-VAX®, at Day 28 (12 mcg total dose JE-PIV) and Day 180 (24 mcg total dose JE-PIV). At the primary endpoint (Day 56), there is convincing evidence that all three JE-PIV groups produced higher titers than the JE-VAX® group by nonparametric analysis; at Day 56, the titer produced by JE-PIV (12 mcg total dose) was estimated to be 4.57 times as large as the titer produced by the licensed vaccine (95% CI: 1.95–10.74). The titer produced by JE-PIV (18 mcg total dose) was estimated to be 3.14 times as large as the titer produced by the licensed vaccine (95% CI: 1.40–7.07). The titer produced by JE-PIV (24 mcg total dose) was estimated to be 8.40 times as large as the titer produced by the licensed vaccine (95% CI: 4.01–17.57).

Differences in GMTs between the two vaccines persisted during the 2 year follow-up period. In this study, the virus from which the vaccine was derived (SA_{14}-14-2) was used to conduct the PRNT assay as the assay can then be conducted under biosafety level 2 conditions. Literature based evidence suggests that homologous neutralization titers may some times be higher than heterologous titers, whereas these differences are not considered significant in controlling JE virus infections [34,35]. This assertion is supported by the correlation of PRNT with protection data [34]. Additionally, broad cross reactivity of antibodies raised against SA_{14}-14-2 has been shown [35] and studies to date suggest the existence of only one serotype of JEV [36]. We plan to test the sera from this study against heterologous viral strains.

This direct comparison of immunogenicity of the two vaccines is also limited by small numbers of subjects. A conservative interpretation of the results of this study would be that JE-PIV is comparable to JE-VAX® with respect to immunogenicity. It also suggests that a two-dose regimen of JE-PIV may elicit similar immunogenicity to the three-dose regimen of JE-VAX®, with possibilities of a shorter dosing schedule and fewer total doses to achieve protection. The durability of the immune response over 2 years is encouraging and supports the possibility that booster doses of JE-PIV will perhaps not be necessary until at least 2 years after primary immunization.

The majority of 73 study subjects immunized with the JE-PIV reported no or few minor symptoms after the cumulative administration of 164 total injections. No subject in the JE-PIV groups developed severe symptoms requiring medication, bed rest, hospitalization or medical intervention. Systemic adverse events of fever and headache were more frequently reported by JE-PIV subjects compared to JE-VAX® recipients. Interestingly, the lower dose JE-PIV groups (12 and 18 mcg groups) were associated with a higher incidence of total systemic adverse reactions than the 24 mcg JE-PIV group. Similarly, there were more JE-PIV recipients with arm pain than those who received JE-VAX®. This could be due to the IM administration of JE-PIV (versus SQ administration for JE-VAX®). However, as noted in Table 6, more severe reactions (moderate to severe swelling and redness at the injection site) were seen more frequently in the JE-VAX® arm. It is therefore difficult to draw hard conclusions based on the small numbers in this study, given the possibility of observer bias due to the sequential and open label design of the trial.

The potential for severe local adverse reactions with JE-VAX® is of concern to physicians and has historically limited the usage of the product. It is of note that in this Phase 2 trial, one subject was medically disqualified after receiving one dose of JE-VAX® due to rash and two subjects experienced severe local reactions at the injection site. No such severe local adverse reactions were observed among any recipients of JE-PIV vaccine.

Results of the JE-PIV Phase 1 study suggested that reactogenicity and immunogenicity rates were likely to be similar for the two JE-PIV dosage groups (6 and 12 mcg) tested in this study and that only a very large study would be able to detect the small differences between the groups. Hence, it was not the objective of this study to demonstrate statistically significant differences between the treatment groups. However, subsequent, larger studies may be used to distinguish small or moderate differences between groups.

In summary, this Phase 2 study showed that JE-PIV in three different dose regimens was tolerable and induced good immune responses to JEV. This immune response appears to be durable, with robust sustained GMTs over at least 2 years. The results of this study suggest that the JE-PIV vaccine can be considered a good candidate for a replacement JE vaccine. Further testing in a large-scale Phase 3 non-inferiority trial to confirm the results of this study is planned. A convenient vaccine dose and schedule is identified for the Phase 3 evaluation (6 mcg IM in two doses, 28 days apart).

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