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Safety and immunogenicity of NYVAC-JEV and ALVAC-JEV attenuated recombinant Japanese encephalitis virus — poxvirus vaccines in vaccinia-nonimmune and vaccinia-immune humans

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Abstract

A controlled, randomized, double-blind clinical trial evaluated whether two attenuated recombinant poxviruses with identical Japanese encephalitis virus (JEV) gene insertions, NYVAC-JEV and ALVAC-JEV, were safe and immunogenic in volunteers. Groups of 10 volunteers distinguished by vaccinia immune status received two doses of each vaccine. The vaccines appeared to be equally safe and well tolerated in volunteers, but more reactogenic than licensed formalin-inactivated JE and placebo vaccines given as controls. NYVAC-JEV and ALVAC-JEV vaccine recipients had frequent occurrence of local warmth, erythema, tenderness, and/or arm pain after vaccination. There was no apparent effect of vaccinia immune status on frequency or magnitude of local and systemic reactions. NYVAC-JEV elicited antibody responses to JEV antigens in recipients but ALVAC-JEV vaccine poorly induced antibody responses. However, NYVAC-JEV vaccine induced neutralizing antibody responses only in vaccinia-nonimmune recipients while vaccinia-immune volunteers failed to develop protective antibodies (5/5 vs. 0/5 seroconversion, $p < 0.01$). These data suggest that preexisting immunity to poxvirus vector may suppress antibody responses to recombinant gene products. © 2000 Published by Elsevier Science Ltd.

Keywords: Japanese encephalitis virus; Recombinant poxviruses; Vaccinia immunity

1. Introduction

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is the leading cause of viral encephalitis in Asia [1]. Approximately 50,000 cases of JEV encephalitis are reported annually. Twenty-five percent of the affected individuals die from their disease and 50% are left with permanent neurologic or psychiatric sequelae [2]. The currently licensed inactivated JEV vaccine (BIKEN, Osaka) is efficacious [3] but is expensive, limited in production, and may require periodic booster doses [4]. Recently, the vaccine has also been associ-

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ated with undesirable hypersensitivity-type reactions [5]. A more readily available and safer vaccine is needed.

New recombinant JEV vaccines have recently been engineered from two highly attenuated poxvirus vectors [6,7]. The NYVAC vector has been derived from vaccinia virus through 18 deletions of genes encoding for virulence factors and human host range replication; the ALVAC attenuated poxvirus vector is canarypox virus, which replicates only in avian species. Four JEV genes (preM, E, NS1, and NS2a) were inserted into each poxvirus parent vector. Both recombinant vaccines, NYVAC-JEV and ALVAC-JEV, have been shown to express premembrane (preM) and envelope (E) structural and nonstructural-1 (NS1) proteins. Subsequently, both attenuated recombinant vaccines have been shown to be immunogenic and protective in animals [8,9].

Attenuated poxvirus vaccines have many desirable properties. They are capable of inducing both humoral and cell mediated responses, and may be less reactogenic and potentially cheaper to produce than inactivated vaccines [10]. Multivalent recombinant vaccines may become possible using large multigene constructs inserted into these vectors [11]. One concern for attenuated poxvirus vaccines is whether previous immunization with vaccinia virus may suppress immune responses to a recombinant vaccinia virus. In a recent study, vaccinia-nonimmune (V-N) individuals had greater antibody and T cell responses to a recombinant HIV-vaccinia virus vaccine than did vaccinia-immune (V-I) volunteers [12].

NYVAC and ALVAC vectored vaccines have been given to a small number of volunteers [13]. Recombinant canarypox-rabies glycoprotein and canarypox-vaccine was safe and immunogenic in Phase I clinical trials [14,15]. A NYVAC-malaria vaccine has also recently been administered to humans and resulted in cytolytic T cell responses [16]. This Phase I study evaluated the safety and immunogenicity of the NYVAC-JEV and ALVAC-JEV poxvirus vaccines in humans. Before the trial, each recombinant vaccine was also tested in rhesus monkeys and found to be safe and immunogenic [17]. Since preexisting vaccinia immunity may influence the immune response to poxvirus vectored vaccines, we studied vaccine responses in volunteers distinguished by their vaccinia virus immune status. Results of the T cell studies have been previously presented [18].

2. Methods

2.1. Volunteers

This was a randomized, controlled, and double-blinded outpatient study. Thirty healthy volunteers were stratified based on the presence or absence of previous exposure to vaccinia virus (documented history of vaccination or presence of vaccination scar). Volunteers were then allocated by block randomization to one of the four vaccine groups: NYVAC-JEV, ALVAC-JEV, BIKEN standard inactivated JEV vaccine, or saline placebo. The study schedule is shown in Table 1.

Written informed consent was obtained from each study volunteer. This protocol was approved by the Human Subjects Research Review Board of the Office of the Surgeon General, US Army. Research was conducted in accordance with Army Regulation 70-25.

2.2. Recombinant vaccines

NYVAC-JEV (vP908) and ALVAC-JEV (vCP107) recombinants were developed at Virogenetics Corporation (Troy, NY) using purified vector viruses. The methods used to generate recombinant JEV vaccines have been previously described [19]. Briefly, cDNA coding for the JEV genes (cloned from Nakayama strain JEV) was inserted into the C5 locus and was regulated by the vaccinia virus H6 promoter. Recombinants were identified and isolated by in situ hybridization, and expression of the insert was confirmed by radioimmunoprecipitation of viral proteins.

The vaccines used in this study were provided by Pasteur Merieux Connaught USA (Swiftwater, PA). Both vaccines were produced in chick embryo fibroblasts derived from pathogen-free chicken eggs. Clarified lysates of infected cells and serum-free media were filtered and combined with stabilizer before lyophilization. Both vaccine products conformed to established requirements for sterility, safety, and identity (unpublished data, on file with Pasteur Merieux Connaught and with the Walter Reed Army Institute of Research, Washington DC).

The NYVAC-JEV and ALVAC-JEV experimental vaccines were administered subcutaneously on study days 0 and 28. The doses, route of administration, and lots of each vaccine used for the Phase I study were the same as those used in the preceding monkey trial. Potencies of NYVAC-JEV and ALVAC-JEV vaccines were 5.8×10^6 and 3.1×10^6 plaque forming units per milliliter (pfu/ml) in chick embryo fibroblast cells, respectively. Reconstituted experimental vaccines were found to be within 0.5 log of described potency (NYVAC-JEV 4×10^6 pfu/ml; ALVAC-JEV 6×10^5 pfu/ml). The vaccines also contain 4.0 and 1.4 mcg/ml of JEV E protein, respectively.

BIKEN formalin-inactivated JEV vaccine (FI-JEV) was given subcutaneously on study days 0, 7 and 28, the conventional immunization schedule [20]. This vaccine was used as a positive control for the validity of

the injection procedure, specimen handling, and laboratory analysis of immunologic responses. Saline placebo recipients served as negative controls to examine the clinical and laboratory effects of the injection procedure alone. Subcutaneous saline injections were administered to volunteers in the placebo group on study days 0, 7 and 28. In order to maintain blinding of investigators and vaccine recipients, volunteers in the experimental vaccine groups received saline injections on study day 7.

Single-dose vials of lyophilized vaccine were reconstituted with 1.2 ml sterile water for injection and drawn into pre-labeled syringes immediately prior to immunization by unblinded study personnel. Inoculations were administered by an investigator who was blinded with respect to vaccine. All volunteers wore a semi-permeable polyurethane dressing (Opsite; Smith and Nephew, Massilon, OH) at the injection site for 1 week after immunization to minimize the possibility of transmission if virus was shed [21].

2.3. Clinical evaluation

Volunteers were seen every other day in the clinic for 2 weeks after each immunization. At each visit, volunteers were interviewed about local and systemic (i.e. constitutional, gastrointestinal, neurologic and musculoskeletal) symptoms. Arm pain was graded as none, mild (full use of arm), moderate (limited use of arm), or severe (inability to use arm). Severity of systemic symptoms was measured on a scale of mild (requiring no change in activity or medication), moderate (requiring change in activity and/or medication), and severe (requiring bed rest or loss of work). Volunteers were asked to monitor temperature twice daily with TempaDots (PyMaH Corp, Somerville, NJ); temperature logs were reviewed and recorded at each visit.

At each visit, the injection site dressing was removed and the site was inspected for the presence of erythema, induration, edema, warmth, tenderness, papules, vesicles or ulcers. Digital calipers were used to measure erythema and induration, if present. Additional clinical evaluation included measuring vital signs and examining skin and regional lymph nodes. Injection site swabs and dressings were cultured for virus isolation at each visit for the first six volunteers; subsequently, virus isolation was performed if there was evidence of inflammation at the injection site.

Laboratory parameters were closely followed for 2 months after initial immunization. These routinely consisted of determination of complete blood count (hemoglobin, hematocrit, white blood cell count and differential, platelet count), liver enzymes (aspartate aminotransferase, alanine aminotransferase), and renal function (serum creatinine).

2.4. Virus isolation

Isolation of NYVAC and ALVAC viruses from swabs of the injection site and dressings was performed on chick embryo fibroblast (CEF) monolayers [22]. Swabs were immediately placed in vials of 1 ml Minimum Essential Medium (MEM). Serial 10-fold dilutions (0.1 ml) were used to inoculate CEF cell monolayers in triplicate. After absorption for 60 min at room temperature, wells were overlaid with Dulbecco's MEM. After 48 h incubation at 37°C under 5% CO_2, the plates were fixed with 10% buffered formalin and stained with 2% crystal violet in methanol. Plaques were counted and values expressed in $\log_{10}$ pfu/ml. Reconstituted experimental vaccines were used as positive controls for the assay. The assays had a sensitivity of as few as 10 pfu/ml NYVAC and 100 pfu/ml ALVAC virions.

Table 1
Study schedule

Study day	Immunization	Clinical evaluation	JEV IgM	JEV PRNT	T cells
−2		×	×	×	×
0	×	×			
2, 5		×			
7	× [a]	×	×		
9, 12, 14, 21, 26		×	×	×	
28	×		×	×	
30, 33		×			
35		×	×		
42		×			
58		×	×	×	×
120				×	
180				×	

[a] NYVAC-JEV and ALVAC-JEV received vaccination with placebo on this day.

2.5. Serology

Antibody responses to JEV antigens were evaluated using a plaque reduction assay for JEV neutralizing antibody (PRNT), enzyme immunosorbent assays (EIAs) for JEV IgM and IgG, and hemagluttination-inhibition (HAI) assays. Serologic followup was completed to 6 months post immunization (Table 1).

PRNT assays for antibodies to JEV were performed in blinded fashion at both the Yale Arbovirus Research Unit (YARU) and at the Armed Forces Research Institute for Medical Sciences (AFRIMS, Bangkok, Thailand) [23]. Sera were heat-inactivated at 56°C for 30 min and serial dilutions mixed in equal volumes with 30–100 pfu of Nakayama-NIH strain virus. After incubation at 37°C for 1 h, the mixture is absorbed for 60–90 min at 37°C in 5% CO_2 onto Vero cell monolayers in 24-well plates. The inoculum was removed and the infected monolayers were overlaid with 1 ml of overlay medium. Plates were incubated for 5–7 days at 37°C in 5% CO_2. A second overlay of agar with 1% neutral red was applied and the plaques counted 24–48 h later. Neutralizing antibody results were interpreted as the highest dilution that inhibits 80% of the number of JEV plaques in Vero cells [24]. Each test is controlled by plaque titration with known positive human serum. Antibody titers $\geq$ 1:10 were defined as positive. PRNT titers measured at AFRIMS were uniformly lower than those obtained at YARU and failed to detect seroconversion in all FI-JIV recipients. The results are reported from YARU only; AFRIMS data are available on request.

The JEV IgM and IgG EIAs were performed at YARU using antibody capture and sandwich EIA, respectively [25]. The test was positive if a 1:50 dilution or greater gives an OD value which is equal to or greater than the mean + three times the standard deviations of three negative control sera. The JEV HAI tests were performed at AFRIMS as described by Clarke and Casals [26]. Antibody titers $\geq$ 1:10 were defined as positive.

PRNT assays for antibodies to vaccinia virus were performed at AFRIMS. Briefly, dilutions of heat-inactivated sera were mixed in equal volumes with 500–1000 pfu of vaccinia virus suspension. After incubation at 37°C for 1 h, the mixture is absorbed for 60 min at 37°C in 5% CO_2 onto Vero cell monolayers in 12-well plates. The inoculum was removed and the infected monolayers were overlaid with 1 ml of overlay medium. Plates were incubated for 48 h at 37°C in 5% CO_2. A second overlay of agar with 2% neutral red was applied and the plaques counted 24–48 h later. Neutralizing antibody results were interpreted as the highest dilution that inhibits 80% of the number of vaccinia virus plaques in Vero cells [24]. Each test is controlled by plaque titration with known positive human serum.

2.6. Statistical analysis

Data analysis is primarily limited to descriptive statistics in the Phase I trial. With five volunteers in each stratum, the power of the study was low (80% power for detecting 75% or greater seroconversion rate in the study groups compared to less than 5% rate in the negative control group, with a Type I error of 5% one-sided) [27]. The frequencies of local and systemic reactions and of seroconversion were calculated for each of the study and control groups. Geometric mean antibody titers (GMT) were derived for each group as well, with neutralizing and HAI antibody titers less than 1:10 interpreted as 1:5 and EIA titers less than 1:50 and greater than 400 interpreted as 1:25 and 1:600, respectively, for computation of GMT.

Analysis of variance was used to compare values of variables between all vaccine groups when appropriate. Fisher's exact t-test was used for such variables when only two sets were compared (e.g. V-N vs. V-I recipients). p-Values of < 0.05 were considered significant in all analyses.

3. Results

3.1. Clinical summary

Thirty volunteers participated in the trial, including 13 V-N and 17 V-I volunteers. Volunteers were allocated to four vaccine groups: NYVAC-JEV ($n = 10$), ALVAC-JEV ($n = 10$), BIKEN standard inactivated JEV vaccine ($n = 5$), or saline placebo ($n = 5$) (Table 2). Distribution of male and female volunteers was similar between vaccination groups. While V-N volunteers were younger than V-I volunteers overall (mean 21 vs. 40 years), mean ages between vaccine groups were similar.

3.2. Safety

There were no severe adverse events, requirements for emergency treatment, hospitalizations, or deaths in the study. No volunteers experienced fever (temperature $\geq$ 38°C) or lymphadenopathy. Three volunteers were medically disqualified from the study: two from the placebo group and one from the ALVAC-JEV group (Table 3). One placebo recipient had symptoms of allergic rhinitis after receiving a single saline immunization and was treated with oral antihistamines; the other had serum transaminase abnormalities following vigorous exercise. The ALVAC-JEV recipient had persistently low white blood cell counts attributed to

Table 2
Composition of vaccine groups

Vaccine	Vaccinia-immune	Number	Gender (male:female)	Mean age in years (SD)
SALINE	No	2	1:1	23 (2)
	Yes	3	3:0	34 (14)
FI-JEV	No	2	1:1	21 (1)
	Yes	3	3:0	43 (13)
NYVAC-JEV	No	5	3:2	21 (2)
	Yes	5	3:2	44 (7)
ALVAC-JEV	No	4	2:2	22 (3)
	Yes	6	3:3	37 (8)

benign ethnic neutropenia. One volunteer from the NYVAC-JEV group required emergency personal leave out of state and was disqualified after receiving a single NYVAC-JEV immunization. These four volunteers received no further vaccinations but were followed clinically for the duration of the trial.

No volunteers developed vesicles, pustules, ulcerations, or urticaria after vaccination. One ALVAC-JEV recipient had two pinpoint scabs near the injection site 1 week after saline immunization. These lesions resolved spontaneously and no virus was recovered from the area or from the overlying dressing.

No recombinant poxviruses were isolated from the injection sites or dressings of any volunteer. There was no dissemination or autoinoculation of virus recorded in vaccine recipients and no volunteers reported spread of virus to household contacts.

3.3. Reactogenicity

NYVAC-JEV and ALVAC-JEV vaccine recipients had more frequent occurrence of local reactions compared to FI-JEV and saline recipients (Table 3). Volunteers reported arm pain on a single visit except for one FI-JEV recipient who had pain on two consecutive study visits. NYVAC-JEV and ALVAC-JEV volunteers reported arm pain after they received active vaccine twice, following their first (5/10 and 8/10, respectively) and third injections (5/9 and 6/9). Arm pain was mild in all but two ALVAC-JEV volunteers, who reported moderate arm pain 2 days following vaccination. In the saline, FI-JEV, and ALVAC-JEV vaccine groups, there were no apparent differences between V-N and V-I individuals reporting arm pain. In the NYVAC-JEV group, fewer V-N recipients (2/5) reported ever having arm pain than V-I volunteers (5/5). All V-I recipients of both investigational vaccines reported arm pain after administration.

Warmth, tenderness, and edema were noted at the injection site in volunteers who received NYVAC-JEV, ALVAC-JEV, or FI-JEV but were not found in saline recipients (Table 3). Erythema and/or induration at the injection site were noted in all NYVAC-JEV and nearly all ALVAC-JEV volunteers. The same signs were infrequently found in FI-JEV recipients and not at all in placebo recipients.

The area of erythema (vertical × horizontal dimensions) was computed for all volunteers after each vaccination and the maximum value selected for each individual. Placebo recipients and the majority of FI-JEV recipients had no detectable erythema after vaccination. A single FI-JEV recipient had a maximum area of erythema of 15.4 cm^2. In contrast, NYVAC-JEV and ALVAC-JEV vaccines elicited erythema

Table 3
Local reactogenicity following immunization

Vaccine	Vaccinia-immune	Number	Arm pain	Warmth	Tenderness	Edema	Erythema[a]	Induration[b]
SALINE	No[c]	2	0	0	0	0	0	0
	Yes[c]	3	1	0	0	0	0	0
FI-JEV	No	2	2	1	2	1	1	1
	Yes	3	1	0	2	0	0	1
NYVAC-JEV	No[c]	5	2	4	4	1	5	5
	Yes	5	5	4	4	3	4	5
ALVAC-JEV	No[c]	4	3	4	3	2	2	2
	Yes	6	6	5	5	1	5	3

[a] Greater than 1 cm^2 in area.
[b] Greater than 1 cm in diameter.
[c] One volunteer did not receive the complete vaccine serie.

(NYVAC-JEV: mean 30.8 cm^2, standard deviation (SD) 8.4 cm^2; ALVAC-JEV: mean 24.1 cm^2, SD 9.5 cm^2). Induration was also noted in recipients of investigational vaccines: NYVAC-JEV (mean 2.9 cm, SD 0.5 cm) and ALVAC-JEV (mean 1.3 cm, SD 0.6 cm). Both responses were greater in NYVAC-JEV and ALVAC-JEV recipients after their second active vaccination.

Within each group, there was little difference in frequency or magnitude of local reactogenicity between V-N and V-I volunteers. However, there was a difference in time of onset of local responses after vaccination between V-N and V-I recipients of NYVAC-JEV vaccine. Four of the five V-N volunteers manifested erythema, swelling and induration without much pain or tenderness 6–9 days after the first dose; these resolved in 5–7 days. All other volunteers, including V-I recipients of NYVAC-JEV vaccine, evidenced inflammatory responses within the first 2 days following vaccination.

Several systemic symptoms were reported by volunteers on at least one clinic visit; most were mild and short-lived. The most common included headache and arthralgias. There were no differences in occurrence and severity of systemic symptoms among the four groups of volunteers and after stratification for V-I status.

3.4. Laboratory abnormalities

No abnormalities were noted in platelet counts. Several volunteers from all vaccine groups experienced transient depression of hematocrit after repeated venipunctures. Two volunteers had abnormalities noted in peripheral leukocyte blood counts: one placebo recipient had occasional elevations of leukocyte counts and an ALVAC-JEV recipient had borderline low leukocyte counts which decreased from 4.2×10^3 to 3.3×10^3 mm^{-3} after his first vaccination, with absolute neutrophil counts greater than $1500/mm^3$. He was diagnosed as having benign ethnic neutropenia and maintained stable total white blood cell counts of $3.5–4.5 \times 10^3$ mm^{-3} for the remainder of the trial.

No abnormalities were discovered in serum creatinine. The sole chemical abnormality noted was elevations in liver enzymes. One placebo recipient developed striking elevations of serum enzyme levels 2 days following strenous physical exercise (maximum aspartate aminotransferase 451 and creatinine phosphokinase 8314 with normal myocardial and brain band fractions). These abnormalities resolved without intervention within 1 week and did not recur. Two volunteers (1 NYVAC-JEV, 1 ALVAC-JEV) had less than two-fold liver enzyme elevations 2–3 weeks after their first immunization, which was also attributed to physical exercise; tests returned to normal within 3–5 days and no subsequent elevations were noted.

3.5. Serology

Immune responses in the vaccine groups varied depending on the test used (Table 4). No volunteers immunized with saline placebo had JEV-specific antibody responses detected by any of the assays. There was no effect of V-I status on antibody responses following immunization with saline or FI-JEV vaccine, hence the data from V-I and V-N volunteers are combined for each control group.

There was a significant effect of vaccination group on seroconversion with JEV PRNT antibody ($p = 0.002$). All FI-JEV vaccine recipients developed PRNT antibodies on day 58. PRNT antibody responses after NYVAC-JEV vaccination were significantly different in V-N vs. V-I volunteers: all V-N recipients seroconverted while no V-I recipients did ($p = 0.004$). The GMT of 1:61 for V-N NYVAC-JEV recipients was less than the GMT of 1:211 for all FI-JEV recipients. Only one ALVAC-JEV recipient, a V-I individual, developed PRNT antibody and this was of low titer.

IgG antibody responses determined by EIA were not prominent in any vaccination group, yet NYVAC-JEV recipients demonstrated the greatest seroconver-

Table 4
Serologic responses following immunization

Vaccine	Vaccinia-immune	PRNT		IgG EIA		IgM EIA	
		Seroconversion	GMT_{58}	Seroconversion	GMT_{58}	Seroconversion	GMT_{28}
SALINE	Combined	0/5	< 1:10	0/5	< 1:50	0/5	< 1:50
FI-JEV	Combined	5/5	1:211	1/5	1:47	1/5	1:29
NYVAC-JEV	No	5/5	1:61	3/5	1:168	3/4[a]	1:59
	Yes	0/5	< 1:10	2/5	1:50	0/5	< 1:50
ALVAC-JEV	No	0/4	< 1:10	0/4	< 1:50	0/4	< 1:50
	Yes	1/6	1:7	2/6	1:48	0/6	< 1:50

[a] Excludes volunteer without day 28 specimen.

sion rates on day 58 (5/10 volunteers). There was no apparent difference between V-N and V-I NYVAC-JEV recipients, although V-N individuals had the highest GMT. Only one of the five FI-JEV and two of the 10 ALVAC-JEV recipients developed detectable IgG antibodies after immunization (GMT 1:47 and 1:37, respectively); all the three were V-I individuals.

Similarly, NYVAC-JEV recipients had the highest seroconversion rate as determined by IgM EIA (3/9 evaluated volunteers). Three of the four V-N NYVAC-JEV recipients tested had detectable IgM antibody responses on day 26 while only one of the five FI-JEV recipients did (GMT 1:59 and 1:29 respectively). Neither V-I NYVAC-JEV nor ALVAC-JEV recipients developed detectable JEV IgM responses.

HAI antibody responses were low but demonstrated seroconversion in FI-JEV, NYVAC-JEV, and ALVAC-JEV recipients (data not shown). Three of the five FI-JEV vaccine recipients had detectable HAI antibody by study day 35 (GMT 1:10). Two of the five NYVAC V-N volunteers and two of the six ALVAC-JEV V-I volunteers (GMT 1:9 and 1:6, respectively) seroconverted. However, none of the NYVAC-JEV V-I or ALVAC-JEV V-N volunteers developed HAI antibody after immunization.

No volunteer developed anti-vaccinia neutralizing antibodies following immunization with recombinant poxvirus vaccines.

4. Discussion

We conducted a Phase I study to determine whether NYVAC-JEV and ALVAC-JEV, two attenuated recombinant poxviruses with identical JEV gene insertions, are safe and immunogenic in humans, and to detect the effect, if any, of preexisting vaccinia immunity on vaccine reactogenicity and immunogenicity. In this preliminary study, both NYVAC-JEV and ALVAC-JEV vaccines appeared to be equally safe and well tolerated in humans. NYVAC-JEV and ALVAC-JEV vaccine recipients had more frequent occurrence of local reactions (warmth, tenderness, erythema, and/or arm pain) compared to FI-JEV and saline recipients. There was no apparent effect of V-I status on frequency or magnitude of local and systemic reactogenicity to these vaccines. NYVAC-JEV elicited antibody responses to JEV antigens while ALVAC-JEV vaccine induced poor antibody responses in recipients. However, the most pronounced antibody responses were observed in V-N volunteers who received NYVAC-JEV while V-I volunteers failed to develop neutralizing antibodies.

We found no evidence of virus replication at the injection site after subcutaneous immunization with either experimental vaccine and observed no cutaneous or systemic complications suggesting dissemination of vaccine virus. These data, coupled with the lack of abnormal clinical or laboratory findings, support a high degree of attenuation of these experimental poxvirus vectors. Despite this apparent decreased viral virulence, NYVAC-JEV and ALVAC-JEV vaccines were more reactogenic than FI-JEV. The degree and frequency of local reactogenicity was similar for NYVAC-JEV and ALVAC-JEV vaccines. These responses are consistent with those observed in volunteers receiving two doses of 5.5 logs of ALVAC-rabies vaccine [28].

There were no significant differences between V-N and V-I volunteers in the frequency and intensity of local and systemic responses to the study vaccines. V-I recipients of NYVAC-JEV had early local reactions, consistent with prominent anti-vaccinia T cell responses. In contrast, 80% of V-N volunteers experienced a 5–7-day delay in onset of local responses after their first dose of NYVAC-JEV vaccine, suggesting a delayed primary immune response. There was no correlation between the frequency or intensity of local inflammatory responses and the antibody titers achieved. However, the timing of appearance of local reactions in V-I recipients may have limited vector replication and expression of JEV antigen, resulting in decreased anti-JEV antibody responses.

Immunogenicity of NYVAC-JEV and ALVAC-JEV vaccines was assessed using three antibody tests. All sera were tested in blinded fashion and included internal controls. The PRNT is a functional assay that most directly correlates with immunity sufficient to prevent disease after natural infection; a neutralizing antibody titer of $\geq$1:10 is presumed to confer protection [29]. Immunization with FI-JEV induced substantial levels of neutralizing antibody as expected in all recipients, with no differences between V-N and V-I individuals. NYVAC-JEV vaccine induced neutralizing antibodies in 50% of all recipients, but only in volunteers that had not been previously immunized with vaccinia virus. ALVAC-JEV vaccine induced poor neutralizing antibody responses in recipients regardless of V-I status. The two other tests (IgM and IgG EIAs, HAI assay) demonstrate antibodies to JEV antigens which may not be protective, and the low levels of JEV-specific antibodies detected using these assays make them less helpful in establishing differences between vaccine groups or between V-N and V-I volunteers within a group.

Cell mediated immune responses to NYVAC-JEV and ALVAC-JEV vaccines were assessed using T cell proliferation and cytotoxicity assays [18]. These tests were chosen to complement the ability of antibody assays to detect specific JEV responses and provide accessory data for interpreting responses in V-N and V-I volunteers. Cellular immunity to JEV was detected

in 45% (9/20) of recipients of experimental vaccines. ALVAC-JEV recipients developed proliferative T cell responses to JEV antigens regardless of their V-I status; positive assays in three V-I recipients suggest that sufficient expression of JEV genes occurred to stimulate immune responses, despite undetectable neutralizing antibody responses. Similarly, NYVAC-JEV vaccine induced JEV specific T cell responses in one V-I volunteer who did not have an anti-JEV antibody response. As poxviruses are potent cellular immunogens, we were also concerned that T cell responses to the vectors may occur without apparent production of JEV-specific antibodies. Volunteers had T cell proliferative responses to the NYVAC and ALVAC vectors (Konishi, personal communication). In particular, V-I individuals that received NYVAC-JEV had more prominent cellular responses to NYVAC than V-N volunteers did. This is in direct contrast to the JEV serologic responses, where V-N volunteers had significantly greater PRNT responses than did V-I volunteers.

The licensed inactivated JE vaccine produced the most consistent antibody responses overall, while the investigational vaccines produced lower antibody responses. Decreased immunogenicity of recombinant poxvirus vaccines may reflect differences in V-I status: individuals with preexisting memory T cells to vaccinia virus may be at a disadvantage for vaccinia vectored antigens, as responses to vaccinia vector thwart the initial immune response to expressed viral antigens [30]. The route of vaccine administration selected (subcutaneous vs. intramuscular or scarification) may provide an alternative explanation for decreased immunogenicity of the experimental poxvirus vaccines. Volunteers received subcutaneous injections as recommended for licensed inactivated JEV vaccine in order to allow comparability of study vaccines. In addition, this route of vaccine administration paralleled that established with preclinical studies and in an ALVAC-RG clinical study [14]. It is known, however, that vaccinia virus replicates to a much higher degree and induces higher levels of neutralizing antibody responses when administered by the scarification method [31]. The doses used in the study were the maximal yields achievable in culture, but these doses are lower than titers delivered by scarification. Moreover, the lack of a booster third dose of vaccine may have affected immune responses to the experimental vaccines. The relative poor immunogenicity of ALVAC-JEV compared to NYVAC-JEV may reflect decreased efficiency of JEV protein expression (Konishi, personal communication). Differences in antigen expression may be critical for a vector with limited replication, because cross-reactive memory responses to the vector may decrease vaccine immunogenicity.

V-N and V-I individuals were equally allocated to each of the four vaccine groups, and both age and gender did not vary significantly between groups. However, the average age of V-N volunteers was less than that of V-I volunteers, consistent with the cessation of routine smallpox vaccination in the general population about 25 years ago. This difference in age may have had an independent effect upon immune responses following vaccination.

In summary, NYVAC-JEV and ALVAC-JEV recombinant poxvirus vaccines appear to be safe and tolerable. NYVAC-JEV induces protective antibodies with local reactogenicity in V-N individuals in this preliminary study, and is worthy of further investigation. ALVAC-JEV vaccine was not very immunogenic in either V-N or V-I volunteers. The potent effect of preexisting vaccinia immunity on responses to NYVAC-JEV vaccine suggests that future trials of recombinant vaccinia vectored vaccines should identify the V-I status of volunteers and include both V-N and V-I individuals. These data may have important implications for the use of other vector virus vaccines, such as adenovirus or herpes simplex virus, where preexisting immunity may suppress responses to recombinant gene products.

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