SAFETY, IMMUNOGENICITY, AND PROTECTIVE EFFICACY OF NYVAC-JEV AND ALVAC-JEV RECOMBINANT JAPANESE ENCEPHALITIS VACCINES IN Rhesus MONKEYS

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Abstract. Two poxvirus-vectored vaccines for Japanese encephalitis (JE), NYVAC-JEV and ALVAC-JEV, were evaluated in rhesus monkeys for safety, immunogenicity, and protective efficacy. The vaccines were given to four monkeys each on study days 0 and 28 along with saline placebo on day 7. For controls, the licensed BIKEN JE vaccine and a saline placebo were given to other groups of four monkeys on days 0, 7, and 28. No systemic effects were observed. All injection site reactions were mild. All vaccines elicited appreciable JE-specific neutralizing antibody responses. However, a more rapid increase and higher peak level of antibody were seen in the BIKEN group as compared with the NYVAC-JEV and ALVAC-JEV groups. The peak neutralizing antibody level in the NYVAC-JEV group was higher than that of the ALVAC-JEV group. Antibody persisted in all four BIKEN recipients through 273 days of follow-up, whereas, the antibody level decreased to the threshold of detection in two NYVAC-JEV and all four ALVAC-JEV recipients by day 120. On day 273, all monkeys were given a booster dose. A rapid increase in neutralizing antibody was seen in all vaccine recipients by seven days. Two months after the booster dose, all monkeys were challenged intranasally with one 90% effective dose of JE virus. Four recipients of saline, three of ALVAC-JEV, one of NYVAC-JEV, and one of BIKEN experienced encephalitis. This study suggests that the NYVAC-JEV and ALVAC-JEV vaccines are safe and immunogenic in monkeys and that the NYVAC-JEV and BIKEN vaccines are effective in protecting monkeys from encephalitis.

Japanese encephalitis virus (JEV), a member of the family Flaviviridae, is the most common cause of viral encephalitis in Asia, affecting an approximately 35,000 people annually.1-3 Virus transmission to humans occurs through the bite of culicine mosquitoes that have been infected by feeding on viremic swine or birds. Infection in humans is usually subclinical with a rate of overt encephalitis of only one of 20 to 1,000 cases. However, clinical disease is often severe with case fatality rate of 25% and neurologic sequelae in 50% of the survivors. This virus also affects domestic livestock causing fetal wastage in swine and encephalitis in horses.

Several JE vaccines are available for humans.2,3 Inactivated JE vaccine (P3 strain) and live attenuated vaccine (SAa214-2 strain), which are produced in primary hamster kidney cells, have been used only in China.4-6 An inactivated JE vaccine purified from infected mouse brains is more widely used and has been produced in some Asian countries including Japan, Korea, India, Taiwan, Thailand, and Vietnam. An efficacy study conducted in Thailand in 1984-1985 using monovalent (Nakayama strain) and bivalent (Nakayama-Beijing strains) mouse brain vaccines demonstrated 91% efficacy for both formulations with minimal side effects.7 In 1992, the BIKEN mouse brain vaccine was licensed in the United States. Although efficacious, the vaccine is relatively expensive, requires booster doses for long-term immunity, and may be overly reactogenic. Allergic reactions following vaccination have been reported from Denmark,8 Australia,9 Canada,10 and the United Kingdom.11 Recently, concerns have been raised about the possibility of post-vaccination encephalitis due to contaminating mouse brain tissue.12,13

Live recombinant poxviruses have been evaluated as vectors to deliver proteins of other pathogens. These systems not only provide a means of mimicking viral infection that induces both humoral and cell-mediated immunity, but also have additional advantages such as the capacity to express genes from multiple pathogens and to yield high levels of recombinant viruses in appropriate cell cultures. A major obstacle of using poxvirus vectors has been the safety concern, especially in immunocompromised individuals. The issue has been addressed by the development of a highly attenuated strain of vaccinia virus (NYVAC) and the use of a host-range restricted canarypox virus (ALVAC).14,15 A number of recombinant vaccine candidates based on these vectors have been developed, including vaccines against human immunodeficiency viruses,16,17 rabies virus,18,19 JEV,20,21 and malaria.22 The NYVAC and ALVAC recombinants encoding premembrane (prM), envelope (E), and nonstructural protein-1 (NS1) genes of JEV have been shown to protect mice against lethal JEV challenge.20,21 In addition, immunizing swine with the NYVAC-JE virus recombinant could also prevent JE viremia upon virus challenge.20 Here, as a prelude to clinical development of these vaccines in humans, we evaluated whether NYVAC-JEV and ALVAC-JEV vaccine candidates are safe and immunogenic in rhesus monkeys. Data on protective efficacy against wild-type JEV challenge is also presented.

MATERIALS AND METHODS

Study protocol. A primate protocol to assess the safety, immunogenicity, and efficacy study of NYVAC/JEV and ALVAC/JEV vaccines was developed and approved by an Institutional Animal Care and Use Committee (United States Army Medical component, Armed Force Research Institute of Medical Sciences) and by the Animal Use Review Office,
Monkeys. Sixteen healthy rhesus monkeys (Macaca mulatta) 3–7 years of age of either sex, weighing 4.1–8.0 kg, were used. Animals had no neutralizing antibody against JE and dengue viruses. The monkeys were caged individually in a double-screened housing facility. The monkeys were randomized into groups of four to receive one of the four test articles.

Vaccine dose and schedule. The test articles included NYVAC-JEV, ALVAC-JEV, a commercially available BIKEN JE vaccine (positive control), and normal saline (negative control). The NYVAC-JEV (vP908 recombinant JEV-vaccinia virus) and ALVAC-JEV (vCP107 recombinant JEV-canary poxvirus) vaccines were produced in chicken embryo fibroblasts in accordance with good manufacturing practice guidelines by Pasteur-Mérieux-Connaught (Swiftwater, PA). Both vaccine lots were shown to be safe and immunogenic in mice and rabbits. One-milliliter doses containing 5.8 × 10^6 plaque-forming units (pfu) of NYVAC-JEV or 3.1 × 10^6 pfu of ALVAC-JEV were given subcutaneously on study days 0 and 28. Physiologic saline (1 ml) was given on day 7 to conform with the BIKEN control vaccine schedule to allow appropriate masking of vaccine administration.

The BIKEN inactivated JE vaccine (JE-VAX™; Research Foundation of Microbial Diseases of Osaka University, Osaka, Japan) was used as a vaccine control because it has been previously shown to be effective in humans. The vaccine (1 ml, Lot EJN 058) was given subcutaneously on study days 0, 7, and 28. Unimmunized controls were given 1 ml of physiologic saline on study days 0, 7, and 28. On study day 273, all 16 monkeys were given a booster dose of the appropriate vaccine or saline control. All immunizations were performed under code.

Following each vaccination, monkeys were kept in individual cages and observed daily for local reactions at the injection site (warmth, erythema, swelling, induration, and necrosis) and for systemic toxicity (activity, appetite, and weight) for seven days after each vaccination. Blood samples were collected on study days 0, 3, 7, 14, 21, 28, 31, 35, 42, 49, 56, 84, 120, 150, 210, 240, 269, 273, 276, 280, 287, 294, 301, and 329 to assess blood chemistry, hematology, and antibody responses.

Clinical laboratory studies. Blood urea nitrogen, creatinine, and hemoglobin levels, hematocrit, total white blood cell count, and platelet count were determined in a clinical laboratory at the Bangkok Children’s Hospital (Bangkok, Thailand). Aspartate aminotransferase and alanine aminotransferase levels were measured using commercial Dri-STAT kits (Beckman Instruments, Brea, CA). Normal ranges of blood chemistry and hematology values for rhesus monkeys were established using 64 blood samples obtained from all 16 monkeys on study days −34, −14, −7, and 0 (prior to immunization).

Immunologic assays. The JE-specific neutralizing antibody titer was determined using the 50% plaque-reduction neutralization test. Immunoglobulin M to JEV in the cerebrospinal fluid (CSF) was determined by ELISA. The normal range for CSF IgM to JEV was determined using 28 prechallenge monkey samples. The mean ± SD antibody level was 1.4 ± 2.5 ELISA units. A value of CSF IgM to JEV greater than 10 units was considered positive.

Intranasal challenge. Two months after the booster dose, all 16 monkeys were challenged intranasally with one 90% effective dose of JEV strain KE-93 (AP61-1, C6/36-1, Mm-1, SM-2) by a procedure described elsewhere. Evaluation of the protective efficacy of the two candidate poxvirus vaccines was performed at the same time as the validation of the intranasal challenge model described in the accompanying paper using the same four saline and four BIKEN vaccine control monkeys. Clinical evidence of encephalitis (i.e., anorexia, weakness, depression, tremor, paralysis, or coma) was sought daily for 28 days following challenge. Blood was collected on challenge day 0 just before virus inoculation and days 1–8 after challenge, and again on days 10, 12, 14, 21, and 28, to assess the extent of viremia and immune responses. Cerebrospinal fluid was collected 7–10 days before challenge and on challenge days 14 and 28 or at the time of necropsy. Necropsy, virus isolation from the blood, CSF, or tissues, and detection of JEV RNA by the reverse transcription–polymerase chain reaction (RT-PCR) were performed as described. Encephalitis due to JEV was defined as the occurrence of focal neurologic signs or depressed consciousness plus one of the following: the presence of JEV in the CSF, IgM to JEV in the CSF, or detection of JEV in tissues of the central nervous system (CNS) if the monkeys were killed.

RESULTS

Local reactogenicity. After each vaccination, injection site reactions were minor for all test vaccines. There was no swelling or necrosis at the injection site for any vaccines tested. Induration was found in one monkey receiving ALVAC-JEV on study days 4 and 7 after the first dose of vaccine. To analyze the reactogenicity data statistically, the frequency of occurrence was scored collectively for all four monkeys within the same group. Totals of 12 times of injection for NYVAC-JEV or ALVAC-JEV group (three injections each × four monkeys) and 16 times for BIKEN or saline group (four injections each × four monkeys) were considered. Erythema was found to be more common for monkeys receiving NYVAC-JEV and ALVAC-JEV vaccines when compared with monkeys receiving saline placebo (12 of 12 and 11 of 12 versus 5 of 16; P = 0.0002 and 0.0049 by Fisher’s exact test, respectively). However, the numbers of vaccinations associated with erythema were not different between the BIKEN vaccine and saline, and among the three vaccine groups (P > 0.05). The NYVAC-JEV, ALVAC-JEV, and BIKEN vaccine recipients also experienced an extended duration of erythema compared with saline recipients (mean = 2.3, 2.9, and 1.8 days versus 0.6 days, respectively; P < 0.05 by Student’s t-test).

Systemic toxicity. Activity, appetite, and body weight of all 16 monkeys remained normal after each dose of vaccine. Deviation of hematology and blood chemistry values beyond the normal range was determined using samples collected three and/or seven days after each vaccine injection. Normal ranges for each assay were established as means ± 2 SD
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FIGURE 1. Japanese encephalitis virus (JEV)-specific neutralizing antibody after vaccination and after challenge with JEV. Four groups of four monkeys were immunized with A, NYVAC-JEV, B, ALVAC-JEV, C, BIKEN, and D, saline on study days 0, 7, 28, and 273. Saline was given to monkeys in the NYVAC-JEV and ALVAC-JEV groups on day 7 instead of vaccine. Thin and dotted arrows indicate immunization with test vaccines and saline placebo, respectively. Reciprocal neutralizing antibody titers (neutralizing titer) are plotted for each monkey. The baseline antibody level of less than 1:10 is plotted as 5. Monkey identifications are shown in each panel. Monkeys that developed encephalitis are shown in a shaded box. All monkeys were challenged intranasally with one 90% effective dose of JEV on day 339 (thick arrows).

Calculated from the 64 prevaccination sera (16 monkeys, four samples per monkey). The normal ranges were blood urea nitrogen = 11-33 mg/dL, creatinine = 0.7-1.2 mg/dL, alanine transaminase = 0–71 IU/L, aspartate transaminase = 17–43 IU/L, hemoglobin = 10.6–15.5 g/dL, hematocrit = 34.2–50.3%, white blood cell count = 3,900–12,900 /μL, and platelet count = 188,000–494,000 /μL. There were a number of injections associated with abnormal values (slightly higher or lower) observed in the NYVAC-JEV, ALVAC-JEV, and BIKEN vaccine groups compared with the saline group; however, they appeared to be associated with particular monkeys. In addition, the frequency of injections associated with abnormal values were not statistically different between the groups (P > 0.05).

Immunogenicity. Japanese encephalitis virus–specific neutralizing antibody was followed for 339 days after vaccination (Figure 1). In the NYVAC-JEV group, an increase in antibody level was detected on study day 21 in three monkeys. By day 28 before the second vaccine dose, antibody responses were observed in all four monkeys. Thereafter, the
antibody level peaked on study day 35 and decreased to undetectable levels by study day 120 in two monkeys (DA254 and DA479) and by day 210 in one monkey (DA353). Neutralizing antibody persisted in one monkey (DA361) until the booster dose on day 273. All four monkeys responded markedly to the booster vaccine, indicating that immunologic memory had been established by the primary vaccination series.

Lower levels of neutralizing antibody were seen in the ALVAC-JEV vaccine group. Antibody appeared on day 28 after the first vaccine dose, peaked around day 35, and returned to baseline levels on day 120 in all four monkeys. All monkeys given the ALVAC-JEV vaccine responded to the booster dose given on day 273, suggesting a functional immunologic memory in all four monkeys.

With a three-dose schedule (days 0, 7, and 28), a more rapid increase and higher magnitude of neutralizing response was seen in the BIKEN vaccine group compared with the previous two vaccine groups. Antibody was detected on day 14 and peaked on day 35 after the third vaccine dose. High levels of neutralizing antibody persisted in all four monkeys until study day 273. Neutralizing antibody levels increased following the fourth vaccine dose. Mean neutralizing titers of neutralizing antibody in an animal that withstood the JEV challenge was 1:7–12 days postchallenge. The lowest titer of neutralizing antibody in an animal that withstood the JEV challenge was 1:46 (monkey DA479) and the maximum titer that failed to protect monkeys from encephalitis severe enough to require killing was 1:30 (monkey DA400). In the NYVAC-JEV group, viremia was detected by the PCR in monkey DA353 on days 2, 3, 6, and 8 postchallenge and in monkey DA361 on day 7 postchallenge. In the ALVAC-JEV group, viremia was also detected by the PCR in monkey DA398 on day 5, 8, and 12 postchallenge and in monkey DA400 on day 8 and 12 post challenge. However, no JEV was isolated from these blood samples. There was no neutralizing antibody response seen in the saline group after challenge but IgM to JEV could be detected in the sera of three monkeys a day before or at the day of necropsy. More details of postchallenge responses of monkeys in the saline and BIKEN groups are presented as part of the validation of the intranasal challenge model in an accompanying paper.25

Infection of the CNS by JEV was examined by virus isolation, JE PCR, and JE IgM assay using CSF specimens collected 14 and 28 days postchallenge. Virus isolation and JE PCR were also performed on necropsied CNS tissues. Table 1 summarizes the data from the NYVAC-JEV and ALVAC-JEV vaccine groups. Data from the BIKEN vaccine and saline control groups are presented in an accompanying paper.25 No JEV was isolated from the CSF of any monkey. Viral RNA was detected in the CSF of only one monkey in the saline group (DA378). For monkeys that survived the challenge, CSF JE IgM was detected in two NYVAC-JEV monkeys (DA353 and DA479), one ALVAC-JEV monkey (DA398), and one BIKEN monkey (DA422), indicating CNS infection. For the monkeys that succumbed to encephalitis, CSF JE IgM was detected in one ALVAC-JEV monkey (DA445) and in all four monkeys in the saline group. Virus was isolated and JEV RNA was also detected in the necropsied CNS tissues of all monkeys with encephalitis.

**DISCUSSION**

Many live recombinant vaccine candidates have been developed based on a highly attenuated strain of vaccinia (NYVAC) and a host-range restricted canary poxvirus (ALVAC).14-22 In the present study, we evaluated the safety, im-

| Monkey | Vaccine   | Day of killing* | CNS tissues³ | | | | |
|---------|-----------|----------------|-------------| | | | |
| DA254   | NYVAC-JEV | 11             | –           | | | |
| DA353   | NYVAC-JEV | –              | ND          | | | ND |
| DA361   | NYVAC-JEV | –              | ND          | | | ND |
| DA479   | NYVAC-JEV | –              | –           | | | ND |
| DA350   | ALVAC-JEV | 12             | –           | | | + |
| DA398   | ALVAC-JEV | –              | ND          | | | ND |
| DA400   | ALVAC-JEV | 11             | –           | | | + |
| DA445   | ALVAC-JEV | 13             | –           | | | + |

* Monkeys that developed encephalitis were killed on days postchallenge as indicated. – = no encephalitis.
† Japanese encephalitis virus (JEV) isolation and a J E polymerase chain reaction (PCR) was performed on CSF collected on study day 14 or at the time of necropsy. JE IgM was determined in CSF collected on study days 14 and 28 or at the time of necropsy. + = positive; – = negative; ND = not done.
‡ The CNS tissues include cerebral cortex, cerebellum, brain stem, spinal cord (cervical, thoracic, and lumbar). Plus sign denotes J E virus isolated or JE PCR positive for all tissues listed above.
munogenicity, and protective efficacy of two vaccine candidates against JE derived from these poxvirus vectors (NYVAC-JEV and ALVAC-JEV) in rhesus monkeys.

The NYVAC-JEV and ALVAC-JEV vaccines were relatively safe compared with the BIKEN vaccine. However, erythema seemed to be common among the monkeys that received these two vaccines. It is interesting to note that on study day 7 when saline was given to monkeys in the NYVAC-JEV and ALVAC-JEV groups instead of vaccines, erythema was seen in only one monkey in each group. Therefore, erythema often seen in monkeys receiving the NYVAC-JEV and ALVAC-JEV vaccines was likely to be due to the vaccines themselves. Since both are live vaccines, we speculate that it might be a result of immunologic reactions to cells at the injection sites that were infected by the vaccine viruses.

The NYVAC-JEV and BIKEN vaccines appeared comparable in protecting monkeys from the JEV challenge. Why did the ALVAC-JEV vaccine apparently fail? One possibility is that ALVAC-JEV is less immunogenic in primates than NYVAC-JEV. It is evident in Figure 1 that neutralizing antibody levels in the group receiving ALVAC-JEV decreased sooner after each vaccination than those receiving the NYVAC-JEV vaccine. Considering that the NYVAC vector is an attenuated strain of vaccinia and that the ALVAC vector is an avian poxvirus, we speculated that cells of primate species would probably favor the expression of foreign proteins (in this case, JEV proteins) of the NYVAC rather than the ALVAC vector.

Four saline recipients, three ALVAC-JEV recipients, one NYVAC-JEV recipient, and one BIKEN recipient developed encephalitis. Aside from the monkey that received BIKEN vaccine (DA422), all others developed signs of diffuse cerebral injury that progressed to deep coma, prompting killing. Monkey DA422 represented an unusual case in that it developed encephalitis in spite of a relatively high titer of serum neutralizing antibodies (1:1,150) at the time of challenge. The clinical signs included depression, anorexia, and paralysis of limbs during 9–18 days postchallenge, but they did not progress to coma. The monkey recovered completely. An increase of CSF IgM to JEV was also seen in this monkey, confirming that the virus did invade the CNS. Factors contributing to disease in this monkey are unknown. From this study, if this unusual surviving case was excluded, the minimal level of neutralizing antibody required to protect the monkeys from lethal challenge was between 1:30 (monkey DA400) and 1:46 (monkey DA479).

A previous study has shown that vaccinating swine with $10^9$ pfu of NYVAC-JEV vaccine 28 days apart induces JE neutralizing antibody responses. It should be noted that in that study, JE neutralizing antibody levels peaked on day 7 after the first vaccination; thereafter the antibody levels decreased gradually to undetectable levels on day 28, suggesting a short-lived immunity. In our monkey study, JE neutralizing antibodies of all four monkeys inoculated with NYVAC-JEV vaccine (5.8 $\times$ $10^8$ pfu) continued to increase from study days 14–28 after primary vaccination. The difference may reflect the permissiveness of a primate species that favors the expression of viral antigens after NYVAC-JEV vaccination.

Earlier studies on the immunogenicity of ALVAC-JEV vaccine have been performed only in mice. Mice immunized with $10^9$ pfu of ALVAC-JEV (vP107) three weeks apart did not show detectable JE neutralizing antibodies three weeks after the primary vaccination. However, JE neutralizing antibodies could be detected three weeks after the second dose. Similarly, a slow JE neutralizing antibody response was also seen in our study. Antibodies were first detected in monkeys immunized with ALVAC-JEV (3.1 $\times$ $10^8$ pfu) on study day 28; the levels of these antibodies increased somewhat after the second vaccine dose and decreased to undetectable levels by study day 120 (Figure 1). The data suggested that at this vaccine dose and regimen, ALVAC-JEV could induce a transient JE neutralizing antibody response that lasted about three months. Compared with NYVAC-JEV, ALVAC-JEV is less effective in inducing and maintaining the protective level of JE neutralizing immunity. However, both candidate vaccines failed to match the BIKEN vaccine in these regards. Rapid and long-lasting JE immunity seen in the BIKEN vaccine recipients may be due to the activation of the immune system by a relatively large amount of purified whole JEV immunogens compared with the JEV proteins expressed after infection of the host cells by the vector vaccines.

Pre-existing immunity against vaccinia may clear vaccinia-vectorized vaccines before the virus infects cells and produces desired viral proteins enough to stimulate the immune response. Therefore, some investigators have speculated that use of a live recombinant virus for boosting may not be an effective vaccination strategy if a homologous recombinant virus was used for priming. In our study, boosting is not clear after the second dose of NYVAC-JEV or ALVAC-JEV vaccines on day 28 since the levels of neutralizing antibodies continue to increase from the first dose (Figure 1). However, it is clear that both NYVAC-JEV and ALVAC-JEV are capable of boosting the JE neutralizing antibody response after the third vaccination on study day 273. Whether JE proteins that boost the immune response are from the newly synthesized proteins after vaccine virus infections or from the JEV proteins that may appear on the envelope of infecting live vaccine viruses is not known. Similar boosting ability by homologous recombinant virus has also been reported for ALVAC-RG vaccine expressing the rabies virus glycoprotein.

A number of JEV-vaccinia recombinants have been engineered to express the prM/E, and NS1 genes of JEV. Of interest is that these recombinant viruses can also direct the synthesis of subviral extracellular particles (EPs) containing prM/E and E proteins. These particles are immunogenic in mice and can provide protective immunity against lethal JE virus challenge. Similarly, infection of NYVAC-JEV vaccine into cells in vitro produces an extracellular particulate form of E proteins and cells infected with ALVAC-JEV vaccine release M, E, and NS1 proteins into the culture media. Whether these particles are produced in NYVAC-JEV- or ALVAC-JEV-infected mice, swine, or monkeys is not known. Nevertheless, it has been shown that mice immunized with a mixture of ALVAC-JEV and EPs produce neutralizing antibody titers higher than mice immunized with ALVAC-JEV or EPs alone. A similar combination immunizing strategy may be applied in future
studies to increase the potency of both NYVAC-JEV and ALVAC-JEV vaccines.

In the present study, we demonstrated the safety, immunogenicity, and protective efficacy of NYVAC-JEV vaccine comparable with BIKEN JE vaccine and marginal immunogenicity and efficacy of ALVAC-JEV vaccine in rhesus monkeys. These data were used to support a decision to commence clinical development of NYVAC-JEV (Kanestha-Thasan N, unpublished data). Further evaluation of NYVAC-JEV protective efficacy in field trials will be considered when an optimal dose and vaccination schedules are further defined in volunteers.

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