JAPANESE ENCEPHALITIS VACCINE (INACTIVATED, BIKEN) IN U. S. SOLDIERS:
IMMUNOGENICITY AND SAFETY OF VACCINE ADMINISTERED IN TWO
DOSing REGIMENS

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Abstract. The safety and immunogenicity of Japanese encephalitis (JE) vaccine (Nakayama strain, monovalent / BIKEN) was studied in 538 U.S. soldiers in 1990. Three doses of vaccine from three consecutively manufactured lots were given on days 0, 7, and either 14 or 30. Serum for antibody determination was drawn at months 0, 2, and 6. Japanese encephalitis plaque reduction neutralization tests were performed by three laboratories on each specimen. Five hundred twenty-eight (98%) participants completed the immunization series. All recipients without antibody before immunization developed neutralizing antibody against JE virus. There were no differences in geometric mean titer among the three test lots at months 2 and 6. Soldiers who received the third dose on day 30 had higher titers at both time points. Antibody to yellow fever had no significant effect on immune response to vaccine. Conclusions drawn from analysis of serologic data from the three labs were nearly identical. Symptoms were generally limited to mild local effects and were reduced in frequency with each subsequent dose in the series (21% to 11%; P < 0.0001). Generalized symptoms were rare (e.g., fever = 5%) with no reported cases of anaphylaxis.

Japanese encephalitis (JE) is a potential threat to U.S. expatriates and military personnel who live in southern and eastern Asia and to the 2–3 million U.S. citizens who travel annually to the region. It is the leading cause of viral encephalitis in Asia, with approximately 50,000 cases reported annually. Approximately 25% of individuals with Japanese encephalitis die and more than one-third of the survivors have persistent neurologic deficits. Although the risk of JE infection among travelers to Asia is low (estimated annual risk is less than 1 in 1 million) for a given individual, the risk may be much higher, depending on season, location, duration of travel, and activities.

Since 1954, a formalin-inactivated, mouse brain–derived vaccine has been manufactured by the Foundation for Microbial Diseases of Osaka University in Japan (BIKEN), but was not licensed in the United States until 1992. The vaccine was initially manufactured as a liquid preparation, but more recently lyophilization, and additional purification steps, such as ultracentrifugation, have been added to the process. The latter vaccine was used in this study. Interest in obtaining a JE vaccine for use in the United States was triggered by deaths of two U.S. citizens due to JE in China in the early 1980s. The U.S. Centers for Disease Control (CDC) sponsored an Investigational New Drug (IND) exemption under which more than 17,000 doses of BIKEN monovalent (Nakayama strain) vaccine were distributed in the United States from 1983 to 1987. An additional 10,000 doses subsequently were administered under this IND to U.S. military personnel deploying to the region for training exercises and to > 35,000 Navy personnel and dependents after an outbreak in Okinawa. Although a two-dose primary series protected native populations in studies in Taiwan and Thailand, the vaccine appeared to be less immunogenic in immunologically naive recipients. Practically all recipients, however, responded well to a third dose given either as a part of the primary series (1–2 weeks after the second injection, or as a booster (6–12 months after the initial series). Although a third dose appears necessary to stimulate presumably protective levels of antibody in previously non-immune individuals, the optimal timing of this dose was unknown.

Although considerable experience with this vaccine had been acquired in Asian populations, consideration for licensure for routine use in the United States required additional data on immunogenicity, safety, and dose schedules in U.S. citizens. Data on several consecutively manufactured lots of the present formulation of the vaccine were also requested to support the product licensure application.

The production of serum neutralizing antibodies against Japanese encephalitis in response to immunization is considered to indicate protection. However, since methods and techniques vary among laboratories performing the plaque-reduction neutralization test (PRNT), results among laboratories may differ.

Immunity to closely related flaviviruses might be expected to influence the immune response to immunization with JE vaccine. Since travelers and military personnel receiving JE vaccine may also be immunized against yellow fever (YF), the interaction between these immunogens is worthy of investigation.

We report results of an open-label, randomized clinical trial of three consecutively manufactured lots of the current vaccine in U.S. soldiers preparing for travel to Asia in which we 1) evaluated the vaccine for its ability to stimulate neutralizing antibody for up to six months, 2) compared two dosing schedules, and 3) measured the potential influence of antibody to YF on the immune response to JE vaccine.
Study volunteers. The study protocol was reviewed and approved for implementation by the Human Subjects Research Review Board, Office of the Surgeon General, U.S. Army. Exclusion criteria for screening potential volunteers included acute febrile illness, malignancy, pregnancy, allergy to vaccine components, chronic cardiac, hepatic, renal, or immunologic diseases, immunosuppressive therapy, and history of receiving JE vaccine. Five hundred thirty-eight healthy active duty soldiers at Schofield Barracks in Oahu, Hawaii were eligible to participate and gave written informed consent. Four hundred thirty-two participants traveled to countries where JE infections occur (Thailand, Bangladesh, the Philippines, or Korea) for military training between study months 2 and 6. All soldiers in the study received immune serum globulin (ISG) (2 ml) before departure on these trips; some also received immunizations against hepatitis B, typhoid fever, and plague.

Vaccine. Three lots of JE vaccine (monovalent Nakayama strain) consecutively manufactured by BIKEN were selected for use in this study. The vaccine is a lyophilized, partially purified extract of infected weanling mouse brain. Lot EJN029 was dated April 15, 1989, and lots EJN030 and EJN031 were dated April 20, 1989. Test vaccine passed tests for product sterility, inactivation, potency in mice (neutralization step of their assays), and thimerosal (0.007%) and formaldehyde content (<0.001%) performed by BIKEN and the Japanese National Institute of Infectious Diseases (Shinjuku-ku, Tokyo, Japan).

Single-dose vials of lyophilized vaccine were stored at 4°C. Each dose was reconstituted immediately before administration with 1.3 ml of sterile water solvent supplied by the manufacturer. One milliliter of vaccine was given subcutaneously in the triceps skinfold. Recipients were observed for 20 min after each injection.

Allocation to study groups. Each participant was assigned to receive three doses from one of the three vaccine lots in one of two dosing regimens: days 0, 7, and 14 (short regimen), or days 0, 7, and 30 (long regimen). Vaccine lot assignment was determined by a computer-generated randomized block design. Participants were arbitrarily allocated to dosing regimen groups depending on the availability of their military unit.

Symptoms/adverse reactions. Symptoms occurring within 72 hr after each injection were determined by questionnaire. Participants were specifically questioned about local reactions (soresness or redness at the site of injection) and systemic reactions (fever, headache, and rash). Participants reporting symptoms were asked to estimate the severity and duration in hours of each. A symptom was defined as mild if it was barely noticeable, moderate if it was present but did not interfere with work, and severe if it interfered with work.

Symptoms following the first dose of vaccine were determined by interview in person one week after the injection. Symptom information after the second dose was gathered at the time of the third injection. Third dose–symptom data were collected by mailed, self-administered questionnaires. Data from participants not responding by mail were collected by in-person or telephone interview 30 days after the third dose.

Serology. Serum was drawn for neutralizing antibody determination on study days 0 (baseline), 60 (month 2), and 180 (month 6), and stored at −24°C. At the conclusion of the study, serum specimens were sent to the laboratories at Yale Arbovirus Research Unit (YARU) (New Haven, CT), the Division of Vector-Borne Infectious Diseases at CDC (Fort Collins, CO), and the BIKEN-Kanoji Institute (Osaka, Japan). Specimens were labeled to mask the date that each specimen was drawn (i.e., study day 0, month 2, or month 6), participants’ vaccine lot group, or dosage regimen.

Neutralizing antibody was determined by PRNT. Nakayama strain (Lot #JEV-N-9) JE virus from BIKEN was the challenge strain. Each of the three laboratories performed the PRNT according to its customary protocol outlined in Table 1. There were several notable differences in the methods used by the laboratories. For example, YARU and CDC used Vero cell cultures in plastic multiple-well microplates, while BIKEN used chick embryo fibroblast cultures in glass Petri dishes. In addition, YARU and CDC performed an enhanced PRNT by adding human serum (negative for antibody against YF, JE, eastern equine, western equine, Venezuelan equine, and St. Louis encephalitis viruses) in the neutralization step of their assays.

The neutralization endpoint in the BIKEN protocol was a 50% reduction in plaque formation, the YARU protocol used 80%, and the CDC protocol used 90%. The initial dilution was 1:10 at YARU and 1:5 at CDC and BIKEN. Specimens with no antibody detected at these dilutions were considered negative.

All specimens from each participant were tested in the same run. To detect run-to-run variation and allow for comparisons of results among the three laboratories, a positive control (serum no. 057A from a vaccine recipient) was included in each run. An additional positive control (immune mouse serum NAbC-4, provided by BIKEN) was included at the beginning and end of each run to ensure within-run consistency.

Serologic results from each laboratory were analyzed separately. For each laboratory, participants with any detectable JE neutralizing antibody at day 0, or a month 6 titer greater than a two-fold dilution higher than month 2 titer, indicating possible natural exposure to JE during travel or passive immunization from ISG administration, were excluded from all analyses of immunogenicity for that laboratory.

Effect of YF immunity. All serum specimens drawn at study day 0 were tested for presence of antibody to YF with an ELISA performed at YARU. Each specimen with a negative ELISA result was confirmed as negative by PRNT. French neurotropic strain YF virus from infected mouse brain was the antigen used for these determinations. Finally, immunization records were reviewed for evidence of having received YF vaccine (17D) during military service.

Statistical analysis. All data were entered into a computerized database, verified for accuracy, and analyzed using SAS (SAS Inc., Cary, NC) software. Categorical data were analyzed by chi-square and Fisher’s exact tests. For analysis of continuous data, Student’s t-test or Kruskal-Wallis, one-way analysis of variance (ANOVA), and two-way ANOVA using the General Linear Models procedure for unbalanced...
designs, Type III sums of squares, were performed with SAS software on a mainframe computer at the Walter Reed Army Institute of Research. Unless stated otherwise, all tests for statistical significance were two-tailed.

RESULTS

Study group allocation, immunization schedules, and demographic data. Results of allocation to vaccine lot and dosing regimen groups are shown in Table 2. A total of 532 soldiers received three doses of vaccine; 270 participants received the third dose given on or about day 14, (median = 7 days, range = 3–9 after dose 2) and 262 received the third dose given on or about day 30 (median 24 = days, range = 13–29 after dose 2). No significant differences in age, sex, or rank were observed among the lot groups. The dosing regimen groups were also similar demographically except that the day 14 group was older (mean age = 23.6 versus 22.1 years; P = 0.002). Of the total sample, 521 had serum specimens drawn at month 2 and 495 at month 6.

Four hundred sixty-five (86.4%) participants had received YF vaccine and 472 (87.7%) had antibody to YF on day 0. The 41 soldiers with neither a history of YF vaccination nor antibody to YF comprised the YF-naive group in the analysis of the effect of YF immunity on response to JE vaccine.

Symptoms. Symptom data were collected from all but one of 538 recipients of the first dose. Completed symptom forms were received from all 535 second dose recipients. All but five of 532 recipients of the third injection completed symptom reports. Four of these were contacted later and were in good health. The fifth left the Army for reasons unrelated to the vaccine.

Soreness and redness at the site of injection were the most commonly reported symptoms (Table 3). No subject reported generalized anaphylaxis or urticaria; however, pruritis at the injection site was reported by eight recipients. One participant reported local itching after each dose of the series, while others reported pruritis after only one dose (two after dose 1, four after dose 2, and one after dose 3). Two recipients reported eye swelling after vaccination. One received a single dose and withdrew from the study; the other completed the series with no complications. None of the soldiers reporting symptoms sought medical attention.

Table 1

Comparison of methodology for the plaque-reduction neutralization antibody test among the three participating laboratories*

<table>
<thead>
<tr>
<th></th>
<th>YARU</th>
<th>CDC</th>
<th>BIKEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
<td>Vero cells</td>
<td>Vero cells</td>
<td>Chick embryo fibroblast</td>
</tr>
<tr>
<td>Vessel for cell culture</td>
<td>Plastic 24-well plate</td>
<td>Plastic 6-well (35 mm)</td>
<td>7-cm glass petri dish</td>
</tr>
<tr>
<td>Cell number (/ml)</td>
<td>8 × 10^3/ml</td>
<td>1.8 × 10^3/ml</td>
<td>2.5 × 10^3/ml</td>
</tr>
<tr>
<td>Plating volume</td>
<td>1.5 ml/well</td>
<td>3.0 ml/well</td>
<td>8 ml/plate</td>
</tr>
<tr>
<td>Cell culture environment</td>
<td>5% CO₂, 37° C, 4 days</td>
<td>Same, 4–7 days</td>
<td>Same, 1 day</td>
</tr>
<tr>
<td>Growth medium</td>
<td>MEM with Earle’s salts and 5% inactivated fetal bovine serum</td>
<td>M-199 with 5% fetal bovine serum</td>
<td>LE medium with 5% CS</td>
</tr>
</tbody>
</table>

Neutralization test

| Positive control serum | 057A human serum | Same | Same |
| Challenge virus strain | Nakayama-NIH strain | Same | Same |
| Diluent for challenge virus and serum | PBS with 2.5% fetal bovine serum and fresh human serum | M-199 with 1% bovine albumin, antibodies, 8% fresh human serum | LE solution with 0.2% bovine albumin |
| Mixture of serum and virus | Equal volumes | Equal volumes | Equal volumes |
| Neutralization interval, temperature, environment | 1 hr 37° C, ambient air | 18 hr, 4° C | 90 min, 37° C |
| Inoculation volume | 0.05 ml | 0.1 ml | 0.4 ml |
| Adsorption interval, temperature, environment | 1 hr, 37° C, 5% CO₂ | Same | Same, 90 min |
| Volume of first overlay | 1.5 ml | 3.0 ml | 8 ml per dish |
| Second overlay | 1.0 ml, 7 days after first overlay | 2.5 ml, day 6–7 after first overlay | 4 ml, 3 days after first overlay |
| Plaque count | 8–9 days after inoculation | 7 days | 3–4 days |
| Calculation of titer | 80% reduction | 90% reduction | 50% reduction |

* YARU = Yale Arbovirus Research Unit; CDC = Centers for Disease Control and Prevention; BIKEN = Kanoji Institute, Research Institute for Microbial Diseases, Osaka University; MEM = minimal essential medium; LE = Earle’s medium with lactalbumin; CS = calf serum; PBS = phosphate-buffered saline.

Table 2

Allocation of study subjects by vaccine lot and dosing regimen groups

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Short (Day 0, 7, 14)</th>
<th>Long (Day 0, 7, 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>270</td>
<td>262</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>Age, years (%)</td>
<td>&lt;20</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>20–24</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>25–29</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>&gt;29</td>
<td>14</td>
</tr>
<tr>
<td>YF* antibody positive</td>
<td>86</td>
<td>90</td>
</tr>
</tbody>
</table>

* YF = yellow fever.
Inj. the vaccine series. Chi-square for trend in total side effects for the vaccine series

versity. Resp. results of the three different laboratories were compared. The

similar overall pattern of differences in GMTs in the specific-¯ed YARU) for this analysis.

differences in overall GMTs among the laboratories were

YARU) for this analysis.

for YARU) for this analysis.

only three of these were identi®ed at all lab-

subjects who received the third dose on day 14

(0.2) 5 (0.9)

(1.2) 2 (0.4)

(1.2) 2 (0.4)

(1.2) 2 (0.4)

and 1:40 at month 6) and BIKEN (1:562 at month 2 and 1:372 at month 6).

In the CDC analysis, all participants had developed at least a 1:10 titer of neutralizing antibody at month 2. By month 6, however, one individual had no detectable antibody (although this specimen was determined to have a titer 1:1280 at YARU and 1:1349 at BIKEN), and 4 had titers of 1:5. All other vaccine recipients (98.9%) had titers of at least 1:10.

Lot consistency. Vaccine from all three lots tested in this study was highly immunogenic. There was a statistically signi®cant (P < 0.05) difference in antibody titer between lot groups EJN029 and EJN030 at month 6 in the analysis performed by YARU. However, this difference was on the order of a 2-fold dilution at most, and statistically significant only in the two-way analysis, which simultaneously examines the effects of dosing regimen and vaccine lot group.

Effect of YF immunity. There was no signi®cant effect of YF immunity on immune response to the JE vaccine. Both groups developed substantial levels of JE neutralizing antibody after immunization. However, in results from one laboratory (BIKEN) at one time point (month 2), YF-immune participants had lower anti-JE titers than those who were not immune to YF (P < 0.05).

DISCUSSION

The pattern of neutralizing antibody response to a three-dose series of this JE vaccine is similar to that reported by Poland and others,7 although geometric mean antibody titers found here by YARU and BIKEN are higher than those previous reported with this vaccine. The differences in antibody titers among the three laboratories may re¯ect varia-

tions in the methods used to determine neutralization titer. However, conclusions concerning vaccine immunogenicity, consistency among lots, and the effects of dosing regimen and YF immunity were the same, despite these methodologic differences.

Vaccine from all three lots was highly immunogenic. The 0, 7, 30 day dosing schedule may be preferable for those who can delay the third dose. The higher antibody titers


<table>
<thead>
<tr>
<th>Dose</th>
<th>Resp./Eng. (%)</th>
<th>Total (%)</th>
<th>Severe (%)</th>
<th>Total (%)</th>
<th>Severe (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>537/53S (99.8)</td>
<td>112 (20.9)</td>
<td>4 (0.7)</td>
<td>44 (8.2)</td>
<td>5 (0.9)</td>
</tr>
<tr>
<td>2</td>
<td>535/53S (100)</td>
<td>66 (12.3)</td>
<td>1 (0.2)</td>
<td>19 (3.6)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>3</td>
<td>527/53S (90.0)</td>
<td>58 (11.0)</td>
<td>1 (0.2)</td>
<td>17 (3.2)</td>
<td>1 (0.2)</td>
</tr>
</tbody>
</table>

* GMT = geometric mean titer. For de®nitions of other abbreviations, see Table 1.

† At all laboratories, the mean log antibody titer in day 0, 7, 30 dosing regimen group was higher at months 2 and 6 (in all cases F < 0.0001).
found in this group at month 2 may reflect the shorter interval between the third dose and the date that serum was obtained (30 days versus 45 days for the 0, 7, 14 group). However, this difference remained at month 6, suggesting that delaying the third dose of the series results in higher antibody titers that persist. The 0, 7, 14 day regimen is safe and immunogenic and can be recommended for those who require a more rapid immunization schedule, although antibody may be expected to be lost sooner after this schedule. The duration of antibody titers after the three dose series using either schedule is unknown, although, in one study of a subset of 39 participants in this study, neutralizing antibody persisted at high levels for three years after primary immunization.18 The need for and timing of additional doses of vaccine and persistence of neutralizing antibody requires additional study in a larger number of vaccinees before definitive recommendations on booster intervals can be given.

The three dose series was safe and well tolerated by soldiers. There were no life-threatening adverse reactions after 1,605 injections administered in this study. Although no formal data on symptoms occurring later than 72 hr after each dose were collected, more severe reactions occurring at any time during the study were likely to have been detected because of repeated contact of investigators with volunteers during vaccine administration, throughout the training exercise in Thailand, and subsequent contacts for serum collection. The incidence of reported symptoms decreased with subsequent doses of the immunization series. There were fewer symptoms reported among soldiers who received their third injection on day 30, suggesting that delaying the third dose of the series for several weeks may have reduced reactivity. However, most symptom data from the group who received the third dose on day 14 were collected by self-administered questionnaire, while the data from most of the longer regimen group were obtained by interview approximately 30 days after the dose. The higher frequency of symptoms reported by the 0, 7, 14 dosing regimen group may therefore reflect more accurate symptom recall as well as the cumulative effect of three injections in two weeks.

Rates of hypersensitivity reactions (anaphylaxis, generalized urticaria, or angioedema) consistently have been reported to occur in 0.5% of JE vaccine recipients.4,5,8,19 Although it has been greatly improved through ultracentrifugation, the vaccine contains components, such as gelatin, which may play a role in hypersensitivity adverse reactions.20 The relationship between vaccination and orbital swelling reported by two subjects in this study is difficult to evaluate. One soldier had reported symptoms of an upper respiratory illness at the time of immunization, and received no additional doses. The other soldier completed the series with no complications. Neither sought medical treatment for any symptoms during the study.

Immunity to YF has been shown to enhance the immune response to inactivated tick-borne encephalitis vaccine, the only other commercially available vaccine designed to protect against a flavivirus.21 In this study, the difference in antibody to JE between the YF immune and non-immune groups attained statistical significance only in the data reported by BIKEN. However, since few of our participants lacked antibody to YF, the statistical power of this study to detect clinically relevant differences was extremely limited.

Acknowledgments: We express our gratitude to the following individuals for their contributions to and support of this study: Stephen L. Jones, Phillip M. Murray, Ronald L. Krogh, F. Stephen Wignall, J. Michael Crutcher, R. Kevin Hanson, Alan H. Mumm, Michael J. Hensley, Walter E. Woods, Paul Albrecht, Leigh Sawyer, Ernest Takafuji, and Walter Brandt.

Financial support: This study was sponsored in part by Pasteur Mérieux Connaught, Inc.

Disclaimer: The views of the authors do not purport to reflect the position of the U.S. Department of the Army or the Department of Defense. None of the authors has any undisclosed conflict of interest in the outcome of this study.

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