Phase III Clinical Trials Comparing the Immunogenicity and Safety of the Vero Cell-Derived Japanese Encephalitis Vaccine Encevac with Those of Mouse Brain-Derived Vaccine by Using the Beijing-1 Strain

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The immunogenicity and safety of an inactivated cell culture Japanese encephalitis vaccine (CC-JEV) were compared with those of an inactivated mouse brain-derived Japanese encephalitis vaccine (MB-JEV) in phase III clinical multicenter trials conducted in children. The vaccines contain the same Japanese encephalitis virus strain, the Beijing-1 strain. Two independent clinical trials (trials 1 and 2) were conducted. Trial 1 was conducted in 468 healthy children. Each subject was injected with 17 μg per dose of either CC-JEV or MB-JEV, and the immunogenicity and safety of the vaccines were investigated. Trial 1 showed that CC-JEV was more immunogenic and reactive than MB-JEV at the same dose. Therefore, to adjust the immunogenicity of CC-JEV to that of MB-JEV, a vaccine that has had a good track record regarding its efficacy for a long time, trial 2 was conducted in 484 healthy children. To improve the stability, CC-JEV was converted from a liquid type to a freeze-dried type of vaccine. Each subject was injected subcutaneously with either 4 μg per dose of CC-JEV, 8 μg per dose of CC-JEV, or 17 μg per dose of MB-JEV twice, at an interval of 2 to 4 weeks, followed by an additional booster immunization 1 to 15 months after the primary immunization. Based on the results of trial 2, 4 μg per dose of the freeze-dried CC-JEV (under the label Encevac) was selected as a substitute for the MB-JEV. Encevac was approved and launched in 2011 and has since been in use as a 2nd-generation Japanese encephalitis vaccine in Japan. (These studies have been registered at the JapicCTI under registration no. JapicCTI-132063 and JapicCTI-080586 for trials 1 and 2, respectively.)

Japanese encephalitis (JE) is an infectious disease caused by the JE virus (JEV), which is mediated by mosquitoes, such as Culex tritaeniorhynchus (1, 2). JE occurs not only in Japan but also in many other Asian countries, including Korea, Taiwan, China, Vietnam, Thailand, Malaysia, Myanmar, and India (3). The number of cases and fatalities due to JE are reported to be about 20,000 and 600 per year, respectively (1). To prevent this infectious disease, a JE vaccine derived from infected mouse brain tissue has been in use for a long time in Japan and other countries. Currently, a live-attenuated vaccine developed from a passaged culture of the JEV SA14 strain in primary hamster kidney cells and animals (mice and hamsters) with successive plaque purifications in primary chicken embryo cells, SA14-14-2, has been in use since 1989 in China and other countries (4). Moreover, an inactivated vaccine produced using the SA14-14-2 vaccine strain has been licensed in the United States, Europe, Canada, and Australia (5).

In Japan, mouse brain-derived JE vaccine (MB-JEV) was initially produced using mouse brains inoculated with JEV Nakayama-NIH as a vaccine virus strain. At that time, MB-JEV was produced by adding formalin to the centrifugal supernatant of a 5% emulsion of mouse brain to inactivate the JE virus (6). Later, the quality of MB-JEV was improved through purification processes. As for the virus strain used for vaccine production, the Nakayama-NIH strain was changed to the Beijing-1 strain in 1989. MB-JEV, using the Beijing-1 strain, showed neutralizing activities against a wide range of domestic and foreign JE viruses. Furthermore, this vaccine strain showed high productivity in vaccine manufacture and high antibody-positive rates and neutralizing antibody titers in vaccinees compared with the Nakayama-NIH strain.

From January 2005 to December 2007, acute disseminated encephalomyelitis (ADEM) occurred after vaccination with MB-JEV at a very low frequency of 0.8 per 100,000 children, according to a national investigation by pediatric departments in Japan (7). The Health, Labor, and Welfare Ministry of Japan admitted in 2005 that the ADEM cases occurring after vaccination with MB-JEV were health hazards, and accordingly, they issued a recommendation to withhold active recommendation of the MB-JEV. The following points were considered to be problems with MB-JEV: (i) a possible risk of it causing ADEM, (ii) difficulties with quality control, and (iii) the use of animals in vaccine production. To address this situation, two new freeze-dried inactivated cell culture JE vaccines (CC-JEV) produced using the Beijing-1 strain were approved as substitutes for the MB-JEV in Japan: JeBKV-V (Biken, The Research Foundation for Microbial Diseases of Osaka University, Kagawa, Japan), approved in 2009, and Encevac (Kaketsuken, the Chemo-Sero-Therapeutic Research Institute, Ku-
mamoto, Japan), approved in 2011. In mice, JeBIK-V showed superior neutralizing antibody titers compared with those of MB-JEV (8). The safety and immunogenicity of JeBIK-V were also shown in children (9). However, as MB-JEV was not used as a comparator in the study, the comparisons of the immunogenicity and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine and safety between the CC-JEV and MB-JEV were not performed simultaneously.
produced using the SA14-14-2 virus strain, which is used for persons ≥17 years of age in North America, Europe, and Australia (under the label Jespect) (11). In all these trials, however, a study on a direct comparison of the immunogenicity and safety between CC-JEV and MB-JEV produced using the Beijing-1 strain has not been reported so far. We report here the results of two series of phase III trials (trials 1 and 2) conducted in children simultaneously using CC-JEV (Encevac) and MB-JEV, produced using the same Beijing-1 strain (these studies have been registered at the JapicCTI under registration no. JapicCTI-132063 and JapicCTI-132064).

**Vaccines.** The CC-JEV was manufactured according to the method described by Sugawara et al. (6). Briefly, Vero cells were passaged in a culture medium containing 2% fetal bovine serum and microcarriers. When cell numbers reached approximately $2 \times 10^7$ cells/ml, cells cultured by microcarriers were isolated and inoculated with the Beijing-1 strain. A serum-free medium was added after virus adsorption, and thereafter, the cells were cultured for 4 days at 37°C. The culture supernatant was then harvested and concentrated by ultrafiltration. After inactivation in order to prepare an inactivated purified virus preparation, the virus particle faction was collected and then treated with formalin for inactivation.

The MB-JEV, used as a comparator vaccine in both trials, was manufactured by the Chemo-Sero-Therapeutic Research Institute (Kaketsuken), Tokyo, Japan, as follows: the Beijing-1 strain was inoculated into the brains of 4-week-old ddy strain mice. The brains of the mice showing symptoms of encephalitis were collected to prepare a brain emulsion. After centrifugation, the supernatant of the emulsion was treated with proteamine to remove the mouse brain-derived materials. The virus fluid was recovered from the supernatant, and the supernatant was subjected to the sucrose density gradient centrifugation. After centrifugation, the virus particle faction was collected and then treated with formalin for inactivation in order to prepare an inactivated purified virus preparation. Finally, the total amount of protein of the MB-JEV was adjusted to 34 μg/ml. In both trials, the liquid MB-JVE containing 17 μg/dose of vaccine antigen in 0.5 ml was used as a comparator vaccine. The vaccine was administered subcutaneously.

**Vaccination design.** Two series of phase III clinical trials, trials 1 and 2, were conducted as double-blinded randomized parallel-group studies in multiple facilities. The subjects were children 6 to 89 months of age without a history of JE vaccine or of JEV infection. The main exclusion criteria were: (i) subjects with acute serious diseases, (ii) subjects who clearly presented anaphylaxis to vaccine components or excipients, (iii) subjects who had received a blood transfusion or administration of gamma-globulin preparation within 3 months before the start of the trial or who had received a massive dose therapy of gamma-globulin preparation (≥200 mg/kg of body weight) within 6 months before the start of the trial, and (iv) subjects for whom enrollment in the trial was judged by the principal investigator or subinvestigators as not appropriate. Written informed consent was obtained from a parent or guardian of each subject before trial enrollment. Prior to the conduction of the trials, the trial-related forms, such as the trial protocols, were investigated by the institu-

**TABLE 1** Background data of subjects in trial 1

| Background factor | Group H (formulation H) (n=218) | Control group (MB-JEV) (n=221) |
|-------------------|---------------------------------|---------------------------------|
| Gender (n [%])    | Value (%)                       | Value (%)                       |
| Male              | 107 (49.1)                      | 115 (52.0)                      |
| Female            | 111 (50.9)                      | 106 (48.0)                      |
| Age (mean [SD]) (mo) | 36.9 (13.4)   | 36.7 (15.5)   |
| Body wt (mean [SD]) (kg) | 13.9 (2.7)   | 13.8 (3.3)   |

| Value | 95% CI |
|-------|--------|
| 42.3–55.9 | 45.2–58.8 |
| 44.1–57.7 | 41.2–54.8 |

*a* Injected vaccine.  
*b* CI, confidence interval.  
*c* NC, not calculated.

**TABLE 2** Seroconversion rates of neutralizing antibody after the 2nd and 3rd injections in trial 1

| Time          | Group H (formulation H) | Control group (MB-JEV) |
|---------------|-------------------------|------------------------|
| % (no. of positive conversions/number of analyzed subjects) | 95% CI | % (no. of positive conversions/number of analyzed subjects) | 95% CI | Noninferiority test |
| After 2nd injection | 100.0 (218/218) | 98.3–100.0 | 99.5 (220/221) | 97.5–100.0 | NC |
| After 3rd injection | 100.0 (218/218) | 98.3–100.0 | 100.0 (221/221) | 98.3–100.0 | P < 0.001 |

*a* CI, confidence interval.  
*b* NC, not calculated.
Trial 1. Trial 1 was conducted from February 2003 to August 2004. A flowchart depicting the dispositions of the subjects in trial 1 is shown in Fig. 1. After obtaining informed consent, 468 qualified subjects were recruited for trial 1; among them, 235 and 233 subjects were assigned to two groups and were injected subcutaneously with a series of 2 doses of 0.5 ml of formulation H or the MB-JEV, respectively, at an interval of 1 to 4 weeks, and an additional injection was given 6 to 15 months after the 2nd injection. For the children <3 years of age, doses of 0.25 ml were given using the same vaccination schedule.

Trial 2. Trial 2 was conducted from June 2008 to May 2009. A flowchart depicting the dispositions of the subjects in trial 2 is shown in Fig. 2. After obtaining informed consent, 480 qualified subjects were recruited for trial 2; among them, 163, 158, and 159 subjects were assigned to three groups, and were injected subcutaneously with a dose of 0.5 ml of formulation M, formulation L, or the MB-JEV, respectively, at an interval of 2 to 4 weeks, and an additional injection was given 1 to 15 months after the 2nd injection. For the children <3 years of age, doses of 0.25 ml were given using the same vaccination schedule.

Randomization. In both trials, randomizations were performed according to a computer-generated algorithm. Trial 1 was performed with a permuted-block design. Trial 2 was performed with a stratified randomization as a stratified factor of the vaccine volumes. The eligible subjects were randomly assigned in a 1:1 ratio for trial 1 and a 1:1:1 ratio for trial 2. In both trials, the statistician generating the randomization algorithm was not involved in determining the eligibility, vaccination course, or determining outcomes of the subjects.

Blinding. In trial 1, the test vaccine and the comparator vaccine were liquid-type vaccines with an identical appearance. However, in trial 2, the test vaccine was a freeze-dried type, while the comparator vaccine was a liquid type. Therefore, to ensure blinding, an investigational drug coordinator who prepared the investigational drug for a doctor and cleared it off after the inoculation instead of the doctor was specially assigned at each hospital.

Safety analysis. In trial 1, a parent or guardian of each subject recorded the presence or absence of adverse events in a health diary every day for 7 days after each inoculation. In trial 2, a parent or guardian of each subject recorded the presence or absence of adverse events in a health diary every day for 13 days after each inoculation. Adverse events were recorded until 27 days after inoculation when they were recognized. Regarding local reactions, including erythema or swelling at the injection site, a reaction of ≥2 cm in diameter was recorded as an adverse event in trial 1, and any reaction regardless of its size was recorded as an adverse event in trial 2. A fever of ≥37.5°C was recorded as an adverse event in both trials. In trial 1, the severity of adverse events was judged by the principal investigator or a subinvestigator and was classified into three levels, mild, moderate, and severe, considered for their interference with normal daily activities. Similarly, in trial 2, the criteria were defined in terms of grades 1 to 4, according to severity. Adverse events for which a possible relationship with one of the test vaccines could not be denied were judged as vaccine-related adverse events.

Immunological analysis. In trial 1, blood serum samples for the measurement of antibody titers were obtained from each subject before the 1st injection and 2 to 6 weeks after the 2nd and 3rd injections. In trial 2, serum samples were obtained from each subject before the 1st injection and 4 to 6 weeks after the 2nd and 3rd injections. The sera were stored at −20°C until the time of measurement. Neutralizing antibodies against a JE virus strain, Beijing-1, were measured by a 50% plaque reduction method using Vero cells and calculated using the 3 points least-squares regression method (3LSRM) (12). Briefly, Vero cells grown in six-well plates (Costar six-well cell culture cluster, flat bottom, with lid, catalog no. 3506; Corning Incorporated, Corning, NY, USA) were used. Serial dilutions of the serum samples were carried out routinely to 1:10, 1:40, 1:160, 1:640, 1:2,560, and 1:10,240, and the challenge virus (Beijing-1 strain) was also diluted to give 100 plaques per well. One more dilution was added and the rest repeated if there was not a 50% plaque reduction. The same volume of diluted serum samples and virus were mixed and added to the cell-seeded wells in triplicate. The control virus was added to 12 wells. The plates were then incubated for 90 min at 37°C, and overlay medium was added. Following 5 days at 37°C incubation, 10% formalin was added, followed by methylene blue tetrahydrate to stain the virus plaques. If the average value of the number of plaques in ≥10 control wells was between 50 and 150, the assay was accepted. The neutralizing antibody titer was expressed as the reciprocal of the dilution of serum that caused a 50% reduction of plaque formation compared to the plaque number of the diluted challenge virus in the absence of antiserum. Antibody positive was defined as the neutralizing antibody titer being ≥1:10 (13).

Statistical analysis of antibody titers. In both trials, the primary endpoint was the seroconversion rate after the 3rd injection based on the

### TABLE 3 Vaccine-related adverse events over three injections (≥5%) in trial 1

| Reaction       | Group H (formulation H) (n = 235) | Control group (MB-JEV) (n = 233) |
|----------------|----------------------------------|----------------------------------|
|                | Value  | 95% CI   | Value  | 95% CI   |
| At injection site |        |          |        |          |
| Erythema       | 22     | 9.4      | 13     | 5.6      |
| Swelling       | 13     | 5.5      | 6      | 2.6      |
| Systemic       |        |          |        |          |
| Fever          | 18     | 7.7      | 23     | 9.9      |

* CI, confidence interval.

### TABLE 4 Background data of subjects in trial 2

| Background factor | Group (injected vaccine type) (n) | M (formulation M) (142) | Control (MB-JEV) (146) |
|-------------------|----------------------------------|------------------------|------------------------|
|                   | Value  | 95% CI   | Value  | 95% CI   | Value  | 95% CI   |
| Gender (n [%])    |        |          |        |          |        |          |
| Male              | 77     | 53.8     | 73     | 51.4     | 68     | 46.6     |
| Female            | 66     | 46.2     | 69     | 48.6     | 78     | 53.4     |
| Age (mean [SD]) (mo) | 48.5  | 18.2     | 48.5  | 16.1     | 47.7  | 17.3     |
| Body wt (mean [SD]) (kg) | 15.7  | 3.9      | 15.5  | 3.1      | 15.4  | 3.4      |

* CI, confidence interval.

* NC, not calculated.

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neutralizing antibody titer. The secondary endpoints were the seroconversion rate after the 2nd injection and the geometric mean antibody titers (GMTs) after the 2nd and 3rd injections. The per-protocol set (PPS) was used to represent the immunogenicity population. Statistical analyses were performed using SAS version 8.2 for trial 1 and SAS version 9.1 for trial 2.

**Trial 1.** In trial 1, the noninferiority in the seroconversion rate after the 3rd injection for the formulation H-injected group (group H) against the MB-JEV group (control group) was statistically analyzed by the method of Dunnett and Gent (14). A sample size of 100 subjects per group was calculated to verify the noninferiority in the seroconversion rate (assumed to have an expected seroconversion rate of 95%) in each group ($\alpha = 0.025$, 90% power, and noninferiority limit $\delta = 10\%$). Assuming that the seropositive rate was 45% before the trial and the withdrawal rate was 10%, 204 subjects in each group, for a total of 408 subjects, were required.

**Trial 2.** In trial 2, the noninferiority in the seroconversion rates after the 3rd injection for the formulation L-injected group (group L) and the formulation M-injected group (group M) against the control group were statistically analyzed by the Farrington-Manning method (15). A sample size of 81 subjects for each group was calculated to verify noninferiority (assumed to have an expected seroconversion rate of 98% against JE virus after the 3rd injection in both groups ($\alpha = 0.025$ [1-tailed test], $\beta = 0.10$, 90% power, and noninferiority limit $\delta = 10\%$). Assuming that the seropositive rate was 10% before the trial and that the withdrawal rate was 10%, 100 subjects in each group, for a total 300 subjects, were required.

### Results

**Trial 1.** The main purpose of trial 1 was to verify the noninferiority in the seroconversion rates of group H to the control group after the 3rd injection. The subjects were injected with either formulation H or the MB-JEV. Among the 468 subjects enrolled in this trial, 235 subjects and 233 subjects were assigned randomly to either group H or the control group, respectively, and all of them were included in the safety population, while 439 subjects were included in the PPS immunogenicity population (group H, 218; control group, 221) (Fig. 1). The main protocol deviations were from specified inoculation and blood collection time points; subjects with deviations were excluded from the PPS. In trial 1, the seropositive rate was much lower than our a priori assumption of 45% based on the results of the National Epidemiological Surveillance of Vaccine-Preventable Diseases by the Ministry of Health, Labor, and Welfare. Although the number of subjects was greater than the planned sample size, the objective of the trial was achieved. The background data of the subjects are shown in Table 1. No statistically significant differences were observed in the baseline factors between the two groups.

**Immunogenicity in trial 1.** The seroconversion rates after the 3rd injection were 100% in both groups. The results analyzed by the Dunnett and Gent test verified that group H was not inferior to the control group in terms of the seroconversion rate, which met the primary endpoint. On the other hand, the seroconversion rate of group H after the 2nd injection was 100%, while that of the control group was 99.5% (Table 2). The GMTs of group H after the 2nd and 3rd injections were statistically higher than those of the control group (Fig. 3). The differences in the GMTs of subjects in group H and the control group were assessed using a $t$ test ($P < 0.001$).

**Safety in trial 1.** In trial 1, vaccine-related adverse events with an incidence of $\geq 5\%$ are listed in Table 3. The incidences of vaccine-related adverse events, such as injection site erythema and injection site swelling, were higher in group H than those of the control group; however, there were no statistically significant differences between the two groups. In group H, erythema exudativum multiforme (a series of diseases in which erosion develops in the mucosa, such as the lips, oral cavity, and eyes, or multiple erythema or erosion lesions develop on the skin of the entire body) occurred in one subject 9 days after the 2nd injection with formulation H. The subject had recovered well 10 days after hospitaliza-

### Table 5: Seroconversion rates of neutralizing antibody after the 2nd and 3rd injections in trial 2

| Group | % (no. of positive conversions/no. of analyzed subjects) | 95% CI | % (no. of positive conversions/no. of analyzed subjects) | 95% CI | % (no. of positive conversions/no. of analyzed subjects) | 95% CI |
|-------|----------------------------------------------------------|-------|----------------------------------------------------------|-------|----------------------------------------------------------|-------|
| Group L (formulation L) | After 2nd injection 100.0 (143/143) 97.5–100.0 | | After 3rd injection 100.0 (143/143) 97.5–100.0 | | After 3rd injection 100.0 (143/143) 97.5–100.0 | |
| Group M (formulation M) | After 2nd injection 100.0 (141/141) 97.4–100.0 | | After 3rd injection 100.0 (140/140) 97.4–100.0 | | After 3rd injection 100.0 (146/146) 97.5–100.0 | |
| Control group (MB-JEV) | After 2nd injection 94.5 (138/146) 89.5–97.6 | | After 3rd injection 100.0 (146/146) 97.5–100.0 | | 95% CI NC | |

a CI, confidence interval.
b NC, not calculated.
tion. The doctor who took care of this subject commented that a relationship between this case and formulation H could not be completely denied, although a viral infection was suspected because fever after pharyngeal erythema was observed. Based on this doctor’s comment, this case was judged as a serious vaccine-related adverse event.

The immunogenicity of formulation H was greater than that of the MB-JEV. However, as the incidence of injection site vaccine-related adverse events for formulation H was higher than that of the MB-JEV and a serious vaccine-related adverse event occurred after the injection of formulation H, we conducted an additional trial, trial 2, to adjust the immunogenicity of the CC-JEV to match that of the MB-JEV.

**Trial 2.** The main purpose of trial 2 was to verify the noninferiority in the seroconversion rates of group L and group M to the control group after the 3rd injection. The subjects were injected with either formulation L, formulation M, or the MB-JEV. Among the 484 subjects enrolled in this trial, the subjects were assigned randomly into group L (n = 163), group M (n = 158), or the control group (n = 159), and group L (n = 163), group M (n = 157; one subject withdrew), or the control group (n = 159) were included in the safety test population. For the PPS immunogenicity test population, the numbers of included subjects were as follows: group L, 143; group M, 142; and the control group, 146 (Fig. 2). The main protocol deviations were the use of prohibited medications and deviations from the specified enrollment procedures; subjects with deviations were excluded from the PPS. In trial 2, the seropositive rate was much lower than our a priori assumption of 10%, which was made based on the results of trial 1. Although the number of subjects was greater than the planned sample size, the objective of the trial was achieved. The background data of the subjects are shown in Table 4. No statistically significant differences were observed in the baseline factors among the three groups.

**Immunogenicity in trial 2.** The seroconversion rates of all groups after the 3rd injection were 100%. The results analyzed by the Farrington-Manning test verified that groups L and M were not inferior to the control group in terms of the seroconversion rate, which met the primary endpoint. On the other hand, while the seroconversion rates of groups L and M after the 2nd injection were 100%, the seroconversion rate of the control group was 94.5% (Table 5). The GMTs of groups L and M after the 2nd and 3rd injections were statistically higher than those of the control group (Fig. 4). The differences in the GMTs of subjects in group L, group M, and the control group were assessed using a t test (P < 0.001). These results showed that the neutralizing antibody titer

### TABLE 6 GMTs after the 3rd injection in four patterns different in vaccine volume in trial 2

| Vaccine volumes at 1st/2nd/3rd injections (ml) | Group (injected vaccine type) (n) | No. receiving | GMT (log10) (95% CI) | No. receiving | GMT (log10) (95% CI) | No. receiving | GMT (log10) (95% CI) |
|---------------------------------------------|----------------------------------|--------------|----------------------|--------------|----------------------|--------------|----------------------|
| 0.25/0.25/0.25 | L (formulation L) (143) | 21 | 3.830 (3.612–4.047) | 15 | 3.793 (3.518–4.069) | 19 | 3.211 (2.944–3.478) |
| 0.25/0.25/0.5 | M (formulation M) (142) | 10 | 3.939 (3.744–4.134) | 10 | 3.999 (3.801–4.197) | 9 | 3.452 (3.109–3.796) |
| 0.25/0.25/0.5 | Control (MB-JEV) (146) | 0 | NA | 2 | 4.535 (NA) | 0 | NA |
| 0.5/0.5/0.5 | L (formulation L) (158) | 112 | 3.866 (3.795–3.937) | 113 | 3.962 (3.898–4.026) | 118 | 3.428 (3.348–3.508) |

* GMT, geometric mean titer; CI, confidence interval; NA, not applicable.

### TABLE 7 Vaccine-related adverse events over three injections (≥5%) in trial 2

| Reaction | Group (injected vaccine type) (n) |
|----------|----------------------------------|
|          | L (formulation L) (163) | M (formulation M) (157) | Control (MB-JEV) (159) |
| Local reactions | No. with adverse reaction | % | 95% CI | No. with adverse reaction | % | 95% CI | No. with adverse reaction | % | 95% CI |
| Erythema | 27 | 16.6 | 11.2–23.2 | 39 | 24.8 | 18.3–32.4 | 33 | 20.8 | 14.7–27.9 |
| Swelling | 11 | 6.7 | 3.4–11.8 | 13 | 8.3 | 4.5–13.7 | 13 | 8.2 | 4.4–13.6 |
| Induration | 3 | 1.8 | 0.4–5.3 | 8 | 5.1 | 2.2–9.8 | 4 | 2.5 | 0.7–6.3 |
| Itching | 1 | 0.6 | 0.0–3.4 | 2 | 1.3 | 0.2–4.5 | 13 | 8.2 | 4.4–13.6 |
| Systemic reactions | No. with adverse reaction | % | 95% CI | No. with adverse reaction | % | 95% CI | No. with adverse reaction | % | 95% CI |
| Fever | 35 | 21.5 | 15.4–28.6 | 44 | 28.0 | 21.2–35.7 | 23 | 14.5 | 9.4–20.9 |
| Grade ≥3 (≥39.0°C) | 3 | 1.8 | 0.4–5.3 | 8 | 5.1 | 2.2–9.8 | 2 | 1.3 | 0.2–4.5 |
| Coughing | 13 | 8.0 | 4.3–13.3 | 9 | 5.7 | 2.7–10.6 | 11 | 6.9 | 3.5–12.0 |
| Nasal drainage | 11 | 6.7 | 3.4–11.8 | 11 | 7.0 | 3.5–12.2 | 8 | 5.0 | 2.2–9.7 |
| Rash | 9 | 5.5 | 2.6–10.2 | 4 | 2.5 | 0.7–6.4 | 4 | 2.5 | 0.7–6.3 |
| Diarrhea | 6 | 3.7 | 1.4–7.8 | 6 | 3.8 | 1.4–8.1 | 8 | 5.0 | 2.2–9.7 |
| Grade ≥3 | 0 | 0 | 0.0–2.2 | 0 | 0 | 0.0–2.3 | 1 | 0.6 | 0.0–3.5 |
| Headache | 4 | 2.5 | 0.7–6.2 | 4 | 2.5 | 0.7–6.4 | 8 | 5.0 | 2.2–9.7 |

* CI, confidence interval.

**a** Diarrhea grade of ≥3 defined as an increase in stool frequency of ≥9 times/day.
against the Beijing-1 strain induced by formulations L or M was higher than that by the MB-JEV. Table 6 shows the GMTs after the 3rd injection according to four patterns of injection volume (1st/2nd/3rd injection): 0.25/0.25/0.25 ml, 0.25/0.25/0.5 ml, 0.25/0.5/0.5 ml, and 0.5/0.5/0.5 ml. The number of subjects for whom the dose was changed from 0.25 ml to 0.5 ml was small. Comparing sets of two patterns, such as 0.25/0.25/0.25 ml and 0.5/0.5/0.5 ml of formulations L and M, respectively, there were no statistically significant differences between them. Based on these results, it is considered acceptable to inoculate children <3 years of age with 0.25 ml of any CC-JEV.

Safety in trial 2. In trial 2, vaccine-related adverse events with an incidence of ≥5% are listed in Table 7. In the local reactions, the most common vaccine-related adverse event was injection site erythema. There were no statistically significant differences in the incidences of injection site erythema among group L, group M, and the control group. In the systemic reactions, the most common vaccine-related adverse event was fever. The incidence of fever in group M was statistically higher than that in the control group (confidence interval for group M, 21.2 to 35.7; control group, 9.4 to 20.9). Most of the adverse events in both group L and group M were lower than grade 3 (<39.0°C) (Table 8). No serious vaccine-related adverse event was observed in trial 2.

Based on these results, we selected formulation L containing 4 μg of CC-JEV per dose as the optimum dose.

Table 9 shows the vaccine-related adverse events after the 3rd injection according to four patterns of injection volume (1st/2nd/3rd injection): 0.25/0.25/0.25 ml, 0.25/0.25/0.5 ml, 0.25/0.5/0.5 ml, and 0.5/0.5/0.5 ml. The number of subjects for whom the dose was changed from 0.25 ml to 0.5 ml was small. Comparing sets of two patterns, such as 0.25/0.25/0.25 ml and 0.5/0.5/0.5 ml of formulations L and M, respectively, there were no statistically significant differences between them. Based on these results, it is considered acceptable to inoculate children <3 years of age with 0.25 ml of formulation L.

**DISCUSSION**

We have developed a new type of JE vaccine, a freeze-dried CC-JEV vaccine derived from Vero cells instead of from mouse brain. The new type of JE vaccine contains a more highly purified antigen than in the previous vaccines, produced by adding an affinity column chromatography step to the manufacturing process used for the production of the MB-JEV. To evaluate the new type of JE vaccine, two series of phase III clinical trials, trials 1 and 2, were conducted as double-blinded randomized parallel-group studies. The trials showed that 4 μg per dose of formulation L has the same immunogenicity as 17 μg per dose of the MB-JEV, and formulation L was renamed Encevac. This is the first report on a direct comparison of immunogenicity and safety in children inoculated with the CC-JEV and MB-JEV derived from the Beijing-1 strain in phase III clinical trials.

After the launch of Encevac in 2011, an additional clinical study using Encevac was recently conducted to investigate its safety and immunogenicity, focusing on the 2nd stage of the Japanese public immunization program in children 9 to 12 years of age (K. Okada, personal communication). The GMT of 21 subjects who had received Encevac at the 1st stage was 10^{2.68} when measured prior to the booster injection at the 2nd stage, and the GMT had increased to 10^{3.37} by about 1 month after the booster injection of Encevac at the 2nd stage. On the other hand, the GMT of 34 subjects who had received MB-JEV at the 1st stage was 10^{2.37} when measured prior to the booster injection at the 2nd stage, and the GMT had increased to 10^{3.65} by about 1 month after the booster injection of Encevac at the 2nd stage. No serious vaccine-related adverse events were reported for any of the groups. Thus,}

**TABLE 8** Vaccine-related adverse event of fever over three injections in trial 2

| Group (injected vaccine type) (n) | L (formulation L) (163) | M (formulation M) (157) | Control (MB-JEV) (159) |
|----------------------------------|------------------------|------------------------|------------------------|
| Fever grade                      | No. with fever (%)     | No. with fever (%)     | No. with fever (%)     |
|                                  | 95% CI                  | 95% CI                 | 95% CI                 |
| Total                            | 35 (21.5)               | 44 (28.0)               | 23 (14.5)               |
| 1                                | 10 (6.1)                | 20 (12.7)               | 14 (8.8)               |
| 2                                | 3 (1.2)                 | 8 (5.1)                 | 7 (4.4)                |
| 3                                | 1 (0.6)                 | 1 (0.6)                 |                           |
| 4                                | 0                       |                         |                         |

* The most severe grade was counted when fever in the same subject occurred at different grades over the three injections. Grade 1, ≥37.5°C; grade 2, ≥38.0°C; grade 3, ≥39.0°C fever continued for less than a day; grade 4, ≥39.0°C fever continued for ≥2 days.

**TABLE 9** Adverse vaccine reactions over three injections in four patterns different in vaccine volume in trial 2

| Vaccine volumes at 1st/2nd/3rd injections (ml) | No. with adverse reaction/total no. receiving dose | % (95% CI) |
|----------------------------------------------|--------------------------------------------------|------------|
| 0.25/0.25/0.25                              | 14/25                                           | 56.0 (34.9–75.6) |
| 0.25/0.25/0.5                                | 9/11                                            | 81.8 (48.2–97.7) |
| 0.25/0.5/0.5                                 | 0 NA                                            | 50.0 (6.8–93.2) |
| 0.5/0.5/0.5                                 | 61/127                                          | 48.0 (39.1–57.1) |

* Two subjects were excluded because they did not receive the 3rd injection.

* CI, confidence interval.

* NA, not applicable.
this clinical study targeting the booster injection at the 2nd stage has further confirmed the safety and immunogenicity of Encevac.

In the 1880s, incidents of ADEM related to a rabies vaccine that was prepared from rabbit spinal cord were reported, and protein contaminants deriving from the spinal cord were considered to be the cause of ADEM (16, 17). Incidents of ADEM after inoculation with a Japanese encephalitis vaccine were also suspected to be caused by protein contaminants derived from mouse brain. Based on these considerations, attempts have been undertaken to reduce the rate of ADEM by changing the materials of the vaccine from mouse brain to cell culture product. However, the World Health Organization (WHO) finally concluded (18) that no causal relationship exists between ADEM and inoculation with the Japanese encephalitis vaccine derived from mouse brain. Furthermore, the WHO Global Advisory Committee on Vaccine Safety concluded that no evidence exists regarding an increased risk of ADEM associated with administration of the inactive JE vaccine. In fact, as the ADEM incidence rate of the newly approved CC-JEV in Japan was one case per approximately 1.3 million injections, while the ADEM incidence rate of the MB-JEV was one case per 0.7 to 2.0 million injections, the number of incidents of ADEM has not reduced after the introduction of the CC-JEV in Japan. However, the CC-JEV has the advantages of being able to reduce a possible risk caused by the contamination of unknown adventitious agents derived from mouse brain and to save animals necessary for manufacturing the JE vaccine.

Although the CC-JEVs used in Europe and the United States contain an aluminum hydroxide adjuvant (5), Encevac shows high immunogenicity even at a low vaccine antigen dose, without any adjuvant. Furthermore, Encevac has a 3-year shelf life under refrigerated conditions without containing any preservative, such as thimerosal. As JE is still the most common kind of viral encephalitis in Asia, especially in tropical and subtropical countries, the JE vaccine Encevac is expected to contribute to the prevention of JE.

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