Semi-Commercial-Scale Production of Japanese
B Encephalitis Virus Vaccines from Tissue
Culture

BALWANT SINGH, IK CHIN CHANG,1 AND W. McD. HAMMON
Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh,
Pittsburgh, Pennsylvania 15213

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This study was undertaken to modify and develop procedures for tissue culture-inactivated Japanese B encephalitis (JBE) virus vaccine production in large quantities. Various types of glass bottles were tried and, considering many advantages, long cylindrical roller (CR) bottles were selected. Several variables were investigated including number and volume of trypsinized cells to be seeded, volume of growth medium required for optimum cell growth, amount of calf serum, and volume of harvest medium for a high-titer virus yield. A good confluent cell sheet in CR bottles was obtained within a week by increasing the calf serum from 4 to 10% and when such tissue in a CR bottle was inoculated with 45,000 viral mean tissue culture infective doses directly into the medium, the cytopathological effects (CPE) appeared on day 5. High-titer virus yields were obtained when the harvests were made at 4°C CPE using medium 199 with 2% human albumin at pH 8.3 to 8.5. No appreciable gain in titer was found from such harvests by blending to release intracellular virions. The production methods finally adopted gave consistently good results, and several inactivated JBE virus vaccine lots with minimum immunizing doses, ranging from 0.005 to 0.017 ml, were prepared using a large number of CR bottles in a simulated commercial-scale production system.

Considering the advantage of tissue culture as a safer and possibly more potent source for vaccine production, Rohitayodhin and Hammon utilized primary hamster kidney cells (HKC) for obtaining a temperature-sensitive, attenuated strain of Japanese B encephalitis (JBE) virus from a virulent isolate by serial passage at reduced temperature and plaque selection (13, 14). Its biological properties and stability were tested; various characteristics and special markers were defined. Further evidence of modification of this virus strain was derived from mosquito infection and transmission tests and its very low neurovirulence for laboratory animals. This change in neurovirulence occurred with maintenance of high cell culture titers when incubated at a permissive temperature, <37°C. It has extremely low or absent infectivity and pathogenicity by the intracerebral (i.c.) route for monkeys, burros, “pathogen-free” weanling mice, and hamsters, and usually has an absence of even infectivity by the subcutaneous (s.c.) route for chimpanzees and man (1, 9, 13, 14). After thorough testing in several species of animals and proper identification of this strain as JBE virus, it was then inactivated with Formalin, for additional safety, to produce an inactivated vaccine for man (3, 5, 16). Repeated doses of this vaccine gave good antibody responses of relatively long duration when injected in different laboratory animals, then in limited trial in man, all without local or systemic reactions (5, 6, 7, 9). The Investigational Drug Review Board (IDRB) of the U.S. Army reviewed and approved the production protocols. The Surgeon General of the Army authorized these tests after the protocols were forwarded to the Division of Biologics Standards, National Institutes of Health and recommended them for approval.

The response to this vaccine in animals appeared to be essentially the same as that of the live virus given in the same manner, apparently due to the extreme temperature sensitivity of the selected mutant and its inability to

1 Present address: Michigan Department of Public Health Laboratories, Lansing, Mich. 48924.
replicate at the restricting body temperature. Since an inactivated vaccine prepared in cell culture by using an attenuated strain of JBE virus would be safer, both for those producing it and the recipients, than one of equal animal potency test similarly prepared in the mouse brain or chicken embryo with a virulent strain, the former was selected for production.

The development of methods to prepare vaccine in larger quantities was undertaken so that its efficacy might be tested in an adequate field trial and so that a commercial production contract might be readily and quickly obtained should further large-scale use be needed at any time. Large-scale production required several modifications from the pilot procedures described previously (2, 5). These mainly concerned the cell culture growth, the virus inoculum, the size, shape, and movement of the container, and the method of virus harvest, all designed to obtain a high-titered yield in large quantity with consistency and economy of time and materials. This report describes the results of this developmental research.

MATERIALS AND METHODS

Virus strain. The attenuated JBE virus strain OCT-541, line 35 to 24 C, plaque 4 to 5 described previously (1, 7, 14), had two additional passages in cell culture at 24 C. The seed virus suspension was stored at \(-65^\circ\text{C}\) and contained \(10^8\) mean tissue culture infectivity doses (TCID\(_{50}\)) per ml.

Cell culture. The procedure for preparation of primary HKC monolayers was the same as described previously (2, 16). The trypsinized packed cells were measured and suspended first in 10 to 15 ml of outgrowth medium containing 10% inactivated calf serum and 0.5% lactalbumin hydrolysate. Vigorous mixing under a hood was required to break the clumps of cells. The cell suspension was diluted further prior to seeding according to the type of bottles used and the glass surface area available for cell growth.

For 32-oz (about 945 ml), flat-surface, prescription-type bottles, 1.0 ml of packed trypsinized cells was suspended in 300 ml of outgrowth medium. Each bottle was seeded with a 50-ml sample, and the medium was changed twice prior to virus inoculation (5). Proportionately smaller amounts, 30 and 10 ml, were used for smaller bottles, 16- and 3-oz sizes (about 473 and 89 ml, respectively), and larger amounts, 400 ml, for large bottles (Pfizer bottles; 28 by 23 by 6 cm). Incubation of these as well as other types of bottles described below, during cell growth, was at 37 C.

Other vessels used most were cylindrical roller (CR) bottles 685 mm in length with 1,200-cm\(^2\) surface area (Flow Laboratories Inc., Rockville, Md.). For CR bottles the trypsinized cells were dispersed at a concentration of 1.5 ml of packed trypsinized cells per 100 ml of outgrowth medium. Other concentrations of trypsinized cells (1 and 2 ml/100 ml of outgrowth medium) were also tried but were less satisfactory. The diluted cells were mixed for 15 to 20 min on a magnetic stirrer, and then 100 ml of suspended cells was delivered into each CR bottle with a 25-ml automatic syringe. The cells were distributed throughout the bottle by gentle shaking and rolling before placing it on a rotating drum (0.2 rpm). After 4 to 6 h, the bottles were removed from the drum and again gently shaken to disperse cells more evenly and to prevent the tendency for aggregation of cells at certain areas on the glass surface. At 18 to 20 h after seeding, an additional 100 ml of outgrowth medium was added to each bottle. The first complete change of outgrowth medium was made 4 to 5 days after seeding, replacing it with 200 ml of the same medium per bottle. A second medium change was made 2 to 3 days later with 200 ml of maintenance medium. A good cell sheet was usually obtained 8 days after seeding.

The outgrowth medium consisted of 10% calf serum; 1% vitamin stock (Microbiological Associates, x100 concentration); and 0.02 g NaHCO\(_3\) (0.5% of a 4% stock solution), 0.0292 g L-glutamine (1% of a 2.92 g stock solution), and 86.5% of a 0.5% solution of lactalbumin hydrolysate in Hanks balanced salt solution (HBSS) and 1% antibiotic mixture consisting of 20,000 U of Penicillin G, 1,000 U of polymyxin B sulfate, 4 mg of streptomycin, and 2,500 U of mycostatin. The maintenance medium differed from outgrowth medium only in the amount of calf serum (4%) and the pH (7.5). The exact composition of these media has been reported previously also (10).

Virus inoculum. Dilutions of the seed virus were prepared in HBSS containing 0.2% bovine albumin. The maintenance medium was removed from bottles, each 32-oz (about 945 ml) bottle was inoculated with 0.5 ml of virus diluted to contain approximately 5,000 TCID\(_{50}\), and each CR bottle was inoculated with 4.5 ml of virus dilution containing 45,000 TCID\(_{50}\). The virus was left to adsorb for 1.5 h at 30 C, as the bottles were rocked and turned periodically. Each 32-oz bottle then received 50 ml of maintenance medium and each CR bottle received 200 ml of the same. The incubation was carried out at 30 C in the rotator. After 3 days, the bottles were observed daily for cytopathological effects (CPE).

Virus harvest. About 5 days after virus inoculation, the first signs of CPE appeared in each type of container. The maintenance medium was then removed, and the monolayer was washed twice with HBSS by using about 20 ml for each 32-oz bottle, and 50 ml for each CR bottle. The bottles were replenished with the final harvest medium consisting of Parker medium 199 (stock concentrate X10, Microbiological Associates) with 2% human albumin (HAL); the pH was adjusted to 8 to 8.5. Thirty milliliters of harvest medium was added for each 32-oz bottle and 100 ml was added for each CR bottle. The bottles were incubated again at 30 C. After 10 to 16 h, the tissue monolayer showed CPE over more than 75% of the area, and virus was harvested after shaking to loosen the remaining cells. For CR bottles, a closed system was devised for carrying out virus harvest and all
changes of medium. The contents of all bottles were pooled and centrifuged at 100 × g for 30 min at 4 C, and the supernatant fluid was filtered through a single membrane filter disk (0.22-μm pore size; 142-mm diameter; Millipore Corp.). Alternatively, centrifugation was performed at 4,080 × g for 30 min in a Sorvall centrifuge and was not filtered afterwards.

**Inactivation.** The filtered or clarified virus harvest was inactivated by using 10% Formalin at a final concentration of 1:4,000 in a water bath at 37 C with mechanical circulation and precision temperature control. The required amount of 10% Formalin was added drop by drop as the flask was shaken constantly for proper mixing (16). The inactivation was allowed to proceed for three times longer than preceding trials had demonstrated was the minimum period required to inactivate all viral infectivity of a lot of similar titer as measured by a test on 0.4 ml (four 0.1-ml tubes). The length of inactivation varied with the titer of virus harvest and was calculated accordingly on the basis of recorded experience (3). During inactivation, samples were removed at various intervals, ampoules were prepared, and the shell was frozen and kept at −65 C for titrations of infectivity.

**Virus assay. (A) Hemagglutination titers.** The test was carried out according to a modified method of Clarke and Casals described previously (2).

**B) Infectivity titrations.** Half-log virus infectivity titrations were carried out in tubes of secondary HKC cells. HBSS containing 0.2% bovine albumin was used as a diluent to prepare the serial virus dilutions. Four secondary HKC tubes were inoculated from each of the series of half-log dilutions, with 0.1 ml of inoculum per tube, and were incubated at 30 C. For tests during the latter part of inactivation, when little or no virus activity was expected, only undiluted material was tested. The maintenance medium was changed after about 5 days. The cultures were observed until CPE was complete in all positive tubes. The TCID₅₀ was calculated by the Reed Muench method as reported previously (2).

**Potency test.** The minimum immunizing dose (MID) of the vaccine was determined by the mouse challenge test as developed by Sabin et al. (15) and modified only slightly by Darwish and Hammon (4). In brief, groups of young adult mice of standard age and weight are given two doses of vaccine intraperitoneally (i.p.) at intervals of 3 days in dilutions ranging from 1:20 to 1:1,280; subsequently, all were challenged by i.p., together with uninoculated controls, with a highly virulent strain of JBE virus. The challenge virus dose was adequate to kill 90 to 100% of the control mice as reported previously (4, 17, 18).

**Characterization and safety tests.** The method for identification of the live virus has been reported in earlier publications of this series (5, 9, 14). Safety tests, including those to detect contaminating viruses, were performed on each vaccine lot to be used for human inoculation. These included tests prior to and after inactivation. Requirements of the Division of Biologics Standards, National Institutes of Health (12), were followed. As reported previously (5, 7, 8), virus harvest both prior to and after inactivation was tested. These tests included inoculation of various cell cultures and a variety of laboratory animals as required for poliomyelitis and measles vaccines.

**RESULTS**

**Effect of concentration of calf serum in growth medium.** The calf serum was inactivated at 56 C for 30 min and added to the outgrowth medium in concentrations of 4 or 10%. Calf serum at the 10% level visibly enhanced the cell growth and particularly improved the visibility of the cells by microscopy as compared with lower concentrations. The cell sheet was completely confluent in 8 days. This effect was more evident in the CR bottles. The virus yield from such tissue was also improved. In three different experiments, slightly higher infectivity and HA titers were obtained when 10% calf serum was used (Table 1).

**Effect of size of virus inoculum and volume of medium.** A series of experiments was performed with various sizes and types of bottles, different input multiplicity doses, changes in the cell growth medium, volumes of maintenance medium, and time of change to 199 medium for virus harvest. In general, with larger inocula of virus the CPE appeared earlier than reported previously (2), and with lower inocula the first appearance of CPE was 1 to 2 days later than in the earlier study. The infectivity titers of virus harvest obtained from different types of bottles under what approached optimal conditions for each are shown in Table 2. The highest titer was obtained from CR bottles. This might be attributed to the small volume of harvest medium used in relation to the area of the cell sheet which was made possible with rotation.

**Time required for virus absorption.** Time required for virus absorption before adding maintenance medium was investigated with CR bottles. As shown in Table 3, the results of experiment 1 and 3 indicated that virus could be added directly to the medium without any loss in final titer.

| Calf serum (%) | log₁₀ TCID₅₀/mi of harvest | Reciprocal of HA titer per 0.4 ml |
|---------------|---------------------------|-----------------------------------|
|               | Exp 1 | Exp 2 | Exp 3 | Exp 1 | Exp 3 |
| 4             | 8.9   | 8.4   | 8.6   | 256   | 512   |
| 10            | 9.3   | 9.1   | 9.1   | 512   | 1024  |

*Infectivity titrations done in HKTC using four tubes per dilution and half-log dilution factor. The culture tubes were incubated at 30 C.
Effect of cell scraping and blending, and pH on the virus harvest. The necessity of scraping off the remaining cells with a rubber policeman to obtain unreleased virus at the time of harvest was investigated in a harvest medium with a pH of 8.4. Three different experiments showed that by shaking the bottle most of the cells could be dislodged from the glass. The addition of cells broken and removed by scraping did not significantly improve the titer of the harvests (Table 3). It was also found that blending to rupture cells, as reported previously (2, 5), was unnecessary under these conditions.

Previous studies proved that a medium of pH 8 placed on the cells 6 to 24 h before harvest gave a higher virus titer yield than a medium of pH 7 (2). The effect of pH from 7.61 to 8.85 was explored. As illustrated in Table 4, the results indicate that virus titers were highest when a

| Table 4. Effect of pH of 199 harvest medium* on virus yield in 32-oz bottles |
|-----------------|----------------------|-----------------|
| Expt no. | pH | Log_{10} TCID₅₀/ml* |
| 1 | 7.61 | 8.2 |
| | 7.90 | 9.0 |
| 2 | 7.95 | 9.0 |
| | 8.35 | 9.5 |
| | 8.52 | 9.47 |
| | 8.60 | 9.5 |
| | 8.85 | 9.0 |
| 3 | 8.0 | 9.0 |
| | 8.3 | 9.3 |
| | 8.5 | 9.2 |

* Includes 2% bovine or human albumin depending on whether the lot was expected to be used for man.

**Titrated at 30 °C in secondary HKTC using four tubes for each half-log interval dilution factor.

| Table 2. Virus yield from different types of bottles |
|-----------------|-----------------|-----------------|
| Type of bottle | Surface area for tissue (cm²) | Inoculum* per bottle (dilution) | Vol of maintenance medium (ml) | Vol of harvest medium (ml) | Virus yield* (log₁₀ TCID₅₀/0.1 ml) |
| 3 oz (ca. 89 ml) | 24 | 0.1 ml of 10⁻⁸ | 10 | 7 | 8.0 |
| 16 oz (ca. 473 ml) | 75 | 0.3 ml of 10⁻⁸ | 30 | 20 | 8.0 |
| 32 oz (ca. 945 ml) | 118 | 0.5 ml of 10⁻⁸ | 50 | 30 | 8.2 |
| Pfizer | 640 | 2.0 ml of 10⁻⁸ | 400 | 300 | 7.7 |
| CR | 1200 | 4.5 ml of 10⁻⁸ | 200 | 100 | 8.8 |

* 10⁻₸ dilution contains 1,000 TCID₅₀/0.1 ml.
** For infectivity titrations four HKTC tubes were used per dilution with half-log dilution factor; tubes were incubated at 30 °C.
*** CR, Cylindrical roller.

| Table 3. Effect of virus absorption and scraping the remaining cells from CR bottles on virus harvest in 199 medium, pH 8.4 |
|-----------------|-----------------|-----------------|-----------------|
| Expt. No. | No. of bottles pooled | Virus* absorbed or not | Treatment of cells | Virus yield* (log₁₀ TCID₅₀/0.1 ml) |
| 1 | 3 | Absorbed | Scraped* | 8.5 |
| | 3 | Absorbed | Not scraped** | 8.33 |
| | 3 | Not absorbed | Scraped | 8.5 |
| | 3 | Not absorbed | Not scraped | 8.4 |
| 2 | 3 | Absorbed | Scraped | 9.0 |
| | 3 | Absorbed | Not scraped | 9.0 |
| | 3 | Absorbed | Scraped | 8.84 |
| | 2 | Not absorbed | Not scraped | 8.92 |

* For virus absorption, bottles were incubated at 30 °C for 1.5 h and rolled by hand every 15 min prior to adding maintenance medium.
** For infectivity titrations four HKTC tubes were used per dilution with half-log dilution factor; tubes were incubated at 30 °C.
*** Remaining cells were scraped by using a rubber policeman.
**** Virus harvest was made after shaking the bottles to loosen the remaining cells.
pH from about 8 to 8.5 was used. A pH above 8.5 did not appear to improve the final virus harvest. At a pH of 8 to 8.5 most of the cells had detached from the glass surface at the time of harvest. Apparently, this pH also helped to release virus particles from the cells, since blending did not improve the titers.

**Effect of filtration.** Different methods of removing cell debris from the harvest fluid were tried. In previous studies, after centrifugation, Millipore filtration, using a 0.45-µm pore membrane in combination with a 0.22-µm pore membrane, was found most suitable. This observation was further investigated and, as illustrated in Table 5, a 0.22-µm pore disk was tried with and without a 0.45-µm disk in different experiments. In these selected sets, and others, when the filter was not primed, the loss of virus infectivity during filtration varied from 0.2 to 0.5 log/0.1 ml. However, these losses were reduced when filters were primed with about 10 ml of Parker 199 medium containing 2% HAL.

**Amount of virus lost by discarding the centrifuged pellet of the final harvest.** Previous tests indicated no appreciable difference in virus content between the supernatant of the centrifuged fluid harvest (without cells scraped off) and the homogenized supernatant of the total harvest (fluid harvest plus cells scraped off). The virus in the sediment of the total harvest after centrifugation at 4,080 × g for 30 min was now determined. The sediment was treated by blending for 5 min to rupture any intact cells, then diluted to the original volume for titration. The infectious titer of the original supernatant fluid was 10^8.9/ml and was 10^8.7 for the resuspended sediment. The HA titers were 1:512 and 1:32, respectively. It was thus concluded that over 99.9% of the total infectivity was present in the supernatant fluid and that an insignificant proportion of the virus was discarded in the sediment.

**Consistency of virus production in CR bottles.** Suitability of CR tube bottles for producing high titers of virus for inactivated vaccine was investigated. Virus harvest was obtained using the optimal methods described above, i.e., 10% calf serum in outgrowth medium, virus inoculum containing 45,000 TCID_{50}/CR bottle, harvest medium with high pH (8.3 to 8.5), and centrifugation of virus harvest at 4,080 × g, but no filtration. In repeated experiments, the virus yields were 10^{4.3} TCID_{50}/0.1 ml or higher as shown in Table 6.

The inactivated vaccine prepared from such virus harvest gave MID values ranging from 0.005 to 0.017, which are comparable to those reported previously when the vaccine was produced in small lots using 3- or 16-oz bottles. These tests were performed by challenge inoculation of mice in an antigenic extinction type test (17).

**DISCUSSION**

For an inactivated vaccine a high-titer virus yield with minimum foreign protein is necessary. It is also most desirable that the method of vaccine production be as simple as possible and require minimum handling and processing.

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**Table 5. Effect of Millipore filtration on infectivity titer of virus harvest in 199 medium at pH 8.0 to 8.2**

| Expt no. | Type of harvest* | Before or after filtration | Filters with or without priming* | Porosity of millipore filters | Log_{10} TCID_{50}/ml |
|----------|------------------|----------------------------|----------------------------------|-------------------------------|-----------------------|
| 1        | Fluid            | Before                     | Unprimed                         | 0.22 µm + 0.45 µm             | 9.60                  |
| 2        | Fluid            | After                      | Unprimed                         | 0.22 µm                       | 9.40                  |
| 3        | Fluid            | Before                     | Unprimed                         | 0.22 µm                       | 8.72                  |
| 4        | Fluid            | After                      | Unprimed                         | 0.22 µm                       | 8.50                  |
| 5        | Complete         | Before                     | Primed                           | 0.22 µm                       | 8.83                  |
| 6        | Complete         | After                      | Unprimed                         | 0.22 µm                       | 9.50                  |
| 7        | Fluid            | Before                     | Unprimed                         | 0.22 µm                       | 9.07                  |
| 8        | Fluid            | After                      | Primed                           | 0.22 µm                       | 9.17                  |

* Complete harvest was made by blending fluid and cell harvest.

* A 10-ml sample of 199 medium containing 2% human albumin was passed through Millipore filters (to prime) before virus harvest.
The experiments reported in this vaccine development project were designed to achieve these aims. A systematic study of growth experiments, particularly in large bottles using Pfizer-type and CR bottles with 640 and 1,200 cm² surface areas, respectively, was undertaken in order to adapt vaccine production from small to semi-commercial scale. A consistently high-titered virus yield from Pfizer bottles could not be obtained. We believe the difficulties were (i) the lack of an adequately flat bottom, resulting in the need to employ a large volume of medium to cover all tissue adequately, and (ii) a high ratio of air volume to tissue surface. In both respects, the long CR bottles offered an advantage. The CR bottles provided a large surface-to-medium volume ratio (1,200 cm² per 100 ml of medium) and a low ratio of air volume to tissue surface. The rotation of bottles insured contact of a minimum amount of medium with all cells.

The tremendous tissue surface in CR bottles necessitated a change in volume of outgrowth medium and in the concentration of calf serum needed for high-quality contiguous cell growth. The exact function of calf serum for HKC growth was not studied but, as reported in Results, increasing the calf serum in the outgrowth medium beyond 4% produced cells which gave an increased yield of virus after inoculation. Many workers have attempted to explain the function and the role of serum for the growth of mammalian cells. It has been suggested that serum acts as a reservoir for essential nutrients and that serum proteins protect cells against an unfavorable environment. It has also been reported that serum is required to inactivate the residual trypsin remaining from enzymatic digestion of monkey kidney cells and the proteolytic enzymes subsequently synthesized by the cells (19). Experience with HKC culture showed that the monolayers were adversely affected when serum-free medium was used from the time of inoculation, resulting in lower virus yield, and that 2% HAL could not substitute for serum during the period of early virus growth (2). Consequently, virus was inoculated in the presence of calf serum, but at the first sign of CPE the serum-containing medium was removed and substituted with a serum-free Parker 159 medium containing 2% HAL to be harvested after 10 to 16 h.

It was shown earlier that the JBE virus is released gradually over a considerable period of time from a cell sheet and that the stability of this released virus is enhanced by an alkaline pH (2). It was shown that a medium with a pH of 8, placed on the cells 6 to 24 h before harvest, yielded higher titers of virus than at a low pH. This was examined further to see if the optimum pH was being used. It was found that a peak yield occurred between pH 8.3 and 8.5. This range is now standard. General practice in vaccine manufacture is, at the time of harvest, to scrape any remaining cells from the glass surface and then rupture them by some means to obtain unreleased virus particles. However,
these steps require more handling time and risk contamination. The high pH (8.3 to 8.5) detached most of the cells and probably ruptured most cells. In any case, no significant increase in infectious titer was obtained by further mechanical scraping or rupturing.

Since we found that the immunogenicity of an inactivated viral vaccine was directly proportionate to the infectious virus titer of the harvest prior to inactivation, it was of prime importance that whatever method was used for the removal of cell debris and viral aggregates should not decrease the titer (17). In our studies, virus harvest was originally filtered but, despite a great variety of methods used, there was usually a substantial loss in infectivity. It was found that centrifugation for 30 min at 4,080×g was sufficient to accomplish the same purpose without any detectable loss in virus titer. Because centrifugation was permitted to substitute for filtration in the manufacture of inactivated measles virus vaccine in the United States, this change was considered acceptable, particularly since the JBE virus (OCT-541) used is a very highly attenuated, temperature-sensitive strain shown to be essentially non-pathogenic for man and various laboratory animals (1, 8, 13, 14). We have, therefore, adopted centrifugation as a substitute for filtration. This is one of the reasons we favor selection of avirulent strains for inactivated vaccines.

The acceptable MID for licensing requirements of the U.S. Public Health Service of the previously licensed vaccine is 0.02 ml or less. The inactivated JBE virus vaccine prepared for using these modifications in production method gave a product ranging from 0.005 to 0.017, well within this limit.

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