Reprinted from THE AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE
Vol. 19, No. 1, January 1970
pp. 119-122
Printed in United States of America

AN INACTIVATED EASTERN EQUINE ENCEPHALOMYELITIS VACCINE PROPAGATED IN CHICK-EMBRYO CELL CULTURE

I. PRODUCTION AND TESTING*†

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ABSTRACT: A formalin-inactivated Eastern equine encephalomyelitis vaccine was prepared from the PE-6 strain virus, propagated in primary chick-embryo cell cultures maintained with Medium 199 containing 0.25% human serum albumin. To effect an increase in antigenic mass per unit volume of fluid harvest, we used the pooled fluids from the first tissue-culture passage as maintenance medium for two additional passages. A formalin concentration of 1:2,000 (0.05%) at 37°C was used for inactivation of the virus suspension. Tests on the "in-process" and final vaccine documented the safety and sterility. Tests of potency on the final lyophilized product in hamsters and guinea pigs yielded ED_{50} values of 0.009 ml and 0.012 ml, respectively. Recent assays have revealed no change in potency after storage of the final product for 17 months at −20°C.

Currently there is no commercial source of Eastern equine encephalomyelitis (EEE) vaccine approved for use in man. For many years investigators and field workers at risk to infection with EEE virus could obtain only limited quantities of a vaccine prepared from infected whole-chick embryos by the method of Randall *et al.*[1] When production and distribution of this vaccine was abruptly halted in 1965–66, the U. S. Army Medical Unit assumed responsibility for production and testing of an EEE vaccine for limited use in man. As part of an over-all program concerned with vaccine research and development, production of the EEE vaccine progressed smoothly and, by necessity, rapidly. This paper, the first of a series, describes the production and testing of the new EEE vaccine.

MATERIALS AND METHODS

Virus

The PE-6 strain of EEE virus was obtained from the Walter Reed Army Institute of Research. The pedigree of the virus is not complete; however, it had been used in the past to produce vaccines for use in man.[1] The virus was passaged twice in embryonated chicken eggs. Seed virus was prepared as a 20% suspension of whole embryo (minus head and limbs) and used for preparation of vaccine as well as the challenge virus in assays of potency.

Virus Assay

Albino mice, Bagg strain, 1 to 3 days old, were obtained from the Fort Detrick animal colony and used for titration of virus suspensions. Serial 10-fold dilutions of virus-containing fluids were inoculated in 0.02-ml amounts via the intracerebral (ic) route into groups of six mice. Titers are expressed as the maximum dilution containing sufficient virus to cause death of 50% of the inoculated mice ($ICLD_{50}$) and were calculated by the method of Reed and Muench.[2]

Cell Cultures

Nine-day-old chick embryos were minced and trypsinized according to conventional methods. The resulting cell suspensions were washed two times in Earle's balanced salt solution (EBSS) and resuspended to a final concentration of 2×10^6 cells per milliliter in a growth medium consisting of Eagle's basal medium in EBSS containing 20% calf serum and 1% glutamine plus 200 units of penicillin G, U. S. P., and 50 μg of streptomycin, U. S. P., per ml. Thirty-ml portions of the cell suspension were dispensed into 250-ml plastic tissue-culture vessels (Falcon), which were then incubated at 37°C until confluent cell sheets were obtained (22 to 24 hours). Once cell confluency

* These studies were conducted in conjunction with a program for development and testing of vaccines for, and the therapy of, acute infection. The investigations were supervised by the Commission on Epidemiological Survey of the Armed Forces Epidemiological Board.

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was achieved the growth medium was decanted and replaced with antibiotic-free Medium 199. The chick-embryo cell (CEC) cultures were held an additional 20 to 24 hours at 37°C. Before infection the medium was removed and the residual fluid drained from the culture.

Assay of Potency

Groups of ten Lakeview strain Golden Syrian hamsters (85 to 95 g), obtained from Lakeview Hamster Colony, were inoculated intraperitoneally (ip) on days 0, 7, and 14 with 0.5 ml of fivefold dilutions of vaccine. At 21 days after the last vaccine dose the animals were challenged ip with 10^3 to 10^4 hamster median lethal doses (LD_{50}) of virulent EEE virus. Animals were observed for death for 14 days. Titration endpoints were determined by the method of Reed and Muench.[2] The potency of a vaccine was expressed as the median effective dose (ED_{50}), *i.e.*, the volume of undiluted vaccine given in each dose of the three dose series that protected 50% of the hamsters from death after challenge. Potency was also assayed by the method of Robinson *et al.*[3] in 250 to 350 g Hartley strain guinea pigs obtained from the Fort Detrick animal farm.

RESULTS–DISCUSSION

Growth and Harvest of EEE Virus

In an attempt to produce the highest antigenic mass in the smallest fluid volume, we employed the following procedure: CEC cultures were drained of Medium 199 and inoculated with about 300 suckling-mouse $ICLD_{50}$ of EEE virus per 1,000 cells. After adsorption for 2 hours at 37°C, the residual inoculum was removed, and 10 ml of serum- and antibiotic-free Medium 199 containing 0.25% human serum albumin, U. S. P., were added to each flask. After incubation at 37°C for 30 hours, 30 to 50% of the cells in all cultures exhibited cytopathologic changes. At this time the culture fluids were harvested, pooled, adjusted to pH 7.4–7.6, and centrifuged at 4°C to remove debris. The pooled harvest was then used in 10 ml amounts as maintenance medium for two additional, consecutive sets of fresh CEC cultures. At each passage the fresh cultures were infected with seed virus before the addition of 10 ml of the pooled virus-containing culture fluids from the previous passage. The fluids from the third (final) passage were pooled and further processed into vaccine.

To produce a substantial volume of the final vaccine, we prepared three separate batches of "three-passage," virus-containing fluids by the method described. The infectivity titer of the third-passage harvest for the batches varied from $10^{8.2}$ to $10^{8.6}$ suckling-mouse $ICLD_{50}$ per ml. Each batch was processed separately into vaccine. After tests of potency revealed significant ED_{50} values for each batch, they were pooled to produce a single lot of vaccine.

Clarification and Formalin-Inactivation of Virus Harvest

Pooled culture fluids from the third CEC passage were centrifuged at 900 × G for 30 minutes at 4°C and then passed through a 0.45 μ membrane filter (Millipore) to remove cellular debris that might adversely affect inactivation of the virus. Studies on numerous lots of EEE vaccine have indicated that little or no loss of infectiousness occurs after clarification of virus harvests with this procedure.

Formalin was added to the clarified virus harvest to a final concentration of 1:2,000 (0.05%). The fluid was thoroughly mixed by shaking and was placed at 37°C for 24 hours. During this period the fluid was agitated frequently. After 24 hours at 37°C the material was held in sealed vessels at 4°C for 15 days, during which time the vessels were shaken at least twice daily.

Samples of vaccine taken at regular intervals during the first 72 hours of inactivation at 37°C were titrated in suckling mice ic. Shown in Figure 1 are the results of such an assay for residual live virus. As indicated, viable virus could not be detected after 4 hours. Thus, the inactivation period of 24 hours was six times that necessary for reduction of infectiousness to a nondetectable level.

Assay of Vaccine Potency

The three batches of EEE vaccine and a pool prepared from them were tested for potency before lyophilization. The hamster-protection test was employed rather than the more cumbersome guinea-pig test heretofore used for EEE[1] and WEE[3] vaccine potency assays. Table 1 is a summary of the data obtained from assays of the three batches and pooled vaccine. The graded responses and ED_{50} values obtained with hamsters indicate that this animal is suitable as a substitute

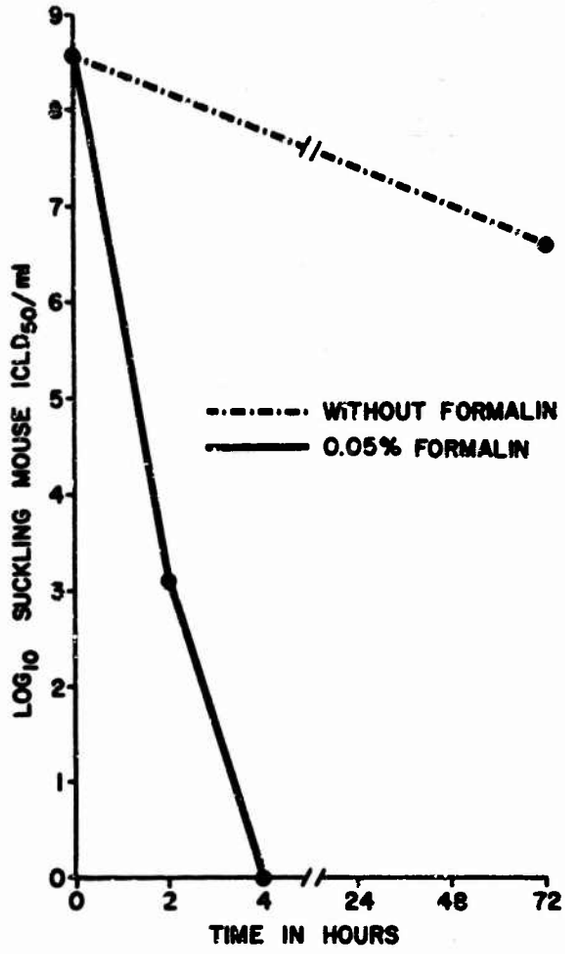

FIGURE 1. Graph showing inactivation at 37°C of EEE virus by 0.05% formalin.

for the guinea pig in assays of potency of EEE vaccine. Studies are in progress to evaluate further the use of hamsters for assays of potency of other group A arbovirus vaccines.

Control Tests: Safety, Toxicity, and Sterility

Using Public Health Service Regulations[4] as a guide, we subjected 10% of the total volume of pooled vaccine to rigorous testing. Residual formalin was neutralized with 10% sodium bisulfite, and the vaccine was then dialyzed against EBSS for 24 hours at 4°C.

Assay for residual live virus. Undiluted and $\log_{10}$ dilutions (10^{-1} through 10^{-4}) of vaccine were subjected to three blind ic passages in suckling mice (10 animals per dilution) with 0.02 ml inoculum. The test was also performed with 21-day-old mice, with the exception that these mice received simultaneous ic and ip inoculations with 0.03 and 0.3 ml, respectively. Assays were also performed in 6- to 12-hour-old Rhode Island Red chicks (Duckworth), by the method of Berman *et al.*[5] No residual live virus was detected by any of the tests employed.

TABLE 1

Potency assays in hamsters of EEE vaccines produced in CEC cultures

Vaccine dilution	Vaccine batch			
	1	2	3	Pool*
Undiluted	10/10†	10/10	10/10	10/10
1:5	10/10	10/10	10/10	10/10
1:25	10/10	10/10	9/10	10/10
1:125	9/10	6/10	6/10	5/10
1:625	2/10	0/10	0/10	3/10
ED_{50} (ml)	0.0016	0.0031	0.0035	0.0024

* Mixture of equal volumes of batches 1, 2, and 3.
† Numerator, survivors; denominator, total.

Tests for adventitious agents. Culture fluids from control CEC cultures were assayed in suckling mice and tissue culture. Twenty suckling mice were inoculated both ic and ip with 0.02 and 0.1 ml, respectively, of undiluted supernatant fluid from control cultures. The animals were observed 10 days. At this time half of the animals were killed, and a blind passage was made with brain suspension. The original animals and those receiving the brain suspension were observed for an additional 24 days. No evidence of infection was observed in any of the animals.

Undiluted control culture fluids were also tested in CEC cultures. After observation for 14 days for cytopathologic changes, fluids from these cultures were passaged into fresh CEC cultures and observed for an additional 14 days. No evidence of infection was noted.

Test of toxicity. Hartley strain guinea pigs, 350 to 450 g, were each inoculated ip with 5.0 ml of vaccine in a ratio of 10 animals per liter of bulk vaccine. These animals remained free of temperature increases and showed normal weight gains for the 10-day observation period.

Tests of sterility. Standard procedures were used to test for the presence of bacteria, fungi, and PPLO. Media employed included fluid thioglycollate broth, Sabouraud's broth, PPLO broth, blood-agar plates, Sabouraud's agar plates, and PPLO agar. Incubation was at 37°C and room

temperature. All tests showed the vaccine to be microbiologically sterile.

Lyophilization and Final Testing

Upon completion of potency and control tests the pooled vaccine was subjected to final processing. Residual formalin, as determined by the NIH method, was neutralized with 35% sodium bisulfite to a level of <0.01% just before lyophilization. The vaccine was then dispensed into glass vials and lyophilized in a cabinet dryer. After being dried, the vials were sealed under vacuum with rubber stoppers and aluminum caps and stored at −20°C.

Previously described tests on an appropriate sampling of the final product showed it to be safe, sterile, and nontoxic. Assays of potency performed after lyophilization with both the guinea pig and the hamster tests gave ED_{50} values of 0.012 ml and 0.009 ml, respectively. Periodic tests of potency on the lyophilized vaccine have revealed no decrease in potency after storage for 17 months at −20°C.

ACKNOWLEDGMENTS

The capable technical assistance of Mrs. Helen T. Emory is gratefully acknowledge. Appreciation is extended to Dr. Joseph P. Lowenthal and Dr. Sanford Berman for packaging and lyophilizing the EEE vaccine.

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