Inactivated Eastern Equine Encephalomyelitis Vaccines Prepared in Monolayer and Concentrated Suspension Chick Embryo Cultures

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Eastern equine encephalomyelitis vaccines were prepared with virus propagated in stationary monolayer cultures and in concentrated suspension cultures of primary chick embryo cells. Virus pools for vaccine preparation were inactivated by three different methods: 0.05% formalin, 41°C heat, and 0.16% β-propiolactone. Heat- and β-propiolactone-inactivated vaccines maintained high hemagglutinating titers in the fluid state for at least 10 months, whereas formalin-inactivated vaccines lost their hemagglutinating activity within a few hours after treatment. The hemagglutinin of β-propiolactone-inactivated virus particles was more dense than the hemagglutinin of the parent virus particles, as determined by sucrose density gradient centrifugation. The increase in density may be due to the degradation or removal of the lipid from the virus envelope. When administered to guinea pigs, all three vaccines stimulated hemagglutination-inhibiting, complement-fixing, and neutralizing antibodies and afforded protection against a live virus challenge. Test results showed that vaccines prepared with virus propagated in concentrated suspension cultures were more immunogenic and stimulated greater serologic responses in guinea pigs than vaccines derived from monolayer-propagated virus. The β-propiolactone-inactivated vaccine was most protective, the heat-inactivated (41°C) vaccine next, and the formalin-inactivated vaccine least potent.

In recent years, renewed interest in vaccine production for group-A arboviruses has developed (3, 6, 13, 18) due to the fact that there are continually wide-spread cases, especially with Eastern equine encephalomyelitis (EEE) viruses.

A variety of techniques has been used to prepare vaccines against arboviruses (3, 6, 11, 18). Early in EEE vaccine development, it was shown that effective formalin-inactivated vaccines, relatively free of side effects, could be prepared (12) by using infected whole chick embryos. The procedure was modified in 1947 (16) to reduce the high content of egg components in the product. Further improvements were made by Lowenthal et al. (11), who produced EEE vaccine in monolayer primary chick embryo cultures (CEC) free of cellular components. Subsequently, Maire et al. (13) developed a potent EEE vaccine propagated in monolayer CEC. However, to increase the viral content of the vaccine, pooled fluids from the first tissue culture passage were used as maintenance medium for two additional passages. Recently, White et al. (20) reported on the high titers of EEE virus obtained from suspension CEC. The current report describes the preparation of EEE vaccines with virus propagated in monolayer CEC and in suspension CEC and compares the immunogenic potencies of these vaccines as determined in guinea pigs.

MATERIALS AND METHODS

Virus. A mouse-brain-adapted PE-6 strain of EEE virus was used in this study. The strain was obtained originally from Cutter Laboratories, Berkeley, Calif., as sixth egg passage material.

Cell cultures. For monolayer cultures, 9- to 11-day-old chick embryos were minced by using conventional methods (15). The resulting cell suspensions were washed two times in Hanks balanced salt solution (HBSS) and suspended to a final concentration of 4 × 10^6 cells per ml in Gey's tris(hydroxymethyl)aminomethane (Tris) growth medium, supplemented with 0.5% lactalbumin hydrolysate and 5% fetal bovine serum (Microbiological Associates Inc., Bethesda, Md.). The cell culture medium also contained penicillin (100 units/ml) and streptomycin (100 µg/ml). Cell suspensions were planted in 40-ml
were cell concentration of the then closed for added technique previous along with the cell suspension, together with the seed virus.

Concentrated suspension cultures were prepared by the method of White et al. (20). Essentially, cells were processed in the same manner as monolayer cultures, except that, after the final washing, cells were suspended to a final concentration of 75 × 10⁶ cells per ml in Eagle’s minimal essential medium containing 1% glucose and 4 mM glutamine. Fifty milliliters of the cell suspensions, together with the seed virus, was dispensed into 250-ml Erlenmeyer flasks, which were then closed with rubber stoppers, agitated on a Therm-O-Shake shaker (Precision Scientific Co., Chicago, Ill.) at 200 strokes (0.5 cm) per min, and incubated at 37 C.

Virus propagation and inactivation. The results of previous studies in this laboratory (20) with the PE-6 strain of EEE virus in CEC (monolayer and concentrated suspension) showed that infection with a multiplicity of 10 plaque-forming units (PFU) per cell produced the maximum quantity of infectious virus and hemagglutinin in 18 hr after infection. Thus, all results in this report were obtained by using that multiplicity and by harvesting at the 18th hr.

Before inactivation, virus suspensions were clarified by centrifugation at 2,000 × g in an International refrigerated centrifuge for 20 min and then were passed through a membrane filter (0.45 μm pore size; Millipore Corp., Bedford, Mass.) to remove cellular debris. The following three methods of inactivation were compared.

(i) Formalin inactivation. Formalin was added to the clarified virus suspension to a final concentration of 1:2,000 (0.05%). The suspensions were thoroughly mixed by shaking and stored at 22 C for 4 days and for an additional 10 days at 4 to 6 C with additional daily shaking.

(ii) β-Propiolactone. Clarified virus suspensions were inactivated with β-propiolactone (BPL) by the technique of French and McKinney (9). A stock 1.6% solution of BPL was made in 0.05 M Na₂HPO₄ and added to the virus suspension to give a final BPL concentration of 0.16%. After the addition of BPL, virus pools were held at 4 to 6 C for 4 days to allow for virus inactivation and hydrolysis of BPL.

(iii) Heat inactivation. Clarified virus suspensions, in sealed glass bottles, were inactivated by incubating at 41 C for 72 hr (20).

Virus titration. Virus was titrated by the hemagglutination (HA) technique (15), the plaque technique described by Porterfield (15) on monolayer CEC, and by intracerebral inoculation (0.03 ml) of suckling Swiss mice. The median lethal dose (LD₅₀) was calculated by the method of Reed and Muench (17).

Safety test. All vaccines were tested for infectious virus by intracerebral inoculation of 0.03 ml into each of 10 litters of suckling Swiss mice (average of 10 mice per litter) and by inoculation of 0.1 ml onto 20 tissue culture tubes of monolayer CEC. No residual virus was detected in any of the inactivated vaccine pools.

Sterility of vaccines was determined by inoculation of 0.5-ml samples into five culture tubes containing 20 ml of fluid thioglycollate medium (Difco), and on Sabouraud dextrose agar plates.

Density gradient centrifugation. Rate-zonal centrifugation of clarified EEE virus or vaccines was performed on gradients of 10 to 40% sucrose in phosphate-buffered saline. Layers of sucrose were allowed to diffuse overnight at 4 to 6 C to obtain a smoother gradient. One milliliter of virus was overlaid onto a 4.0-ml sucrose gradient and centrifuged for 90 min in a Beckman model L-4 preparative ultracentrifuge with an SW39 rotor at 105,000 × g. After centrifugation, five-drop fractions (0.05 ml per drop) were collected from the bottom of the tube, immediately diluted 1:10 with cold medium 199, and assayed. Generally, every fifth fraction was held undiluted so that its refractive index could be determined. Density was calculated from the empirical formula

\[ \text{Density} = 10.8601 \times (\text{HD}^{25°} - 13.4974) \]

Assay of vaccines. Potency assays were performed in guinea pigs (Hartley strain, 350 to 450 g), which were obtained from the Department of Laboratory Animals, Walter Reed Army Institute of Research. Vaccines were serially diluted, and groups of guinea pigs were inoculated subcutaneously with 0.5-ml doses of vaccine on days 0 and 7. All animals were challenged intracerebrally with 100 to 1,000 guinea pig LD₅₀ of the virus 14 days after the second inoculation. Median effective dose (ED₃₀) values were calculated by the method of probit analysis (8).

Antibody assays were performed on postvaccination guinea pig sera by serum neutralization (SN),

\[ \text{VIRUS TITER (log₁₀) PER ML) } \]

**FIG. 1.** Inactivation curves of vaccines and control virus suspension at various temperatures.
complement fixation (CF), and hemagglutination inhibition (HI) tests. Male guinea pigs (350 to 450 g) were inoculated intraperitoneally with two 0.5-ml doses of vaccine, spaced 7 days apart. All animals were bled by cardiac puncture after the second dose. Bleedings were pooled within groups of guinea pigs, and sera were stored at -20°C. The CF-52 method described by Kent and Fife (10) was used to detect CF serum antibodies, as well as CF antigens in sucrose gradient fractions. Hemagglutination-inhibiting antibodies were measured by the method of Clarke and Casals (4), and SN antibodies were measured by the plaque reduction system in CEC as described by Dulbecco et al. (5).

RESULTS

Vaccine preparation and assay. A pool of EEE virus propagated in suspension culture was obtained and divided into three portions. One portion was inactivated with heat, another with BPL, and the third with formalin. A formalin-inactivated vaccine was also prepared with virus grown in monolayer cultures to compare its immunogenicity in guinea pigs with that of the formalin-inactivated vaccine propagated in concentrated suspension cultures.

Samples taken at regular intervals during the first 72 hr of infection were titrated intracerebrally in suckling mice. Shown in Fig. 1 are the results of such assays for residual live virus. As indicated, BPL treatment inactivated virus most rapidly (8 hr), followed by formalin (48 hr) and then 41°C heat (72 hr). Infectious virus, HA, and CF activity levels, before and after treatment by the different methods, are summarized in Table 1. The titer of infectious virus from suspension cultures was 2 logs greater than that obtained from monolayer cultures and was correspondingly accompanied by considerably higher HA activity and a twofold increase in CF activity. All treatments completely inactivated the virus and none of the inactivation procedures affected CF

| Vaccine and cell prepn | Antibody response | ED₅₀ (ml) |
|------------------------|-------------------|-----------|
| SN                    | HI                | CF        |          |
| β-Propiolactone-inactivated vaccine; suspension cultures | 640 | 640 | 320 | 0.0030 |
| Heat-inactivated (41°C) vaccine; suspension cultures | 640 | 1280 | 320 | 0.0046 |
| Formalin-inactivated vaccine; suspension cultures | 160 | 320 | 80 | 0.0208 |
| Formalin-inactivated vaccine; monolayer cultures | 40 | 160 | 40 | 0.1839 |

* Reciprocal of serum dilution. SN, serum neutralization; HI, hemagglutination-inhibition; CF, complement fixation.

* ED₅₀ is the total volume of vaccine protecting 50% of vaccinated guinea pigs against a lethal challenge.
activity. However, HA activity was reduced approximately 10-fold by PBL and to below detectable levels by formalin. Original levels of HA were maintained only in those virus pools treated with heat (41 C).

Of the four vaccines tested (Table 2), the lowest serologic responses and the least protective potencies were obtained with formalin-inactivated vaccine propagated in monolayer cell cultures. The formalin-inactivated virus pool derived from concentrated suspension cultures, compared with the formalin-inactivated virus pool propagated in monolayer cultures, elicited a two- to fourfold higher antibody response in guinea pigs; this response was accompanied by an increase (approximately 10-fold) in protective potency. The BPL and heat-inactivated virus pools derived from concentrated suspension cultures gave similar responses, and, compared with the formalin-inactivated material from the same virus pool, elicited two- to fourfold greater antibody responses and approximately five- to sevenfold greater protective potencies.

Rate-zonal density gradient centrifugation. As a result of the differences in antigenic and immunogenic characteristics of the vaccines prepared from a common virus pool, it was decided to examine untreated and treated virus preparations by sucrose density gradient centrifugation for possible physical alteration of virus particles.

Previous reports (7, 9) have shown that BPL-inactivated arboviruses may have increased HA titers as a result of BPL treatment. Furthermore, it has been shown in this and other laboratories (6, 20) that formalin-inactivated arboviruses lose all of their HA activity within a few hours after treatment (Table 1). Untreated and treated virus preparations were sedimented in linear, 10 to 40% sucrose density gradients. With untreated virus, plaque-forming and HA activity sedimented at different densities. The infectivity peak was 1.20 to 1.22 g/cm³ and the HA peak was 1.18 g/cm³ (Fig. 2). These results are in accord with those of Aalestad et al. on the fractionation by CsCl of the Louisiana strain of EEE virus (1). Although there were differences in the density peaks of infectious virus and HA particles, there were significant titratable virus and HA activity over a broad range of fractions. Also, plaques were relatively uniform in size and shape. From Fig. 3, it can be seen that untreated and heat-inactivated HA particles sedimented at a density of 1.18 g/cm³, whereas BPL-inactivated particles sedimented at a density of 1.22 g/cm³. The increase in density may be due to the degradation or breakdown of lipids in the virus envelope.

**DISCUSSION**

A comparative study was made of the immunogenic properties of EEE vaccines inactivated by three different methods, with virus grown under two different sets of conditions. The investigation attempted to answer two basic questions: (i) does concentrated suspension CEC serve as a better source of EEE virus production than monolayer CEC (11, 13), and (ii) do the methods of virus inactivation have any effect on the immunogenicity of inactivated vaccines (6, 7, 11)?

Concentrated suspension cultures produced at least 2 logs more infectious virus, 8 times more
HA activity, and 2 times more CF activity than the monolayer cultures at the time of harvest (Table 1). After inactivation, higher antibody titers and greater protection against a challenge were produced in guinea pigs with suspension culture-grown virus than with monolayer culture-grown virus. The higher serological responses and the protective characteristics of the vaccines prepared with virus propagated in concentrated suspension cultures indicate that the total mass of viral particles was far greater than that in vaccine prepared with virus propagated in monolayer cultures. This suggests that concentrated suspension cultures should be further investigated as a method for the production of other viruses for vaccine and diagnostic antigen preparations. Also of interest are the differences in immune response of the three vaccines prepared with virus from a common pool grown in concentrated suspension cultures. Since the heat- and BPL-inactivated vaccines afforded greater protection than the formalin-inactivated vaccine, and since high antigenic activities remained after heat- and BPL-inactivation, there appears to be a possible correlation between antigenicities and immunogenicities of these EEE virus preparations.

Sucrose density gradient centrifugation revealed that BPL-inactivated virus-hemagglutinating particles were more dense than mature uninactivated virus particles. This may indicate degradation of the virus capsid or, in some way, an effect on the lipid content of the envelope. This observation is similar to that made with Tween-ether-extracted Sindbis and Chikungunya arboviruses (6, 14).

Of prime interest in this report is the production of more potent vaccines with virus propagated in concentrated suspension CEC than with that produced in monolayer CEC. Since concentrated suspension cultures are infected at the time of planting in serum-free medium, there is no time available for the cells to divide. Thus, the concentrated suspension cultures may have a distinct advantage over monolayer and Maitland-type cultures as a source of virus growth for the preparation of vaccines for human use.

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