Inactivated Venezuelan Equine Encephalomyelitis Vaccine Prepared from Attenuated (TC-83 Strain) Virus

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Formalin-inactivated Venezuelan equine encephalomyelitis virus vaccine was prepared from virus propagated in rolling-bottle cultures of chicken embryo cells. The attenuated, TC-83 strain of virus was propagated in these cultures with a maintenance medium consisting of serum-free medium 199 containing 0.25% human serum albumin and antibiotics. By adjustment of maintenance medium volume (100 to 300 ml) and multiplicity of inoculum (0.04 to 0.00004), high-titered yields of virus were obtained with minimal cell destruction at convenient harvest times, viz, 18 to 24 h postinoculation. Inactivation of virus at 37°C was complete between 8 to 10 h with 0.05% Formalin and within 6 to 8 h with 0.1% Formalin. Antigen extinction potency tests in mice indicated that potent vaccines could be produced at both Formalin concentrations and, furthermore, that potency was not adversely affected by inactivation periods of up to 96 h.

In an earlier paper, we outlined the usefulness of an inactivated Venezuelan equine encephalomyelitis (VEE) vaccine and described the preparation of such a vaccine from the Trinidad strain of VEE virus (3). However, in view of the reluctance to use a product prepared from a highly virulent, "exotic" strain of VEE virus, studies were initiated to determine if an equally potent vaccine could be prepared from the now widely used attenuated TC-83 strain of VEE virus (8, 12).

Reported here are results of studies on the parameters of production of a Formalin-inactivated VEE vaccine prepared from the attenuated, TC-83 strain of VEE virus grown in rolling-bottle cultures of chicken embryo cells (CEC).

MATERIALS AND METHODS

Viruses. The attenuated, TC-83 strain of VEE virus (8) was used to prepare the vaccine. Virus seed for these studies was prepared by a single passage of the TC-83 strain in CEC culture. Trinidad strain VEE virus was used as challenge virus for potency assays (10).

Virus titration. Trinidad strain VEE virus was titrated intraperitoneally (i.p.) in 3-week-old white mice (CD-1 strain of Charles River Mouse Farms, Wilmington, Mass.) as previously described (3). TC-83 strain virus was titrated intracerebrally (i.c.) in 1-to-3-day-old CD-1 mice (3). Titration end points for both strains were determined after a 14-day period by the Reed and Muench method (11). Results are expressed as mean lethal doses (LD₅₀) per ml.

Cell culture. Rolling-bottle (280 mm, Belco Glass Inc., Vineland, N.J.) cultures of CEC were prepared as previously described (1). Twenty-four hours prior to inoculation, the growth medium was replaced with a maintenance medium (MM) consisting of serum-free medium 199 containing 0.25% human serum albumin plus 100 μg each of neomycin (USP) and streptomycin (USP) per ml.

Inoculation of cell cultures. MM was removed from cultures, and the residual liquids were drained from the cell sheets. Infecting virus in 10 ml of MM then was added to each culture and adsorbed for 1 h at 35°C with the roller apparatus set at 0.7 rpm. The effect of multiplicity of inoculum (MOI) was determined by inoculating cultures at a MOI of 0.04 to 0.00004 (i.e., 0.04 to 0.00004 mouse LD₅₀ per cell) and maintaining the cultures with 200 ml of MM. Culture fluids were removed at 24 h postinoculation and assayed for virus content as described. In all other studies an empirical MOI of 0.0004 was employed; this was a 10⁻⁴ dilution of the CEC virus seed.

Preparation of vaccine. Fluids from infected cultures were centrifuged at 1,000 × g for 30 min at 4°C and passed through a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.) to remove intact cells and debris. Formalin (formaldehyde, 37% assay) was added to a final concentration of 1:2000 (0.05%) or 1:1000 (0.1%). The fluids were mixed thoroughly, and the closed vessels were placed in a reciprocating 37°C water bath (New Brunswick Scientific Co., New Brunswick, N.J.) set at 150 rpm to maintain a gentle, constant movement of the fluids throughout the 24 to 96 h inactivation periods. Upon
completion of the desired 37 C inactivation period, the material was stored in closed vessels at 4 C for 15 days, during which time the contents were gently mixed twice daily.

**Potency test.** Vaccine potency was determined as previously described by using a single dose, antigen extinction test in 3-week-old CD-1 white mice (3). Intraperitoneal challenge with 10^4 mouse i.p. LD_{50} of Trinidad strain virus was performed 14 days post-vaccination. Titration end points and mean effective dose values were determined by previously described methods (2).

**Tests for safety and sterility.** Randomly selected lots of vaccine were tested in accordance with current U.S. Government regulations (6).

### RESULTS

**Effect of MOI on virus yield.** Based on results of prior work with Trinidad strain VEE virus (3), a harvest time of 24 h was selected to determine the effect of MOI on virus yield (Table 1). Although similar yields were obtained with all MOI tested, a MOI of 0.0004 was selected for all subsequent studies, because this represented a 10^{-4} dilution of the vaccine seed virus and would therefore decrease the amount of extraneous material carried over from seed.

**Effect of volume of MM.** Replicate cultures were inoculated at a MOI of 0.0004 and maintained with 100 to 300 ml of MM. The results of titrations performed with fluids removed from these cultures at regular intervals are shown in Table 2. As indicated, between 12 and 24 h postinoculation there were no substantial differences in the titers achieved with the three MM volumes. However, considerably less cell destruction occurred in those cultures maintained with 200 or 300 ml of MM. In all cases, cell destruction was moderate at 12 to 18 h post-inoculation, but increased greatly by 24 h, most notably in those cultures maintained with 100 ml of MM.

**Formalin inactivation of virus.** To determine the rate of inactivation, samples were taken at regular intervals during the inactiva-

### Table 1. Effect of MOI* on propagation of TC-83 strain VEE virus in rolling-bottle chicken embryo cell cultures

| MOI   | Log_{10} LD_{50}/ml by MOI at 24 h postinoculation |
|-------|-----------------------------------------------|
| 0.04  | 10.3                                          |
| 0.004 | 10.8                                          |
| 0.0004| 10.4                                          |
| 0.00004| 10.5                                         |

* Multiplicity of inoculum. Titer of virus inoculum/total number of cells.

### Table 2. Effect of maintenance medium volume on propagation of TC-83 strain VEE virus in rolling-bottle chicken embryo cell cultures

| Time post-inoculation (h) | Log_{10} LD_{50}/ml by maintenance medium volume* |
|---------------------------|-----------------------------------------------|
|                           | 100 ml | 200 ml | 300 ml |
| 6                         | 6.9     | 5.7     | 6.1    |
| 12                        | 10.5    | 9.8     | 10.0   |
| 18                        | 10.3    | 9.8     | 10.1   |
| 20                        | 10.4    | 10.1    | 10.3   |
| 24                        | 10.3    | 9.9     | 9.6    |

* Multiplicity of inoculum was 0.0004.

### Table 3. Formalin inactivation* of TC-83 strain VEE virus at 37 C

| Time after Formalin (h) | Log_{10} LD_{50}/0.03 ml by Formalin concn | Virus* control |
|-------------------------|--------------------------------------------|----------------|
|                         | 0.05% | 0.1% |                               |
| 0                       | 8.4   | 8.3  | 8.3                           |
| 2                       | 2.0   | <1.0 | c                             |
| 4                       | <1.0  | <1.0 |                               |
| 6                       | <1.0  | <1.0 |                               |
| 8                       | <1.0  | 0*   | 7.5                           |
| 12                      | 0     | 0    |                               |
| 10                      | 0     | 0    |                               |
| 14                      | 0     | 0    |                               |
| 16                      | 0     | 0    | 7.3                           |
| 18                      | 0     | 0    |                               |
| 20                      | 0     | 0    |                               |
| 22                      | 0     | 0    |                               |
| 24                      | 0     | 0    | 7.2                           |

* Determined by intracerebral inoculation of suckling mice with 0.03 ml of log_{10} dilutions.

* Virus was subjected to 37 C only.

* Blanks, not tested.

* Zero indicates no evidence of virus-induced death in mice inoculated; ≥50 mice were inoculated with undiluted vaccine at each time interval from 8 to 24 h.
TABLE 4. Effect of Formalin concentration and length of inactivation period on potency of killed TC-83 strain VEE vaccine

| Formalin concn (%) | ED₅₀ (ml) by hour of inactivation |
|-------------------|---------------------------------|
| 0.05              | 0.047* (0.022-0.085)             |
|                   | 0.022* (0.003-0.120)             |
|                   | 0.023* (0.019-0.028)             |
|                   | 0.042* (0.001-0.029)             |
| 0.1               | 0.027* (0.002-0.046)             |
|                   | 0.011* (0.001-0.029)             |
|                   | 0.069* (0.038-0.110)             |
|                   | 0.038* (0.003-0.042)             |

* Mean of three lots; values in parentheses are range of values for ED₅₀; when no range is shown, only one lot was tested.

Effect of Formalin concentration and length of inactivation period on vaccine potency. Because extended contact with Formalin or exposure to temperature above 0°C may result in destruction of virus antigens, 30 small lots of experimental vaccine were prepared from 18-24-h harvest fluids by using either 0.05 or 0.1% Formalin and inactivation periods of 24 to 96 h. Summarized in Table 4 are the results of single-dose potency assays performed on these vaccine lots. These data indicate that killed TC-83 strain is stable antigenically, even after inactivation for periods as long as 96 h. The volume of MM and preinactivation titers of the fluids used to prepare these vaccine lots varied from 100 to 300 ml and 10⁴ to 10⁶ suckling mouse i.c. LD₅₀ per ml, respectively. There was no correlation between these values and the potency test results.

Tests on selected lots of vaccine indicated that the methods employed resulted in a product that would meet current U.S. standards for safety and sterility (6).

DISCUSSION

In this study we have employed the previously developed rolling-bottle technique (1) to propagate the attenuated, TC-83 strain of VEE virus. We showed that use of 200 or 300 ml of MM resulted in decreased cell destruction without a concomitant decrease in titer when harvests were made 12 to 24 h postinoculation. Furthermore, clarification by centrifugation and filtration also was used to remove additional cellular material. Studies on the effect of using various MOIs demonstrate that high dilutions of seed virus (i.e., low virus input) may be used without affecting the optimal time to harvest or the virus yield. Indeed, high-titered virus material with decreased amounts of cellular debris can be obtained in this system at several MOIs and at harvest times of 12 to 24 h by manipulating virus input and MM volume. For large-scale production, this latitude would be advantageous, because several factors could be adjusted to permit flexible production schedules.

Studies on Formalin inactivation at 37°C showed that TC-83 strain VEE virus was similar to the virulent Trinidad strain in terms of heat stability and sensitivity to Formalin (3). Because the standards for biological products of the Code of Federal Regulations require Formalin treatment at 37°C for a period three times that required to reduce virus to a nondetectable level, this would indicate a total inactivation period of between 24 and 30 h for vaccines made with 0.05% Formalin and a period of between 18 and 24 h when 0.1% Formalin is employed (6). Data on 30 lots of vaccine prepared with both 0.05 and 0.1% Formalin and inactivated at 37°C for 24 to 96 h indicate that the potency of this vaccine is not adversely affected by prolonged contact with Formalin. Thus, for additional safety, the U.S. standards may easily be exceeded in terms of inactivation period without endangering the quality of the final product. Similar results were obtained with the Trinidad strain (3).

Because no correlation could be made between preinactivation titers and vaccine potencies, one must assume that even the harvests with the lowest titer (i.e., 10⁴ to 10⁶ suckling mouse i.c. LD₅₀ per ml) contained sufficient antigenic mass for the production of a potent product. As was true for the Trinidad strain vaccine (3), high virus titers were consistently obtained in cultures maintained with 300 ml of MM, and thus large volumes of high-titered virus could easily be obtained by this method for large-scale production of vaccine.

In our earlier paper (3) we reviewed the problem of incomplete inactivation that plagued the earliest attempts to produce a Formalin-killed VEE vaccine. The usefulness and limitations of the live, attenuated TC-83 vaccine, which was developed to replace the old killed product, were also discussed (3). There are additional situations in which use of the live, attenuated product is undesirable. For example, in countries where the disease is not present, but may be a threat, use of the live vaccine could result in the development of an enzootic focus of the vaccine virus in a rodent-mosquito-rodent cycle. The isolation of the vaccine virus from mosquitoes in Louisiana by Pedersen et al. (9), in concert with laboratory data on reversion to virulence of the attenuated
strain in rodents (7, 8), indicates the danger inherent in using a live product in areas where disease is not present and where habitats exist that have potential reservoir hosts and insect vectors.

Use of a killed product would present none of the problems associated with live vaccine. Moreover, because the danger resulting from residual live virus is greatly minimized through the use of the attenuated, TC-83 strain virus, the vaccine described in this paper represents an improvement in safety as compared with the killed Trinidad strain product (3). Use of such attenuated strains for killed products has been advocated for several years by Darwish and Hammon (4, 5).

As with the killed Trinidad product (3), this vaccine will soon be tested in Equidae to establish dosage and persistence of immunity. Moreover, upon approval of the appropriate U.S. government regulating agencies, vaccine now being commercially produced by our method will be tested in volunteers to establish parameters for optimal dose and schedule of administration, as well as for persistence of immune response.

The killed product described here obviously cannot replace live, attenuated VEE vaccine under conditions where a rapid abatement of raging epizootics or epidemics of the disease must be affected. However, the killed product can be extremely useful in those situations where the live product is contraindicated.

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