Volume Production of Reference Seed Virus and Immune Ascitic Fluids for Six Arboviruses

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Seed virus reagent and control and immune ascitic fluids were prepared for six arboviruses: buttonwillow, epizootic hemorrhagic disease of deer, Turlock, anopheles B, Kern Canyon, and Semliki Forest. Pilot studies were initiated for each reagent to determine a satisfactory method for their production. Results of homologous and heterologous tests are reported.

Standard reference reagents are available for many viruses, but until recently very few were made for the arbovirus group. The desirability of having reference grade viral diagnostic reagents has been demonstrated for groups of viruses other than the arboviruses, e.g., the human enteroviruses and adenoviruses (3, 4–8, 10, 15, 17). Difficulties associated with preparing and testing reagents for such large groups of viruses make this an impractical task for the small laboratory. The provision of large quantities of these reagents with adequate testing, therefore, provides a uniform source of supply and helps eliminate the errors sometimes associated with the casual exchange of materials among laboratories. Data offered here supplement those distributed by the Research Resources Branch, National Institute of Allergy and Infectious Diseases (3).

MATERIALS AND METHODS

Viruses. Prototype, source, and passage history of the seed viruses used in production of these reference reagents are listed in Table 1. The viruses used in reagent production were: buttonwillow, epizootic hemorrhagic disease of deer (EHD), Turlock, anopheles B, Kern Canyon, and Semliki Forest. All viruses were titrated in suckling mice before reagent production.

Mice. To avoid contamination of the seed virus reagent with murine viruses, Webster strain mice obtained from the National Laboratory Animal Co., Creve Coeur, Mo., and found to be free from the murine viruses pneumonia virus of mice, minute virus of mice, Sendai, reovirus 3, mouse encephalomyelitis (GDVII), K virus, polyoma, simian virus 5, lymphocytic choriomeningitis virus, mouse hepatitis, and mouse adenovirus were used initially. Because of the need for increased numbers of mice, it was necessary to change to another supplier during production. No other source of mice, however, was found to be completely free from these viruses. CD1 strain mice from the Charles River Breeding Laboratory had antibodies to Sendai and minute virus of mice. These mice were used for four of the six reagents made; buttonwillow was made with the Webster strain, and EHD was made with both sources of mice.

**Master seed and seed virus reagent.** A master seed for each virus was prepared in suckling mice inoculated intracerebrally (ic) with 0.02 ml of the source virus. This master seed was then used to prepare the virus reagent and immunizing antigen. Approximately 20 litters of mice were used for each virus. Brains of moribund mice were prepared as a 20% suspension in 4% bovine serum albumin, fraction 5 (BSA) and phosphate-buffered saline (PBS), pH 7.9.

Master seeds were titrated in suckling mice and authenticated with a standard serum neutralization test with reference antisera or immune ascitic fluid provided by the Yale Arbovirus Research Unit (YARU). Samples of all preparations were sent to Microbiological Associates, Bethesda, Md., for detection of murine viruses by the mouse antibody production (MAP) testing procedure (12).

Seed virus reagents (SVR) were prepared by inoculating suckling mice ic with 0.02 ml of the master seed with 25 to 39 litters used for each virus pool. Moribund mice were stored at −90 C, and before use, the brains were removed by a modification of the method described by Strome (16). To obtain a sterile product, the mice were rinsed several times in tap water, once in Merthiolate (1:1,000), and once in 70% alcohol and then dried on a sterile towel. With a sterile scalpel, the skin on the skull was incised, and a 15-gauge needle attached to a vacuum system was inserted into the exposed skull. By this mechanism, the brains were drawn directly into a Sorvall blender. Brains were weighed, made into a 20% suspension with PBS, emulsified in the blender at 2 C for 1 min and then centrifuged at 1,500 × g for 30 min. Each SVR contained a minimum of 250 ml of a 10% suspension of brain material in PBS to

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after had anopheles with suspension Freund's Tables week-old in 0.2 ml.

| Virus | Strain          | Donor; identification         | Passage history* |
|-------|-----------------|-------------------------------|-----------------|
|       |                 |                               | Donor | SFRE   |
|       |                 |                               | James Hardy, Univ. of California at Berkeley | SM₂, SM₁ |
|       |                 |                               | D. O. Trainer, Univ. of Wisconsin | SM₂, SM₁ |
|       |                 |                               | ATCC VR328 | E, SM₂, SM₁ |
|       |                 |                               | ATCC VR86 | M₁, SM₂, SM₁ |
|       |                 |                               | ATCC VR-551 | SM₂, SM₁ |
|       |                 |                               | ATCC VR-57 | SM₁, SM₁ |

* SM, suckling mouse; E, egg; M, mouse; SFRE, Southwest Foundation for Research and Education.

which had been added a final concentration of 4% BSA as a stabilizer, with a final pH of 7.9. Samples were tested for sterility in the following: fluid thioglycolate medium, blood-agar, PPLO agar (Difco), and Saboraud dextrose medium, both at 37 C and at room temperature. Potency or infectivity titers were performed in newborn mice. Reference antisera or immune ascitic fluids provided by YARU were used for identity tests and for serum neutralization breakthrough tests to check for purity of the products. Neutralization breakthrough tests were performed to determine the presence of virus contaminants in the SVR. Moribund mice were selected from virus dilution or dilutions in the neutralization test at which level the antiserum failed to neutralize. Viruses isolated in this breakthrough zone were then identified.

**Immunizing antigen.** All virus preparations, either killed or live, were prepared in infant or newborn mice inoculated ic with the master seed. Moribund mice were frozen at -90 C. For live virus immunizing antigen, a 10% suspension of infected mouse brains without a protein stabilizer was used. Killed antigens consisted of a 20% suspension of infected mouse brains in PBS rendered inactive by diluting the virus pool with equal parts of 0.1% beta propiolactone (BPL) in PBS, giving a final concentration of 10% mouse brain antigen and 0.05% BPL (9). The suspension was incubated at 37 C for 1 hr and immediately used to immunize mice for production of immune ascitic fluid. Immune ascitic fluid. Two groups of 5- to 6-week-old female mice (350 per group) were inoculated intraperitoneally with an equal portion of Freund's complete adjuvant (0.25 ml) and a 10% suspension of either live or killed virus (0.25 ml).

The schedules for inoculation of mice are given in Tables 2 and 3. Ascitic fluid was produced in mice with approximately $4 \times 10^6$ cells of sarcoma 180/TG in 0.2 ml (13). Sarcoma cells obtained from Philip Coleman at the Center for Disease Control (CDC) were used for buttonwillow, EHD, Turlock, Kern Canyon, and Semliki Forest virus reagents; for anopheles B virus, cells were obtained from Microbiological Associates. Approximately 10 to 15 days after injection of sarcoma 180/TG cells, most mice had distended abdomens. The abdomen was then

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**Table 1. Origin and passage histories of arboviruses used in production of seed virus reagent and immune ascitic fluid**

| Virus                  | Strain     | Donor; identification         | Passage history* |
|------------------------|------------|-------------------------------|-----------------|
| Buttonwillow           | A7956      |                               | SM₂, SM₁       |
| Epizootic hemorrhagic  | NJ-55      |                               | SM₂, SM₁       |
| disease of deer        |            |                               | SM₂, SM₁       |
| Turlock                | S-1954-847-32 | ATCC VR328               | E, SM₂, SM₁   |
| Anopheles B            | Original   | ATCC VR86                    | M₁, SM₂, SM₁   |
| Kern Canyon            | M-206      | ATCC VR-551                  | SM₂, SM₁       |
| Semliki Forest         | Original   | ATCC VR-67                   | SM₁, SM₁       |

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**Table 2. Schedule for production of reagent immune ascitic fluids for buttonwillow, Turlock, anopheles B, and Kern Canyon viruses**

| Time (day) | Vol of antigen (ml) | Other material | Additional information |
|------------|---------------------|----------------|-----------------------|
| 0          | 0.25                | 0.25 ml of FCA | 10% suspension of LV  |
| 7          | 0.25                | 0.25 ml of FCA | 10% suspension of LV  |
| 14         | 0.2 ml of sarcoma   | 180/TG         | 10% suspension of LV  |
| 21         | 0.25                | 0.25 ml of FCA | 10% suspension of LV  |

* Abbreviations: FCA, Freund's complete adjuvant; LV, live vaccine. Antigen and other materials were administered by the intraperitoneal route.

**Table 3. Schedule for production of reagent immune ascitic fluids for EHD and Semliki Forest viruses**

| Time (day) | Vol of antigen (ml) | Other material | Additional information |
|------------|---------------------|----------------|-----------------------|
| 0          | 0.25                | 0.25 ml of FCA | 10% suspension of LV  |
| 4          | 0.25                | 0.25 ml of FCA | 10% suspension of LV  |
| 21         | 0.25                | 0.25 ml of FCA | 10% suspension of LV  |
| 24         | 0.2 ml of sarcoma   | 180/TG         | 10% suspension of LV  |
| 28         | 0.25                | 0.25 ml of FCA | 10% suspension of LV  |

* Abbreviations: FCA, Freund's complete adjuvant; LV, live vaccine; KV, killed vaccine. Antigen and other materials were administered by the intraperitoneal route.
disinfected with iodine, and ascitic fluids were withdrawn with a 15- or 18-gauge needle and a 30-ml syringe. If the mice survived, they were tapped a second time. Multiple tappings yielded hemolyzed or bacterially contaminated material so no attempt was made to tap more than twice. Fluids were pooled and centrifuged at 16,000 \times g in a RC2-B Sorvall centrifuge with a GSA head for 1 hr. The supernatant fluid was withdrawn and stored at −20 C overnight. The next day, the fluid was thawed, again centrifuged at 16,000 \times g for 1 hr, and then poured through a gauze funnel; the fibrinous clot was broken up with a pipette. Immune ascitic fluids ranged in volume from 4,730 to 2,500 ml. These were then divided into lots of approximately 600 ml and stored at −90 C. Each lot was tested for sterility. Any found to contain fungi, mycoplasma, or more than 10 bacteria per ml was not used.

**Control ascitic fluid.** Approximately 50 5- to 6-week-old female mice were inoculated intraperitoneally using the same schedule as that for immune ascitic fluid (Tables 2 and 3) but using a 10% suspension of normal suckling mouse brain immunizing antigen. In the case of control ascitic fluid for Semliki Forest virus, the same method of inactivating virus was used on normal suckling mouse brains. Withdrawing the fluid and processing were the same as these procedures for immune ascitic fluid. Sterility tests were performed as for immune ascitic fluid and with the same standards.

**Serology.** Complement fixation (CF) tests were performed by the microtiter (14) LBCF system (1) on both control and immune ascitic fluid. The method of Clark and Casals (2) was used in preparing hemagglutinating antigens and performing hemagglutination-inhibition (HI) tests. Neutralization tests were done in infant mice with the constant immune ascitic fluid-virus dilution technique after incubation for 1 hr at 37 C. The neutralization index was calculated by the method of Reed and Muench (11). CF and HI tests, both homologous and heterologous, were done on individual lots of immune and control ascitic fluids. Neutralization tests, both homologous and heterologous, were done on small proportional pools of reagent immune ascitic fluids.

**Safety tests.** Each control ascitic fluid and a pool of reagent immune ascitic fluid were tested for homologous and extraneous encephalitis-producing viruses by inoculating at least 15 suckling mice ic with 0.02 ml of undiluted and diluted material (1:100). Also approximately 18 3- to 4-week-old mice were inoculated ic with 0.03 ml of the above reagents. Mice were held for 21 days, and the brains of dead mice or those suspected of illness from any of the four groups were passed ic in suckling and weanling mice. No virus was found.

**RESULTS AND DISCUSSION**

The immune ascitic fluid for each of the reagents had a homologous CF antibody titer of not less than 1:128 (Table 4), a log neutralization index of not less than 3.7, except for anopheles B (Table 5), and, where applicable, an HI titer of not less than 1:320 (Table 6). To determine preferable immunization schedules for obtaining the above desired titers, pilot studies were initiated before the production of any reagent. Pilot studies included the schedules used for production (Tables 2 and 3), but for the sake of brevity production schedules are not listed in Tables 7 through 11.

Of the three pilot studies initiated for the production of buttonwillow immune ascitic fluid, the rejected two (Table 7) gave smaller volumes of ascitic fluid and lower titers. A third of the mice never produced ascites, whereas all mice but one of the schedule used for production did. In studies with the remaining viruses, Freund's complete adjuvant plus sarcoma 180/TG cells were used because a larger volume of ascitic fluid was produced compared to the use of sarcoma 180/TG cells alone. The CF titer for the rejected buttonwillow immune ascitic fluid was 1:128, whereas the pilot schedule, eventually used for production, resulted in fluid with a titer of 1:512. The immunization schedule used in the production of buttonwillow reagent (Table 2) was suggested by R. Shope of YARU (personal communication).

Two of the three pilot studies for EHD virus are shown in Table 8. Although these schedules gave approximately the same volume of ascitic fluid as the schedule eventually used for production, they were rejected because of the low CF titer produced (1:32 as compared to 1:128). The final schedule for EHD virus ascitic fluid production (Table 3) was one suggested by the arbovirus unit of CDC (personal communication).

Two of the three tests for Turlock immune ascitic fluid are shown in Table 9. At this time, it was decided to determine whether storing the immunizing antigen at −90 C as a 10% suspension in PBS without a protein stabilizer would affect the titers. The CF and HI titers were identical whether the immunizing antigen was stored as whole frozen mice (harvesting only enough brains for immunizing at the specified time needed) or as a 10% suspension in PBS without a protein stabilizer and maintained at −90 C. Identical titers were also obtained in similar studies for anopheles B and Kern Canyon. The schedule indicated in Table 2 was used for the production of Turlock reagent immune ascitic fluid.

Preliminary production of anopheles B immune ascitic fluid was similar to those for Turlock (Table 10). The two schedules shown were essentially the same, in volume of ascitic fluid produced and CF titer, as the schedule
Table 4. Potency of arbovirus immune ascitic fluids: results of cross-CF tests

| Antigen                  | St. Louis encephalitis | Buttonwillow | EHD  | Turlock | Anopheles B | Kern Canyon | Semliki Forest |
|--------------------------|------------------------|--------------|------|---------|-------------|-------------|----------------|
| Buttonwillow             | <8                     | 128<sup>a</sup> | <8   | <8      | <8          | <8          | <8             |
| EHD                      | <8                     | <8           | <8   | <8      | <8          | <8          | <8             |
| Turlock                  | <8                     | <8           | <8   | <8      | <8          | 256         | 16             |
| Anopheles B              | <8                     | <8           | <8   | <8      | 512         | <8          | <8             |
| Kern Canyon              | <8                     | <8           | <8   | <8      | 256         | <8          | 512            |
| Semliki Forest           | <8                     | <8           | <8   | <8      | <8          | <8          | 512            |
| St. Louis encephalitis   | 256                    | <8           | <8   | <8      | <8          | <8          | <8             |
| Western equine encephalitis | <8                 | <8           | <8   | <8      | <8          | <8          | <8             |
| Eastern equine encephalitis | <8                | <8           | <8   | <8      | <8          | <8          | <8             |

<sup>a</sup> Numbers in boldface represent reciprocal of the highest serum dilution fixing 70% of the complement.

Table 5. Potency of immune ascitic fluids: results of cross-neutralization tests<sup>a</sup>

| Ascitic fluids              | Western equine encephalitis | St. Louis encephalitis | Buttonwillow | EHD  | Turlock | Anopheles B | Kern Canyon | Semliki Forest |
|-----------------------------|-----------------------------|------------------------|--------------|------|---------|-------------|-------------|----------------|
| Buttonwillow                | 0                           | 0.4                    | >6.0<sup>a</sup> | —    | —       | —           | —           | —              |
| EHD                         | —                           | 0.8                    | 0.2          | 3.7  | 0       | <0.5       | <0.5       | —              |
| Turlock                     | —                           | 0.9                    | 0.3          | 3.2  | <0.6   | 1.5        | 0.1        | —              |
| Anopheles B                 | —                           | 0                      | —            | 0.3  | 1.5     | 0.5        | 4.1        | 0.5            |
| Kern Canyon                 | —                           | 0.2                    | —            | —    | 0.5     | 4.1        | 0.5        | —              |
| Semliki Forest              | —                           | 0.4                    | —            | —    | —       | 0.2        | 6.3        | —              |

<sup>a</sup> Log neutralization index.

<sup>b</sup> Numbers in boldface represent homologous titer.

Table 6. Potency of arbovirus immune ascitic fluids: results of cross-HI tests

| Immune ascitic fluids       | Western equine encephalitis | Eastern equine encephalitis | St. Louis encephalitis | Buttonwillow | Turlock | Semliki Forest |
|-----------------------------|-----------------------------|-----------------------------|------------------------|--------------|---------|----------------|
| Buttonwillow                | <10                         | <10                         | <10                    | 320<sup>a</sup> | <10     | <10            |
| EHD<sup>a</sup>             | <10                         | <10                         | <10                    | <10          | <10     | <10            |
| Turlock                     | <10                         | <10                         | <10                    | <10          | <10     | <10            |
| Anopheles B<sup>a</sup>     | <10                         | <10                         | <10                    | <10          | <10     | <10            |
| Kern Canyon<sup>a</sup>     | <10                         | <10                         | <10                    | <10          | <10     | <10            |
| Semliki Forest              | 40                          | 40                          | 10                     | <10          | <10     | 640            |

<sup>a</sup> HA antigen not available or the virus does not produce an HA antigen.

<sup>b</sup> Numbers in boldface represent reciprocal of highest serum dilution inhibiting four to eight units of antigen used in reagent production (Table 2).

A problem arose with anopheles B virus, with reference to temperature sensitivity, that was not encountered with any of the other viruses. In neutralization tests of the reagent immune ascitic fluid, the control titer of the virus dropped approximately 2.5 logs. After incubation at 37 C for 1 hr in control ascitic fluid, the titer of the master seed dropped from 9.2 to 6.5 logs per g, and in a similar test with 4% BSA as the control diluent a drop of 2.1 logs was observed. To test the effect of temperature, duplicate neutralization tests were performed at room temperature (about 23 C) and at 37 C for 1 hr with the seed virus reagent in control ascitic fluid. After incubation under those conditions, the control titer at room temperature was 8.5 logs, whereas the control titer at 37 C was 5.9 log units compared to the original titer of 9.0.

Kern Canyon pilot studies consisted of the reagent production schedule given in Table 2, except for the method of storage of the immunizing agent. As stated earlier, identical titers were obtained.

Results with Semliki Forest are shown in
### Table 7. Pilot study schedules for buttonwillow virus

| Time (day) | Vol of antigen (ml) | Other material | Route | Titer | Additional information |
|------------|---------------------|----------------|-------|-------|------------------------|
| 0          | 0.25<sup>b</sup>    |                | sc    |       | 10⁻⁸⁻⁸<sup>c</sup>    | 10% suspension of LV |
| 4          | 0.25                | 0.2 ml of sarcoma 180/TG | sc    |       | 10% suspension of LV   |
| 14         | 0.25                | 0.25 ml of FCA  | ip    |       | 10% suspension of LV   |
| 21         | 0.25                |                | ip    |       | 10% suspension of LV   |
| 0          | 0.25<sup>b</sup>    |                | ip    |       | 10⁻⁸⁻⁸<sup>c</sup>    | 10% suspension of LV |
| 7          | 0.25                | 0.2 ml of sarcoma 180/TG | ip    |       | 10% suspension of LV   |
| 14         | 0.25                | 0.25 ml of FCA  | ip    |       | 10% suspension of LV   |
| 15         | 0.25                |                | ip    |       | 10% suspension of LV   |
| 21         | 0.25                |                | ip    |       | 10% suspension of LV   |

<sup>a</sup> Production schedule not included; see Table 2. Abbreviations: FCA, Freund’s complete adjuvant; sc, subcutaneous; ip, intraperitoneal; LV, live vaccine.

<sup>b</sup> Stored as 10% suspension in PBS.

<sup>c</sup> SMLD<sub>50</sub>/gram.

### Table 8. Pilot study schedules for EHD virus

| Time (day) | Vol of antigen (ml) | Other material | Route | Titer | Additional information |
|------------|---------------------|----------------|-------|-------|------------------------|
| 0          | 0.25<sup>b</sup>    |                | sc    |       | 10⁻⁸⁻⁸<sup>c</sup>    | 10% suspension of LV |
| 4          | 0.25                | 0.25 ml of FCA  | ip    |       | 10% suspension of LV   |
| 21         | 0.25                | 0.25 ml of FCA  | ip    |       | 10% suspension of LV   |
| 24         | 0.25                | 0.2 ml of sarcoma 180/TG | ip    |       | 10% suspension of LV   |
| 28         | 0.25                | 0.25 ml of FCA  | ip    |       | 10% suspension of LV   |
| 0          | 0.25<sup>b</sup>    | 0.25 ml of FCA  | ip    |       | 10⁻⁸⁻⁸<sup>c</sup>    | 10% suspension of LV |
| 7          | 0.25                | 0.25 ml of FCA  | ip    |       | 10% suspension of LV   |
| 14         | 0.25                | 0.2 ml of sarcoma 180/TG | ip    |       | 10% suspension of LV   |
| 21         | 0.25                | 0.25 ml of FCA  | ip    |       | 10% suspension of LV   |

<sup>a</sup> Production schedule not included; see Table 3. Abbreviations: FCA, Freund’s complete adjuvant; sc, subcutaneous; ip, intraperitoneal; LV, live vaccine.

<sup>b</sup> Stored as 10% suspension in PBS.

<sup>c</sup> SMLD<sub>50</sub>/gram.

### Table 9. Pilot study schedules for Turlock virus

| Time (day) | Vol of antigen (ml) | Other material | Route | Titer | Additional information |
|------------|---------------------|----------------|-------|-------|------------------------|
| 0          | 0.25<sup>a</sup>    | 0.25 ml of FCA  | ip    |       | 10⁻⁸⁻⁸<sup>c</sup>    | 10% suspension of LV |
| 7          | 0.1                 | 0.1 ml of FCA   | ip    |       | 10% suspension of LV   |
| 14         | 0.1                 | 0.2 ml of sarcoma 180/TG | ip    |       | 10% suspension of LV   |
| 21         | 0.1                 | 0.1 ml of FCA   | ip    |       | 10% suspension of LV   |
| 0          | 0.25<sup>a</sup>    | 0.25 ml of FCA  | ip    |       | 10⁻⁸⁻⁸<sup>c</sup>    | 10% suspension of LV |
| 7          | 0.25                | 0.25 ml of FCA  | ip    |       | 10% suspension of LV   |
| 14         | 0.25                | 0.2 ml of sarcoma 180/TG | ip    |       | 10% suspension of LV   |
| 21         | 0.25                | 0.25 ml of FCA  | ip    |       | 10% suspension of LV   |

<sup>a</sup> Production schedule not included; see Table 2. Abbreviation: FCA, Freund’s complete adjuvant; ip, intraperitoneal; LV, live vaccine.

<sup>a</sup> Stored as whole brains.

<sup>c</sup> SMLD<sub>50</sub>/gram.

<sup>a</sup> Stored as 10% suspension.
Table 10. Pilot study schedules for anopheles B virus*

| Time (day) | Vol of antigen (ml) | Other material | Route | Titer   | Additional information     |
|------------|---------------------|----------------|--------|---------|-----------------------------|
| 0          | 0.25                | 0.25 ml of FCA | ip     | $10^{-0.2}$ | 10% suspension of LV       |
| 3          | 0.25                | 0.25 ml of FCA | ip     | $10^{-0.2}$ | 10% suspension of LV       |
| 21         | 0.25                | 0.25 ml of FCA | ip     | $10^{-0.2}$ | 10% suspension of LV       |
| 24         | 0.25                | 0.25 ml of FCA | ip     | $10^{-0.2}$ | 10% suspension of LV       |
| 28         | 0.25                | 0.25 ml of FCA | ip     | $10^{-0.2}$ | 10% suspension of LV       |
| 0          | 0.25                | 0.25 ml of FCA | ip     | $10^{-0.2}$ | 10% suspension of LV       |
| 14         | 0.25                | 0.25 ml of FCA | ip     | $10^{-0.2}$ | 10% suspension of LV       |
| 21         | 0.25                | 0.25 ml of FCA | ip     | $10^{-0.2}$ | 10% suspension of LV       |

* Production schedule not included; see Table 2. Abbreviations: FCA, Freund's complete adjuvant; ip, intraperitoneal; LV, live vaccine.

SMLD$_{30}$/gram.

Table 11. Pilot study schedules for Semliki Forest virus*

| Time (day) | Vol of antigen (ml) | Other material | Route | Titer | Additional information     |
|------------|---------------------|----------------|--------|-------|-----------------------------|
| 0          | 0.5*                | 0.5 ml of FCA  | ip     | $10^{-0.4}$ | 10% suspension of BPL-killed vaccine |
| 7          | 0.1                 | 0.1 ml of FCA  | ip     |       | 10% suspension of LV       |
| 14         | 0.1                 | 0.2 ml of sarcoma 180/TG | ip |       | 10% suspension of LV       |
| 21         | 0.1                 | 0.1 ml of FCA  | ip     |       | 10% suspension of LV       |
| 0          | 0.5*                | 0.5 ml of FCA  | ip     | $10^{-0.4}$ | 10% suspension of BPL-killed vaccine |
| 7          | 0.25                | 0.25 ml of FCA | ip     |       | 10% suspension of LV       |
| 14         | 0.25                | 0.2 ml of sarcoma 180/TG | ip |       | 10% suspension of LV       |
| 21         | 0.25                | 0.25 ml of FCA | ip     |       | 10% suspension of LV       |

* Production schedule not included; see Table 3. Abbreviations: FCA, Freund's complete adjuvant; ip, intraperitoneal; BPL, beta propiolactone; LV, live vaccine.

SMLD$_{30}$/gram.

Table 11. These results were rejected because of the marginal CF and HI titers produced (1:32 and 1:40). In a repeat pilot study with the schedule given in Table 2, with the exception of the first injection consisting of killed virus, acceptable titers were obtained (CF 1:128). Perhaps the large initial injection of 0.5 ml of killed vaccine resulted in a state of partial tolerance or immune paralysis. Starting with a lower initial dose (0.25 ml), the titers obtained were much higher, even though the total amount of immunizing agent administered was roughly comparable. This schedule, however, was not used in the reagent production (Table 3) because the results were obtained after production had begun.

The titer of the seed virus reagents for the six arboviruses produced are presented in Table 12. All had titers of not less than $10^4$.

Table 12. Seed virus titers after storage at $-90^\circ$ C

| Virus              | Titer/mg* | Time (days) in storage* |
|--------------------|-----------|------------------------|
| Buttonwillow       | $10^{-5.6}$ | 25                     |
| EHD                | $10^{-5.2}$ | 5                      |
| Turlock            | $10^{-6.0}$ | 8                      |
| Anopheles B        | $10^{-6.4}$ | 12                     |
| Kern Canyon        | $10^{-6.4}$ | 2                      |
| Semliki Forest     | $10^{-11.7}$ | 17                     |

*Two- to 4-day-old mice inoculated ic with 0.02 ml of virus.

* Stored in 10% mouse brain, 4% BSA.

median lethal doses (LD$_{50}$) per gram.

Reagents for the buttonwillow virus are listed in the Catalog of Research Reagents, 1970 (3) as V-534-001-522 (virus) and V534-701-562 (IAF). The remaining viruses and their
immune ascitic fluid (IAF) will become available as confirmatory testing and packaging are completed.

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