Development of Four Arboviruses in Mice and Application to Rapid Test Procedures

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Mice were inoculated with St. Louis encephalitis (SLE), Flanders (FLAN), California (CE), or Tensaw (TEN) viruses. At fixed intervals after inoculation, brains from these mice were collected and assayed for infective virus and complement-fixing, hemagglutinating, and precipitating antigens. Detectability of these antigens was correlated with the appearance of signs of illness in the mice. Infective virus appeared 64, 48, 48, and 40 hr before signs of illness and 90, 86, 64, and 56 hr before death in mice inoculated with SLE, FLAN, CE, and TEN viruses, respectively. Diagnostic antigens were also detected well before signs of illness appeared. These findings were applied to the isolation of viruses from field-collected specimens. It was shown that by harvesting tissues at appropriate intervals these viruses could be detected and identified more rapidly than by conventional techniques with mice.

The growth of a number of arboviruses and their associated proteins has been studied in detail but generally with emphasis on development at the cellular or subcellular level (1, 4, 6, 13). Few studies have been conducted to determine the rate of development in host animals and, in particular, the time of development of usable antigens in mouse brain. The present report concerns kinetic studies on the development of infectivity and antigenicity of four North American arboviruses, St. Louis encephalitis (SLE), Flanders (FLAN), California encephalitis (CE), and Tensaw (TEN), in the laboratory mouse.

These four viruses are mosquito-borne and are commonly collected in various parts of the United States. CE and SLE have been prominently associated with human illnesses (5, 7). The suckling laboratory mouse (SM) is a sensitive host system for these viruses and is used to isolate them from field-collected specimens. However, so little is known of the replicative properties and antigen development of these common viruses in the mouse that studies were undertaken to determine the time-course for appearance of infective virus and complement-fixing (CF), hemagglutinating (HA), and precipitating antigens in relation to the appearance of signs of illness. In addition, these findings were applied in a more rapid identification scheme than is now routinely used.

MATERIALS AND METHODS

Virus strains. The following virus strains were used in the growth rate studies: SLE (TBH-28), isolated from human brain tissue, Florida, 1962 (3), used in the fourth SM passage; FLAN (A9-1551), from Caliseta melanura, Alabama, 1960 (10), fourth SM passage; CE (La Crosse), from human brain tissue, Wisconsin, 1960 (11), in fourth SM passage; and TEN (A9-171B), from Anopheles crucians, Alabama, 1960 (10), fifth SM passage. Field strains of viruses, used as original mosquito suspensions in detection studies on unpassaged viruses, were SLE (TD6-3AS), Culex quinquefasciatus, Texas, 1966; FLAN (TD6-688C), C. quinquefasciatus, Texas, 1966; CE (NMS-7AD), Aedes species, New Mexico, 1965; and TEN (WX3-4AD), Psorophora confinis, Georgia, 1963.

These field strains were chosen because they all produced signs of illness when inoculated into SM. Other field strains, composed of suspensions of a few to 100 pooled, infected mosquitoes collected by members of the Arbovirus Unit and shown in previous studies to contain one or another of these viruses, were also used.

Antigens. Crude CF antigens were prepared by centrifuging 20% (v/v) infected SM brain at 11,000 × g for 30 min at 4 C. The supernatant fluid was diluted 1:5 and used in serial twofold dilutions against 8 units of homologous immune mouse serum or immune mouse ascitic fluid and 7 units of complement. The end point was judged to be the greatest antigen dilution giving more than 50% fixation with the immune preparation. Veronal buffer was used as the diluent in all CF tests.

The method recommended by Work was used to
prepare crude HA antigens (14). These were tested at 37 C for 30 min with standardized [optical density (OD) at 450 nm] goose cells suspended in buffers to give pH values of 6.0, 6.2, 6.4, 6.6, and 6.8. Titer was determined as the greatest antigen dilution giving complete agglutination.

Antigens for precipitin-in-gel tests were undiluted, frozen-thawed breis of SM brain (8). Results of these tests were recorded on a 0 to + scale depending upon intensity of the arc of precipitation: 0 = no visible precipitation; 1+ = a trace; 2+ = a weak but readily visible arc; 3+ = a high degree of precipitation; 4+ = optimally intense arc, as compared with a known positive control preparation. Replicate samples were used for all tests.

**Immune sera and immune ascitic fluids.** Hyperimmune ascitic fluids (HIAF) were produced by the method of Tikasinski et al. (12). In general, 8-week-old female Swiss mice were immunized with four doses of 0.5 ml of a clarified 10% infected SM brain suspension, given intraperitoneally on days 0, 5, 21, and 28. The first two doses of SME virus were inactivated with a final concentration of 0.05% beta-propiolactone. Sarcoma 180 cells were given by the same route on day 25, and fluids were removed as the abdomens became extended. Only HIAF with homologous CF titers of at least 1:256 were used.

Immune rabbit sera were used in precipitin-in-gel tests for detecting CE and TEN viruses. For production of these antiserum, rabbits were given two intraperitoneal doses of 0.5 ml of live, 10% SM brain suspensions and then bled 2 weeks after the second dose. For the SME and FLAN virus precipitin-in-gel and other serological tests, we used mouse HIAF.

**Determination of infective virus.** Serial 10-fold dilutions of infected SM brain clarified by centrifugation at 18,000 × g for 30 min were inoculated into two litters of 2- to 4-day-old mice, 0.02 ml per mouse, by the intracranial (ic) route. End points were calculated by the method of Reed and Muench (9). Mice were observed daily and according to the visible signs of illness were recorded as sick, paralyzed, prostrate, or moribund. These designations indicated stages of disease progression based on past experience with these and other viruses.

**Growth curves.** Litters of eight SM each were inoculated with either the SME, FLAN, CE, or TEN virus. Each mouse was inoculated ic with 0.02 ml of inoculum containing an estimated 500 LD50 of indicated virus. At appropriate 8-hr intervals after inoculation, two litters for each virus were sacrificed and stored at −60 C until harvested. For harvesting, the mice were thawed; their brains were collected by mechanical aspiration, pooled, and stored at −60 C. The same individual titrated crude HA and CF antigens of all viruses on a single day. Infectivity tests were carried out on different days, but all infectivity titrations and precipitin-in-gel tests of a given virus were done on the same day.

**Detection of viruses in field-collected specimens.** Each of several infected suspensions of pooled, macerated mosquitoes was inoculated into two litters of SM by the ic route. At 40, 64, 88, and 107 hr after inoculation, two mice from each litter were selected at random and stored at −60 C. The remainder of the mice were collected when they became moribund. In addition, a further passage of brains was made, and the brains were collected 48 hr later or when signs of illness were evident. Brains of all mice were harvested separately, pooled by group, and tested for infectivity or CF antigen, or both, and agar gel precipitin.

**RESULTS**

**Replication of laboratory-adapted strains.** Figure 1 shows the progressive development of infectious SLE virus (strain TBH-28) and its CF and HA antigens in SM brain. The times precipitating antigen and illness appeared are noted. From a nadir between 0 and 16 hr, infectious virus increased to a peak titer of 106.1/0.02 ml at 24 to 32 hr. Thereafter, the quantity of recoverable virus remained static until the death of the mouse on the day 4 to 5. CF and HA antigens could not be detected as early as infectious virus. Infectious virus and CF antigen were detected at 16 and 40 hr, respectively, at which times signs of illness were not apparent. HA and precipitating antigens were first detected at the 56th hr, 24 hr before the first signs of illness.

Like SLE virus, Flanders virus (strain A9-155L) did not kill mice until more than 4 days after inoculation (Fig. 2).

However, infectious virus was isolated as early as the 16th hr, and CF and precipitating antigens were observed by the 32nd and 40th hr, respectively. Signs of illness lagged behind detection of infectious virus by 48 hr and behind appearance of CF and precipitating antigens by 32 and 24 hr, respectively. No HA antigen was detected. Optimum virus titer (106.9/0.02 ml) was observed by 32 to 40 hr after inoculation.

HA antigen was not detected through the course of infection with the La Crosse strain of California virus (Fig. 3). Virus replication was evident as early as the 8th hr after inoculation; CF antigen was detected by the 24th hr, and precipitating antigen was detected by the 40th hr. Infectious virus and CF and precipitating antigens were detected well before signs of illness could be observed: 48, 32, and 16 hr before illness, respectively. Optimum virus titer (106.9/0.02 ml) was observed by 40 hr after inoculation.

As seen in Fig. 4, infectious Tensaw virus (strain A9-171 B) was first detected 16 hr after inoculation, approximately 16 hr before CF antigen appeared, 32 hr before precipitating antigen, and 40 hr before signs of illness. Again, no HA antigen was noted. Peak infectivity (approximately 106.5/0.02 ml) was observed 40 to 56 hr after inoculation.

**Detection of virus in suspensions of pools of infected mosquitoes.** Determining the earliest
FIG. 1. Appearance of infective virus, hemagglutinating, complement-fixing, and precipitating antigens and their relation to signs of illness in suckling mice inoculated with St. Louis encephalitis virus.

FIG. 2. Appearance of infective virus, hemagglutinating, complement-fixing, and precipitating antigens and their relation to signs of illness in suckling mice inoculated with Flanders virus.

intervals at which the four virus types could be detected from infected, pooled mosquitoes was of practical interest. Mice were inoculated with infected mosquito suspensions, and the appearance of infective virus and CF and precipitating antigens and the symptomatology were noted. The results are presented in Tables 1 to 4.

Infectivity and CF and precipitating antigen titers increased rapidly with passing time. The virus in the suspensions of pooled, infected mosquitoes was of low titer, ranging from 1.7 to 2.2 SM ic LD50/ml, and reacted in neither the CF nor precipitin-in-gel tests. By the 40th hr post-inoculation, replication of CE, TEN, and FLAN viruses was evident; SLE virus replication was first clearly detected by the 64th hr.

That optimum infectivity and CF antigen titers of FLAN and TEN viruses were lower than optimum titers with SLE and CE viruses is consistent with findings with reagents prepared by more conventional techniques.

Passage of brains from mice showing signs of illness was done to parallel the technique presently utilized in most laboratories. With all four viruses, infectivity and appreciable CF and precipitating antigens were detected at the time when mice showed signs of illness (Tables 1 to 4, passage level 2). Somewhat higher titers of CF and pre-
Fig. 3. Appearance of infective virus, hemagglutinating, complement-fixing, and precipitating antigens and their relation to signs of illness in suckling mice inoculated with California (La Crosse) virus.

Fig. 4. Appearance of infective virus, hemagglutinating, complement-fixing, and precipitating antigens and their relation to signs of illness in suckling mice inoculated with Tensaw virus.

cipitating antigens noted at the second passage level probably reflect adaptation to replication in the mouse host.

Results of a sample application of the presymptomatic mouse brain harvest technique in detecting the four viruses in mosquito suspensions are shown in Table 5. In some instances, the presence of infective virus could be shown as early as 48 to 64 hr, although symptoms and death would not be expected earlier than 100 hr.

DISCUSSION

Infectious virus was isolated from infected SM brain well in advance of CF and precipitating
were viruses precipitins. used case, of of minants indicated. If these antigens are different determinants on the same particle, the assay systems used in these studies for detecting infectious viruses must have been more sensitive than the systems used for detecting CF antigens and precipitins. Possibly, the time course of synthesis of whole, infectious virus is more rapid than that of associated subviral particles. Whichever the case, it is of interest that infectious virus and CF, HA (when detected), and precipitating antigens were detected before signs of illness were observed in the mice. Maximal infective virus titers were attained in SM brain in as few as 32 hr (CE) and in as many as 74 hr (SLE) before the mice died. In terms of production or detectability, infectious virus was followed chronologically by CF, HA, and precipitating antigens and signs of illness. Under these testing conditions, determinations of HA antigens for these viruses in crude suspensions of infected SM brains obviously are of little diagnostic value. Preparation of crude HA antigens, nevertheless, is a recommended procedure for routine arbovirus identification.

### Table 1. St. Louis encephalitis virus (strain TD6-3AS) infectivity for mice and antigenicity at different periods of incubation

| Passage level | Time after inoculation | Infectivity titer of brain (log SM ic LD50/ml) | CF titer (reciprocal) | Agar gel reaction |
|---------------|------------------------|---------------------------------------------|-----------------------|------------------|
|               | hr                     |                                             |                       |                  |
| 0             | 0                      | 1.7                                         | <5                    | 0                |
| 1             | 40                     | ≤2.5                                        | <5                    | 0                |
| 1             | 64                     | 3.5                                         | 10                    | 1                |
| 1             | 88                     | ≥8.5                                        | 80                    | 2                |
| 1             | 107                    | 8.5                                         | 320                   | 3                |
| 1             | 192a                   | +c                                          | 320                   | 4                |
| 2             | 48                     | +                                           | 160                   | 2                |
| 2             | 96a                    | +                                           | 1,280                 | 4                |

a Results shown were determined by testing a brain pool of four SM harvested at the time indicated.

b These mice showed signs of illness.

c Not titrated; virus isolated from 1% brain suspension.

### Table 2. Flanders virus (strain TD6-688C) infectivity for mice and antigenicity at different periods of incubation

| Passage level | Time after inoculation | Infectivity titer of brain (log SM ic LD50/ml) | CF titer (reciprocal) | Agar gel reaction |
|---------------|------------------------|---------------------------------------------|-----------------------|------------------|
|               | hr                     |                                             |                       |                  |
| 0             | 0                      | 2.0                                         | <5                    | 0                |
| 1             | 40                     | 3.5                                         | <5                    | 0                |
| 1             | 64                     | 4.3                                         | <5                    | 0                |
| 1             | 88                     | 6.4                                         | 10                    | 0                |
| 1             | 107                    | 6.1                                         | 40                    | 1                |
| 1             | 168b                   | +c                                          | 80                    | 3                |
| 2             | 48                     | +                                           | 160                   | 2                |
| 2             | 96b                    | +                                           | 320                   | 3                |

a Results shown were determined by testing a brain pool of four SM harvested at the time indicated.

b These mice showed signs of illness.

c Not titrated; virus isolated from 1% brain suspension.

### Table 3. California virus (La Crosse type, strain NM5-7AO) infectivity for mice and antigenicity at different periods of incubation

| Passage level | Time after inoculation | Infectivity titer of brain (log SM ic LD50/ml) | CF titer (reciprocal) | Agar gel reaction |
|---------------|------------------------|---------------------------------------------|-----------------------|------------------|
|               | hr                     |                                             |                       |                  |
| 0             | 0                      | 2.2                                         | <5                    | 0                |
| 1             | 40                     | 5.4                                         | 40                    | 0                |
| 1             | 64                     | 6.7                                         | 160                   | 1                |
| 1             | 88                     | 6.5                                         | 320                   | 3                |
| 1             | 107                    | 7.3                                         | 160                   | 3                |
| 1             | 120b                   | +c                                          | 640                   | 4                |
| 2             | 48b                    | +                                           | 1,280                 | 4                |

a Results shown were determined by testing a brain pool of four SM harvested at the time indicated.

b These mice showed signs of illness.

c Not titrated: virus isolated from 1% brain suspension.

### Table 4. Tensaw virus (strain WX3-4AD) infectivity for mice and antigenicity at different periods of incubation

| Passage level | Time after inoculation | Infectivity titer of brain (log SM ic LD50/ml) | CF titer (reciprocal) | Agar gel reaction |
|---------------|------------------------|---------------------------------------------|-----------------------|------------------|
|               | hr                     |                                             |                       |                  |
| 0             | 0                      | 2.2                                         | <5                    | 0                |
| 1             | 40                     | 5.5                                         | 40                    | 0                |
| 1             | 64                     | 7.8                                         | 80                    | 0                |
| 1             | 88                     | 8.5                                         | 160                   | 1                |
| 1             | 107                    | 8.5                                         | 80                    | 2                |
| 1             | 192b                   | +c                                          | 160                   | 3                |
| 2             | 48b                    | +                                           | 160                   | 2                |
| 2             | 72b                    | +                                           | 80                    | 3                |

a Results shown were determined by testing a brain pool of four SM harvested at the time indicated.

b These mice showed signs of illness.

c Not titrated; virus isolated from 1% brain suspension.
TABLE 5. Detection of infective virus in brains of suckling mice at various hours of incubation after inoculation with infected mosquito suspensions

| Virus          | Mosquito pool no. | Infective virus detected* after incubation for | Parallel group of 8 mice |
|---------------|-------------------|-----------------------------------------------|--------------------------|
|               | 48 hr 64 hr 72 hr 88 hr |                                              |                          |
| St. Louis encephalitis | 1                | +                                              | +                         |
|               | 2                | +                                              | +                         |
|               | 3                | +                                              | +                         |
|               | 4                | +                                              | +                         |
|               | 5                | +                                              | +                         |
| Flanders      | 1                | +                                              | + + +                     |
|               | 2                | +                                              | + + +                     |
|               | 3                | +                                              | + + +                     |
|               | 4                | +                                              | + + +                     |
|               | 5                | +                                              | + + +                     |
| California    | 1                | +                                              | +                         |
|               | 2                | +                                              | +                         |
|               | 3                | +                                              | +                         |
|               | 4                | +                                              | +                         |
|               | 5                | +                                              | +                         |
|               | 6                | +                                              | +                         |
| Tensaw        | 1                | +                                              | +                         |
|               | 2                | +                                              | +                         |

* All suspensions killed SM at an earlier time when first tested. Failure to kill mice in current tests was attributed to loss of infectivity during storage.

* Results based upon sacrifice of two mice per time period.

* Virus isolated from all mice showing signs of illness during a 14-day observation period.

* No virus isolated.

* Virus isolated from one mouse found prostrate on postinoculation day 10, reflecting low titer of inoculum.

Sucrose-acetone (2) or other extraction procedures probably would increase to useful titers HA antigens for TEN and possibly CE viruses; no HA activity has been reported for FLAN virus. Removal of nonspecific inhibitors by most methods is time-consuming but often necessary for determining serological relationships to known types.

The knowledge derived from studies of temporal production of viral antigens in mice inoculated with suspensions of infected mosquitoes has a practical application in preventing unnecessary delays in viral detection and identification. Identification of a virus during a potential epidemic or epizootic situation depends upon obtaining a usable antigen as rapidly as possible, and success in this regard is abetted by knowledge of the time diagnostic viral antigens and infective virus appear in the laboratory host. The present study has shown that antigens and infective virus are present in diagnostically usable levels well before signs of disease are evident.

Unfortunately, considerable variation exists in virus and antigen development in presymptomatic hosts, and tissues may be harvested before the quantity of virus or antigen has reached a level sufficient for diagnostic purposes.

Several of the commonest arboviruses in the United States, Eastern equine encephalitis (EEE), Western equine encephalitis (WEE), SLE, FLAN, CE, and TEN viruses, are often isolated concurrently from mosquito pools and must be distinguished from each other. The speed with which this is done is of obvious importance if human or animal disease is involved. EEE and WEE viruses usually pose no great problem, although they of course must be differentiated from each other, since clues of their identity are obtained from the species of mosquito involved, the geographic location, and the brief survival time of mice or cell cultures inoculated.

However, recent outbreaks of SLE (7) and CE (5) in this country have demonstrated the need to differentiate these viruses rapidly from the nonpathogenic FLAN and TEN viruses with which they occur concomitantly. SLE and FLAN viruses, for example, are frequently isolated from the same species of Culex mosquitoes collected in the same geographic localities at the same time of year. Mixed infections in the same mosquito pool are also encountered occasionally. Both can kill mice in 7 to 10 days on second passage. Likewise, CE and TEN viruses may be confused, since they often occur together in the same population of Aedes mosquitoes. The time needed to kill SM is also similar, 5 to 9 days on the first passage and 2 to 5 days on the second passage.

Standard techniques for arbovirus isolation and identification involve inoculation of SM via the ic route with suspensions of pooled, macerated blood-sucking arthropods or with blood, cerebrospinal fluid, or autopsy material from humans or other vertebrates. Brain tissue from mice subsequently showing signs of illness is then usually passed to other mice to adapt the virus to the new host, with a resultant increase of virus titers to levels more certain to be useful for diagnosis. The present study indicates, at least for SLE, FLA, CE, or TEN viruses, the practicality of sacrificing one or two of the inoculated mice at about the 88th hr of incubation and testing them for infective virus by neutralization with group-specific or
type-specific antiserum. An alternative procedure is to collect the mice about 107 hr after inoculation and test them by the precipitin-in-gel or CF test. Results of a program to use this method on a regular basis have been promising but suggest that similar evaluation studies should be conducted for each virus or virus group considered. The value of this technique appears greatest when the saving of 2 to 5 days in diagnosis or identification is of obvious importance.

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