Virulence of Venezuelan Equine Encephalomyelitis Virus Subtypes for Various Laboratory Hosts

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Mice, guinea pigs, and duck embryo cell cultures were inoculated with known subtypes of Venezuelan equine encephalomyelitis (VEE) virus and the attenuated (TC-83) strain of VEE. With the exception of TC-83, all strains were highly pathogenic for suckling mice by either intracranial or intraperitoneal routes of inoculation used. Virulence for older mice and guinea pigs provided a means to distinguish strains. In addition, virulence or lack of virulence for adult mice or guinea pigs provides a rapid method for separating epizootic subtype IB from TC-83 VEE virus isolates.

During the 1971 epizootic of Venezuelan equine encephalomyelitis (VEE) in south Texas (9), an intensive, equine vaccination campaign was carried out in the field, with a large proportion of the laboratory support work done at the Arbovirus Section, Center for Disease Control, Atlanta, Ga. Since the vaccine contained a live, attenuated VEE virus (strain TC-83) (3), either epizootic VEE virus subtype IB (8) or the vaccine strain itself might be isolated from equine specimens submitted for diagnosis. In addition to these two strains of VEE virus, eastern equine encephalitis and western equine encephalitis viruses, also members of arbovirus serogroup A, were known to occur in and were isolated from the areas studied. Obviously, prompt and correct identification was an epidemiological necessity. Distinguishing eastern and western equine encephalitis viruses from each other and from both of the VEE virus strains present is a relatively straightforward serological procedure, but rapid differentiation of the epizootic subtype IB from the TC-83 vaccine strain by serological methods posed a more complex problem. A reliable and rapid screening method was sought to distinguish between these two, based on easily observed differences in pathogenicity for available laboratory hosts.

Mice, guinea pigs, and duck embryo cell cultures were inoculated with known subtypes of VEE virus (including TC-83) in attempts to show easily recognized virulence markers. VEE subtype IB was confirmed to be uniformly highly pathogenic for weaned guinea pigs and adult mice inoculated by the peripheral route, whereas TC-83 was not.

MATERIALS AND METHODS

Viruses. Subtype IA (VEE Trinidad donkey), type II (Everglades), type III (Mucambo), and type IV (Pixuna) were obtained from the arbovirus reagent repository in the Arbovirology Section, Center for Disease Control (Atlanta, Ga.). Subtypes IB (Ica and PTF-39), IC (P-678), ID (3880), and IE (Mena II) were obtained from K. M. Johnson, Middle America Research Unit, Gorgas Memorial Laboratories, Canal Zone, Panama. Subtype IB (Three Rivers), used as the prototype VEE virus of the Texas epizootic, was isolated from serum drawn on 30 June 1971 from a 3-month-old colt; this animal was pastured at the time in the vicinity of Three Rivers, Live Oak County, Tex. Subtype IB (GJ9-1BJ) had been isolated during an equine epizootic of VEE in Guatemala (1). None of these VEE viruses had undergone more than four suckling mouse passages in the laboratory. Stocks of the various viruses or virus subtypes were prepared by passing each of them once in Vero cells and harvesting the infected, cell culture supernatant fluids. These were stored at −60 °C until used in the various tests.

Viral inoculations. Serial, 10-fold dilutions of each virus were inoculated into litters of suckling mice by the intracranial (ic; 0.02 ml) or intraperitoneal (i.p.; 0.03 ml) route, into 3-week-old or weaned mice (WM) by the ic (0.03 ml) or i.p. (0.05 ml) route, into guinea pigs (150 to 250 gm) by the i.p. (0.1 ml) route, or onto Pekin duck embryo cell monolayers (0.1 ml) (6). Animals were observed for 14 days, after which time survivors of VEE virus inoculations were challenged by the i.p. route with 200 to 600 suckling mice ic mean lethal doses of VEE virus (strain GJ9-1BJ) and observed for an additional 14 days. The mean infective dose was calculated on the basis of challenge survivors. For controls, uninoculated animals of the same species and age were inoculated i.p. with the challenge dose.

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Cell cultures were overlayed for plaques and observed until the controls spontaneously degenerated (about 10 to 12 days).

When field-collected equine specimens were received at the Center for Disease Control, sera, whole blood clots, or 10% tissue suspensions were inoculated into litters of six suckling mice by the ic (0.02 ml) route. The suckling mice were then observed twice daily for 10 to 14 days. Those mice with signs of illness were frozen at -80 C; subsequently, their brains were collected by aspiration and pooled to prepare a crude complement-fixing antigen. This antigen was tested against a number of immune, mouse ascitic fluids prepared against arboviruses known to occur in the area from which the specimen originated. Reaction of crude complement-fixing antigens with VEE virus-immune mouse ascitic fluid was taken as evidence that the virus isolated was VEE virus; without prior quantitation, the original, suckling-mice brain pools were then inoculated as 10% suspensions without clarification into guinea pigs by the i.p. (0.1 ml) route to assess virulence. However, it was assumed from preliminary studies that a single mouse passage would amplify the IB strain in equine blood or tissue to yield at least 10^4 to 10^5 suckling mice ic mean lethal doses.

**RESULTS**

As shown in Table 1, all strains of VEE virus (including TC-83) were virulent for both suckling mice (inoculated ic) and duck embryo cells. When suckling mice were inoculated ic, only TC-83 had a titer less than 9 Dex (1 per ml), where Dex is the Order of Magnitude based on the mean lethal dose. When WM were inoculated ic, the titer of TC-83 was only 4.2 Dex and type IV (Pixuna), ≤ 2.4 Dex, whereas all other viruses tested titered > 9 Dex. In WM inoculated i.p., strain TC-83 and type IV (Pixuna) had similarly low virulence. Titers of subtype IE (Mena II) and type III (Mucambo) inoculated i.p. into WM were slightly to appreciably less than those in suckling mice. When adult mice were inoculated i.p. with all strains, titers were essentially the same or slightly lower than they were in WM inoculated i.p.

Inoculation of guinea pigs i.p. gave results approximating those with adult or WM, with three exceptions: subtype IE (Mena II) failed to kill guinea pigs in any concentration used, VEE type II (Everglades) failed to kill except in the very lowest dilutions, and type III (Mucambo) had a higher titer in guinea pigs than in WM or adult mice inoculated i.p.

When surviving animals of all VEE virus titrations were challenged i.p. with VEE subtype IB, variations were noted in the degree of protection which had been conferred. Despite species of host, age, or route, animals inoculated either TC-83 or type II (Everglades) survived the challenge dose. Subtype IE (Mena II) protected all WM and adult mice inoculated i.p., and those guinea pigs which had received the higher dilutions were to some degree protected but not completely. Type III (Mucambo)

**Table 1. Susceptibility of mice, guinea pigs, and duck embryo cells to infection with various subtypes of VEE virus**

| Type | Subtype | Strain       | Passage level* | SM ic | SM i.p. | WM ic | WM i.p. | AM* i.p. | GP* i.p. | Log₁₀ LD₅₀ per ml | Log₁₀ plaque-forming units per ml in duck embryo cells |
|------|---------|--------------|----------------|-------|---------|-------|---------|----------|----------|----------------|--------------------------------------------------|
| I A  | Trinidad donkey (TC-83) | E₁V₂ | >9 >9 >9 >9 >9 >9 NT* | >8 |
| B    | Ica | WM,SM,V₂ | >9 >9 >9 >9 >9 >9 >9 NT | >8 |
|      | PTF-39 | SM, V₂ | >9 >9 >9 >9 >9 >9 >9 NT | >8 |
| C    | Three Rivers | SM₂ | >9 >9 >9 >9 >9 >9 NT | >8 |
| D    | P-676 | SM₂ | >9 >9 >9 >9 >9 >9 NT | >8 |
| E    | Mena II | SM₂, V₂ | >9 >9 >9 >9 >9 >9 NT | >8 |
| II Everglades | FF-7C | SM₂, V₂ | >9 >9 >9 >9 >9 >9 NT | >8 |
| III Mucambo | BeAn 8 | WM,SM₄ | >9 >9 >9 >9 >9 >9 NT | >8 |
| IV Pixuna | BeAr 35645 | SM₄ | >9 >9 ≤2.4 ≤2.4 ≤2.5 ≤2.5 NT | >8 |

* E, egg; V, Vero cells; SM, suckling mice; WM, weaned mouse; subscripts, number of passages.
* AM, Adult mice (8 to 10 weeks old).
* GP, Guinea pigs.
* NT, Not tested.
* —, No additional passages from vaccine status.
* Titer in parentheses, mean infective dose per ml obtained by challenging surviving animals i.p. with 200 to 500 suckling mice mean lethal doses (LD₅₀) of VEE (GJ9-1BJ) virus.
protected WM and adult mice inoculated i.p., but no difference was seen between the mean lethal and infective doses in guinea pigs. Type IV (Pixuna) conferred protection upon WM inoculated i.c or i.p. and adult mice inoculated i.p.; the guinea pig mean infective dose was \( \geq 3.0 \) Dex greater than the mean lethal dose. None of the previously uninoculated control animals survived challenge with VEE subtype IB.

One of the purposes of this study was to confirm the utility of the guinea pig i.p. inoculation test for differentiation of epizootic (IB) and vaccine (TC-83) strains of VEE virus isolated during the 1971 epizootic. Therefore, 176 VEE virus isolates were inoculated into guinea pigs by the i.p. route. The results by history of vaccination are given in Table 2. All of 62 VEE virus isolates from unvaccinated equines were virulent for guinea pigs, often killing them within 18 to 24 h after inoculation (dose approximately 9 Dex). Of 114 VEE virus isolates from vaccinated equines, 25 were virulent for guinea pigs and 89 were not. All 25 of the virulent strains had been obtained from Texas, the only state in the United States where epizootic VEE was reported. In addition to these 176 isolates, 17 VEE virus isolates from VEE-vaccinated horses outside of the epizootic area and 274 VEE isolates from mosquitoes collected within the epizootic area in 1971 were tested; all 17 isolates from equines and 100 of the isolates from mosquitoes were tested for virulence in adult mice and guinea pigs, as described above. The remaining 174 isolates from mosquitoes were tested only in adult mice. None of the 17 equine isolates killed either host inoculated whereas the 100 isolates from mosquitoes killed both adult mice and guinea pigs, and the remaining 174 VEE isolates from mosquitoes killed adult mice.

**DISCUSSION**

Although all virus strains used in this study were pathogenic for duck embryo cells and mice, the observed pathogenicity varied greatly by age of the mice and route of inoculation. No differences were noted between strains inoculated into duck embryo cells. Even though inoculation by the i.p. route produced lower titers than i.c inoculation, the survival of at least some challenged animals, originally inoculated with one of the VEE virus strains, provided evidence of infectivity without overt disease. Such infectivity, if characteristic of the so-called endemic strains, may explain the continued occurrence of these viruses in the absence of overt disease in nature. Gross protection between subtypes might explain why certain areas are free of epizootic VEE. It is to be noted, however, that VEE type III (Mucambo) virus, which has been isolated in the past from man, rodents, birds, opossums, mosquitoes, sentinel mice, and monkeys, appears by these tests to be a potentially virulent strain, because its pathogenicity by route and age for mice and guinea pigs is similar to that of VEE virus subtypes IA-D. However, studies in equines have not demonstrated such virulence (7).

Virulent (IB) and attenuated (TC-83) VEE virus strains can quickly be separated by peripherally inoculating WM, adult mice, or guinea pigs to provide epizootic data during the course of an outbreak. The IB strain is lethal, whereas the TC-83 strain is not. Such host virulence tests can be most useful to determine whether the virulent strain or the TC-83 strain is being transmitted in nature after mass vaccination. Since 274 VEE virus isolates from mosquitoes collected in Texas and northeastern Mexico during the epizootic period of 1971 were shown to be virulent for guinea pigs (W. D. Sudia, V. F. Newhouse, L. D. Beadle, D. L. Miller, J. G. Johnston, Jr., R. Young, C. H. Calisher, and K. S. C. Maness, Amer. J. Epidemiol., in press), and/or adult mice by i.p. inoculation, they were assumed to be epidemic strains. In contrast, two viruses isolated from mosquitoes collected in Louisiana in 1971 (4) and in Mexico in 1972 (T. H. Work, personal communication) were identified serologically as VEE virus but were not virulent for guinea pigs and therefore appear to be more closely related to the TC-83 strain. Based on epidemiological grounds, it is highly unlikely that these two isolates are subtype IE or subtype IV, although this is a possibility.

The origin of epidemic strains is still uncertain; it is not known whether they are enzootic in hidden ecological niches or whether they

| Location | Unvaccinated | Vaccinated |
|----------|--------------|------------|
|          | +*           | -*         |
| Mexico   | 6 0          | 0 0        |
| Texas    | 56 0         | 25 46      |
| Other    | 0 0          | 0 43       |

\*+, Kills guinea pigs.
\*-, Guinea pigs survived.
mutate from "endemic" strains of low pathogenicity. Therefore, periodic monitoring of the pathogenicity of naturally occurring endemic strains by the methods described here seems appropriate. It is possible that pathogenic variants may occasionally arise and serve to initiate epidemics. Recent isolation of a strain of type II (Everglades) virus with increased virulence may support this possibility (5).

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