Virulence Heterogeneity of a Predominantly Avirulent Western Equine Encephalitis Virus Population

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SUMMARY
Selective removal of small plaque (SP) Western equine encephalitis (WEE) virus from a population heterogeneous with respect to virulence and plaque morphology permitted direct detection of a small sub-population of virulent large plaque (LP) WEE virus. Selective removal of SP-WEE virus was achieved by intracardiac (i.c.a.) inoculation of hamsters; plasma obtained 60 min after inoculation was proportionately enriched for LP-WEE virus since only the SP-WEE virus was cleared. By this method, the proportion of LP- to SP-WEE virus, in a population of SP-WEE virus which appeared to be homogeneous by conventional plaquing methods, was calculated to be 1 LP- to 25,000 SP-WEE virions. The presence of a virulent LP-WEE virus sub-population explains why a single passage of a high but not low dose of SP-WEE virus in hamsters resulted in the emergence of an LP-WEE virus population with enhanced virulence.

INTRODUCTION
A virus population is often composed of heterogeneous sub-populations with a spectrum of virulence characteristics. The virulence of a particular virus pool is often determined by the sub-population of infectious virions with the greatest virulence, although this sub-population may constitute only a small fraction of the whole (Bradish, Allner & Maber, 1972; Jahrling, 1975). Moreover, the proportion of virulent to avirulent sub-populations can readily be shifted by passage depending on the substrate used for virus replication.

We have observed that high doses of a small plaque (SP) variant of Western equine encephalitis virus (WEE) inoculated subcutaneously into hamsters produce viraemias consisting of large plaque (LP) virus with greater virulence for hamsters than SP-virus. This suggests either the presence of virulent LP-WEE virus in the original inoculum or a high rate of mutation by the small plaque variant to the virulent large plaque type. In this paper, we present a novel method to measure the proportion of LP- to SP-WEE virus in the predominantly SP-virus population. The method involves the selective removal of SP-virus from the population to a level which permits detection of the remaining LP-virus.

METHODS
Isolation and assay of plaque variants. Western equine encephalitis virus (WEE), strain 72VI880 was initially isolated in duck embryo cell cultures from the blood of a nestling house sparrow in Texas. The first duck embryo cell culture passage was obtained from Dr Charles Calisher, Center for Disease Control, Fort Collins, Colorado and was passed
twice at terminal dilution in chick embryo cell cultures (CEC) by Dr Robert Robey, Merrill National Drug Co., Swiftywater, Pa. We obtained the second CEC passage pool, and plaqued it on CEC. Large plaques (LP) measuring 4 to 5 mm in diam. and small plaques (SP) measuring 1-0 mm developed in a ratio of approx. 2:1, after incubation at 37 °C for 36 h. Five other WEE virus strains with identical isolation and passage histories, produced only LP on CEC. Therefore, to obtain purified clones of SP- and LP-WEE from a single source, strain 72V1880 was selected as the starting material. Unpurified strain 72V1880, in second CEC passage, killed all suckling mice and all adult hamsters, and infected but did not kill adult mice inoculated s.c. with 1000 p.f.u.

Progeny virus from individual LP- and SP-clones were harvested as described (Jahrling & Scherer, 1973a) and in CEC to obtain stock virus suspensions, which appeared to be homogeneous with respect to plaque morphology (see Results). All virus titrations were performed by counting p.f.u. on CEC monolayers grown in 10 cm² wells of plastic plates as described previously (Jahrling, Dendy & Eddy, 1974).

**Determination of virus clearance rates in hamsters.** Hamsters weighing 90 to 100 g (Lakeview Hamster Colony, Newfield, NJ) and lightly anaesthetized with sodium pentobarbitol were inoculated via the intracardiac (i.c.a.) route with 1-0 ml of virus suspensions diluted in 1 % bovine serum albumin in Hanks’ balanced salts solution (BA/H), pH 7-6, containing 1-0 mg of Evans’ blue. The actual amounts of SP- and LP-WEE viruses inoculated were 10-1 and 10-0 log₁₀ p.f.u. respectively. Hamsters were serially bled from the orbital sinus 1, 5, 30 and 60 min after inoculation to obtain plasma which was immediately assayed for virus. The specific clearance of virus from the blood was calculated by first determining the blood volume of each hamster (on the basis of Evans’ blue concentration), and then comparing the observed concentration of virus with the concentrations expected if no clearance had occurred (Jahrling & Scherer, 1973b).

**Virulence testing of stock viruses and isolates.** Virus suspensions or plasma specimens were diluted in BA/H and were inoculated s.c. into 90 to 100 g hamsters in 0-2 ml volumes. Hamsters caged in groups of five were observed for 21 days, and deaths recorded. All hamsters were bled under ether anaesthesia from the orbital sinus 3 days after inoculation and plasma was tested for viraemia. In the clearance experiments, five hamsters were bled from the heart 1 and 60 min after i.c.a. inoculation of SP-WEE; each plasma sample was inoculated s.c. without dilution or diluted 1:1000 prior to inoculation into five additional hamsters to determine its virulence.

**RESULTS**

**Selective clearance of SP-WEE virus from hamster plasma**

The rates at which SP- and LP-WEE viruses were cleared from hamster plasma after i.c.a. inoculation of 10-1 and 10-0 log₁₀ p.f.u. respectively were compared (Fig. 1). The concentration of LP-virus in plasma did not decrease during the 60 min test period. In contrast, the concentration of SP-virus decreased rapidly; 60 min after inoculation more than 3-5 log₁₀ p.f.u./0-2 ml of plasma had been cleared.

Both the LP- and SP-virus populations inoculated appeared to be homogeneous with respect to plaque morphology. Of 500 plaques measured from each population, all plaques developing from the LP-virus stock measured between 4-3 and 6-1 mm, while all plaques from the SP-virus stock measured 1-0 mm or less. However, when plasma samples obtained from hamsters 30 and 60 min after inoculation of SP-virus were titrated, LP variants were observed in cultures crowded with SP (Fig. 2). Since it was possible to detect LP variants superimposed on monolayers containing approx. 400 SP, large and small plaques
Fig. 1. Clearance of Western encephalitis virus large plaque (LP) and small plaque (SP) variants from the plasma of hamsters inoculated via the intracardiac route with 10.0 and 10.1 log_{10} p.f.u. respectively. Each point is a geometric mean ± s.e. based on 15 hamsters.

Fig. 2. Large and small plaques detected in a titration of plasma obtained from a representative hamster 60 min after i.c.a. inoculation of 10.1 log_{10} p.f.u. of SP-WEE virus.
Table 1. Detection of LP variants in plasmas of hamsters after intracardiac inoculation of SP-WEE virus*

<table>
<thead>
<tr>
<th>Min after inoculation</th>
<th>log_{10} p.f.u./0.2 ml plasma ± 1 s.e.†</th>
<th>SP</th>
<th>LP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.04 ± 0.05</td>
<td>&lt; 5.5‡</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>5.64 ± 0.16</td>
<td>3.30 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>5.19 ± 0.13</td>
<td>3.32 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

* 10⁻¹ log_{10} p.f.u. SP-WEE inoculated in 1.0 ml, i.c.a.
† Geometric mean titres based on blood samples from 15 hamsters.
‡ LP could not be detected by direct plaquing when the proportion of SP:LP exceeded 2.6 log_{10} p.f.u.

Table 2. Virulence and infectivity titrations of LP- and SP-WEE viruses in hamsters inoculated s.c.

<table>
<thead>
<tr>
<th>Virus</th>
<th>log_{10} p.f.u.</th>
<th>Dead/total</th>
<th>% dead</th>
<th>no. positive/total</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP</td>
<td>7.2</td>
<td>20/25</td>
<td>80</td>
<td>25/25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>17/23</td>
<td>74</td>
<td>23/23</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>16/25</td>
<td>64</td>
<td>25/25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>2/15</td>
<td>13</td>
<td>8/15</td>
<td>53</td>
</tr>
<tr>
<td>SP</td>
<td>8.5</td>
<td>8/10</td>
<td>80</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>9/15</td>
<td>60</td>
<td>13/15</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>2/15</td>
<td>13</td>
<td>5/15</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>2/29</td>
<td>07</td>
<td>0/29</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1/15</td>
<td>06</td>
<td>0/15</td>
<td>0</td>
</tr>
</tbody>
</table>

* Hamsters were bled from the orbital sinus 3 days after inoculation.

developing from plasmas of SP-virus-inoculated hamsters were scored separately (Table 1). The LP-virus titre could not be determined for samples obtained 1 min after inoculation, because of the high proportion of SP-virus; however, by 30 and 60 min, sufficient SP-virus had been cleared to permit detection of LP-virus, present at a mean titre of 3.3 log_{10} p.f.u./0.2 ml of plasma at both times.

Calculation of the LP:SP ratio for the SP-virus population

On the basis of Fig. 1 and Table 1, it was tentatively assumed that the level of LP-virus (3.3 log_{10} p.f.u./0.2 ml plasma) detected in plasma 30 and 60 min after inoculation reflected only a dilution of LP-virus, present in the original SP-virus population, by the blood volume of the inoculated hamsters (5.0 ml). The total number of LP-viruses in the 1.0 ml inoculum was thus calculated to be 4.7 log_{10} p.f.u. The SP-virus population inoculated contained 10⁻¹ log_{10} p.f.u. Therefore, the ratio log_{10} (LP p.f.u.: SP p.f.u.) was −5.4. In arithmetic terms, the LP:SP ratio was 1:250000.

Confirmation of the calculated LP:SP ratio by inoculation of hamsters

The virulence of SP-WEE virus was compared with that of LP-WEE virus by titration in hamsters inoculated s.c. (Table 2). LP-virus, in doses of 1.2 log_{10} p.f.u. or greater, infected all hamsters inoculated (as measured by the detection of LP-virus in plasma 3 days after inoculation), although not all hamsters infected with LP-virus died. The lowest
Virulence heterogeneity of WEE virus

Table 3. Infectivity and virulence of plasma obtained from hamsters
1 and 60 min after i.c.a. inoculation of SP-WEE virus*

<table>
<thead>
<tr>
<th>Min after SP-virus inoculation</th>
<th>Mean log_{10} SP-p.f.u. of plasma inoculated</th>
<th>Mean log_{10} p.f.u. inoculated</th>
<th>% of hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP</td>
<td>LP</td>
<td>Dead</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LP-viraemic</td>
</tr>
<tr>
<td>60</td>
<td>5.2</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.3</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>8.0</td>
<td>0</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.3</td>
<td>100</td>
</tr>
</tbody>
</table>

* Five hamsters were bled 1 and 60 min after i.c.a. inoculation of 10^1 log_{10} p.f.u. SP-WEE virus. Each plasma sample was inoculated s.c. without dilution or diluted 1:1000 into five additional hamsters to determine virulence and infectivity. The percentage of hamsters dead and percentage LP-viraemic are therefore based on 25 hamsters for each dilution of plasma tested.

LP-virus dose tested, 0.2 log_{10} p.f.u., infected 53% and killed 13% of hamsters inoculated s.c. In contrast, only high doses of SP-virus were lethal for the majority of hamsters inoculated. Doses of 8.5 and 6.5 log_{10} p.f.u. of SP-virus killed 80 and 60% of hamsters respectively. Most significant, however, was the observation that in all of 10 plasma samples obtained from the highest dose group, and in 13 of 15 from the 6.5 log_{10} p.f.u. dose group, LP-virus was detected in plasma obtained 3 days after SP-virus inoculation. The LP-virus titres in these plasma samples ranged from 6.3 to 7.1 log_{10} p.f.u./ml. No SP-virus was detected in these same plasma samples. Presumably, LP-virus present in the original SP-virus inoculum, had been selectively amplified and the progeny virus population was enriched for LP-virus by inoculation of hamsters. All hamsters that died following SP-virus inoculation developed an LP-viraemia. Higher dilutions of SP-virus killed significantly less hamsters and produced an LP-viraemia less frequently. None of 29 hamsters inoculated with 4.5 log_{10} p.f.u., and only 5 of 15 hamsters inoculated with 5.5 log_{10} p.f.u. of SP-virus developed an LP-viraemia. No hamsters ever developed an SP-viraemia, either in the presence or absence of LP-virus. Since hamsters which failed to develop an LP-viraemia following SP-virus inoculation also failed to develop neutralizing antibody to either SP- or LP-virus, and failed to survive s.c. challenge with 1000 p.f.u. of LP-virus, it appeared that SP virus failed to infect hamsters inoculated s.c.

One interpretation of the results in Table 2 is that LP-virus, present at a low level in the SP-virus population, was diluted to a minimally infectious level when the SP-virus titre was between 4.5 to 5.5 log_{10} p.f.u. Assuming that SP-WEE (which apparently does not replicate in the hamster) also does not interfere with LP-virus infectivity when inoculated simultaneously (see below), we concluded that, in the dilution of SP-virus containing 5.5 log_{10} p.f.u. SP-virions, approx. 0.2 log_{10} p.f.u. LP-virus was present, corresponding to a log_{10} ratio of LP p.f.u.:SP p.f.u. of -5.3.

Evidence that SP-WEE does not interfere with LP-WEE infectivity

Plasma samples obtained from five hamsters 1 and 60 min after i.c.a. inoculation of SP-WEE were tested for virulence (Table 3). Undiluted plasma obtained 60 min after inoculation contained mean titres of 5.2 and 3.3 log_{10} p.f.u. of SP- and LP-WEE respectively, as determined by direct counting of both plaque types. All 25 hamsters inoculated with undiluted 60 min plasma developed LP-viraemia, and 80% died. Dilution of 60 min plasma to contain 0.3 log_{10} p.f.u. LP resulted in only 28% LP-viraemic, and 4% dead.
In 1 min plasma samples, $8 \cdot 0 \log_{10}$ p.f.u. SP-WEE failed to interfere with the infectivity or the lethality of the $3 \cdot 3 \log_{10}$ p.f.u. LP calculated to be present.

In addition to demonstrating that SP-WEE did not interfere with the expression of LP-WEE virulence, these data further indicated that the high lethality of undiluted 60 min blood depended on the presence of LP-virus, and not on the $5 \cdot 2 \log_{10}$ p.f.u. of SP-WEE present; the $10^{-3}$ dilution of 1 min plasma, also containing $5 \cdot 0 \log_{10}$ p.f.u. of SP-virus but only $0 \cdot 3 \log_{10}$ p.f.u. of LP-virus, was of low lethality.

**DISCUSSION**

By taking advantage of the observation that an avirulent SP-WEE virus is rapidly cleared from the plasma of hamsters inoculated i.c.a., while a virulent LP-WEE virus is not detectably cleared, we have demonstrated directly the presence of LP-WEE in a population predominantly comprised of SP-WEE virions. The ratio of LP-p.f.u.:SP-p.f.u. for the population tested was $1:250000$, calculated from the proportions of LP- and SP-p.f.u. observed in virus titrations of plasma obtained 30 and 60 min after i.c.a. inoculation of SP-WEE.

The validity of this calculation was partially supported by the observation that dilutions of the SP-virus population in excess of $1:250000$ did not kill hamsters and did not produce an LP-viraemia, while dilutions less than $1:250000$ did kill hamsters and did produce an LP-viraemia. Detection of LP-virus by the latter method depended on the expression of virulence by progeny LP-virus which presumably emerged from a pre-existing sub-population and had a selective advantage to replicate in the intact hamster. The former method depended on the selective removal of SP-virus to a level low enough to permit detection of LP-virus in the original virus population, and was a more direct demonstration of the relative magnitude of the LP-virus sub-population.

The sensitivity of the selective clearance method to detect LP-WEE virus in SP-virus populations could probably be increased to better than $1:250000$ if plasma samples, obtained 60 min after SP-virus inoculation, were titrated under conditions which enhanced definition of LP-relative to SP-virus plaques. Stanton, May & de St Joer (1974) have recently described a method for detecting 2 LP-WEE plaques in the presence of 2200 SP-WEE plaques, by employing high bicarbonate concentrations in the agar overlay medium and by increasing the concentration of CO$_2$ during incubation. In contrast, under our standard plaquing conditions we could detect approx. 1 LP in the presence of 400 SP. Therefore, combination of the methods might increase sensitivity about fivefold, to 1 LP in 750000 SP-WEE virions.

The selective clearance method described may have broad application to detection of low levels of virulent LP-virus in populations of other virus strains of low virulence. Benign strains of Venezuelan encephalitis virus (VEE) are rapidly cleared from hamster plasma while virulent VEE strains are cleared much more slowly (Jahrling & Scherer, 1973b; Jahrling & Gorelkin, 1975). A similar correlation between rapid clearance and low virulence has been reported for Mengo virus clones (Campbell, Buera & Tobias, 1970). A general feature of virus clearance curves is that clearance does not continue at a uniform rate; rather, the curve flattens out, leaving an ‘uncleared tail’ (Mims, 1964). One possible explanation for this ‘uncleared tail’ is that the remaining virus is not representative of the predominant virus population inoculated. Although the uncleared virus might not always be easily differentiated on the basis of plaque size, it might still have different virulence characteristics. For example, in the present study, $5 \cdot 2 \log_{10}$ p.f.u. of virus
(enriched for LP) was isolated from 60 min blood and was virulent, while $5.5 \log_{10}$ p.f.u. of the original SP-WEE inoculum was not.

The primary purpose of this paper is to report the potential utility of the selective clearance method to detect low levels of virulent virus in predominantly avirulent virus populations. However, we have also identified the SP- and LP-variants of WEE virus as interesting candidates for comparative pathogenesis studies in preliminary experiments, we have shown that the SP-virus diluted to contain no LP-virus produces no viraemia following s.c. inoculation and fails to infect hamsters at all, since they fail to seroconvert or to resist challenge with WEE-LP-virus. However, following intracranial inoculation, SP-virus grows rapidly in the brain without apparently reverting to the LP type, and produces an encephalitis although still without viraemia (P. Jahrling, unpublished observation). In contrast, LP-virus produces a high level viraemia ($6$ to $8 \log_{10}$ p.f.u./ml) following s.c. inoculation, and invades the brain to produce an encephalitis. The low virulence of SP-virus may be a function of its inability to produce a viraemia, which in turn may be a function not of an inability to replicate per se, but of the rapid clearance of SP-virus from the blood, by mechanisms yet to be identified for WEE virus. For VEE viruses, benign SP-variants are selectively removed from the circulation by hepatic reticuloendothelial cells (Jahrling & Gorelkin, 1975). The exclusion of SP-WEE from the undamaged brain may simply reflect an inability to maintain a viraemia above a threshold level. Alternatively, SP-WEE may require a higher viraemia level to penetrate the 'blood-brain barrier' than LP-WEE. These hypotheses may be tested directly either by inoculating very high doses of purified SP-WEE to produce higher artificial viraemia levels than those attained in the present study, or by inoculating lower doses of SP-WEE into hamsters whose ability to clear virus has been impaired by blockade of the reticuloendothelial system.

Finally, the lack of interference observed for the expression of LP-WEE virulence in the presence of high levels of SP-WEE is similar to the phenomenon described for VEE viruses (Jahrling, 1975) and for some Semliki Forest virus strains (Bradish et al. 1972). The expression of virulence by a mixed virus population depends on the relative efficiency of the virulent and benign sub-populations to produce destructive versus protective responses (Bradish et al. 1972). To explain the lack of interference in the WEE virus system, it will be necessary to understand the destructive versus protective responses induced by pure populations of LP- and SP-WEE viruses. Since low doses of LP-WEE are lethal for the majority of hamsters, the entire plaque progeny of the LP-WEE inoculum used for these studies can be observed for homogeneity. Through the use of the selective clearance method, the homogeneity of the SP-WEE populations, used in higher inoculum doses, may also be ensured.

REFERENCES


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