Features of Cross Protection Between Sindbis and Venezuelan Equine Encephalitis Viruses in Mice—Relationship of Route of Immunization to Protection

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SUMMARY

In the immunization of mice with Sindbis virus, a central nervous system infection elicits greater cross protection against lethal challenges with Venezuelan equine encephalitis (VEE) virus than do infections initiated by extraneural routes. Infective Sindbis virus administered intraperitoneally, subcutaneously or intravenously was considerably less cross protective than a single inoculation of the virus into the cerebrum, yet similar levels of homologous neutralizing antibody at 28 days post-inoculation were elicited by the host, regardless of the route by which the virus was administered. Cross protection was correlated with invasion by, and replication of, Sindbis virus within the central nervous system and histopathological changes in the brain. The lowest levels of VEE virus infectivity were recovered from the brains of mice previously infected with Sindbis virus by the intracerebral route.

INTRODUCTION

Numerous reports have confirmed that the infection of experimental animals with an arbovirus results in a degree of protection against a subsequent infection with another serologically related virus (Hammon & Sather, 1956; Traub, 1961; Allen, 1962; Wisseman et al. 1962; Casals, 1963; Hearn & Rainey, 1963; Price et al. 1963). For example, mice immunized with Mayaro virus may survive a normally lethal challenge with Chikungunya, Semliki Forest or Venezuelan equine encephalitis viruses (Allen, 1962). Such cross challenge studies, with the group A arboviruses, are of particular interest since this cross protection occurs in the absence of close antigenic relationships, as determined by serum neutralization tests (Casals, 1963). Although several hypotheses have been proposed (Traub, 1961; Wisseman et al. 1962; Casals, 1963; Hearn & Rainey, 1963; Third & Price, 1968), the mechanisms of cross protection among group A arboviruses have not been elucidated. The studies described here emphasize the involvement of the central nervous system (CNS) in cross protection.

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Table 1. Comparison of routes and doses of immunization with Sindbis virus for protection against an intraperitoneal challenge with VEE virus

<table>
<thead>
<tr>
<th>Immunizing dose as log (SM i.c. LD_{50})</th>
<th>Immunizing route</th>
<th>Incidence of survival after VEE virus challenge*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>i.c. 4/7 i.p. 2/10 i.v. 4/13 s.c. 0/15</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>i.c. 4/10 i.p. 3/10 i.v. 1/13 s.c. 1/15</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>i.c. 6/10 i.p. 0/10 i.v. 0/13 s.c. 2/15</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>i.c. 7/10 i.p. 0/10 i.v. 1/15 s.c. 0/14</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>i.c. 7/10 i.p. 0/10 i.v. 0/15 s.c. 0/15</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>i.c. 3/10 i.p. 0/10 i.v. 0/15 s.c. 0/15</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>i.c. 0/10 i.p. 0/10 i.v. 0/14 s.c. 0/15</td>
<td></td>
</tr>
</tbody>
</table>

* Challenge contained 10^3 mouse i.p. LD_{50} of VEE virus given 21 to 28 days after immunization.

METHODS

Viruses. The viruses used were passage 27 of strain AR 339 of Sindbis virus (Taylor, 1967) and passage 13 of the virulent Trinidad donkey strain of VEE virus (Taylor, 1967). Virus seeds (Sindbis, 10^{10} SM i.c. LD_{50}/ml; VEE, 10^{11} SM i.p. LD_{50}/ml were prepared as 10% suspensions of suckling mouse (SM) brain in a 1:1 mixture of borate-buffered saline (BS) and heat-inactivated normal rabbit serum (NRS). Virus infectivities in tissues from infected mice were determined by intracerebral (i.c.) inoculation of 21-day-old Swiss-Webster mice with dilutions of tissue suspensions. Challenge with VEE virus consisted of intraperitoneal (i.p.) inoculation of 0.03 ml of appropriate virus dilution on days 28 to 42 after immunization.

Neutralization tests (NT) were performed on L cell monolayers using a plaque inhibition technique (Porterfield, 1960).

Histopathology. Brains removed from killed mice were fixed in 10% neutral formalin, embedded in paraffin, sectioned (6 μm) and stained with haematoxylin and eosin.

RESULTS

Sindbis virus immunization and cross protection against VEE virus

Mice (21 days old) weighing 10 to 14 g were inoculated with log dilutions of Sindbis virus by either the i.c., i.p., subcutaneous (s.c.) or intravenous (i.v.) route. Twenty-one days later the mice were challenged with 10^5 SM i.p. LD_{50}/ml of VEE virus administered i.p. The greatest protection (57 to 70%) was observed in mice infected by the i.c. route with 10^{5.5} or more SM i.c. LD_{50}/ml of Sindbis virus (Table 1), whereas considerably less protection was conferred by a single i.p., s.c. or i.v. inoculation of Sindbis virus, regardless of the dose.

Various immunization routes and schedules of inoculations with Sindbis virus were compared for efficacy of cross protection against graded doses of VEE virus. Routes, schedules and doses of immunization with Sindbis virus included: (i) a single i.c. inoculation with 10^{5.5} SM i.c. LD_{50}/ml, (ii) a single i.c. inoculation as in (i) plus an i.p. inoculation with 10^{5} SM i.c. LD_{50}/ml 14 days later, (iii) a single inoculation i.p. with 10^{5.5} SM i.c. LD_{50}/ml, (iv) two inoculations i.p. with 10^{5.5} SM i.c. LD_{50}/ml 14 days apart, (v) as (iv) plus a third inoculation with 10^{5.5} SM i.c. LD_{50}/ml on day 21. All groups were challenged with log dilutions of VEE virus 42 days after the first inoculation with Sindbis virus. The dose response data in Table 2 show that the greatest protection occurred in the groups that received an i.c. inoculation of Sindbis virus. Virus inoculated i.p. subsequent to an
### Table 2. Comparison of immunizing regimens with Sindbis virus for cross protection against graded doses of VEE virus

<table>
<thead>
<tr>
<th>Immunization regimen</th>
<th>log&lt;sub&gt;10&lt;/sub&gt; (SM i.c. LD&lt;sub&gt;50&lt;/sub&gt;/ml) challenge of VEE virus*</th>
<th>Accumulated survivors/ challenged</th>
<th>% S†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Route</td>
<td>Number of inoculations</td>
<td>Dose</td>
<td>Incidence of survival in challenged mice</td>
</tr>
<tr>
<td></td>
<td>in log&lt;sub&gt;10&lt;/sub&gt; (SM i.c. LD&lt;sub&gt;50&lt;/sub&gt;/ml)</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>i.c.</td>
<td>1</td>
<td>3·5</td>
<td>7/10</td>
</tr>
<tr>
<td>i.c.</td>
<td>3</td>
<td>3·5</td>
<td>3/3</td>
</tr>
<tr>
<td>i.p.</td>
<td>1</td>
<td>5·5</td>
<td>2/6</td>
</tr>
<tr>
<td>i.p.</td>
<td>2</td>
<td>5·5</td>
<td>0/1</td>
</tr>
<tr>
<td>i.p.</td>
<td>3</td>
<td>5·5</td>
<td>2/7</td>
</tr>
<tr>
<td>Non-immunized</td>
<td>—</td>
<td>—</td>
<td>0/25</td>
</tr>
</tbody>
</table>

* Inoculation of 0·03 ml of respective challenge dose on day 42 for all groups immunized by i.c. or i.p. routes.† Per cent survival.
‡ Represents accumulated survivors in groups challenged with ≥ 4 log<sub>10</sub> (SM i.c. LD<sub>50</sub>/ml) VEE.
§ Represents accumulated survivors in groups challenged with < 4 log<sub>10</sub> (SM i.c. LD<sub>50</sub>/ml) VEE.

**Fig. 1.** Mean levels of virus in tissues of mice after infection with Sindbis virus. ■ . . . ■, i.p. inoculated; • — •, i.c. inoculated; ▲ — ▲, s.c. inoculated.
### Table 3. Histological findings in brains of mice inoculated with Sindbis virus

<table>
<thead>
<tr>
<th>Dose and route of inoculation</th>
<th>Day p.i.</th>
<th>No. of animals with lesions/ no. of animals examined</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{3.5}$ SM i.c. LD$_{50}$</td>
<td>0-3</td>
<td>0/12</td>
<td>No significant lesions</td>
</tr>
<tr>
<td>i.c.</td>
<td>4</td>
<td>1/2</td>
<td>Focal mild encephalitis-lymphocytic perivascular cuffing and gliosis</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3/3</td>
<td>Focal mild encephalitis-lymphocytic perivascular cuffing and gliosis</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3/3</td>
<td>Diffuse mild encephalitis-lymphocytic perivascular cuffing and gliosis</td>
</tr>
<tr>
<td>$10^3$ SM i.c. LD$_{50}$</td>
<td>8-10</td>
<td>9/9</td>
<td>Focal mild encephalitis-lymphocytic perivascular cuffing and gliosis</td>
</tr>
<tr>
<td>s.c.</td>
<td>12</td>
<td>0/27</td>
<td>No significant lesions</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0/3</td>
<td>No significant lesions</td>
</tr>
<tr>
<td>$10^4$ SM i.c. LD$_{50}$</td>
<td>1-18</td>
<td>0/33</td>
<td>No significant lesions</td>
</tr>
</tbody>
</table>

### Table 4. Summary of clinical and histological features of routes of Sindbis immunization in relationship to cross protection against VEE virus

<table>
<thead>
<tr>
<th>Route of Sindbis infection</th>
<th>Development of Sindbis virus</th>
<th>Total log$<em>{10}$ (SM i.c. LD$</em>{50}$/ml of CNS lesions)</th>
<th>Days of occurrence of Sindbis virus viraemia</th>
<th>Development of Sindbis neutralizing antibodies* day 28</th>
<th>Survived/ challenged (%)$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.c.</td>
<td>Mild encephalitis</td>
<td>$&gt;5.0$</td>
<td>1-4</td>
<td>1:32</td>
<td>113/159 (0.71)</td>
</tr>
<tr>
<td>i.p.</td>
<td>No significant lesions</td>
<td>$\leq1.0$</td>
<td>2-3</td>
<td>1:32</td>
<td>15/112 (0.13)</td>
</tr>
<tr>
<td>s.c.</td>
<td>Few lesions observed</td>
<td>$\leq2.0$</td>
<td>1-2</td>
<td>1:32</td>
<td>7/174 (0.04)</td>
</tr>
</tbody>
</table>

* Mean titre per 5 mice per group.  
$^\dagger$ Based on survival against $10^6$ SM i.p. LD$_{50}$/ml of VEE virus administered i.p. 28 days after Sindbis immunization.

i.c. inoculation did not enhance the protection against VEE virus. Even though higher doses of Sindbis virus were given in the i.p. inoculations, less protection was elicited than an i.c. inoculation, and multiple i.p. inoculations did not consistently enhance protection over that achieved by a single i.p. inoculation.

Involvement of the CNS in mice inoculated with Sindbis virus

Groups of mice were inoculated with Sindbis virus by either the i.c., i.p., or s.c. route. Mice in each group were selected randomly and the levels of Sindbis virus in the blood and brains of individual animals were determined for up to 10 days after infection. Mouse brains were also examined histopathologically. The results of several experiments are shown in Fig. 1 and Tables 3 and 4.

As seen in Fig. 1 and Table 4, only barely detectable levels of Sindbis virus were recovered from the brains of mice inoculated s.c. and i.p. with $10^3$ SM i.c. LD$_{50}$/ml Sindbis virus, whereas greater than $10^6$ SM i.c. LD$_{50}$/ml of virus were recovered per ml of 10% suspension from the brains of the i.c. inoculated mice during the acute phase of the infection. The results show that Sindbis virus inoculated i.c. proliferated to high levels in the brain, whereas
relatively little or no virus accumulated in the CNS when the virus was inoculated extra-
neurally.

Relatively little circulating virus was recovered from the blood of mice inoculated with
Sindbis virus, regardless of the route of inoculation (Fig. 1).

The brains of mice inoculated with Sindbis virus revealed few, if any, lesions in the s.c.
or i.p. inoculated mice (Table 3). In contrast, specific Sindbis virus lesions were observed
in the brains of i.c. inoculated mice as early as 4 days after infection. Predominant lesions
of mild gliosis and perivascular cuffing were present for 10 days. The lesions eventually
underwent mineralization and the residues persisted for at least 160 days, but infective
virus was not recovered from brains of inoculated mice after 10 days following infection.

Twenty-one days after infection with $10^5$ SM i.c. LD$_{50}$/ml of Sindbis virus inoculated i.c.,
i.p. or s.c., five mice in each group were bled and individual sera were assayed for the
presence of Sindbis-neutralizing antibody. No neutralizing antibodies were detectable in the
sera from any of the groups until day 8 post-inoculation, at which time the mean titre of the
i.c. inoculated group was 1:32 as compared to the i.p. (1:8) and s.c. (1:4) inoculated groups.
The highest mean titre for the i.c. inoculated group was 1:64 on day 21, as compared to
1:16 for the i.p. and s.c. inoculated groups. However, by 28 days post-inoculation, all
groups had equivalent levels of neutralizing antibody with mean titres of 1:32 for the

![Fig. 2. Levels of infective VEE virus in blood and brains of Sindbis immunized and non-immunized
mice after i.p. challenge with $10^5$ SM i.p. LD$_{50}$/ml VEE virus. • •, non-immunized; ●●● i.p.
immunized; ▲▲▲ i.c. immunized.](image-url)
respective groups. On day 28, titres ranged from 1:8 to 1:128 in the s.c. inoculated group. Titres in the i.c. and i.p. groups ranged from 1:16 to 1:64. A summary of the features associated with Sindbis virus infection is given in Table 4 and related to cross protection against i.p. challenge with $10^5$ SM i.c. LD$_{50}$/ml of VEE virus. Using protection against VEE virus challenge as a measure of the effectiveness of immunization, it was apparent that cross protection was more closely correlated with Sindbis virus multiplication in the brain and the incidence of brain-associated lesions than with the observed level, at 28 days after infection, of neutralizing antibodies to the immunogen.

**Challenge with VEE virus of mice previously infected with Sindbis virus**

Two groups of 21-day-old mice were immunized by i.c. or i.p. inoculation with $\geq 10^5$ SM i.c. LD$_{50}$/ml of Sindbis virus, respectively. A third (control) group was not inoculated. Twenty-eight days later the mice were challenged with approx. $10^5$ SM i.c. LD$_{50}$/ml of VEE virus inoculated by the i.p. route. Each day after challenge, three mice from each group were bled and perfused with 10 ml of saline. The brains, liver, spleens and mesenteric lymph nodes were removed, triturated and assayed for infective virus. The blood was heparinized and diluted 1:10 prior to assay.

The results presented in Fig. 2 demonstrate a similarity of the viraemic responses in the three groups. A clearance of infective VEE virus from the blood began after the third day and the blood was not infective by the 6th day in Sindbis immunized animals and in the control mice by the 8th day. Presumably the blood clearance was associated with the appearance of specific neutralizing antibodies detectable after the 4th day. Virus concentrations in the spleen, liver, and lymph nodes paralleled the viraemias, but at lower levels, and no significant differences between groups were discerned. Virus appeared in the brains from all three groups on the first day after challenge. Levels of virus in the brains increased in all three groups until day 3. In control mice and mice immunized i.p. with Sindbis virus, the levels of VEE virus in the brain continued to increase until the 5th day, then remained constant or decreased slightly until death between days 8 and 10. Levels of VEE virus in the brains of mice immunized i.c. with Sindbis virus reached a maximum on day 3. Thereafter the virus levels remained considerably below those in the other two groups. Again, this suppression of the infectious VEE virus in the brain correlated with the previous Sindbis virus infection of the CNS.

**DISCUSSION**

We conclude from these results that cross protection against VEE virus in mice is considerably enhanced if the CNS is primarily infected with Sindbis virus. This effect is not understood, however, a successful hypothesis for the role of the CNS in cross protection must account for the following: (i) the limitation of cross protection to immunologically-related viruses; (ii) greater resistance against challenge by extraneural routes as compared to intracerebral challenge; (iii) absence of detectable neutralizing antibodies to the challenge virus at the time of challenge; (iv) lack of cross protection when immunosuppressants, such as cortisone or cyclophosphomide, are administered simultaneously with the challenge virus.

Mice in our experiments were challenged with VEE virus 3 weeks after infection with Sindbis virus. Transitory non-specific resistance acquired immediately after infection is generally non-operative after 3 weeks (Casals, 1963; Hearn & Rainey, 1963). Cross protection persisting beyond the first week after immunization is more specific: it is limited to antigenically-related viruses. Virus interference was suggested (Traub, 1961) as a possible explanation of this enduring type of cross protection between EEE and VEE viruses in mice.
Route of immunization against VEE virus

Traub postulated that, after primary infection, tissues became latently infected with 'pro-virus' particles, and this infection causes an exclusion of the challenge virus. Even if an intrinsic interference exists, it would only partially explain the localized effect of a CNS infection with Sindbis virus in protecting against VEE virus. At least three experimental findings are unexplained by this hypothesis: (i) the lack of cross protection against an intracerebral challenge with VEE virus (Allen, 1962); (ii) the lack of demonstrable interference against VEE virus in vitro by cell cultures chronically infected with Sindbis virus (D. L. Fine & W. P. Allen, unpublished observations); (iii) the blocking of cross protection by cyclophosphomide, an immunosuppressant, administered simultaneously with the challenge virus (D. L. Fine & W. P. Allen, unpublished observations).

There is convincing evidence that an immunological mechanism plays a role in this cross protection. Neutralizing antibodies against the challenge virus are protective and the delay of the appearance of neutralizing antibodies by immunosuppression also prevent cross protection. We have considered the possibility that cross protection is mediated by an anamnestic response initiated shortly after challenge (Casals, 1963; Hearn & Rainey, 1963). Evidence of a secondary immune response has been described for haemagglutination-inhibition (HI) antibody in mice immunized and challenged with related arboviruses (Casals, 1963). However, no evidence of a secondary response of neutralizing antibodies could be found in Sindbis vaccinated mice that were challenged with either VEE virus (D. L. Fine & W. P. Allen, unpublished observations), or Semliki Forest virus (Brand & Allen, 1961). The possibility of a serum protective factor (SPF) being elicited prior to specific neutralizing antibodies has not been excluded. An SPF was found in 2 to 4 days after challenge with group B arboviruses (Third & Price, 1968) and was related to cross protection among group B tick-borne viruses.

Morgan, Olitsky & Schlesinger (1942) found that levels of specific neutralizing antibodies in the brain were correlated with resistance to intracerebral challenge with EEE or VEE viruses. Failure to detect antibody in the cerebral spinal fluid was associated with a lack of CNS resistance even when antibody was demonstrable in serum. Our studies were unavoidably terminated before such measurements could be made. It is conceivable that a cerebral infection with Sindbis virus primes immunologically responsive cells in the CNS for a rapid, localized immune response after challenge with VEE virus. The need for more direct evidence of a local immune response warrants further investigation of this cross protection. As pointed out by others (Casals, 1963), an understanding of the mechanisms of cross protection may facilitate the development of vaccines against arboviruses pathogenic for man and animals.

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REFERENCES


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