Age-Dependent and Strain-Related Differences of Virulence of Semliki Forest Virus in Mice

By P. FLEMING

Microbiological Research Establishment, Porton Down, Salisbury, Wilts, SP4 0JG

(Accepted 27 April 1977)

SUMMARY

Factors that influence the virulence of Semliki Forest virus for mice have been studied. *In vivo* experiments showed that maximum brain infectivities following i.p. inoculation of adult mice with a virus strain of low virulence were less than those found in mice showing clinical signs in virulent infections. The virulent strains generally caused death before antibody could be detected in brain tissue; the less virulent strain caused an infection that was neuroinvasive, but infectivity increased less rapidly in the brain and allowed antibody to intervene before clinical signs were apparent. IgG3 antibody was first detected in brain tissue coincident with the beginning of the decline of brain infectivities in avirulent infections; other sub-classes of IgG antibody were not detected until later.

*In vitro* experiments showed differences in abilities of SFV strains to replicate in tissue culture and adult mouse brain tissue, and differences in susceptibility to infection of brain tissue from mice of various ages. These *in vitro* results could provide a basis for differences of virulence.

INTRODUCTION

Several isolations have been classified as Semliki Forest virus (SFV) on the basis of serological identity; three such African strains were rated of high (strain L), intermediate (strain V), or low (strain A[74]) virulence according to responses following inoculation of mice, guinea-pigs and rabbits (Bradish, Allner & Maber, 1971).

Although SFV is now a familiar model for the alphaviruses there is still some doubt as to the cause of age-dependent development of resistance in young mice, and of strain-related differences of virulence in adult mice. Bradish & Allner (1972) considered that cerebral multiplication of virus caused death only in those mice infected by strains of SFV that did not trigger an early protective response. Woodward & Smith (1975) proposed that the avirulence of one SFV strain in adult mice was due to the production of defective interfering particles in the brain; Pattyn, De Vleeschauwer & van der Groen (1975) suggested that this same strain was avirulent because it could not replicate in the brain tissue of adult mice. Clearly these possible causes of virulence or avirulence merit the closest possible examination; the synthesis of antibody and its ability to react with virus in the brain are of particular relevance because, as well as nerve cell destruction by multiplication of virus, antigen–antibody or hypersensitivity reactions might contribute to the pathogenesis of these diseases (Webb & Smith, 1966).
METHODS

Virus and virus assay. The origin and derivation of the strains of SFV used in this work have been described by Bradish et al. (1971). Following their notation, passes in mice (no cipher), chick embryo cells (C), and hamsters (H) are indicated after the strain designation. Working stocks were prepared as follows: L10.H6.C2; V14.C2; and A7[74].C2; for simplicity these are referred to as strains L, V and A[74] respectively, with further passages indicated as above. Sindbis virus, strain Egar 339, was passed once in chick embryo cells (CEC) after 11 passes in mouse brain. Stock viruses were stored at −70 °C.

Plaque assays were carried out using CEC suspensions in agar containing Parker's medium 199 plus 5% calf serum (199.5CS); DEAE-dextran (Pharmacia Ltd., Sweden) was added at a concentration that maximized plaque number.

Plaque enhancement ratios. Plaque enhancement ratios are the ratio of the plaque count in the presence of an optimal concentration of DEAE-dextran to that without additive, as determined in CEC suspensions in 0.75% Special Agar-Noble (Difco laboratories, Detroit) containing 199.5CS.

Mouse inoculation. Random-bred Porton mice were inoculated by the intraperitoneal (i.p.) route; mice that then died, or were found to be immune to lethal i.p. challenge 28 days later, were considered to have been infected. Median infective doses were estimated by interpolation of response-log dose graphs. Mice were usually housed in groups of five; experiments showed that cross-infection did not occur within such groups.

Preparation of samples. Blood was collected from the retro-orbital plexus. Serum was prepared after allowing the blood to clot at 4 °C; blood samples were obtained after sedimenting the cells from heparinized blood (17 units/ml). Tissues were homogenized in 199.10CS. Antibody samples were stored at 4 °C.

Interferon assay. Samples were prepared for assay by dialysis at pH 2 for three days at 4 °C, followed by further dialysis to restore the pH to 7-2. Relative interferon activities were determined by reduction of infectivity yields from SFV-infected monolayers of mouse L-cells. Tissue:blood interferon ratios are quoted as the mean ± standard deviation.

Blood samples of small volume and low interferon activity (Fig. 5) were mixed with SFV at a concentration that ensured that the contribution of residual blood infectivity was negligible; yields were compared with that of a control culture with normal, heparinized blood as additive.

Antibody assay. Solutions were inactivated by heating at 56 °C for 30 min. Equal volumes (0.028 ml) of SFV, strain V, at 3 × 10^4 p.f.u./ml and appropriate dilutions of antibody solution were mixed, incubated for 2 h at 34 °C and then diluted 180-fold before assay of residual infective virus. Antibody titres are expressed as the dilution that reduced the plaque count by 50%.

Antibody class identification. Equal volumes (0.028 ml) of antibody solution and anti-mouse globulin serum were mixed and incubated for 2 h at 34 °C before addition of virus as above; after a further 2 h incubation at 34 °C, the mixtures were diluted 120-fold before assay of residual infectivity. Whenever possible, antibody concentrations were chosen to give a plaque reduction of about 80% in the control mixture without anti-mouse globulin; concentrations of anti-globulin sera were sufficient to eliminate anti-SFV activity under this condition.

Anti-mouse globulin sera. Goat anti-mouse γ-globulin, IgM, IgG2a, IgG1 and IgG2b sera were purchased from Flow laboratories, Irvine, Scotland. Purified κ- and λ-chain, and ascites fluid containing myeloma protein IgG3, were obtained from Bionetics.
**Virulence of SFV strains**

Table 1a. Replication of CEC derived virus in cultures of brain fragments from 12-, 26- and 33-day-old mice*

<table>
<thead>
<tr>
<th>Inoculum log p.f.u./ml</th>
<th>Virus yield† as log p.f.u./culture at 0 and 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFV.L</td>
<td></td>
</tr>
<tr>
<td>4.4</td>
<td>2.9</td>
</tr>
<tr>
<td>3.4</td>
<td>1.9</td>
</tr>
<tr>
<td>2.7</td>
<td>1.5</td>
</tr>
<tr>
<td>2.4</td>
<td>0.9</td>
</tr>
<tr>
<td>1.7</td>
<td>0.5</td>
</tr>
<tr>
<td>0.7</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>SFV.A[74]</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>4.1</td>
</tr>
<tr>
<td>4.2</td>
<td>3.1</td>
</tr>
<tr>
<td>3.2</td>
<td>2.1</td>
</tr>
<tr>
<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>1.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**Table 1b. Replication of mouse brain derived virus in cultures of brain fragments from 26-day-old mice**

<table>
<thead>
<tr>
<th>SFV.V</th>
<th>SFV.A[74]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum log p.f.u./ml</td>
<td>Log p.f.u./culture at 0 and 48 h</td>
</tr>
<tr>
<td>8.5‡</td>
<td>7.4§ 6.6</td>
</tr>
<tr>
<td>7.5</td>
<td>6.4 6.2</td>
</tr>
<tr>
<td>6.5</td>
<td>5.4 6.0</td>
</tr>
<tr>
<td>5.5</td>
<td>4.4 6.3</td>
</tr>
<tr>
<td>4.4</td>
<td>3.4 6.0</td>
</tr>
</tbody>
</table>

* Virus infectivities at 48 h in cultures that had clearly supported replication are shown in bold type.
† Virus infectivities less than 2.5 log p.f.u./culture before incubation were estimated by extrapolation.
‡ The highest inocula corresponded to a brain suspension of 0.2 g/ml.
§ Infectivities before incubation should be reduced by 2.4 log p.f.u./culture before comparison with the 48 h infectivity to allow for thermal inactivation.

Laboratory Products, Kensington, U.S.A.; preparation of rabbit anti-mouse IgG3, κ- and λ-light chain sera followed the protocol of Greaves (1971). Anti-mouse immune serum was prepared by intramuscular inoculation of a rabbit with 0.5 ml of convalescent anti-SFV serum with low antibody titre (300) mixed with 0.5 ml of Freund’s adjuvant; antiserum was prepared five weeks later.

**Mouse organ fragment culture.** Muscle from the hind leg, brains and spleens was chopped into 3 mm cubes; these were divided into portions equivalent to one whole organ, or about 0.5 g of muscle, and incubated for 2 h at 34 °C in 199,10CS containing virus. The medium was then removed and the fragments transferred to the surface of 5 ml of soft agar in 199,5CS in a 5 cm Petri dish. These cultures were then incubated for 48 h at 37 °C in a humidified atmosphere of air+5% CO₂. The total contents of the Petri dish were homogenized in 199,10CS and then assayed for virus infectivity.

Fragment cultures for the *in vitro* synthesis of antibody were prepared from spleens of infected mice and used at the equivalent of six spleens per Petri dish; antibody solutions were prepared as above after five days incubation at 37 °C.
RESULTS

In vitro studies

Replication in CEC and mouse L cells

Replication proceeded more quickly in CEC than in L cells; release of infective virus began after 4.5 h in CEC, but not until 7 h from L cell monolayers incubated at 34 °C. The rate of release of infective virus from CEC was reproducibly slower for the avirulent strain A[74] (0.40 log p.f.u./h) than for the virulent strains L and V (0.50 log p.f.u./h). The rates of release of virus from L cells were generally lower than for CEC, but strain differences were not reproducible. Thus the rate of replication was lower for strain A[74] than strains L and V in CEC, and generally lower for all strains in L cells than in CEC.

Replication in organ explant cultures

The results of replication studies in brain fragment cultures are summarized in Table 1. Two results in Table 1a are possibly relevant to age-related differences of virulence of strain A[74], and strain-related differences in adult mice. First, although replication occurred in brains from mice of all ages tested, higher concentrations were needed to infect brains of older mice: for instance, with strain L an inoculum concentration of 1.7 log p.f.u./ml initiated replication in 12-day-old, but not in 26- or 33-day-old cultures. Second, for brains
Virulence of SFV strains

Fig. 2. Mean blood virus infectivity 40 min after i.p. inoculation of 35-day-old mice with SFV strain L (■), V (○), A[74] (△) or V.C7 (○). Each point was determined from a graph as in Fig. 1.

from mice of a given age, the minimum infective concentration was marginally lower for the virulent strains: in 33-day-old brain cultures replication was initiated with an inoculum concentration of 2.7 log p.f.u./ml of strain L but not by 3.2 log p.f.u./ml of strain A[74].

The results of Table 1a were obtained using CEC-derived virus. Table 1b shows the results obtained from cultures of brain fragments from 26-day-old mice inoculated with homogenates of brains of 35-day-old mice taken four days post inoculation (p.i.) with strain V or A[74]. Replication occurred in all cultures except that inoculated with the lowest concentration of A[74]; there was no evidence for the presence of defective interfering particles in A[74] infections in the mouse.

The three CEC-derived strains of SFV used in these studies replicated in muscle, brain and spleen fragment cultures. Exposure of tissue fragments from 25-day-old mice to interferon (a 1:4 dilution of serum heated for 30 min at 56 °C, taken 24 h p.i. of 4-week-old mice with A[74]) before inoculation with 10⁶ p.f.u./ml of SFV V, significantly reduced the infective virus yields from all three fragment culture systems: brain, from 4.8 to 3.5 log p.f.u./culture; spleen, 6.2 to 3.7; muscle, 6.3 to 5.4. Thus all three cell types were susceptible to the action of interferon.

In vivo studies

Initiation of infection

Virus was recovered from the blood of 35-day-old mice almost immediately after i.p. inoculation with high doses of SFV; blood infectivity reached an equilibrium about 40 min p.i. (Fig. 1). Although there was a 10-fold difference between the highest and lowest concen-
Table 2. Properties of SFV strains and outcome of infection following i.p. inoculation of 35-day-old mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plaque enhancement ratio of inoculated virus</th>
<th>p.f.u./ID₉₀</th>
<th>Outcome of infection*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>% deaths (no. tested)</td>
</tr>
<tr>
<td>L</td>
<td>1·8</td>
<td>8</td>
<td>96 (27)</td>
</tr>
<tr>
<td>A[74]</td>
<td>2·0</td>
<td>16</td>
<td>25 (526)</td>
</tr>
<tr>
<td>V</td>
<td>5·7</td>
<td>40</td>
<td>88 (34)</td>
</tr>
<tr>
<td>V.C7</td>
<td>&gt; 10⁴</td>
<td>1100</td>
<td>64 (55)</td>
</tr>
</tbody>
</table>

* These results were obtained after inoculation with various doses greater than 10 ID₉₀.

Trations, a meaningful average log virus infectivity could be determined. The mean blood virus infectivity at 40 min p.i. was proportional to the inoculated dose over the experimentally accessible range (Fig. 2). Extrapolation of the results of Fig. 2 to low doses of inoculum indicated that at least 0·5 p.f.u. would be found in the blood 40 min p.i. with 1 ID₉₀ (Table 2) of any strain of SFV. Fig. 2 also shows that the efficiency of transfer of virus from the peritoneal cavity to the bloodstream and the plaque enhancement ratio of the inoculated virus (Table 2) were inversely proportional. This variation of efficiency of transfer of virus to the bloodstream is of greater significance if a virus population that is heterogeneous with respect to plaque enhancement ratio (Fleming, 1973) is used as the inoculum. Of ten mice inoculated with such a population (strain V.C1) with a mean plaque enhancement ratio of 150, nine were found to have virus with plaque enhancement ratios < 30 in the bloodstream 40 min p.i. A possible consequence of this selection is discussed later.

Outcome of infection

Death invariably occurred 3 to 5 days after i.p. inoculation of 12-day-old mice with infective doses of strains L, V or A[74]. This virulent response to A[74] changed to a predominantly avirulent one in mice aged from 15 to 20 days, as has already been described for the parent strain (Bradish et al. 1971).

The outcome of infection following i.p. inoculation of 35-day-old mice is shown in Table 2 as the percentage of mice dying and the mean day of death. Deaths following infection by the virulent strains L and V occurred slightly but reproducibly earlier the higher the dose; mice occasionally survived infections following inoculation of doses near the ID₉₀ level, but convalescent sera had very low antibody titres (< 10). In contrast, in the predominantly avirulent infections following A[74] inoculation, neither the incidence nor the time of death was related to the inoculated dose, and mortality rates were irregular even at identical doses.

The spread of infection in adult mice

There is little doubt that SFV infections spread to the target organ (the brain) by transport in, and diffusion from, the blood (Mims, 1957). Fig. 3 shows the total infectivity in the brain following i.p. inoculation with strains A[74] or V. That part of the infectivity which can be accounted for by the blood-borne virus (calculated from the data of Kaliss & Pressman, 1950) is indicated. No evidence could be adduced to indicate replication of virus in the brain much before 24 h p.i., nor were there any indications of strain-related differences in the efficiency of transfer from the blood to the brain. Replication in the brain occurred
Virulence of SFV strains

Fig. 3. Total infectivity recovered from the brains of 35-day-old mice following i.p. inoculation with (a) $1.8 \times 10^4$ p.f.u. of strain A[74], or (b) $4.2 \times 10^5$ p.f.u. of strain V. The infectivity that can be accounted for by blood-borne virus is indicated by the broken lines. More than 90% of the observations are within the limits indicated.

Fig. 4. Total infectivity recovered from the spleens of 35-day-old mice following i.p. inoculation with (a) $1.8 \times 10^4$ p.f.u. of strain A[74], or (b) $4.2 \times 10^5$ p.f.u. of strain V. The infectivity that can be accounted for by blood-borne virus is indicated by the broken lines. More than 90% of the observations are within the limits indicated.
with all strains from day 1 p.i. but was much slower for the avirulent strain A[74], so that by
day 4 p.i., brain infectivities for this strain were distinct from those of the virulent strain V.

Brain infectivities increased steadily until death in strain V infections but decreased
rapidly from day 5 p.i. for strain A[74]; maximum brain infectivities in strain A[74] infec-
tions corresponded to those found on days 2 to 3 in virulent infections, at which time
these mice appeared well. Although $10^8$ to $10^{10}$ p.f.u. were recovered from the brains of
mice that had died from strain V infections, only about 500 p.f.u. were found in mice
dead following A[74] inoculation; thus the brain infectivity in these mice also followed
the general pattern indicated in Fig. 3(a).

Infectivities in the spleen rose rapidly during the first 24 h p.i. but thereafter fell gradually
(Fig. 4). Thus, in contrast to the brain, there was no evidence of replication in the spleen
after the peak of viraemia.

Brain and spleen infectivities in strain L infections were similar to those for strain V.

**Interferon in the blood**

Interferon could be detected as early as 5 h after i.p. inoculation with SFV (Fig. 5). This
inhibitor (in pooled serum from L strain infected mice) was identified as interferon by the
usual criteria of species specificity (no activity in CEC), ability to inhibit Sindbis virus
replication in L cells, and stability at pH 2. Fig. 5 and further data showed that this early
interferon appeared in 81% (22/27), 70% (7/10) and 9% (2/23) of mice inoculated with
strains L, V and A[74] respectively. There were clear strain-related differences, but the
occasional early appearance of interferon was not correlated with subsequent deaths from
A[74] infections, nor was its appearance or level correlated with virus infectivities in the
blood that persisted from the inoculum, except that this 5 h interferon only appeared after
Virulence of SFV strains

inoculation with infective doses: sub-infective doses (e.g. 100 p.f.u. of strain V.C7) induced interferon secretion but not until 7 to 10 h after i.p. inoculation. The more common appearance of interferon 5 h after inoculation with virulent strains is of interest primarily because it was almost certainly secreted from the first cells infected with virus after its entry into the bloodstream, and suggests that virulent strains tend to replicate more rapidly in the mouse.

Blood interferon activities reached maxima at about 24 h p.i. and were probably related to the phase and level of viraemia after peripheral replication (Bradish & Allner, 1972).

Interferon in the brain and spleen

Paired brain/spleen and blood samples taken 24 h p.i. of ten mice with strain A[74] showed levels of interferon in the brain which were one-twentieth of those in the blood (interferon per g brain/ml blood = 0.05 ± 0.013) and were therefore higher than could be accounted for solely by interferon in the blood (Kaliss & Pressman, 1950). This extravascular interferon probably diffused from the blood since there was no evidence of virus replication in the brain by this time. Interferon activities in the spleen were ninefold higher than in the blood (interferon per g spleen/ml blood = 9.0 ± 1.15) suggesting that the spleen was a site of interferon secretion.

There was no significant increase in brain interferon activity between days 1 and 2 p.i. with SFV.V despite a 200-fold increase in brain infectivity (Fig. 3). A similar observation was made by Oaten, Webb & Bowen (1976) using the avirulent A[74] strain. It thus appears that brain cells secrete little, if any, interferon in SFV infections.

Protective effects of antiserum and pre-infection with virus

Administration of anti-SFV serum during the acute phase of infection with virulent strains of SFV can produce a measure of protection (Seamer, Boulter & Zlotnik, 1971). In the present investigation it was found that a single dose of rabbit anti-SFV serum (antibody titre = 1000; 0.2 ml i.p. 24 h p.i. with SFV) protected 26% (26/100) and 54% (38/70) of mice infected with the virulent strains L and V respectively. Of the survivors tested 28 days p.i. only 9% (4/44) had failed to develop active immunity, but antibody titres were low (1 to 250).

Although no protection was afforded when Sindbis virus (10⁷ p.f.u. = 1000 ID₅₀) was inoculated more than five days before challenge with 100 ID₅₀ of the highly virulent strain L, 60% (36/60) of mice survived if the challenge was only 24 h later; anti-SFV antibody was found in the convalescent sera of all these survivors but again titres were low (1 to 250). Thus, of these 60 mice pre-infected with Sindbis virus, SFV.L infection was prevented in 12, abated in 24, and unaffected in 24. This protection was possibly interferon-mediated (Hearn & Rainey, 1963).

These low antibody titres should be compared with those of 900 to 8000 found in the convalescent sera of mice following i.p. inoculation with high doses of strain A[74].

Antibody in convalescent sera

The class or subclass of antibody predominating in any sample was determined by the ability of antisera raised against mouse globulins to neutralize the anti-SFV activity; the criteria for identifying the predominant antibody class are shown for representative samples in Table 3. The three antibodies, designated for convenience, IgW, IgX and IgY, can thus be distinguished from the recognized subclasses of IgG and also from IgM since they were not neutralized by anti-IgM serum, but their identities are uncertain. IgM antibody activity
Table 3. Identification of antibody subclasses: representative samples

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>p.f.u./ml after incubation and dilution*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 †</td>
</tr>
<tr>
<td>Control, no antibody</td>
<td>66</td>
</tr>
<tr>
<td>Antibody without antiglobulin</td>
<td>3</td>
</tr>
<tr>
<td>Antibody + anti-immune mouse serum</td>
<td>68</td>
</tr>
<tr>
<td>anti-γ globulin</td>
<td>68</td>
</tr>
<tr>
<td>anti-IgM</td>
<td>79</td>
</tr>
<tr>
<td>anti-IgG2a</td>
<td>3</td>
</tr>
<tr>
<td>anti-IgG2b</td>
<td>4</td>
</tr>
<tr>
<td>anti-IgG3</td>
<td>6</td>
</tr>
</tbody>
</table>

Predominant antibody subclass

- IgM
- IgG2a
- IgG3
- IgY
- IgX
- IgW

* See Methods for details of incubation and dilution; plaque counts are the mean of three assay plates.
† Results were obtained using 35-day-old mice inoculated with strain A [74]; neutralization of anti-SFV activity is indicated by bold type. Sample No. 1 is serum 4 days p.i. Sample 2 is a high titre (23000) serum 8 days p.i. Samples 3 and 4 are paired brain and serum samples 9 days p.i. Samples 5 and 6 are convalescent sera from mice treated with anti-SFV serum 24 h p.i.

Table 4. Predominant antibody in convalescent sera

<table>
<thead>
<tr>
<th>Serum antibody titre</th>
<th>Predominant antibody subclass(es)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–65</td>
<td>IgW or IgX</td>
</tr>
<tr>
<td>65–85</td>
<td>IgX</td>
</tr>
<tr>
<td>85–400</td>
<td>IgX or IgY</td>
</tr>
<tr>
<td>400–1950</td>
<td>IgY</td>
</tr>
<tr>
<td>1950–3000</td>
<td>IgY or IgG2a</td>
</tr>
<tr>
<td>3000–8000</td>
<td>IgG2a</td>
</tr>
</tbody>
</table>

was neutralized by anti-IgM, anti-γ globulin and anti-immune mouse sera; IgM, IgY and IgG2a activities were also neutralized by anti-κ-light chain serum. Anti-λ-light chain serum did not neutralize IgM, IgX, IgY or IgG2a antibody activity but, since it also failed to produce any precipitation lines in immunoelectrophoresis of mouse serum, its potency must be considered doubtful. This technique can only indicate the predominant subclass; it is possible that sera with high antibody titres contain other subclasses with significant in vivo activities.

Analysis of over 100 sera collected 28 days after SFV inoculation revealed a correlation between predominant antibody type and total antibody titre (Table 4); some mice were inoculated with anti-SFV serum 24 h after, or Sindbis virus 24 h before, SFV inoculation in order to obtain convalescent sera with reduced antibody titres (see previous section for details). IgW or IgX predominated in sera with the lowest anti-SFV activity, IgX or IgY at intermediate levels, but IgG2a could only be detected in sera with the highest antibody activities; IgG1 was detected in only two sera (titres ~ 6000) and IgG2b and IgG3 not at all. No virus strain-related differences were noted, but sera taken after inoculation with potentially virulent strains were inevitably of low titre. Repeated inoculation of virus gave rise to higher antibody titres until only the ‘normal’ (IgG2a) antibody predominated. The lowest titres therefore indicated a much reduced primary stimulation.

Characterization of IgW, IgX and IgY was difficult because they were only present in low concentrations. They were considered to be immunoglobulins primarily because they
Virulence of SFV strains

were specific in their ability to neutralize all strains of SFV but not Sindbis virus, and their presence and persistence in serum – even at the lowest levels – was an indication of immunity against challenge with virulent SFV. Also, they lost no activity during 4 h at 56 °C, and, in samples with a sufficiently high activity for this to be determined, their kinetics of virus neutralization and their elution profiles from Sephadex G-200 were similar to those for IgG2a. The ability of IgW to neutralize SFV was enhanced by anti-mouse IgG2a serum; this effect has been observed in other virus–antibody systems (Brown et al. 1969) and was not due to complement. IgX was present in some sera in concentrations sufficiently high to be immunogenic. IgY is clearly identifiable as an immunoglobulin since it was neutralized by anti-mouse κ-light chain serum. The identification of the possible roles of these three antibodies must await determination of their secondary properties and the availability of monospecific antisera to facilitate their detection.

Early synthesis and secretion of antibody

Antibody was first detected and identified in the blood of mice 4 days p.i. with SFV. A[74]; thereafter, antibody levels rose rapidly until about day 15, at which time a steady state was reached. From day 4 to day 6 IgM predominated in serum samples; from day 7 IgM levels dropped and IgY or IgG2a became the predominant antibody, depending on the overall antibody titre.

The preponderance of IgM in the blood during the acute phase of infection made it very difficult to detect other antibody classes that could reach virus in brain tissue. To obviate this, homogenates of brain tissue were assayed for antibody concentration and predominant subclass. Antibody was regularly detected and identified in the brain from day 6 p.i., that is two days later than first detected in the blood. Comparison of antibody titres and antibody subclass in paired brain and serum samples showed that both IgG2a and IgG3 were often identified as the predominant antibody in the brain when they were below detectable levels in the blood (see Table 3). Conversely, IgM was frequently the predominant antibody in the blood when only a minor component in brain homogenates. These qualitative observations clearly indicate that IgG2a and IgG3 permeated the extravascular areas of the brain and suggest that IgM did not.

In eight of nine cases IgG3 was found in the brain tissue from day 6 to day 8 p.i., and was therefore probably secreted at about the same time as IgM; IgG2a was not detected in the brain until day 8.

Antibody from spleen fragment cultures

Mice usually died from virulent SFV infections before appreciable concentrations of antibody appeared in the blood. To obviate this limitation on comparison with avirulent infections, mice were infected with similar doses (1000 ID50) of strains V or A[74] and their spleens removed four days later; fragments were then cultured for a further five days before being homogenized as described in Methods; the resulting antibody solutions were then heated for 30 min at 56 °C and assayed for antibody titre and predominant antibody type. Although both solutions contained some non-specific inhibitor, they both had anti-SFV titres of about 20 with a mixture of IgM and IgG3 predominating; in these respects they were indistinguishable.
DISCUSSION

Adult mice surviving infections induced by small doses of any strain of SFV developed similar low antibody levels; reduced antibody levels were also found if infections initiated by high dose of any strain were abated either by pre-infection with Sindbis virus or by administration of anti-SFV serum. Cultures of spleen fragments from mice inoculated with relatively high doses of virulent or avirulent strains yielded antibody solutions of indistinguishable titre and class. It was thus not possible to demonstrate any difference in antibody synthesis following inoculation of adult mice with virulent or avirulent SFV; virulent strains might therefore induce the same antibody response as avirulent strains if the mice survived. Studies with Sindbis virus (Griffin, 1976) showed that the age-dependent change from virulent to avirulent response was not due to the failure of suckling mice to secrete antibody. Together, these observations suggest that the age-dependent development of resistance to SFV in young mice, and strain-related differences of virulence in adult mice, are not due to differences in antibody response.

Brain cells appeared to secrete little, if any, interferon in SFV infections, although they were susceptible to its protective effects. The high levels of interferon circulating in the blood during the first day after inoculation of virus could thus delay initiation of virus replication in the brain, but interferon could not inhibit the subsequent rise of brain infectivity. In contrast, infections of the spleen, which produced high levels of interferon, are probably self-limiting.

Although there was a wide variation in individual responses, it was often possible to identify significant strain-related differences, as in the ability of virus to reach the bloodstream and thus initiate infection following i.p. inoculation. The correlation between plaque enhancement ratios of SFV populations, surface properties of the virus (Fleming, 1973), and efficiency of transfer to the blood suggests that this transfer might be influenced by similar surface properties. The observed selection in transfer could prevent infection by a small proportion of virulent virus in a population of otherwise avirulent virus particles. The differences in ability of virus strains to reach the bloodstream following i.p. inoculation explain the observed differences in ID₅₀.

However, the only strain-related difference which was correlated with the outcome of infection was the rate at which virus infectivities increased in the brain. In vitro experiments indicated that higher concentrations of SFV were required to initiate infections in brain tissue from older mice, and that virulent strains were possibly more efficient in initiating replication in adult mouse brain tissue; results from tissue culture experiments showed that rates of replication depended on both virus strain and host cell. These differences in ability of virus strains to infect and replicate could correlate with the different rates of increase of virus infectivity in the brain. The more common early appearance of interferon after inoculation of mice with virulent strains of SFV supports the concept that virulent strains replicate more rapidly in the mouse.

The lower rate of increase of brain infectivity in avirulent infections allows an antibody response to develop before levels reach those associated with clinical signs and death. The first antibody found in the extravascular region of brain tissue is of the IgG₃ subclass. Its earliest detection indicated that it was probably secreted from about day 5 p.i., which coincided with the beginning of the sharp decline of brain infectivity in avirulent infections; it could thus be the cause of this decline. IgG₃ is reported to permeate membranes with great facility and does not fix complement (Grey, Hirst & Cohn, 1971; Spiegelberg, 1974) and would therefore be most effective in such a role. IgG₂a was often detected before virus
Virulence of SFV strains

infectivity had been eliminated from the brain and – since this subclass of antibody does fix complement and penetrates the brain tissue – this could cause symptoms by an immunopathogenic mechanism; the irregular incidence of deaths occurring relatively late in strain A[74] infections might be due to such an effect.

Both age-dependent development of resistance, and strain-related differences of virulence of SFV in adult mice may therefore be explained in terms of rate of increase of brain infectivity; in suckling mice this is rapid for all strains, but in adult mice is sufficiently reduced for the less virulent strains to allow antibody to intervene before virus proliferation causes cell destruction leading to clinical signs and eventually death.

The author gratefully acknowledges the expert technical assistance of Miss A. Beeson; the immunoelectrophoresis experiment was performed by Dr W. H. Ford.

REFERENCES


(Received 28 March 1977)