Protection of Mice Infected with a Lethal Dose of Semliki Forest Virus by Defective Interfering Virus: Modulation of Virus Multiplication

By A. D. T. BARRATT, A. R. GUEST, A. MACKENZIE and N. J. DIMMOCK*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K. and *Institute for Research on Animal Diseases, Compton, Berkshire, RG16 0NN, U.K.

(Accepted 1 August 1984)

SUMMARY

Certain defective interfering (DI) Semliki Forest virus (SFV) preparations completely protected the majority of mice inoculated with a normally lethal dose of SFV, and the surviving mice showed no signs of disease during the period of observation. Depending upon which DI SFV preparation was used, the survivors were resistant to challenge with 100 LD50 SFV (DI SFV p13a), or were completely sensitive (DI SFV p4), the latter having evidently failed to establish a protective immunity. In this report we compared the ability of these two DI SFV preparations to inhibit multiplication of infectious virus in mice inoculated with 10 LD50 SFV. The following conclusions emerged: (i) virus multiplication was profoundly inhibited in the majority of mice treated with either of the DI virus preparations although there was significant multiplication in most tissues, including brain. The number of mice showing evidence of reduced infectivity titres (58%) correlated well with the 60% which survived without disease in lethality experiments. (ii) Despite the presence of infectivity, no SFV antigen or histopathological lesions were detected in brain or spinal cord. (iii) The DI virus preparations p4 and p13a altered the distribution of infectivity in the mouse in different ways: during the first 2 days of the infection modulated by DI virus p4, the infectivity titres (in brain, olfactory lobes and spleen) were comparatively high, being > 1% of those in mice inoculated with standard virus alone. However, from day 3, titres declined precipitously and there was little infectivity in any of the tissues investigated. On the other hand, mice treated with DI SFV p13a had, over the entire duration of infection, greatly reduced though significant infectivity in brain, olfactory lobes and spleen and very little infectivity in serum. (iv) In a minority of mice (14.5%), DI virus p13a altered the distribution of infectivity between different tissues so that there was significantly decreased virus in just one or two of the four tissues investigated, suggesting that the infection was being subtly modulated by the DI virus. (v) Interference assays failed to detect DI SFV in any tissue samples although the effects of DI virus on infection in the mouse were obvious.

INTRODUCTION

Defective interfering (DI) virus particles arise by the deletion of a major part of the genome of standard (infectious) virus and as a result can only multiply in cells co-infected with standard virus. Such co-infection results in a reduced yield of standard virus progeny, a phenomenon known as homotypic interference. DI particles are spontaneously produced during serial passage at both high (Perrault, 1981) and low (Barrett et al., 1981) multiplicity of infection of many, if not all, animal viruses (Huang & Baltimore, 1977; Holland et al., 1980; Perrault, 1981) but because of their dependence on standard virus, multiplication of DI virus is optimal at high m.o.i. Under these conditions DI virus particles become enriched and can outnumber those of the standard virus. Interference occurs intracellularly and is specific for the virus from which the DI virus was derived (Perrault, 1981), although interference has been reported between standard virus and DI virus belonging to different serotypes of vesicular stomatitis virus (VSV) (Prevec & Kang, 1970;...
Schnitzlein & Reichmann, 1976) and between DI Semliki Forest virus (SFV) and Sindbis virus (Barrett & Dimmock, 1984a).

To date, most of the work on DI particles has been concerned with the molecular biology (Perrault, 1981; Lazzarini et al., 1981) rather than the modulation of infection by DI particles in vivo, although this possibility was first suggested by Huang & Baltimore (1970). Several workers have shown that animals can be protected from acute virus infections by exogenously administered DI virus, for example with VSV inoculated intracerebrally (Doyle & Holland, 1973; Holland & Villareal, 1975; Rabinowitz et al., 1977; Jones & Holland, 1980) or intraperitoneally (Fultz et al., 1982), with reovirus (Spandidos & Graham, 1976), with influenza virus (von Magnus, 1951; Rabinowitz & Huprikar, 1979), with lymphocytic choriomeningitis virus (Welsh et al., 1977) and with SFV (Dimmock & Kennedy, 1978; Crouch et al., 1982; Barrett & Dimmock, 1984b, c). However, only a few studies have used appropriate controls to demonstrate that these effects are due to interference rather than to the immunogenic effects of DI virus particle antigens (Jones & Holland, 1980; Dimmock & Kennedy, 1978; Crouch et al., 1982; Barrett & Dimmock, 1984b, c).

As a result of administering DI virus, the acute infection may become entirely subclinical (SFV: Dimmock & Kennedy, 1978; Barrett & Dimmock, 1984c) or converted to a chronic wasting condition (VSV inoculated intracerebrally: Doyle & Holland, 1973; reovirus: Spandidos & Graham, 1976), or a long-term persistent infection (VSV inoculated intraperitoneally: Fultz et al., 1982). However, almost nothing is known of how virus and host interact to bring about profound changes in the expression of disease.

In earlier reports we demonstrated that SFV DI virus can protect mice from a lethal SFV encephalitis (Dimmock & Kennedy, 1978; Crouch et al., 1982). However, we subsequently found that DI viruses are heterogeneous in their biological properties and we defined three categories: (1) DI virus which protected mice and left them immune to challenge at 3 weeks after infection with 100 LD_{50} SFV; (2) DI virus which protected mice but failed to establish immunity to challenge; (3) DI virus which did not protect mice even though it had the same interference titre in vitro as the other preparations (Barrett & Dimmock, 1984c). In this report we have examined the extent to which the first two categories of DI SFV modulate the multiplication of SFV in various tissues of the mouse.

**METHODS**

**Viruses.** Virulent SFV (ts'; standard virus) was grown in BHK-21 cells (m.o.i. = 0·1) as described by Dimmock & Kennedy (1978). Avirulent SFV was obtained by passage in BHK-21 cells (m.o.i. = 0·1) for 18 h at 37 °C from the avirulent strain A774 (Bradish et al., 1971). DI SFV preparations were derived by serial undiluted passage of SFV in BHK cells which in later passages were maintained at a constant m.o.i. of 50 p.f.u./cell by the addition of non-infectious virus. Incubation was for 24 h at 37 °C. DI SFV p4 (i.e. four undiluted passages) was generated from the avirulent strain A774 (Bradish et al., 1971). DI SFV pl3a was derived from the DI SFV p8 used by Dimmock & Kennedy (1978).

**Cells.** Chick embryo fibroblast (CEF) monolayers were prepared as described by Morser et al. (1973) and BHK-21 and L929 cells were grown as monolayers by standard techniques.

**Infectivity titrations.** Virus was plaque assayed in CEF (Kennedy & Burke, 1972) at 33 °C, with an agar overlay containing 0·02%, DEAE-dextran.

**Mice.** Male CFLP random bred mice 4 to 5 weeks old weighing about 25 g were obtained from Hacking and Churchill, Wyton, Huntingdon, Cambs., U.K., and were used 3 to 6 days after delivery.

**Inoculation of mice.** Groups of eight mice were inoculated intranasally under light ether anaesthesia with a 20 μl vol. as described by Dimmock & Kennedy (1978). Treatment of mice with DI SFV comprised two inoculations of a DI SFV preparation 2 h apart. The second inoculum contained 10 LD_{50} (6000 p.f.u.) standard SFV. Possible immunostimulation by DI SFV was controlled by using non-infectious u.v.-irradiated standard SFV (UV-SFV) of the same antigenic mass as determined by haemagglutination.

**Tissue sampling.** When tissue samples were required, mice were killed with ether. Blood was obtained from the heart and serum was stored at −70 °C. Spleen, olfactory lobes and brain (minus olfactory lobes) were dispersed in Medium 199 containing 2% newborn calf serum and stored at −70 °C.

**U.v. irradiation.** This was done as described by Dimmock & Kennedy (1978) except that the virus was placed 10 cm below a u.v. lamp (Gelman Sciences, Northampton, U.K.) for appropriate times. The dosage was 14μW/cm² and was checked on each time of usage with a Blak-Ray u.v. meter (Model J225, Ultraviolet Products Inc., San Gabriel, Ca., U.S.A.).

**Haemagglutination assay.** These were performed according to Clarke & Casals (1958). Goose red blood cells were obtained from CAMR, Porton Down, Wilts., U.K.
Interference assays. Two assays, the RNA synthesis inhibition assay (RSIA) (Barrett et al., 1981) and the yield reduction assay (YRA), were used to quantify the interfering activity of DI virus. The latter is a modification of an assay originally described by Bellett & Cooper (1959) and since modified by Kowal & Stollar (1980). DI SFV is diluted in a diluent of maintenance medium [GMEM plus 2% newborn calf serum and 2 μg/ml actinomycin D (to inhibit any induction of interferon)] containing standard virus at an m.o.i. of 5 p.f.u./cell. Samples (250 μl) of each dilution are inoculated onto monolayers of 2 x 10⁵ L929 cells in glass vials and left for 1 h at 37 °C. The inoculum is removed and replaced by 1 ml maintenance medium and incubation continued at 37 °C. At 16 h post-infection incubation is stopped and cultures are assayed for infectivity. The number of defective interfering virus units (DIU) in a DI SFV preparation is defined as the reciprocal of the dilution where standard virus production is reduced to 50% of controls inoculated with standard virus alone.

Histological procedures. Mice were killed with ether and the brains and spinal cords removed intact. Part of the brain was removed for infectivity assay and the remainder fixed in neutral buffered formalin. Tissues were prepared for paraffin embedding and sectioning by standard procedures. Sections were stained with haematoxylin and eosin.

Immunohistochemical staining. Paraffin sections were reacted with convalescent antiserum prepared by infecting rabbits with the avirulent A774 strain of SFV and 3 weeks later with the ts⁺ strain. Positive antibody–antigen reaction was located with biotin-avidin (Vectastain; Vector Laboratories, Burlingame, Ca., U.S.A.) or peroxidase-antiperoxidase (Dakopetts A/S, Guldborgvej, Copenhagen, Denmark) methodology. There was positive immunohistochemical staining with anti-SFV diluted up to 1/120.

RESULTS

In a previous study we have shown that DI SFV preparations p4 and p13a protected mice against infection with 10 LD₅₀ standard SFV by two apparently different mechanisms (Barrett & Dimmock, 1984c). Properties of the two DI virus preparations and the ways in which they modulated various parameters in the mouse are summarized in Table 1. This shows that although the DI SFV preparations contain the same amount of SFV antigen and prevent death of the same proportion of mice, animals inoculated with DI SFV p13a were resistant to challenge with 100 LD₅₀ at 21 days post-infection, whereas DI SFV p4-treated mice were completely susceptible. To investigate these differences we have inoculated mice with 10 LD₅₀ plus one or other of the two DI SFV preparations and determined the distribution of infectious virus in a number of tissues at intervals after infection.

Growth curve of standard SFV in mice after intranasal inoculation

Fig. 1 shows the growth curves of 10 LD₅₀ standard SFV in CFLP mice after intranasal inoculation. The serum, spleen, brain (minus olfactory lobes) and olfactory lobes were assayed for in-
<table>
<thead>
<tr>
<th>Passage history</th>
<th>Antigenic mass (HAU/ml)</th>
<th>Infectivity (p.f.u./ml)</th>
<th>U.v. (s)*</th>
<th>Interference titre (DIU/ml)†</th>
<th>Protection (٪)§</th>
<th>Survivors of challenge (٪)¶</th>
<th>Neutralizing antibody titre¶¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>p4</td>
<td>4</td>
<td>1 x 10⁵</td>
<td>100</td>
<td>56</td>
<td>60</td>
<td>5</td>
<td>&lt;2, &lt;5, &lt;9, &lt;20, 2, 9, 32, 2000, 2000, 3162</td>
</tr>
<tr>
<td>p13a</td>
<td>4</td>
<td>3 x 10⁷</td>
<td>60</td>
<td>50</td>
<td>59</td>
<td>81</td>
<td>&lt;9, &lt;9, &lt;9, 9, 10, 18, 22, 22, 141, 398, 501, 1000</td>
</tr>
</tbody>
</table>

* U.v. irradiation time (in seconds) used to inactivate standard virus in the DI SFV preparation prior to use for protecting mice.
† Protocol for RSIA is given in Methods.
‡ Data from Barrett & Dimmock (1984c).
§ Expressed as a percentage of mice inoculated with 10 LD₅₀ standard SFV plus DI SFV.
¶ Protected mice in the previous column were challenged with 100 LD₅₀ at 21 to 24 days post-infection.
¶¶ Serum at day 13 post-infection, obtained by the plaque reduction assay. Mice inoculated with DI virus alone have no detectable antibody titre.
Inhibition of SFV multiplication by DI virus

Infectivity. Virus was first seen in the olfactory lobes at 12 h and this increased up to 3 days post-infection. Infectivity increased until 4 days post-infection when peak titres (up to \(10^{10}\) p.f.u./brain) were detected. Virus was only detected in spleen and serum from day 2 post-infection where virus titres reached \(10^9\) p.f.u./mouse. Titres between mice were very consistent and the calculated error was correspondingly small. Mice showed clinical signs of disease on day 3 and died either on day 4 or day 5 post-infection. It was rare for an animal to survive until day 6.

Multiplication of virus in mice co-infected with DI and standard SFV

Groups of eight mice were inoculated with DI SFV plus 10 LD\(_{50}\) standard virus or 10 LD\(_{50}\) standard virus plus UV-SFV according to the protocol of Dimmock & Kennedy (1978). UV-SFV [the equivalent of 4 haemagglutinating units (HAU)/ml of SFV antigen] was used to control for any immunogenic activity of the DI virus (also 4 HAU/ml) and also showed that uptake of infectious virus in the nose was not being impeded. Properties of the DI virus preparations used are summarized in Table 1. Mice were killed at intervals after inoculation and the infectivity present in serum, spleen, brain (less olfactory lobes) and olfactory lobes determined. The results obtained for the two DI SFV preparations examined are shown in Fig. 2 and 3. All mice not treated with DI virus died by day 5 post-infection; hence, on this day only reduced numbers were available for sampling.

Mice treated with DI SFV p4

Comparison of the infectivity titres in mice treated with 10 LD\(_{50}\) plus DI SFV p4 with those found in mice inoculated with 10 LD\(_{50}\) plus UV-SFV (Fig. 2) shows that the DI virus-treated mice can be divided into two classes: (i) those which have titres reduced by >99% and (ii) those which have titres that were not significantly different. Overall 58% of the infectivity titres from the four tissues examined were in the former group. This figure compares favourably with lethality studies where 60% survived. Thus, we equate the first group with mice that would be protected from death by DI virus and the second group with mice that would die with the disease following its normal course (Dimmock & Kennedy, 1978). Examination of Fig. 2 shows that up to day 2 post-infection, infectivity titres in the brain, olfactory lobes and spleen were virtually identical in DI-treated and untreated mice whereas infectivity titres in the serum were lower in DI-treated mice than in untreated mice. However, the majority of samples from DI virus-treated mice taken from day 3 onwards had lower infectivity titres than those of untreated mice. Many of these samples, and all samples taken from day 7 post-infection onwards, had undetectable levels of infectivity (< 400 p.f.u./tissue).

Mice treated with DI SFV p13a

Infectivity titres obtained from mice infected with 10 LD\(_{50}\) plus DI SFV p13a can again be divided into two classes (Fig. 3). Sixty-eight% of all the infectivity titres from the four tissues examined were in the group defined as having titres reduced by >99% compared with mice not treated with DI SFV. This figure is close to the 59% survival achieved by treatment with DI SFV p13a in lethality experiments. The pattern of infectivity titres in DI SFV p13a-treated mice was different from that found in DI SFV p4-treated mice. Whereas the latter group had infectivity titres identical to untreated mice up to day 2 post-infection, mice that had been inoculated with 10 LD\(_{50}\) plus DI SFV p13a had reduced infectivity titres at all time points examined. There was little virus in serum (compared with other tissues) of DI virus-treated mice, while infectivity titres in the spleen on days 2 and 3 post-infection showed little reduction compared with mice that did not receive DI virus. These results demonstrate that DI virus not only prevents the encephalitis but also prevents spread of virus in other tissues. Inspection of infectivity titres of mice that comprise the group of individuals in which 'titres were reduced by >99%' showed that the majority contained no detectable virus (<400 p.f.u./tissue). However, in other experiments, a more sensitive assay has shown that many of these mice have levels of infectious virus ranging from 10 to 100 p.f.u./mouse, indicating that the majority of mice were indeed infected; only 27% had less than 10 p.f.u./mouse on day 4 post-infection. At days 7, 11 and 14 post-infection virus
Fig. 2. Multiplication of SFV in mice inoculated with 10 LD₅₀ and treated with DI SFV p4. Eight mice were inoculated with 10 LD₅₀ plus UV-SFV or 10 LD₅₀ plus DI SFV and were sacrificed at various times after infection. (UV-SFV is inactivated standard virus containing the same amount of antigen as DI virus and is included to control for possible immunogenic effects.) Curves represent mean infectivity titres of detectable virus of samples from mice inoculated with 10 LD₅₀ plus UV-SFV. Arrows with an adjacent number refer to the number of mice with undetectable infectivity. ●, 10 LD₅₀ plus UV-SFV; ▲, 10 LD₅₀ plus DI SFV.
Inhibition of SFV multiplication by DI virus

Fig. 3. Multiplication of SFV in mice inoculated with 10 LD50 and treated with DI SFV p13a. Other details are as described in the legend to Fig. 2.
Table 2. Abnormal distribution of infectivity in tissues of some mice inoculated with 10 LD$_{50}$ plus DI SFV p13a

<table>
<thead>
<tr>
<th>Day post-infection</th>
<th>Mouse no.</th>
<th>Brain (log$_{10}$ p.f.u./mouse)</th>
<th>Olfactory lobes (log$_{10}$ p.f.u./mouse)</th>
<th>Spleen (log$_{10}$ p.f.u./mouse)</th>
<th>Serum (log$_{10}$ p.f.u./mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1*</td>
<td>7.3</td>
<td>6.9</td>
<td>3.2</td>
<td>4.4</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>&lt;2.0</td>
<td>&lt;1.7</td>
<td>2.6</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>&lt;1.7</td>
<td>5.7</td>
<td>3.0</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>3</td>
<td>1*</td>
<td>8.8</td>
<td>6.5</td>
<td>4.0</td>
<td>5.2</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>&lt;1.3</td>
<td>&lt;2.0</td>
<td>3.6</td>
<td>4.3</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>&lt;1.3</td>
<td>5.2</td>
<td>3.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>4.0</td>
<td>4.2</td>
<td>3.3</td>
<td>3.1</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>5.2</td>
<td>&lt;1.7</td>
<td>&lt;1.4</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>5</td>
<td>1*</td>
<td>7.5</td>
<td>5.7</td>
<td>2.8</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3.4</td>
<td>5.1</td>
<td>&lt;2.0</td>
<td>4.1</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>&lt;1.6</td>
<td>&lt;1.7</td>
<td>2.7</td>
<td>3.3</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>4.9</td>
<td>&lt;1.7</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
</tbody>
</table>

* Control mice were inoculated with 10 LD$_{50}$ plus UV-SFV. These were not given DI virus.

was not detectable (< 400 p.f.u./mouse) in any of the tissues with one exception at day 14 which had a brain infectivity titre of 10$^{4.9}$, but no virus in the other three tissues examined.

Disproportionate reduction in specific organ infectivity in infections modulated by DI SFV p13a

As discussed above, mice inoculated with DI SFV p13a fell into two classes: (i) those with infectivity titres reduced by > 99% compared with mice infected with 10 LD$_{50}$ plus UV-SFV and (ii) those with titres unaffected by inoculation of DI virus. In the majority of mice, the levels of infectivity in each of the four tissues investigated were either consistently low or consistently high. The latter were indistinguishable from mice inoculated with SFV alone (Fig. 1). However, nine mice treated with DI virus p13a had titres which did not fit either pattern. These had high titres in one or more tissues and low titres in the others (Table 2). For example, mouse 6 on day 2 had no detectable virus in brain and serum and yet there were high titres in the olfactory lobes and spleen whereas the control mouse (mouse 1 on day 2) had similar titres in olfactory lobes and spleen but > 10$^5$-fold more virus in the brain and > 10$^{1.8}$-fold more virus in the serum. It must be emphasized that only 9/62 (14.5%) had this unusual pattern of virus infection and that none of these mice showed clinical signs of sickness. The effect was specific to DI virus p13a and none of the mice treated with DI SFV p4 showed an unusual pattern of virus infectivity. Particularly noteworthy is the presence of virus in brain at 14 days after infection when one would have expected immune processes to have cleared virus completely.

Failure to detect DI virus in DI virus-treated mice

It was of obvious interest to follow the levels of DI virus in the above experiments. In an attempt to do so we used both the YRA and the RSIA as these appear to measure different parameters of interference (Barrett et al., 1984). Both assays failed to detect DI virus in either brain or olfactory lobes of DI virus p4- or p13a-treated mice. This result was unexpected since we have demonstrated elsewhere that inoculation of DI virus prevents death (Dimmock & Kennedy, 1978; Barrett & Dimmock, 1984b, c) and have described above how DI SFV inhibits virus multiplication. Possible explanations are that the assays may not be sufficiently sensitive (the RSIA detects > 10$^{5.15}$ DI particles/250 μl: Barrett et al., 1981), that DI virus which is registered by these assays does not interfere in mice or that DI virus is not propagated in the mouse.

Absence of histopathology and virus antigen in brains of mice treated with DI SFV

Brains from mice treated with either DI SFV p4 or p13a were obtained on days 4 and 11 post-infection and stained sections examined for histopathological changes. Mice infected with 10
Table 3. Immunity generated in mice inoculated with graded doses of virulent and avirulent SFV

<table>
<thead>
<tr>
<th>Dose inoculated (log₁₀ p.f.u.)*</th>
<th>Titre in brain at day 4 (log₁₀ p.f.u./mouse)</th>
<th>Survivors of primary infection</th>
<th>Survivors of challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus</td>
<td>Survivors of survivors in brain at day 4</td>
<td>†</td>
<td>‡</td>
</tr>
<tr>
<td>Virulent 2.8</td>
<td>&lt;1.6, 8.4, 8.3, 8.9</td>
<td>2/5</td>
<td>40</td>
</tr>
<tr>
<td>1.8</td>
<td>All &lt;1.6</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>0.8</td>
<td>All &lt;1.6</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>1.8</td>
<td>All &lt;1.6</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>Avirulent 6.6</td>
<td>7.1, 6.9, 6.5, 5.9</td>
<td>4/5</td>
<td>80</td>
</tr>
<tr>
<td>5.6</td>
<td>6.5, 6.2, 6.2, 6.2</td>
<td>3/5</td>
<td>60</td>
</tr>
<tr>
<td>4.6</td>
<td>6.4, 8.2, 6.2, 6.4</td>
<td>3/5</td>
<td>60</td>
</tr>
<tr>
<td>3.6</td>
<td>6.4, 6.2, 1.3, 6.6</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>2.6</td>
<td>3.0, &lt;1.3, &lt;1.3, &lt;1.3</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>1.6</td>
<td>All &lt;1.3</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>None§</td>
<td>–</td>
<td>5/5</td>
<td>100</td>
</tr>
</tbody>
</table>

* Virus was inoculated intranasally in a vol. of 20 μl as described in Methods. 10²⁻⁸ p.f.u. virulent SFV is 1 LD₅₀.

Virulent SFV is the standard virus used throughout.

† Number of mice surviving/number of mice inoculated.

‡ Number of mice surviving/number of mice inoculated. Challenged with 100 LD₅₀.

§ Mock-infected with medium only.

LD₅₀ + UV-SFV showed extensive neuronolysis throughout the brain including the olfactory bulbs, occasional but pronounced focal degeneration notably in the pyramidal cell layer of the hippocampus and mild perivascular cuffing as described previously (Crouch et al., 1982). However, brains from DI virus-treated mice were indistinguishable from mock-inoculated controls on both days tested. The presence of virus antigen in brains of infected mice not treated with DI virus could be readily demonstrated by immunochemical staining and was sometimes coincident with pathological lesions. However, some apparently healthy neurons also contained antigen, for example in the pyramidal cell layer of the hippocampus and in the cerebral cortex. Positive immunohistochemical staining of virus antigen was also found in the cytoplasm of otherwise healthy neurons and glial cells, particularly in thalamic nuclei, substantia nigra and brain stem. Cells in spinal cord and Purkinje cells in cerebellum were also sometimes positive. Conversely, in some anatomical areas there were sites of histopathological change such as cellular degeneration and perivascular cellular reaction which contained no demonstrable antigen. Similarly, olfactory lobes did not show marked concentrations of antigen by immunohistochemistry. No virus antigen was detected in brain or spinal cord of DI virus-treated mice.

DISCUSSION

In this study we have shown that the two DI SFV preparations examined, p4 and p13a, both inhibit the multiplication of standard virus in the mouse. Inoculation of mice with 10 LD₅₀ plus an amount of UV-SFV equivalent to the DI virus showed that modulation was not due to blocking of cellular receptors or to the immunogenicity of DI SFV. Virulent SFV had low infectivity levels throughout infection, while DI SFV p4-treated mice had infectivity titres identical to untreated mice for the first 2 days of infection (Table 4). Titres in p4-treated mice then declined and from day 3 onwards were similar to those in p13a-treated mice. In both experimental groups virus was cleared by day 7 with one exception which had a high level of brain infectivity at day 14 post-infection. Thus, we have the situation in DI virus-treated mice that standard virus is no longer virulent and multiplication of standard SFV is contained by some unknown mechanism as a subclinical infection. This is all the more remarkable as Table 3 demonstrates that, in the absence of DI virus, any mouse that is infected develops a lethal encephalitis (i.e. ID₅₀ = LD₅₀).

The infectivity data reported above present a paradox since infected mice treated with DI SFV p4 have the same distribution and amount of infectivity at day 2 post-infection as untreated mice and we therefore would expect there to have been a sufficient immunogenic stimulus to
generate a protective immunity. However, these mice were unable to resist challenge by 100 LD₅₀ SFV at 3 weeks after infection and the disease followed its usual lethal course (Barrett & Dimmock, 1984c). Conversely, as mice treated with DI virus p13a had much lower levels of infectivity, one might have expected immunity to be impaired but such mice resisted challenge completely.

Levels of neutralizing antibody in both sets of mice were similar and less than in mice that had experienced infection with avirulent SFV; the distribution of titres in mice treated with DI virus p4 or p13a were also very similar (Barrett & Dimmock, 1984c; Table 1). One possible explanation is that infected mice treated with DI virus p13a synthesize an immunizing amount of non-infectious SFV antigen but this appears unlikely as immunocytochemistry failed to show the presence of SFV antigen in sections of brain and there was no sign of immune cell infiltration. The failure of mice to establish protective immunity may therefore be due to an immunosuppression peculiar to DI virus p4, although this is unlikely to be general immunosuppression. We know of no precedent for this observation.

In an attempt to resolve the problem outlined above, mice were infected intranasally with different quantities of virulent or avirulent SFV to determine whether, in the presence of low or undetectable levels of SFV, mice could develop immunity to challenge (Table 3). Firstly, the results show that for virulent SFV the LD₅₀ is also the ID₅₀ and mice either develop high titres of virus in brain or none at all. This contrasts with the intermediate levels of infectivity seen above in infections involving DI virus. Secondly, mice inoculated with less than 1 LD₅₀ had no detectable virus in brain at day 4 post-infection and were susceptible to subsequent challenge with 100 LD₅₀. (An odd survivor is also seen on occasion in mice with no previous experience of SFV.) Inoculation of mice with > 1 LD₅₀ equivalent of avirulent SFV (i.e. > 10⁶ p.f.u.) resulted in virus titres in brain of over 10⁶ p.f.u. on day 4 post-infection (the peak of infectivity; A. D. T. Barrett & N. J. Dimmock, unpublished observations) and these survived challenge with 100 LD₅₀. The majority of mice inoculated with 10¹⁶ or 10²⁶ p.f.u. did not have virus in brain at day 4 and did not survive challenge. We therefore conclude that, in an SFV infection where DI virus has not been inoculated, mice that have no detectable virus in brain develop no protective immunity and will succumb to challenge. It is self evident that mice treated with DI SFV p13a together with 10 LD₅₀ and which subsequently resist challenge by 100 LD₅₀ must express sufficient SFV antigen to stimulate protective immunity; however, the nature and distribution of this SFV antigen is unknown at present.

Despite clear evidence that administration of DI SFV prevents disease and death (Barrett & Dimmock, 1984b) and inhibits standard virus multiplication, we were unable to detect DI virus in brain or olfactory lobes. It may be that the concentration of DI virus is low and cannot be detected by the assays that register > 10⁵⁻⁷ DI particles/ml. Indeed, in a previous study an amplification step did reveal the presence of DI virus in mouse brain (Dimmock & Kennedy, 1978). A similar problem of low levels of DI VSV after inoculation into adult mice was ascribed to the age of the host (Holland & Villareal, 1975) as DI virus could be propagated and even generated in the brains of newborn animals. Alternatively, the DI virus which interferes in vivo may be different from the DI virus active in tissue culture and undetectable in assays in vitro. The latter seems unlikely but since DI SFV RNA is heterogeneous (Kääriäinen et al., 1981) the possibility cannot be excluded.

Analysis of the infectivity titres in DI SFV p13a-treated mice revealed that 14-5% showed an anomalous distribution of infectivity whereas distribution of infectivity in mice treated with DI
Inhibition of SFV multiplication by DI virus

SFV p4 was always indistinguishable from the untreated control. In these p13a-treated mice one or more tissues contained a disproportionate amount of virus, as if DI virus was failing to inhibit multiplication in that particular tissue. These results indicate that as well as controlling the level of virus multiplication in infected tissues, DI SFV can cause a more subtle, possibly tissue-specific, modulation of infection. Furthermore, as the mice referred to above, including the individual with brain infectivity at day 14, were clinically normal, it is interesting to speculate that they may have survived carrying a persistent infection which has the potential to produce disease in later life.

In the above discussion we have dealt with the ways in which two DI SFV preparations differ from each other without commenting on the possible basis of this difference. However, others have found that SFV DI preparations produced in BHK cells in a similar way to p4 and 13a were physically heterogeneous and had sequences that differed as a result of deletions, sequence rearrangements and repetitions of parts of the standard virus genome (Kääriäinen et al., 1981; Pettersson, 1981; Söderlund et al., 1981; Lehtovaara et al., 1981, 1982). We therefore suggest that it is possible that the two DI preparations p4 and p13a, which we find differ in their interference properties, do so because of differences in the sequence of their RNAs.

A.D.T.B. was supported by an S.E.R.C. studentship and this project was funded in part by the Medical Research Council. Actinomycin D was the kind gift of Merck, Sharp and Dohme.

REFERENCES


A.D.T.B. was supported by an S.E.R.C. studentship and this project was funded in part by the Medical Research Council. Actinomycin D was the kind gift of Merck, Sharp and Dohme.


*(Received 16 May 1984)*