Infection of the Adrenal Gland as a Route to the Central Nervous System after Viraemia with Herpes Simplex Virus in the Mouse

By T. J. HILL,* D. L. YIRRELL AND W. A. BLYTH

Department of Microbiology, The Medical School, University Walk, Bristol BS8 1TD, U.K.

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SUMMARY

Intravenous inoculation of 4-week-old female NIH (inbred) mice with herpes simplex virus type 1 (HSV-1) strain P2C6 (defective in thymidine kinase) produced bilateral hind limb paralysis in nearly all animals by the 5th day after inoculation; very few mice died. In male mice the incidence and severity of paralysis was considerably lower than in females. The parental strain, CL(101), produced similar paralysis but all mice died by day 7. Observations on paralysis and death after intravenous inoculation are given for other strains of HSV-1 and HSV-2. By day 1 after inoculation of P2C6 significant virus replication had occurred in the adrenal glands but in none of the other organs tested. Titres of virus were similar in the adrenal glands of male and female mice. Histology of the adrenals showed most extensive replication in the cortex with some involvement of the medulla, particularly at the corticomedullary junction. By the 2nd and 3rd days, virus was detected in the lower thoracic spinal cord of both male and female animals but clearance was possibly quicker from males. Adrenalectomy proved that virus reached the cord via the adrenals. In the cord the infection was associated with bilateral demyelination in the ventral white matter as early as day 3.

INTRODUCTION

There is extensive evidence for the spread of herpes simplex virus (HSV) within the nerves of the peripheral and central nervous system (Hill, 1985). Although haematogenous spread of virus has been reported, particularly in young animals (Johnson, 1964; Kern et al., 1973; Lascano & Berria, 1980) this route does not appear to be of major importance in experimental or natural infection with HSV. Nevertheless, intravenous inoculation of inactivated or thymidine kinase (TK)-deficient virus has been used experimentally to induce ‘split tolerance’ [suppression of delayed-type hypersensitivity to HSV (Nash et al., 1981; Altmann & Blyth, 1985)].

During such experiments with a TK-deficient virus we observed that virtually all inoculated mice developed hind limb paralysis if a sufficiently high dose of virus was used. The relative avirulence of the TK-deficient viruses has allowed us to make a long-term study of the pathogenesis of this paralysis. The results of these experiments have revealed the extreme susceptibility of the adrenal gland to infection with HSV and the crucial role of this gland in infection of the nervous system after viraemia with this virus.

METHODS

Viruses. The following viruses were used: HSV-1 strain SC16, HSV-2 strain AR11 (Hill et al., 1975); HSV-1 strain KOS (kindly supplied by Dr B. Roizman, Chicago, Ill., U.S.A.); HSV-2 strain HG52 (kindly supplied by Dr G. B. Clements, Institute of Virology, Glasgow, U.K.); HSV-1 CL(101) and two of its TK-deficient mutants, CL(101)TK- and P2C6 (kindly supplied by Dr H. J. Field, Cambridge, U.K.). CL(101)TK- was selected by growth in 5-bromodeoxyuridine (Dubbs & Kit, 1964), P2C6 was selected by growth in acyclovir (Field & Darby, 1980).

Mice. NIH/Ola inbred mice were originally obtained from Olac 1976 Ltd (Bicester, U.K.); they were maintained as a breeding colony in the department. All were used at 4 weeks old and except where stated were female.
**Inoculation.** Unanaesthetized mice were inoculated with 0.2 ml of virus suspension in maintenance medium into a lateral tail vein.

For inoculation into the facial vein, mice were anaesthetized with an intraperitoneal injection of sodium pentobarbitone. A small skin incision was made over the right facial vein and 0.2 ml of virus suspension was inoculated into the vein through a 30-gauge needle. The skin incision was closed with a 4/0 silk suture.

**Scoring system for hind limb paralysis.** The severity of hind limb paralysis was scored as follows: 1, least detectable paralysis; 2, obvious difficulty in movement of hind limbs; 3, severe but incomplete paralysis; 4, severe paralysis with movement limited to the paws; 5, complete paralysis.

**Isolation of virus from tissues.** Mice were killed by intraperitoneal injection of sodium pentobarbitone. The following tissues were removed (not all in every experiment) and ground immediately in 0.4 ml of maintenance medium: the adrenal glands (in some experiments the two glands from each mouse were ground together), slices of liver from separate lobes, a quarter of one kidney (including cortex and medulla), a middle section of the spleen (approximately one-sixth of the whole organ), a quarter of one lung, heart muscle from the tip of the ventricles (approximately one-sixth of the whole organ), a piece of pectoral muscle equivalent in size to the sample of heart muscle, an eye, the cervical spinal cord, thoracic cord from 1st to 5th ribs, thoracic cord from 5th to 11th ribs, thoracic cord either side of the penultimate ribs, thoracolumbar cord (either side of the last ribs), lumbar spinal cord, a coeliac ganglion, cervical ganglia 3, 4, 5 and lumbar ganglia 2, 3, 4 (each from one side) and the brainstem (this was ground in 0.8 ml medium). From each tissue suspension two 50 μl samples and when necessary serial tenfold dilutions were inoculated to Vero cells grown in Multidishes (Sterilin) for assay of p.f.u. Blood was taken from the thoracic cavity and diluted with an equal volume of 2% sodium citrate solution. Two samples each of 100 μl were inoculated to Vero cells. After absorption for 1 h the cultures with brain or blood were washed with 0.5 ml medium before addition of medium with methyl cellulose; others were not washed. A minimum of 4 p.f.u. could be detected from each tissue sample except blood (minimum 10 p.f.u.) and brainstem (8 p.f.u.).

**Adrenalectomy.** Three-week-old mice were anaesthetized by i.p. injection of sodium pentobarbitone and subcutaneous injection of a mixture of fentanyl citrate and fluanisone (Hynorm, Crown Chemical Co. Ltd, Lamberhurst, U.K.). An incision was made through the shaved skin of the abdominal wall just ventral to the left kidney. Fine forceps were clamped firmly across the adrenal blood vessels for 30 s and the left adrenal gland was then removed. The abdominal muscle and skin were closed separately with 4/0 silk sutures. The same procedure was used for the mock operation but the blood vessels were not clamped and the gland was not removed.

**Electron microscopy.** Mice were killed by i.p. injection of sodium pentobarbitone, the thorax was opened and a small incision was made in the right atrium of the heart. Ten ml of saline was perfused through the animal via a 25-gauge needle inserted into the left ventricle. This was followed by 20 ml of a mixture of 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M-cacodylate buffer at pH 7.3. Tissues were then excised and processed for electron microscopy.

**Immunoperoxidase staining.** Adrenal glands were fixed in Bouin’s solution, dehydrated and embedded in paraffin wax. Paraffin sections were processed for staining by the peroxidase-antiperoxidase (PAP) method to demonstrate HSV antigens. A polyclonal rabbit antiserum to HSV-1, swine antiserum to rabbit immunoglobulins, rabbit antiperoxidase complex and normal swine serum were all obtained from Dakopatts Ltd (High Wycombe, U.K.).

**RESULTS**

**Clinical disease after intravenous inoculation of HSV**

Groups of 20 to 30 mice were inoculated intravenously with 10⁶ p.f.u. P₂C₆ and observed daily. On the 4th day after inoculation some (0 to 50% in different groups, mean 33%) developed weakness or paralysis of one or both hind legs. By the 5th day almost all mice were bilaterally affected to some degree varying from slight weakness and loss of movement to complete flaccid paralysis (Fig. 1). In the more severely affected animals the tail was also paralysed and the animals became incontinent with respect to both urination and defaecation. In spite of this the animals did not appear generally ill; indeed, from the thorax forward they appeared normal. From about 10 days after inoculation some animals recovered the ability to move the hind legs. Those less severely affected sometimes recovered completely but there was often some residual weakness. Partial recovery was common even from complete hind paralysis but some animals were permanently paralysed. On occasions, from about 2 weeks to 3 months after inoculation, some animals died. They were usually those which suffered permanent or at least partial paralysis but occasionally animals died after substantial recovery. The proportion of animals that died varied greatly in different groups ranging from 3 to 70% with a mean of 37%.

Decrease in the dose of strain P₂C₆ resulted in decreased severity and incidence of paralysis. With 10⁵ p.f.u., 60 of 119 animals were affected; only 19 had a score of 3 or more. With 10⁴ p.f.u.
only three of 20 animals were affected. Male mice were considerably less likely to suffer paralysis after intravenous injection of virus. With 10⁶ p.f.u. only 23/86 (27%) showed slight hind limb paralysis which was at a maximum 8 days after inoculation. The mean score for affected animals was 2.

Different strains of virus also produced different clinical results (Table 1). Strain CL(101)TK⁻, a further variant of CL(101) (also deficient in TK), induced no clinical signs when 10⁶ p.f.u. were inoculated (except in one of 57 animals with a paralysis score of 1). Strain CL(101) was far more virulent than its derivatives defective in TK activity. Only nine of 24 animals survived an inoculation of 10⁴ p.f.u. Of the survivors, three showed severe or complete paralysis; five were less severely affected. Strain KOS was of intermediate virulence.

Another virulent strain, SC16, was also tested. Animals inoculated intravenously with 10⁵ p.f.u. died, as did virtually all those which showed hind limb paralysis after inoculation with 10⁴ p.f.u. Two relatively avirulent strains of HSV-2 (HG52 and AR11) also produced hind limb paralysis after intravenous inoculation. With both, the clinical signs developed later than with the HSV-1 strains. This was particularly noticeable with strain HG52 which also produced only monolateral paralysis in a proportion of animals.

Fig. 1. Hind limb paralysis in male (○, ○) and female (■, □) mice after intravenous inoculation of 10⁶ p.f.u. HSV-1 strain P3C6. Percentage of mice with paralysis (○, ■) and the mean score of affected mice (○, □) (see Methods) were determined.
Table 1. Paralysis and death in mice inoculated intravenously with various strains of HSV-1 or HSV-2

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Dose</th>
<th>Mean day of onset of paralysis (range)</th>
<th>No. with paralysis/survivors</th>
<th>Mean score of affected mice</th>
<th>% Survivors of total inoculated</th>
<th>No. with paralysis/survivors</th>
<th>Mean score of affected mice</th>
<th>% Survivors of total inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>P,C6*</td>
<td>$10^6$</td>
<td>5.3 (5-6)</td>
<td>20/70 (29)‡</td>
<td>2.0</td>
<td>100</td>
<td>4/23 (17)‡</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>P,2C6</td>
<td>$10^6$</td>
<td>–§</td>
<td>3/20 (15)</td>
<td>1.7</td>
<td>99</td>
<td>16/45 (36)</td>
<td>2.6</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>–</td>
<td>60/119 (50)</td>
<td>2.4</td>
<td>99</td>
<td>60/69 (87)</td>
<td>2.9</td>
<td>95</td>
</tr>
<tr>
<td>CL(101)TK-</td>
<td>$10^6$</td>
<td>4.7 (4-8)</td>
<td>224/257 (87)</td>
<td>3.6</td>
<td>99</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL(101)</td>
<td>$10^4$</td>
<td>5.9 (5-7)</td>
<td>14/15 (93)</td>
<td>3.6</td>
<td>63</td>
<td>7/10 (70)</td>
<td>2.0</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>$10^3$</td>
<td>4.6 (4-6)</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC16</td>
<td>$10^3$</td>
<td>–</td>
<td>0/20</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>–</td>
<td>2/27 (7)</td>
<td>1.5</td>
<td>64</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>5.3 (4-7)</td>
<td>9/10 (90)</td>
<td>4.6</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^6$</td>
<td>–</td>
<td>1/27</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KOS</td>
<td>$10^4$</td>
<td>5.7 (5-7)</td>
<td>7/17 (41)</td>
<td>2.1</td>
<td>81</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^3$</td>
<td>4.8 (4-5)</td>
<td>14/16 (88)</td>
<td>2.6</td>
<td>40</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^6$</td>
<td>4.3 (4-5)</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HG52†</td>
<td>$10^5$</td>
<td>9.8 (6-21)</td>
<td>1/20 (5)</td>
<td>3.0</td>
<td>100</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^6$</td>
<td>9.8 (6-21)</td>
<td>11/98 (11)</td>
<td>2.7</td>
<td>100</td>
<td>19/71 (27)</td>
<td>2.7</td>
<td>72</td>
</tr>
<tr>
<td>AR11†</td>
<td>$10^4$</td>
<td>10.5 (9-14)</td>
<td>0/15</td>
<td>100</td>
<td>9/13 (69)</td>
<td>2.8</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>8.3 (7-12)</td>
<td>3/15 (20)</td>
<td>1.7</td>
<td>100</td>
<td>7/7 (100)</td>
<td>2.7</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>$10^6$</td>
<td>7.7 (7-12)</td>
<td>8/14 (57)</td>
<td>3.3</td>
<td>93</td>
<td>7/7 (100)</td>
<td>4.0</td>
<td>47</td>
</tr>
</tbody>
</table>

* Male mice; all others were female.
† HSV-2.
‡ Numbers in parentheses are percentages.
§ – Not available.
Table 2. *Isolation of P₂C₆ from tissues during the 2 h after intravenous inoculation of 10⁶ p.f.u.*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Isolation/total</th>
<th>Titre*</th>
<th>Isolation/total</th>
<th>Titre*</th>
<th>Isolation/total</th>
<th>Titre*</th>
<th>Isolation/total</th>
<th>Titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>14/14</td>
<td>2.1</td>
<td>6/6</td>
<td>1.7</td>
<td>6/6</td>
<td>1.6</td>
<td>4/6</td>
<td>1.1</td>
</tr>
<tr>
<td>Heart</td>
<td>14/14</td>
<td>2.5</td>
<td>6/6</td>
<td>2.6</td>
<td>6/6</td>
<td>2.2</td>
<td>6/6</td>
<td>1.6</td>
</tr>
<tr>
<td>Adrenal</td>
<td>16/18</td>
<td>2.0</td>
<td>6/6</td>
<td>1.8</td>
<td>6/6</td>
<td>1.8</td>
<td>6/6</td>
<td>1.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>7/8</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>3/12</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0/24</td>
<td>–</td>
<td></td>
<td>ND</td>
<td></td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Lung</td>
<td>8/8</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectoral muscle</td>
<td>8/8</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* log₀ Mean titre of those with virus, per specimen or from blood, per ml.
† ND, Not done.

To find whether the site of paralysis depended on the site of inoculation, 11 animals were inoculated intravenously into the right facial vein with 10⁶ p.f.u. P₂C₆. Ten showed typical hind limb paralysis (two were severely affected); none showed abnormality elsewhere.

**Clinical effects of virus inoculation after removal of one adrenal gland**

Animals were inoculated with 10⁶ p.f.u. P₂C₆ 1 week after removal of the left adrenal gland. All 11 control animals (with mock removal of the gland) showed bilateral paralysis of the hind legs. After removal of the left adrenal gland none of 10 animals showed clinical abnormality of the left hind leg but eight suffered paralysis of the right leg.

**Isolation of HSV after inoculation intravenously**

Animals were inoculated with 10⁶ p.f.u. P₂C₆, and at intervals during the following 2 h tissues were taken for virus isolation (Table 2). Clearly, all the tissues contained blood but no effort was made to separate this. After 2 h the amounts of virus isolated were very small. Attempts to isolate virus were also made at daily intervals during the week after inoculation (Table 3). Virus was never isolated from the spleen and only sporadically from the liver. However, the eye contained virus more frequently. Consistent isolation was obtained only from adrenal glands and spinal cord, and so these tissues were investigated in more detail.

**Isolation of virus from adrenal glands**

One adrenal gland was removed from each of a group of animals daily after inoculation with 10⁶ p.f.u. P₂C₆ and virus in the tissue was titrated (Table 3). Peak titres of about 10⁵ p.f.u. were reached 2 to 3 days after inoculation; thereafter, the amount of virus and the proportion of animals from which it was obtained rapidly decreased. Growth of P₂C₆ in the adrenal glands of male mice was similar to that in female mice. Growth of strains CL(101), CL(101)TK⁻ and SC16 in the adrenal glands of female mice was also studied (Table 4). In more limited experiments virus was isolated from the adrenal glands of five of six animals 1 day after intravenous inoculation of 10⁵ p.f.u. of strain HG52 or 10⁴ p.f.u. of strain KOS. Adrenal glands of all of six animals contained virus 1 day after inoculation of 10⁵ p.f.u. strain KOS.

**Isolation of virus from the spinal cord**

At daily intervals after inoculation of 10⁶ p.f.u. P₂C₆, sections of cord were examined for the presence of virus (Table 5). The cord was frequently infected even 1 day after inoculation intravenously. Subsequently virus was isolated with increasing frequency so that almost all samples of cord from the thoracic region yielded virus from 4 to 7 days after inoculation. Virus was less consistently present in the lumbar cord, and disappeared sooner. It was not isolated from the cervical cord. From male mice, virus was sought from one section of the cord comprising the lower thoracic and upper lumbar regions. Virus was isolated from nearly all
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Table 3. Isolation of $P_2C_6$ from tissues during the 4 days after inoculation of $10^6$ p.f.u. intravenously

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time after inoculation (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Isolation/total</td>
</tr>
<tr>
<td>Blood</td>
<td>5/6</td>
</tr>
<tr>
<td>Eye</td>
<td>1/6</td>
</tr>
<tr>
<td>Kidney</td>
<td>0/6</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/6</td>
</tr>
<tr>
<td>Liver†</td>
<td>15/18</td>
</tr>
<tr>
<td>Lung</td>
<td>3/6</td>
</tr>
<tr>
<td>Heart</td>
<td>0/4</td>
</tr>
<tr>
<td>Adrenal female‡</td>
<td>16/16</td>
</tr>
<tr>
<td>Adrenal male‡</td>
<td>12/12</td>
</tr>
</tbody>
</table>

* log$_{10}$ Mean p.f.u./specimen (blood p.f.u./ml) of those with virus.
† Three specimens from each of six animals.
‡ Females, one adrenal/animal; males, both adrenals together.
§ ND, Not done.
† Also 0/6 on days 5, 6 and 7.
¶ Nine of ten specimens mean log$_{10}$ titre 1.3 (range 0.6 to 1.7). The 10th specimen had log$_{10}$ titre of 3.2.
** Day 5, 7/12 (titre 1.3); days 6 and 7, 0/4.
†† Day 5, 1/12 (titre 1.7); days 6 and 7, 0/12.

animals by the 3rd day after inoculation and for the first 6 days titres were indistinguishable from those in the cords of female animals. No virus was isolated after the 6th day. When strain CL(101)TK$^-$ was sought in the lower thoracic cord of female mice after inoculation of $10^6$ p.f.u. intravenously, one of six mice yielded virus on each of days 2 and 3 (titres 4.5 x $10^2$ and 4.4 x $10^1$ respectively). On the 4th day virus was isolated from one of 12 specimens ($10^3$ p.f.u.) but no isolation was made from six animals tested 1, 5, 6 or 7 days after inoculation.

After inoculation of $10^6$ p.f.u. $P_2C_6$ virus was sought in ganglia and brainstem (Table 5). It was never found in the dorsal root ganglia tested and only rarely (at very low titre) in coeliac ganglia. However, it was isolated from the brainstem of some mice 4 and 5 days after inoculation.

**Histology of the adrenal gland and spinal cord after intravenous inoculation of $10^6$ p.f.u. $P_2C_6**

One day after inoculation, foci of cells productively infected with HSV were seen by electron microscopy in the zona fasciculata and zona reticularis of the adrenal cortex. Sections of adrenal gland stained with PAP revealed that these areas of the cortex were the main sites of infection even at 4 days after inoculation (Fig. 2). However, as early as 1 day after inoculation foci of infection at the corticomedullary junction involved both cortical and medullary cells (Fig. 3).

Viral capsids were seen in the nuclei of endothelial cells in blood-vessels in the adrenal cortex 1 day after inoculation. By day 3 red blood cells were present in tissue spaces in the cortex and medulla. However, even on day 7 there was little evidence of infiltration by inflammatory cells into the cortex or medulla.

Demyelinated axons, unwrapping of myelin sheaths, oedema and cellular infiltration (predominantly macrophages with ingested myelin) were seen in the ventral white matter on both sides of the lower thoracic cord 3 days after inoculation with $P_2C_6$.

A detailed study of the histological changes in the adrenal gland and central nervous system (CNS) will be published separately.

**DISCUSSION**

If small amounts of a virus enter the bloodstream as a single inoculum it is likely that virtually all of it will quickly be removed by adsorption to the endothelium of blood-vessels, and by phagocytic cells of the reticuloendothelial system. However, when relatively large numbers of infective particles are introduced at a given time there is clearly the possibility, as happened in...
Table 4. *Isolation of HSV from adrenal glands of female mice after inoculation of different strains intravenously*

<table>
<thead>
<tr>
<th>Strain (p.f.u./animal)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isolation/total</td>
<td>Titre*</td>
<td>Isolation/total</td>
<td>Titre*</td>
<td>Isolation/total</td>
<td>Titre*</td>
<td>Isolation/total</td>
</tr>
<tr>
<td>SC16 (10^5)</td>
<td>4/5</td>
<td>5.5</td>
<td>4/4</td>
<td>7.0</td>
<td>4/4</td>
<td>7.2</td>
<td>t</td>
</tr>
<tr>
<td>CL(101) (10^5)</td>
<td>4/4</td>
<td>5.3</td>
<td>6/6</td>
<td>7.4</td>
<td>4/4</td>
<td>6.5</td>
<td>t</td>
</tr>
<tr>
<td>CL(101)TK- (10^6)</td>
<td>14/14</td>
<td>3.5</td>
<td>14/14</td>
<td>4.6</td>
<td>18/20</td>
<td>2.2</td>
<td>3/6</td>
</tr>
<tr>
<td>P₂C₆ (10^6)</td>
<td>16/16</td>
<td>4.8</td>
<td>24/24</td>
<td>4.8</td>
<td>12/12</td>
<td>3.3</td>
<td>7/12</td>
</tr>
</tbody>
</table>

* P.f.u./adrenal gland. For P₂C₆ only one gland was tested; for other strains both glands were tested together.
† Too few survivors to test.
Table 5. Isolation of \( P_2 C_6 \) from nervous tissues during the week after intravenous inoculation of \( 1 \times 10^6 \) p.f.u.

<table>
<thead>
<tr>
<th>Time after inoculation (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tissue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brainstem</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>0/12 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
</tr>
<tr>
<td>Coeliac ganglion</td>
<td>0/6 -</td>
<td>1/6 0.6</td>
<td>1/14 0.6</td>
<td>0/8 -</td>
<td>ND§</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cervical ganglia 3, 4, 5</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lumbar ganglia 2, 3, 4</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sections of spinal cord†</td>
<td>A 0/6 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>0/12 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
<td>0/6 -</td>
</tr>
<tr>
<td>B 1/5 0.6</td>
<td>5/5 1.9</td>
<td>9/11 3.8</td>
<td>6/6 5.1</td>
<td>6/6 3.6</td>
<td>11/12 3.4</td>
<td>9/9 2.1</td>
<td></td>
</tr>
<tr>
<td>C 1/5 0.6</td>
<td>2/5 2.3</td>
<td>9/11 4.4</td>
<td>6/6 4.3</td>
<td>6/6 3.7</td>
<td>12/12 2.9</td>
<td>8/9 1.6</td>
<td></td>
</tr>
<tr>
<td>D 3/9 0.6</td>
<td>5/9 1.0</td>
<td>18/21 4.1</td>
<td>20/20 5.1</td>
<td>15/16 4.1</td>
<td>11/16 3.8</td>
<td>7/13 2.5</td>
<td></td>
</tr>
<tr>
<td>E 3/4 1.2</td>
<td>2/4 1.1</td>
<td>4/10 1.5</td>
<td>12/14 1.9</td>
<td>9/10 1.5</td>
<td>0/4 -</td>
<td>2/4 0.8</td>
<td></td>
</tr>
<tr>
<td>F 0/12 -</td>
<td>0/12 -</td>
<td>3/12 0.6</td>
<td>7/18 0.8</td>
<td>2/6 2.7</td>
<td>0/6 -</td>
<td>1/6 1.7</td>
<td></td>
</tr>
<tr>
<td>One section of spinal cord comprising C, D and E‡</td>
<td>5/12 1.3</td>
<td>4/6 1.9</td>
<td>10/12 4.1</td>
<td>3/6 3.6</td>
<td>5/6 2.3</td>
<td>4/6 2.2</td>
<td>0/6 -</td>
</tr>
</tbody>
</table>

* \( \log_{10} \) Mean p.f.u./specimen which contained virus.
† A, Cervical; B, thoracic, 1st to 5th rib; C, thoracic 5th to 11th rib; D, thoracic either side of 12th rib; E, thoracolumbar junction; F, lumbar cord. In addition, section B, day 8, 6/6 (2.1); day 9, 6/6 (1.5); day 10, 2/6 (1.6); section C, day 8, 4/6 (1.5); day 9, 5/6 (1.6); day 10, 5/6 (0.9); section D, day 8, 6/6 (2.3); day 9, 6/6 (1.7); day 10, 5/6 (1.7). In a further experiment virus was not isolated from the cords of 12 mice tested on each of days 10 to 14, 21 or 28; specimens were cervical cord, whole thoracic cord, lumbar cord.
‡ Male mice, all others were females.
§ ND, Not done.
Fig. 2. Section of whole adrenal gland stained by the PAP method from a female mouse 4 days after intravenous inoculation with 10⁶ p.f.u. HSV-1 strain P₂C₆. Viral antigens (dark staining) are mainly in the inner cortex (arrows).

Fig. 3. Corticomedullary junction of adrenal gland from a female mouse 1 day after intravenous inoculation with 10⁶ p.f.u. HSV-1 strain P₂C₆. C, Cortical cells of the zona reticularis with intranuclear herpesvirus capsids; M, medullary cell with intranuclear capsids. Bar marker represents 2 μm.
this study, that some will reach many organs albeit in small amounts. The titres of virus found
15 min after inoculation suggest some localization of virus particularly in the heart, lung and
adrenal gland above that to be expected from the blood within the tissue. However (as was
expected), during the ensuing 2 h titres of infectious virus fell.

The clinical signs seen in animals inoculated with 10^6 p.f.u. of HSV-1 P2C6 suggested strongly
a bilateral lesion in the middle to lower section of the spinal cord. The complete absence of signs
from the thorax forward made it likely that virus was gaining access to the cord only via a
specific route rather than throughout its length from a variety of organs. Since inoculation of
virus into the facial vein produced identical clinical signs to those after inoculation into the tail
vein, tissue around the site of inoculation could not be the source of virus that induced the cord
lesion. The occurrence of unilateral rather than bilateral paralysis after removal of one adrenal
gland suggests strongly that this organ provides the main source of virus entering the cord.
Previously, Goodpasture & Teague (1923) reported that rabbits suffered hind limb paralysis
after inoculation of HSV directly into the adrenal gland. Our observations may also explain
those of Cook & Stevens (1976) who noted temporary hind limb paralysis in mice inoculated
intravenously with HSV.

Infection at sites other than the adrenal gland was neither so consistent nor so long-lived as
occurred in this organ and titres of infectious virus were at least 1000-fold lower in other organs.

Amounts of infective virus found in the adrenal glands within 2 h of inoculation were very
small so there is little evidence for major preferential sequestration of virus in the gland.

With HSV-1 P2C6, at least up to 6 days after inoculation (when practically all clinical disease
has developed), the time course of infection and the titres to which virus grows are virtually
identical in adrenal gland and spinal cord of male and female mice although the clinical signs
are dramatically more severe in female mice.

In a similar manner the difference in clinical response after inoculation of 10^6 p.f.u. of P2C6
(which produces severe paralysis) or CL(101)TK^- (virtually no paralysis) is hardly reflected in
the titres of virus found in adrenal glands. However, with strain CL(101)TK^- isolation of virus
from the cord was far less common and titres were far smaller than with strain P2C6 so that less
damage (and less paralysis) would be expected. These differences between the two strains could
reflect differing ability to spread to the medulla of the adrenal gland (see below) or to replicate
within the nervous system.

From the timing and incidence of isolation of virus from the spinal cord no conclusions can be
drawn as to the precise site of entry. However, once in the CNS virus can presumably spread
widely by axonal flow and by replication in glial cells (Hill, 1983). The route of entry is presumed
to be via the sympathetic nerve supply to the adrenal gland which enters the cord in the middle
to lower thoracic region. This route might explain the occasional isolation of very small amounts
of virus from the coeliac ganglion since nerve fibres from the gland pass through the ganglion
complex. This isolation might result from virus in transit along axons rather than from actual
infection of the ganglion.

Clinical, virological and preliminary histological observations provide evidence on the site of
damage in the spinal cord. Mice with complete hind limb paralysis had a reflex response, albeit
reduced, to pinching of a hind foot. This suggests that the sensory and lower motor neuron
supplies to the limb were intact or only partly affected. Furthermore, virus was isolated less
frequently, was present in smaller amounts and was cleared more rapidly from the lumbar cord
(the region giving rise to motor and sensory nerves of the hind limbs) than the thoracic cord.
Therefore, the most likely series of events leading to paralysis after intravenous inoculation of
HSV is as follows. (i) After replication in the adrenal gland virus enters the preganglionic nerve
fibres which supply the medulla. [The existence of a nerve supply to cortical cells is still
controversial (Neville & O'Hare, 1982). The fibres to medullary cells arise from neurons in the
intermediolateral column in the grey matter of the middle to lower thoracic cord and emerge via
the ventral roots.] (ii) Virus spreads through tracts of autonomic fibres in the ventral white
matter of the thoracic cord. We have observed demyelination and oedema in this region of the
cord as early as 3 days after intravenous inoculation of P2C6. Such demyelination is a common
sequel to HSV infection in the CNS (Hill, 1983). (iii) The infection and consequent
demyelination spreads from autonomic tracts to adjacent motor neuron tracts in the thoracic cord, thereby producing an upper motor neuron paralysis of the hind limbs.

The fact that no mice died during the period of primary infection with strain P2C6 presumably reflects failure of infection to spread to vital sites within the CNS even though CNS infection is established in all animals. Others (Field & Darby, 1980) have found that strains P2C6 and CL(101)TK- are relatively avirulent even when injected intracerebrally. Preliminary investigation of the later deaths suggests that they result from opportunistic bacterial infection of the urinary tract rather than from viral infection.

The parent strain [CL(101)] of the two TK- mutants and the wild-type strain of HSV-1 (SC16) tested were far more virulent than the mutants after intravenous inoculation as they are by other routes (Field & Darby, 1980). The higher titres attained by these virulent strains in adrenal glands could result from greater replication of TK+ viruses in the neural tissue of the medulla. By the 4th day after inoculation, interpretation of the titres of virus in adrenal glands is complicated by the possibility of re-seeding of the gland by virus from the nervous tissue (Field & Hill, 1974; Kapoor et al., 1982). The two virulent strains probably killed the animals by virtue of more efficient replication and spread within the CNS so that far more virus may have been available to re-seed the adrenal glands.

The involvement of the adrenal gland in experimental infection with HSV has been commented on by Smith (1931) who inoculated rabbits intravenously, subcutaneously, intracerebrally or into the testis, Slavin & Berry (1943) (intranasally in suckling mice), Cook & Stevens (1976) (intravenously in mice), Kapoor et al. (1982) (subcutaneously in nude mice), and Nachtigal & Caulfield (1984) (intranasally in mice). Further instances have been noted from clinical medicine [see Nachtigal & Caulfield (1984) for references].

Our observations and those of others (with presumed TK+ virus) (Slavin & Berry, 1943; Nachtigal & Caulfield, 1984) suggest that the cortex is the major site of infection. The high concentration of steroid hormones in the cortex, and in particular their immunosuppressive effects, may make part of the gland especially susceptible to infection with diverse organisms (Frenkel, 1960). Such local immunosuppression might explain the paucity of inflammatory cell infiltration seen during infections of the adrenal gland. However, it is noteworthy that intravenous inoculation of 'avirulent' strains of HSV causes changes perhaps quite separate from infection in the adrenal gland, namely induction of tolerance to delayed-type hypersensitivity through the stimulation of suppressor T lymphocytes (Nash et al., 1981).

Nevertheless, although viruses such as P2C6 and CL(101)TK- reached high titres in the gland, the infection was cleared completely by the 6th day after inoculation. This rapid clearance may have been facilitated by the early damage observed in the endothelial cells of blood-vessels of the cortex which would allow rapid access of antibody to the infected tissue. In their study, Nachtigal & Caulfield (1984) did not comment on such damage but in generalized HSV infection in humans adrenal haemorrhage is common (Tucker & Scofield, 1961). The decreased severity of clinical disease in male as compared with female mice may be connected with the possibly faster clearance of virus from the cord in males. However, the clinical signs develop well before differences in clearance are apparent. In general (Lahita, 1984) and with HSV (Knoblich et al., 1983), female mice have a more vigorous immune response than males so that faster clearance might have been expected in females. In view of this the more severe clinical signs in females suggest the possibility of some immunopathology.

Although in many human infections with HSV viraemia is not important, in neonatal infection (Bahrani et al., 1966; Arvin et al., 1982) and in immunosuppressed people (Montgomery et al., 1969) it probably plays a significant role in the disease. Involvement of the adrenal gland and the possibility of damage in the cord should be borne in mind when considering explanations of nervous system dysfunction associated with HSV infections (Klastersky et al., 1972; Craig & Nahmias, 1973; Shturman-Ellstein et al., 1976; Caplan et al., 1977; Oates & Greenhouse, 1978; White et al., 1984).

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REFERENCES


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