Atypical Patterns of Neural Infection Produced in Mice by Drug-resistant Strains of Herpes Simplex Virus

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SUMMARY

Mice inoculated intracerebrally (i.c.) with a mutant strain of HSV were found to develop cataracts 1 to 2 months after inoculation. Cataract formation was subsequently shown to follow an acute retinitis which commenced within 1 week of inoculation. The mutant had been selected for high resistance to the nucleoside analogue acyclovir and has been shown previously to be defective in the induction of thymidine kinase and also to express an altered DNA polymerase. The LD₅₀ for mice inoculated i.c. was >10⁵ p.f.u. compared with approx. 7 p.f.u. for the parental strain. Studies of virus replication following i.c. inoculation with a sublethal dose of the mutant revealed that only small amounts of infectious virus were produced in the brain, but during a period from 6 to 12 days after inoculation vigorous replication occurred in retinal tissue, producing very high titres of virus.

INTRODUCTION

It is now well-established that mutants of HSV which are defective in the induction of the virus-specified thymidine kinase (TK) have an impaired ability to replicate in vivo (Field & Wildy, 1978; Field & Darby, 1980; Tenser & Dunstan, 1979; Tenser et al., 1979). In particular, the TK viruses replicate poorly in the epidermal cells of the skin and in the cells of the nervous system. One of the most marked features of all the TK-defective viruses we have examined is their reduction in lethality following intracerebral (i.c.) inoculation (Field & Wildy, 1978; Field & Darby, 1980). Over 20 similar strains have been examined to date and, in all, the reduction in ‘neurovirulence’ has been >200-fold when compared with the parental virus.

One such virus is the HSV-1 mutant, SC16 R₉C₂, which was selected for high resistance to acyclovir (ACV). The biochemical properties of this mutant have been described previously (Field et al., 1980) and it was shown that the virus induced low levels of TK (approx. 3% of the parental strain) and there was an additional lesion, most likely in the virus-induced DNA polymerase. [The latter was suggested by the fact that the mutant was co-resistant to phosphonoacetic acid and also was resistant to ACV in transformed mouse L cells which express HSV TK (Darby et al., 1980).]

The mutant R₉C₂ appeared to grow very poorly in the skin of intradermally inoculated mice and following i.c. inoculation the LD₅₀ was >10⁵ p.f.u. compared with the parental strain, SC16, for which the LD₅₀ i.c. was 7 p.f.u. in Balb/c mice. However, it was noted that surviving mice which had been inoculated i.c. with relatively large doses of the mutant developed cataracts 1 to 2 months after inoculation. The present study was designed to elucidate this curious observation and describes the virological events in tissues leading to ocular damage. Some comparative data were also obtained using different routes of inoculation and several other virus mutants.
Methods

Viruses. The principal virus strain used was SC16 R₉C₂ (Field et al., 1980) derived from HSV-1 SC16 (Hill et al., 1975) following 10 passages of the parental strain in the presence of increasing concentrations of ACV. Other viruses used were: SC16 R₉C₁ and SC16 S₆ both ACV-selected mutants of SC16 (Field et al., 1980; Darby et al., 1981), Bry TK⁻ which is a bromodeoxyuridine-selected mutant of the HSV type 2 strain, Bry (Thouless, 1972; Field & Wildy, 1978) and finally the strain C1(101)TK-p7. The latter was selected for increased resistance to ACV (Field et al., 1980) and was derived from the bromodeoxyuridine-selected mutant of C1(101) first isolated by Dubbs & Kit (1964).

Virus inoculation. For i.c. inoculation, 20 μl virus suspension were injected into the left cerebral hemisphere (to a depth of 1 to 2 mm) of anaesthetized 3-week-old female Balb/c mice. Groups of 10 mice were inoculated i.c. with doses of virus from 10¹ to 10⁴ p.f.u./mouse. For the sequential histological study a single group of 30 mice was inoculated with 10⁴ p.f.u./mouse. For intravenous inoculation 0.1 ml virus suspension were injected into the tail vein.

Isolation of virus from tissue. Tissue samples were homogenized in Glasgow-modified Eagle's medium (GM) using 3 ml glass grinders. For brain samples the debris was removed by centrifuging at 250 g for 10 min. The fluid was then diluted and the amount of infectious virus present determined by plaque titration using BHK-21 cells. For the reactivation of infectious virus from latently infected tissue the samples were cultured at 37 °C in GM supplemented with 1% calf serum for 6 days then tested for virus as above. Brain was cultured either whole or in small fragments. These techniques have been described previously in more detail (Field et al., 1979).

Microscopy. Thirty mice were sacrificed at intervals between 4 days and 8 weeks post-inoculation. The brain, eyes and optic nerves were dissected free from the skull and fixed in buffered formol-saline for light microscopy. Prior to processing and embedding in paraffin wax, the surface of the left cerebral hemisphere was lightly painted with Indian ink, so that it could be identified with certainty in histological sections. The eyes with optic nerves in continuity were serially sectioned at 5 μm intervals. The 5 μm sections were taken at 100 μm intervals through each brain. Alternate sections were stained with haematoxylin and eosin. The following special stains were also employed: solochrome cyanin and Weil's haematoxylin for myelin, elastic ponceau S for collagen, Mallory's phosphotungstic acid haematoxylin for glia, Gordon and Sweets' method for reticulin fibres and periodic acid–Schiff and Palmgren's silver stain for axons.

For immunofluorescent staining, unfixed tissue was frozen by immersion in liquid nitrogen and sectioned on a cryostat (Cambridge Instruments). The tissue sections were then fixed for 15 min in cold acetone and stained by an indirect technique (Coons & Kaplan, 1950) using hyperimmune rabbit anti-HSV-1 serum and fluorescein isothiocyanate conjugated to sheep anti-rabbit IgG (Wellcome Reagents). Specimens were examined on a Leitz Orthoplan microscope using incident illumination.

Test for delayed-type hypersensitivity (DTH). Antigen was prepared by heating a preparation of SC16 for 30 min at 50 °C. The material was then diluted in GM to give a dose of 10⁶ erstwhile p.f.u. and injected into the ear pinna in a volume of 20 μl. The thickness of the ear pinna was then measured after 24 and 48 h. Negligible ear thickening was observed in control (previously uninfected) mice while the positive reaction was manifested by an increase in ear thickness of two- to threefold (Nash et al., 1980).

Results

Production of ocular opacity in mice inoculated i.c. with SC16 R₉C₂

Following i.c. inoculation with SC16 R₉C₂ the onset of obvious lens opacity occurred in all
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Table 1. Incidence of cataract formation in mice following i.e. inoculation with various doses of SC16 RgC₂

<table>
<thead>
<tr>
<th>Inoculum dose i.e. (p.f.u./mouse)</th>
<th>No. showing corneal opacity*/*</th>
<th>no. in survivors† group</th>
</tr>
</thead>
<tbody>
<tr>
<td>10¹</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>10²</td>
<td>2/9</td>
<td>2/9 (20%)</td>
</tr>
<tr>
<td>10³</td>
<td>3/7</td>
<td>3/7 (43%)</td>
</tr>
<tr>
<td>10⁴</td>
<td>9/26</td>
<td>9/26 (35%)</td>
</tr>
</tbody>
</table>

* Observed up to 3 months post-inoculation.
† All mice survived except in the 10⁴ p.f.u. inoculated group.
‡ Fifty-nine mice were inoculated; two died during the 2nd week post-inoculation. Thirty-one mice were sampled randomly during the first 3 weeks for virus isolation and histological examination.

cases between the 7th and 12th week. In almost all cases the condition was bilateral but occasionally whiteness appeared in the left eye (inoculated side) 1 to 7 days before the right. The total numbers of mice which developed ocular opacity after several different inoculum doses are shown in Table 1. All mice survived the smaller doses but, of the mice inoculated with 10⁴ p.f.u., two mice died during the 2nd week post-inoculation, i.e. during the acute phase of the infection. Subsequently, during the period 2 to 3 months post-inoculation 2 of the 26 remaining mice (both blind) showed acute signs of neurological disease. One of these mice survived; the other became progressively affected and was killed in extremis. At this stage all virus cultures of homogenates of ocular and CNS tissue were negative. This intriguing observation of late neurological sequelae will be followed up in a future study which requires larger numbers of mice.

Microscopy

Intracerebral injection of the mutant virus SC16 RgC₂ elicited a low grade encephalitis, concentrated in the vicinity of the injection site, in the left cerebral hemisphere. Six days post-inoculation there was perivascular cuffing by chronic inflammatory cells and a few polymorphonuclear neutrophil leukocytes, around the haemorrhagic needle track, which usually extended from the cerebral cortex into the thalamus. There was also a light chronic inflammatory cell infiltrate in the leptomeninges and scattered subependymal inflammatory foci particularly in the walls of both third and lateral ventricles. The localized perivascular cuffing persisted for at least 3 weeks, but none of the animals showed a generalized encephalitis. Although a peak of virus replication in the eyes was demonstrated at 8 days post-inoculation (see below), the onset of histological changes was more variable, being detectable in several animals 7 to 8 days post-inoculation, but in others delayed, and in two animals only just developing at 14 days. The sequence of ocular inflammation was almost simultaneous in both eyes. The initial changes were sparse perivascular polymorphonuclear neutrophil leukocytes in the ganglion cell layer and spreading into the vitreous and scattered tiny foci of necrosis, chiefly in the inner plexiform layer. Between 9 and 14 days post-inoculation there were larger foci of retinal necrosis, becoming confluent and involving all layers of the neural retina. Polymorphonuclear neutrophil leukocytes were predominant in the initial inflammatory response, but were rapidly succeeded by lymphocytes and plasma cells, which formed a dense infiltrate in both retina and choroid. Detachment of the neural retina occurred with the first signs of inflammation, after which the pigment epithelial cells showed obvious phagocytic activity and became multilayered. Macrophages also appeared amongst the necrotic retinal cells. In the majority of mice destruction of the entire neural retina was complete by 21 days (Fig. 1a). In only a few animals the process remained focal. Necrotic cell debris was phagocytosed and the retina replaced by a granulomatous chronic
Fig. 1. (a) Mouse eye. Twenty-one days after i.c. inoculation with SC16 R$_4$C$_2$. Posterior aspect of the eye showing inflammatory destruction of the retina and dense lymphocytic infiltration of the nerve head (l). There is an abnormal proliferation of epithelial cells (ep) beneath the capsule of the lens. In comparison with man, the lens of the mouse is a relatively large structure and, as shown here, following inflammation the retinal tissue may become adherent to the posterior lens capsule. The optic nerve (N) and lens (L) are indicated and the sclera (S) may be used for cross-reference with (b). Section was haematoxylin and eosin stained. (b) Mouse eye. Similar field of view to (a) taken from a different mouse 5 days after i.c. inoculation with SC16 R$_4$C$_2$, and stained by the indirect immunofluorescence technique to reveal HSV antigens. The lens (L) is in the bottom left of the picture and the crescent of confluent fluorescence corresponds to the retina (see a). The sclera (S) is plainly visible (compare with a). Both bar markers represent 20 $\mu$m.
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Inflammatory cell infiltrate, composed predominantly of epithelioid macrophages. After 2 months, the lymphocyte and plasma cells had largely disappeared, but the layers of macrophages persisted and sometimes developed fine, granular calcification. Inflammatory cell infiltration of the optic nerves coincided with the onset of retinitis and, following retinal destruction, there was almost complete degeneration of the myelinated axons.

Changes in the lens began in some animals during the phase of acute retinitis. Between 9 and 14 days post-inoculation the lens epithelial cells at the equator showed increased mitotic activity and a layer of cells extended beneath the posterior capsule, which is normally devoid of an epithelial lining. Proliferation of the lens epithelium was accompanied by swelling and fragmentation of the posterior cortical lens fibres. Between 14 and 21 days post-inoculation small clusters of epithelial cells appeared beneath the posterior capsule (Fig. 1). In some animals the changes did not progress further. At 2 to 3 months, in animals with cataracts easily visible to the naked eye, the lens capsule was wrinkled. The proliferating lens epithelium had undergone fibrous metaplasia and there was patchy, granular calcification of degenerate lens fibres.

Immunofluorescence

Indirect immunofluorescence for HSV antigens in sections of the eyes revealed intense confluent staining of the inner layers of the retina 5 days after inoculation (Fig. 1 b). Antigen-containing cells were also observed in the optic nerve.

Tests of latent infections in i.c. inoculated mice

Attempts were made to reactivate virus from i.c. inoculated mice by the culture in vitro of explanted tissue. The techniques employed for these tests had proved highly successful for the reactivation of the parental virus, SC16, from dorsal root ganglia following peripheral or intraneural inoculation (Field & Wildy, 1978; Field et al., 1979). Reactivation of virus from R9C2-inoculated mice was attempted 3 months post-inoculation from mice both with and without obvious ocular opacity. Left and right cerebral hemisphere, trigeminal ganglia and ocular tissues were cultured. In all cases the cultures yielded negative results; no infectious virus was recovered.

Development of immunity in i.e. inoculated mice

Two tests for immunity were applied to mice following an initial infection with 10^4 p.f.u. R9C2 i.c. Firstly, mice were challenged with 10^3 p.f.u. of the wild-type virus injected i.c. (this corresponded to the injection of 100 times the LD_{50} of SC16). All the test mice survived this challenge. Secondly, further mice were injected subcutaneously in the ear pinna with a dose of virus antigen prepared by heating the parental strain. The mice responded to this challenge with a DTH reaction, which was similar in magnitude and timing to that produced in mice latently infected with the wild-type virus. The latter response has been previously characterized by ourselves (Nash et al., 1980). The DTH response observed in i.e. inoculated mice did not differ significantly between mice with and without obvious signs of blindness.

Patterns of virus replication following i.c. inoculation with R9C2

In order to elucidate the production of ocular pathology by the mutant, further mice were inoculated using 10^4 p.f.u. SC16 R9C2 inoculated into the left cerebral hemisphere. Groups of mice were killed at various times post-inoculation and the amounts of infectious virus present were determined. The results (Fig. 2) show that low, but detectable, levels of virus replication occurred in the cerebrum and cerebellum. Even at early times (1 and 3 days post-inoculation) very small amounts of virus were detectable in the left eyes but, during the period 7 to 12 days post-inoculation, an extraordinary burst of virus replication occurred in the eye samples and
only relatively slowly did this virus replication decline to undetectable levels. In further experiments the lacrymal gland and associated tissue were dissected free from the eyeball. Almost all the infectivity was found to reside in the eye samples with no significant virus replication detectable in the lacrymal gland.

The yield virus obtained from ocular tissue at the time of peak virus replication (8 days post-inoculation) was tested for signs of reversion to the wild-type phenotype. Samples were tested from three mice independently. In each case, the virus was found to retain high resistance to ACV and failed to induce TK (all <3% wild-type). It was therefore concluded that the virus replication which was observed in ocular tissue could not be accounted for by reversion of the mutant virus to wild-type.

**Patterns of virus replication following i.c. inoculation of different virus strains**

The phenomenon of ocular opacity as a late sequel to i.c. inoculation was noted to occur sporadically following inoculation with other wild-type and mutant strains of HSV. These were observed very occasionally and the phenomenon could not readily be quantified. However, a number of HSV strains were tested to see if they produced ocular replication comparable with SC16 RgC² during the acute phase of the infection. The samples tested were the entire brain and the combined left and right eye tissues. Mice were examined at days 6 and 8 post-inoculation, these being the times of maximum ocular virus replication in the case of SC16 RgC².

Wild-type strains of both HSV type 1 and type 2 exhibited a similar distribution of virus during the acute disease. However, these animals were examined in extremis (Table 2). In these mice considerable virus replication also occurred in brain and there were no survivors. Several different TK⁻ mutants derived from the parental strains were tested, but, as shown in Table 2, none of these resembled SC16 RgC². The other mutants either produced considerable and invariably lethal virus replication in the brain, or only very low levels of replication in both brain and eyes.

**Patterns of virus replication following inoculation of SC16 and SC16 RgC² by other routes**

Previously published work (Field & Wildy, 1978; Field et al., 1979) showed that, following subcutaneous inoculation into the ear pinna, the wild-type virus replicated locally in the skin, invaded the peripheral nervous system and established latent infections in the cervical dorsal root ganglia. In contrast, the mutant SC16 RgC² replicated poorly in the skin and the
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Table 2. Virus replication in the brains and eyes of mice following i.c. inoculation with various strains of HSV

<table>
<thead>
<tr>
<th>HSV strain</th>
<th>SC16</th>
<th>SC16 RgC2</th>
<th>SC16 RgC1</th>
<th>SC16 S6</th>
<th>Bry TK</th>
<th>C1(101)TK-</th>
<th>C1(101)TK-p7</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK phenotype</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neurovirulence, i.c. inoculation (p.f.u./LD50)</td>
<td>10^3</td>
<td>10^3</td>
<td>10^3</td>
<td>10^3</td>
<td>10^3</td>
<td>2 x 10^2</td>
<td>2 x 10^4</td>
</tr>
<tr>
<td>Inoculum (p.f.u., i.c.)</td>
<td>3 x 10^3</td>
<td>&gt;10^3</td>
<td>&gt;10^3</td>
<td>&gt;10^3</td>
<td>&gt;10^3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 6 post inoculation</td>
<td>3.5+</td>
<td>0.5</td>
<td>&lt;0</td>
<td>0.2</td>
<td>3.4+</td>
<td>0.6</td>
<td>5.5+</td>
</tr>
<tr>
<td>Eyes (left + right)</td>
<td>4.5</td>
<td>4.0</td>
<td>0.1</td>
<td>&lt;0</td>
<td>5.0+</td>
<td>&lt;0</td>
<td>3.4+</td>
</tr>
<tr>
<td>Day 8 post inoculation</td>
<td>3.3+</td>
<td>&lt;0</td>
<td>&lt;0</td>
<td>&lt;0</td>
<td>ND</td>
<td>1.3</td>
<td>ND</td>
</tr>
<tr>
<td>Eyes (left + right)</td>
<td>5.0+</td>
<td>6.0</td>
<td>0.8</td>
<td>&lt;0</td>
<td>ND</td>
<td>2.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Log10 p.f.u./sample; geometric mean titre obtained from two, three or four mice, samples tested independently.
† Mice dying.
‡ 2/4 mice positive; mean of positives.
§ ND, Not done (no survivors).
†† Titre of 1/4 mice positive (3/4 were negative).

Table 3. Isolation of virus from the organs of mice following tail vein inoculation

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Virus titre* with HSV strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC16†</td>
</tr>
<tr>
<td></td>
<td>1 day post-inoculation</td>
</tr>
<tr>
<td>Lung (left lobe)</td>
<td>-‡</td>
</tr>
<tr>
<td>Adrenal glands (left + right)</td>
<td>-‡</td>
</tr>
<tr>
<td>Whole brain</td>
<td>-‡</td>
</tr>
<tr>
<td>Liver (approx. 1/4)</td>
<td>All samples negative on all days</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
</tr>
<tr>
<td>Kidney (left)</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>Eyes (left + right)</td>
<td></td>
</tr>
</tbody>
</table>

* Geometric mean titre (log10) obtained from two mice tested independently at each time.
† SC16, 10^4 p.f.u. inoculated; SC16 RgC2, 10^6 p.f.u. inoculated.
‡ No virus plaques in the neat sample.
§ SC16-inoculated mice died between days 7 and 10 post-inoculation.

establishment of latent infections could not be demonstrated (Field & Darby, 1980). During the course of this previous work ocular involvement was never observed although such mice were observed for up to 1 year post-inoculation.

In order to look for other susceptible target tissues for the replication of the mutant, virus was inoculated intravenously and a range of organs tested for evidence of virus replication. Mice were inoculated into the tail vein with a relatively large dose of SC16 RgC2 (10^6 p.f.u.) but this dose produced no mortality. Mice were similarly inoculated with the wild-type SC16 but using a smaller dose (10^4 p.f.u.) which represented approx. 100 times the LD50.

The observed patterns of virus replication are shown in Table 3. It was conspicuous that the mutant virus revealed no capacity to replicate in any tissue, except that low levels of
infectious virus were detected in the lung. In particular, no virus replication was detected in the ocular samples. In the case of the wild-type virus the prominent tissues for early replication were the adrenal glands. The infection progressed to the CNS, although the eyes were spared 8 days post-inoculation when the mice were dying. However, recent observations in this laboratory (Kapoor et al., 1982) have shown that when athymic nude mice were inoculated subcutaneously with SC16, producing a progressive infection, virus was isolated from the eyes from 14 days post-inoculation.

**DISCUSSION**

The observation that sublethal i.c. inoculation of mice with a mutant strain of HSV produced late ocular sequelae was investigated. The direct explanation was found to be a brief period of virus replication in the eye. Failure of intravenous inoculation to produce ocular infection and the early inflammatory cell infiltration observed in the optic nerve suggest that the virus spreads to the retina along the nerve. Virus replication in the eye reached a peak around 8 days post-inoculation. Subsequently, there was complete destruction of the neural retina and Wallerian degeneration in the optic nerve. The development of cataracts may also be a consequence of direct virus infection of the lens epithelium. However, it could be simply a non-specific sequel to intra-ocular injury, as seen in the human eye, e.g. after silicone injection for retinal detachment. An attempt to resolve the question will be made in a further study.

Three wild-type strains of HSV were found to exhibit a similar pattern of ocular infection following i.e. infection. However, this was overshadowed by a rapidly fatal encephalitis. Several different non-lethal mutants were also examined but, while these failed to replicate well in the CNS, they showed an equally limited capacity to replicate in ocular tissues. In the case of SC16 R_{9}C_{2} the eye was the only site in which active virus replication could be demonstrated in the mouse, although this mutant grows well *in vitro* to produce yields equal to its parental strain in cultured BHK cells.

Although, for a short period, ocular infection with SC16 R_{9}C_{2} was intense, it did eventually regress. Histology roughly paralleled virus isolation and, following a florid inflammatory response, lymphocytes and plasma cells in the retina became scanty. There was no evidence of a persistent virus infection. No infectious virus was isolated at later times, no fluorescent antigens were observed in brain or eye, and no latent infections could be detected in the same tissues. However, the latter negative results may be because the methods employed were not sensitive enough to detect low levels of latent mutant virus, although similar methods readily detect latent infections of the peripheral nervous system with wild-type viruses. Alternatively, the mutant R_{9}C_{2} may be defective in its ability to establish latent infections or to reactivate from the latent state in explant culture.

It is recognized that the intracerebral inoculation of mice with a mutant virus is a highly artificial experimental situation. However, it has revealed certain properties of the mutant SC16 R_{9}C_{2}. It is conceivable that such mutants will arise spontaneously in man when the use of systemic nucleoside analogues becomes more widespread. Furthermore, a mutant strain with similar biochemical properties (TK-defective, phosphonoacetic acid-resistant) is being developed as a possible attenuated vaccine (Gauri et al., 1981). The work described here suggests that unexpected patterns of insidious virus replication may result when such mutated viruses interact with man. Furthermore, severe retinitis has been reported to be a complication of herpes simplex encephalitis in man (Greer, 1980; Johnson & Wisotzkey, 1977; Minckler et al., 1976) and it is reasonable to speculate that such complications may be observed more frequently in patients surviving encephalitis, following therapy with more effective antiviral compounds. Ocular involvement is a more regular feature of several animal herpes virus infections, e.g. natural outbreaks of infectious bovine rhinotracheitis in cattle.
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(Gibbs et al., 1975) or experimental infection of dogs with canine herpes (Percy et al., 1971). Inoculation of mice with the present mutant provides a useful easily reproducible animal model in which to study the development of ocular disease.

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Note added in proof. It was noted recently that cataracts occurred in approx. 10% of surviving Sprague-Dawley rats 1 to 3 months after i.c. inoculation with $10^6$ p.f.u. wild-type or laboratory strains of HSV-1 and HSV-2 (A. Vahlne, personal communication).

REFERENCES


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