In vivo complementation studies of a glycoprotein H-deleted herpes simplex virus-based vector

P. G. Speck,† S. Efstathiou and A. C. Minson

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

The utilization of herpes simplex virus (HSV) as a vector for gene delivery to the nervous system or as a live vaccine delivery system is dependent on the construction and characterization of disabled virus mutants which are unable to cause disease. Under certain circumstances, however, replication-defective vectors may carry a potential risk if they can be efficiently complemented by a co-infecting wild-type virus. Stocks of defective vectors should, therefore, be free from replication-competent virus, and helper cell lines should be incapable of generating replication-competent virus by recombination between the vector and the complementary gene. We describe a glycoprotein H-negative (gH-) virus/helper cell line combination which generates helper-free defective virus stocks containing replication-competent virus at a frequency no higher than 1 in $10^9$ p.f.u. This virus/helper cell system provides a suitable background for the construction of safe replication-defective gene delivery vectors. In vivo studies demonstrate that gH- virus is unable to initiate disease in mice and establishes latency at low efficiency compared to wild-type HSV. To determine whether gH- virus can be complemented by wild-type virus in vivo, mice were infected with a variety of mixtures of these viruses. Complementation was observed in a minority of animals infected with more than $10^9$ p.f.u. of both wild-type and defective virus but the most common observation was that the presence of defective virus suppressed entry of wild-type virus into the nervous system.

Introduction

Herpes simplex virus type 1 (HSV-1) is a common pathogen carried in a latent form by at least 70% of the human population. In the majority of individuals infection is inapparent, while a minority suffer mild recurrent disease episodes. Despite its benign behaviour in the majority of infected people, HSV-1 has the potential to cause severe damage as evidenced by its ability to lytically infect a wide variety of cell types in the neonate, its ability to cause severe disease in the immune suppressed and its ability, in apparently normal individuals, to cause a severe or fatal encephalitis. The cytopathic and neurotropic characteristics of HSV are significant factors in assessing the potential risks involved in the genetic manipulation of the virus for practical or experimental purposes. Analysis of the molecular basis of host cell restriction and latent infection requires modifications of viral coding sequences or regulatory sequences designed to alter pathogenic behaviour. In addition, attenuated or disabled HSV constructs have been proposed as vaccines or vaccine delivery vehicles, and the ability of HSV to establish stable latent infection of neurones has led to the development of HSV vectors for gene delivery to the CNS, both as tools in neurophysiological studies (Craig et al., 1995) and as potential delivery vehicles in human gene therapy (Glorioso et al., 1995). In each of these settings, the use of a wild-type genetic background is unacceptable and considerable effort has, therefore, been made to construct attenuated or disabled mutants. A variety of replication-competent, avirulent mutants of HSV have been constructed by deletion or inactivation of ‘dispensable’ genes or functions including the thymidine kinase (TK) gene (Sanders et al., 1982), dispensable glycoprotein genes (e.g. Meignier et al., 1988), the RL1 neurovirulence gene (Chou et al., 1990), the immediate-early transactivator, Vmw110 (Stow & Stow, 1986) and the transactivating function of the virion tegument protein Vmw65 (Ace et al., 1989). Most engineered mutants of this type are substantially attenuated, at least in rodents, but for many purposes replication-incompetent viruses, complemented in trans by a helper cell line, offer advantages. Several
defective virus/helper cell line combinations have been described including viruses lacking essential regulatory genes or essential structural proteins (e.g. De Luca et al., 1985; Weinheimer et al., 1992). Replication-incompetent mutants of HSV are, almost by definition, incapable of causing disease but, if used as vectors for biologically active molecules, may be perceived in some circumstances as carrying potential risk. In particular, the ability of replication-defective vectors to be ‘helped’ by co-infecting wild-type viruses leads to the view that stocks of defective vectors should be free of replication-competent helper viruses and that defective virus/helper cell combinations should be incapable of generating competent viruses by recombination between the vector and the complementing gene resident in the helper cell line. In addition, since more than 70% of the human population is infected with HSV, active infection in a recipient could provide helper function for a defective HSV vector.

Forrester et al. (1992) described the construction and properties of an HSV-1 mutant, SCAgH-lacZ, in which part of the glycoprotein H (gH)-coding sequence was replaced by a lacZ expression cassette, and a helper cell line, F6, which provided gH in trans. This virus, when infecting normal cells, completes the productive cycle, but produces gH- non-infectious particles. Farrell et al. (1994) showed that SCAgH-lacZ was replication-incompetent in vivo but noted that stocks of this defective virus contained detectable levels of replication-competent virus, probably reflecting the fact that the helper cell line contained substantial viral sequences in common with the defective virus, thus allowing rescue of the gH gene by homologous recombination. In this paper we describe an analogous gH- virus/helper cell line combination in which the gH-flanking sequence.

Methods

Viruses and Cells. Vero, BHK-21 and CR1 cells (Boursnell et al., 1995) were grown in Glasgow Modified Eagle's Medium (GMEM) containing 10% newborn calf serum. CR1 cells are derived from Vero cells and were a gift from M. Boursnell, Cantab Pharmaceuticals, Cambridge, UK. These cells contain the gH-coding sequence (UL22) and 3’ untranslated sequence (nucleotides 43781-46382; McGeoch et al., 1988) under the control of the HSV-1 gD promoter (−392 to +11) derived from plasmid pGDBr (Everett, 1994). This cell line is analogous to the F6 helper cell line (Forrester et al., 1992) but carries substantially less gH-flanking sequence.

HSV-1 strain SC16 is a well-characterized low-passage isolate of moderate virulence (Hill et al., 1975). HSV-SCZgH-pA is a derivative of SC16 in which the gH-coding sequence (nucleotides 43875-46386) is replaced by a cytomegalovirus IE-lacZ expression cassette derived from plasmid pMV10 (Forrester et al., 1992). The virus retains the 3’ untranslated sequence and poly(A) addition site of the gH gene. Details of the construction of this virus are described elsewhere (Browne et al., 1996). HSV-1 TK-DM21 is derived from strain SC16 and contains a deletion in the TK gene (Efstathiou et al., 1989).

Virus stocks were grown at an m.o.i. of 0.01 p.f.u. per cell on BHK cells and assayed on Vero cells. SCZgH-pA was grown and assayed on CR1 cells. TK- virus was identified by plaque assay in the presence of 5 μg/ml acyclovir (ACV), a gift from G. Darby, Glaxo Wellcome Pharmaceuticals, UK. This concentration of ACV reduced the titre of strain SC16 by > 99.8%, but had no effect on the titre of mutant TK DM21. Virus expressing lacZ (LacZ) was identified by staining assay plates for β-galactosidase as follows. Monolayers were fixed in 0.5% glutaraldehyde in PBS for 15 min at room temperature and were then permeabilized by incubation in PBS containing 0.01% sodium deoxycholate, 0.02% NP40 for 10 min at room temperature. The monolayers were then incubated at 37 °C in the same solution containing 5 mM-potassium ferricyanide–ferrocyanide plus 0.2 mg/ml 5-bromo-4-chloro-3-indol β-D-galactopyranoside (X-Gal). After 1 h the monolayers were counterstained with neutral red. Where LacZ* virus plaques were found following titration of virus from mouse tissue, the gH+ phenotype of these progeny was confirmed by showing that they could not be detected by assay on Vero cells.

Mice. Female BALB/c mice were obtained from Harlan (Bicester, UK) at 4–5 weeks of age and were used after 1 week. Mice were lightly anaesthetized with Metofane (c-Vet, Leyland, UK) and were infected by sub-dermal injection of a 20 μl inoculum in GMEM into the ear pinna. Animals were subsequently killed by intraperitoneal injection of sodium pentobarbitone and ear pinnae and innervating sensory ganglia (cII, cIII, cIV) were stored in GMEM at −70 °C prior to homogenization and assay.

Mice which had been infected for more than 30 days were tested for the presence of reactivatable latent virus by incubation of fresh dissected ganglia in GMEM plus 10% fetal calf serum for 8 days. The tissue was then homogenized and assayed. Latency associated transcripts (LATs) in fixed ganglionic tissue sections were detected by in situ hybridization using T7 RNA polymerase transcripts from plasmid pBS-0 (Speck & Simmons, 1991) with digoxigenin as a detection system as described by Arthur et al. (1993).

Results

Efficiency of complementation by CR1 cells

In order to demonstrate that the CR1 (helper) cells fully complement the defect in the gH-null mutant SCZgH-pA, the one-step growth characteristics of this virus, propagated on CR1 cells, were measured and compared to those of wild-type SC16 propagated on the same cell type. The results (Fig. 1) show that the growth in vitro of SCZgH-pA on CR1 cells is not different from that of SC16. Further, plaques produced by this mutant virus resemble those of SC16 in size and morphology, and stocks of SCZgH-pA grown and assayed on CR1 cells attain a high titre, typically in the order of 2–4 x 10⁸ p.f.u./ml. We conclude that CR1 cells provide full complementation of the defect in SCZgH-pA.

Stocks of mutant SCZgH-pA contain no detectable replication-competent virus

Replication-defective deletion mutants may occasionally be rescued by recombination with the viral gene resident in the
helper cell line. Since it is a desirable feature of replication-defective vectors that they should remain 'helper-free', it is important to assess the frequency with which replication-competent virus is generated in any defective virus/helper cell combination. The extent of sequence homology between SCZgH-pA and CR1 cells is small, comprising 4 bp on the 5' flank and 94 bp on the 3' flank of the gH-coding sequence, plus 403 bp of the gD promoter used to control expression of the gH gene in CR1 cells. It seemed unlikely that this would allow significant recombination between viral and helper cell sequences, and preliminary examination of virus stocks confirmed that inocula containing ~ 10^6 p.f.u. SCZgH-pA (as assayed on CR1 cells) failed to produce plaques on Vero or BHK cells. In order to place an upper limit on the level of competent virus in a SCZgH-pA stock, monolayers of BHK cells in 50 large (150 cm²) flasks, comprising 5 x 10^9 cells in total, were infected with 10^9 p.f.u. of a SCZgH-pA working stock (i.e. at an m.o.i of 0·2). An additional flask was infected with an equivalent inoculum (i.e. 2 x 10^7 p.f.u.) plus ~ 300 p.f.u. SC16 and a further flask was inoculated with ~ 300 p.f.u. SC16 alone, to ensure that competent virus could be detected against this background of defective virus. The flasks were fixed and stained with toluidine blue after 3 days. Microscopical examination of all flasks infected with SCZgH-pA alone revealed no plaques but control flasks infected with SCZgH-pA plus SC16 or with SC16 alone contained 317 and 312 plaques respectively. We conclude that the SCZgH-pA/CR1 cell combination generates replication-competent virus at a rate no higher than 1 in 10^9 p.f.u.

**Table 1. Establishment of latent infection**

Forty-five days after mice were infected with virus in the left ear, animals were killed and sensory ganglia (cII, cIII, cIV) were removed, pooled, fixed and sectioned. The presence of viral genomes was confirmed by detection in situ of HSV-1 LATs.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dose (p.f.u.)</th>
<th>No. of ganglionic sections examined</th>
<th>LAT-positive neurones (per ganglionic section)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC16</td>
<td>2 x 10⁶</td>
<td>35</td>
<td>511 (14·6)</td>
</tr>
<tr>
<td>SCZgH-pA</td>
<td>2 x 10⁶</td>
<td>104</td>
<td>8 (0·076)</td>
</tr>
<tr>
<td>SCZgH-pA</td>
<td>2 x 10⁷</td>
<td>71</td>
<td>15 (0·21)</td>
</tr>
</tbody>
</table>

**SCZgH-pA fails to replicate in spinal ganglia but establishes latent infection**

Groups of four mice were infected, via the ear pinna, with 2 x 10^6 or 2 x 10^7 p.f.u. SCZgH-pA and, after 4 days, the innervating cervical ganglia (cII, cIII, cIV) were dissected and pooled ganglia from each mouse were assayed for infectivity on CR1 cells. No infectivity was found, consistent with previous results using a gH⁻ virus (Farrell et al., 1994), and no animals suffered detectable disease symptoms. Mice infected with this range of doses of the parental virus yielded titres of 10^2-10^3 p.f.u. from cervical ganglia (Table 2). These data establish, predictably, that a gH⁻ virus cannot replicate within the nervous system. Several reports have shown, however, that replication is not required for the establishment of latency and that replication-defective viruses can achieve latency (reviewed by Ho, 1992). To show that SCZgH-pA establishes latent infection in sensory neurones, groups of mice were infected by injection of 2 x 10^6 or 2 x 10^7 p.f.u. into the left ear and, 45 days after infection, the animals were killed and dorsal root ganglia (cII, cIII, cIV) were removed, pooled, fixed and processed into paraffin wax blocks. Sections (6 µm) were subjected to in situ hybridization to confirm the presence of HSV genomes by revealing virally encoded LATs. Enumeration of LAT-positive neuronal profiles (Table 1) showed that SC16-infected tissue contained 14·6 LAT-positive neuronal profiles per ganglionic section, which is about 200 times the number of LAT-positive profiles counted in SCZgH-pA-infected tissue from animals infected with the same dose. Thus, notwithstanding its inability to produce infectious virus in ganglia during acute infection, SCZgH-pA establishes latency in sensory ganglia, albeit at low efficiency compared with wild-type HSV-1.

The presence of latent SCZgH-pA in spinal ganglia was confirmed in explant co-cultivation and superinfection experiments. A group of 20 mice was infected in the left ear with 2 x 10⁶ p.f.u. of SCZgH-pA and 30 days later the animals were killed and cervical ganglia (cII, cIII, cIV) removed. Ganglia from
10 mice were superinfected by placing in tissue-culture medium containing $2 \times 10^6$ p.f.u. of HSV-1 strain SC16 and were then incubated at 37 °C for 8 days. Ganglia from the remaining mice were also incubated, but in the absence of wild-type virus. After incubation, homogenates of ganglia were assayed for the presence of infectious virus and LacZ virus on CR1 cells. Ganglia from 4/10 mice yielded β-galactosidase-positive ‘blue’ plaques after superinfection, homogenization and plaque assay, with 0.1% - 0.7% of total plaques being blue in the presence of X-Gal. No infectivity was recovered in assays of ganglia that were incubated in the absence of wild-type virus.

Latent SCZgH-pA is therefore incapable of yielding infectious progeny following reactivation but can be rescued, at least in vitro, by wild-type virus. We could not achieve similar rescue by wild-type superinfection in vivo. However, this experiment is compromised by the fact that mice infected with the gH− virus are substantially resistant to superinfection (Farrell et al., 1994).

**Complementation of SCZgH-pA by wild-type virus in vivo**

Although SCZgH-pA is replication-defective in vitro, and stocks of the virus are free of replication-competent helper virus, the potential presence of active HSV infection in humans provides a source of helper virus. We attempted to assess the potential of wild-type helper virus to support the growth of SCZgH-pA in the nervous system by infecting groups of mice with a variety of mixtures of SC16 plus SCZgH-pA using different ratios from 1:10 to 10:1. After 4 days the cervical ganglia were dissected and assayed for the presence of wild-type (LacZ+) and mutant (LacZ−) infectivity on CR1 cells. Where LacZ+ progeny were observed, their gH− phenotype was confirmed by re-assay on Vero cells. Previous studies have shown that HSV mutants lacking functional TK are incapable of replication in the nervous system but can be complemented by wild-type virus (Efstathiou et al., 1989). We therefore included, for comparison, mixed infections of SC16 and TK−DM21, a mutant of strain SC16 carrying a deletion in the TK gene, and assayed virus yields in cervical ganglia in the presence and absence of ACV. The results of this series of experiments are given in Table 2, in which the yield of virus from each mouse is expressed as log10 and the proportion of gH+ (LacZ+) virus or TK− (ACV-resistant) virus is given in brackets as a percentage of total yield. As reported previously, the TK deletion mutant is unable to replicate in sensory ganglia, even after infection at very high doses, but is complemented efficiently by wild-type virus when mice were infected with equal doses. Mixed infections of wild-type and SCZgH-pA gave different results. The most consistent finding was that the gH− mutant, when used at the same dose as wild-type or in excess, suppressed replication of wild-type virus in the ganglia of most mice. This phenomenon was not observed when the wild-type virus was in excess, nor was it observed in mixed infections of wild-type and TK− viruses. In a small number of animals SCZgH-pA failed to prevent wild-type replication in sensory ganglia and in these instances the yields of virus approximate to those found in animals infected with the corresponding dose of wild-type virus alone. Although the data are few in number, these results imply an ‘all-or-nothing’ effect, and are difficult to interpret as ‘interference’ by the defective virus. To determine whether suppression of virus replication in sensory ganglia was reflected in growth at the periphery, ear tissue from some animals was assayed for infectivity. Table 3 gives the ear and ganglia titres from individual animals 4 days after infection. SCZgH-pA appears to have no effect on growth of wild-type virus at the periphery and there is no suggestion that suppression of growth in the ganglia correlates with reduced growth in the ear. The data suggest that SCZgH-pA interferes with the entry of wild-type virus into the sensory neurones and that if wild-type virus overcomes this ‘bottleneck’, then normal yields of virus in sensory ganglia are achieved.

Although we have no satisfactory explanation for these results, the consequence is that complementation of SCZgH-pA is rarely observed. The results in Table 2 can be summarized as follows: where wild-type virus is in large excess over the gH− mutant, wild-type replication in sensory ganglia is unaffected and no complementation of the defective virus is

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>SC16</th>
<th>SCZgH pA+</th>
<th>Yield in pooled dl, clll, clIV ganglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x 10^6</td>
<td>5 x 10^6</td>
<td>1 x 10^7</td>
<td>2 x 10^6</td>
</tr>
<tr>
<td>5 x 10^6</td>
<td>2 x 10^6</td>
<td>1 x 10^7</td>
<td>2 x 10^6</td>
</tr>
<tr>
<td>1 x 10^7</td>
<td>5 x 10^6</td>
<td>5 x 10^6</td>
<td>3 x 10^6</td>
</tr>
</tbody>
</table>

* Yields from individual mice are expressed as log_{10}. Where no infectivity was detected the yield is given as zero. The limit of detection is 5 p.f.u.
+ Percentage of progeny with a TK+ phenotype.
# Percentage of progeny with a LacZ+ phenotype.
In vivo properties of an HSV gH deletion mutant

Replication-defective virus/helper cell systems have been described for many virus groups, both as experimental tools and as vectors for vaccine or gene delivery. It is a general requirement of such systems that they should be free of replication-competent virus and it is an obvious advantage, in preparing high-titre stocks, if the helper cell compensates fully for the engineered defect in the vector. Although a number of HSV-1 defective/helper cell systems have been described, there are few data describing their biological properties. Our own experience, however, is that several of these defective virus/helper cell combinations generate replication-competent virus at significant frequency and that competent virus is rapidly amplified in virus stocks, suggesting that the competent virus has a selective advantage. The properties of HSV-SCZgH-pA described in this paper show that the helper cell line CRI compensates fully for the absence of gH in the defective virus and that replication-competent virus arises at a frequency of less than 1 in $10^3$ p.f.u. This presumably reflects the very limited sequence homology between defective virus and helper cells.

Consistent with previous studies of gH- herpesviruses (Farrell et al., 1994; McLean et al., 1994), HSV-SCZgH-pA caused no detectable disease in infected animals and failed to replicate in sensory ganglia innervating the inoculation site. The virus was, however, capable of establishing latent infection in sensory neurones, but with about 1% the efficiency of an equivalent dose of the parental wild-type virus. This is not surprising given previous findings that virus replication is not required for establishment of latency (reviewed by Ho, 1992) and we presume that SCZgH-pA establishes latency following direct entry of the inoculum into sensory nerve endings, an event whose frequency is likely to depend upon the site of inoculation and the degree of trauma involved.

The ability of a wild-type helper virus to complement a replication-defective virus in vivo is an unquantifiable factor in assessing the safe use of defective viruses. This is particularly significant in the case of herpesviruses because more than 70% of the population is latently infected and asymptomatic shedding is frequent. Recombination between different mutants of HSV in vivo is well established (Javier et al., 1986; Sederati et al., 1988) and has also been reported using mutants of pseudorabies virus (Christensen & Lomniczi, 1993), but complementation is less well studied. Efstathiou et al. (1989) demonstrated that a TK deletion mutant could be complemented by a wild-type virus, a finding which we confirm in this paper, but complementation of a replication-defective virus by a wild-type helper in vivo has, to our knowledge, not been addressed. We show in this paper that HSV-1 SCZgH-pA can be complemented in vivo by wild-type helper virus, but our results are, nevertheless, reassuring in that, despite the use of a mixed inoculum, complementation was never observed at low virus doses and occurred only rarely at high doses of both helper and defective viruses. The most common outcome of mixed infection at high doses was that the presence of defective virus suppressed entry of wild-type virus into the nervous system. We accept that these conclusions must be treated with caution. The data have been obtained from experiments using a limited range of dose combinations, a single route of inoculation and, most importantly, using single times post-infection to assess the virus replication during the acute phase. It is possible that in vivo complementation would be more apparent if a larger number of variables was examined.

HSV-1 SCZgH-pA is a lytic virus that, in normal cells, produces non-infectious virus particles and is, therefore, limited to a single cycle of replication. Disabled single cycle herpes simplex viruses of this type have been proposed as vaccines or vaccine delivery vehicles (McLean et al., 1994) but clearly are not suitable for other applications, such as foreign gene delivery where survival of the infected cell is required. Since CRI cells complement efficiently and SCZgH-pA is helper-

---

### Table 3. Effects of defective gH- virus on wild-type virus replication at the periphery and in sensory ganglia

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>SC16</th>
<th>SCZgH-pA</th>
<th>Ears</th>
<th>Ganglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \times 10^6$</td>
<td>-</td>
<td></td>
<td>5.46</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>5.76</td>
<td></td>
<td>2.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.32</td>
<td></td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.47</td>
<td></td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.50</td>
<td></td>
<td>2.47</td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^6$</td>
<td>$1 \times 10^6$</td>
<td></td>
<td>5.59</td>
<td>&lt;5 p.f.u.</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td></td>
<td>&lt;5 p.f.u.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.38</td>
<td></td>
<td>&lt;5 p.f.u.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.28</td>
<td></td>
<td>&lt;5 p.f.u.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.53</td>
<td></td>
<td>&lt;5 p.f.u.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.32</td>
<td></td>
<td>&lt;5 p.f.u.</td>
<td></td>
</tr>
<tr>
<td>$2 \times 10^6$</td>
<td>$2 \times 10^7$</td>
<td></td>
<td>5.59</td>
<td>&lt;5 p.f.u.</td>
</tr>
<tr>
<td></td>
<td>5.51</td>
<td></td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.54</td>
<td></td>
<td>&lt;5 p.f.u.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.83</td>
<td></td>
<td>1.77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.53</td>
<td></td>
<td>&lt;5 p.f.u.</td>
<td></td>
</tr>
</tbody>
</table>

Observed: where the gH- virus is present at equal concentration or in excess over the wild-type virus, wild-type replication is usually suppressed but where wild-type replication occurs, complementation of the defective virus, albeit at low levels, is observed. In addition to the experiments summarized in Table 2 we have infected animals with lower doses of virus (total p.f.u. $2 \times 10^4$ or $2 \times 10^5$) comprising SC16:SCZgH-pA mixtures with ratios of 1:1 or 1:10. The lower doses gave inconsistent detection of wild-type replication at the periphery and in sensory ganglia. Nevertheless, no LacZ + virus was detected in sensory ganglia in these experiments.

---

**Discussion**

Replication-defective virus/helper cell systems have been described for many virus groups, both as experimental tools and as vectors for vaccine or gene delivery. It is a general requirement of such systems that they should be free of replication-competent virus and it is an obvious advantage, in preparing high-titre stocks, if the helper cell compensates fully for the engineered defect in the vector. Although a number of HSV-1 defective/helper cell systems have been described, there are few data describing their biological properties. Our own experience, however, is that several of these defective virus/helper cell combinations generate replication-competent virus at significant frequency and that competent virus is rapidly amplified in virus stocks, suggesting that the competent virus has a selective advantage. The properties of HSV-SCZgH-pA described in this paper show that the helper cell line CRI compensates fully for the absence of gH in the defective virus and that replication-competent virus arises at a frequency of less than 1 in $10^3$ p.f.u. This presumably reflects the very limited sequence homology between defective virus and helper cells.

Consistent with previous studies of gH- herpesviruses (Farrell et al., 1994; McLean et al., 1994), HSV-SCZgH-pA caused no detectable disease in infected animals and failed to replicate in sensory ganglia innervating the inoculation site. The virus was, however, capable of establishing latent infection in sensory neurones, but with about 1% the efficiency of an equivalent dose of the parental wild-type virus. This is not surprising given previous findings that virus replication is not required for establishment of latency (reviewed by Ho, 1992) and we presume that SCZgH-pA establishes latency following direct entry of the inoculum into sensory nerve endings, an event whose frequency is likely to depend upon the site of inoculation and the degree of trauma involved.

The ability of a wild-type helper virus to complement a replication-defective virus in vivo is an unquantifiable factor in assessing the safe use of defective viruses. This is particularly significant in the case of herpesviruses because more than 70% of the population is latently infected and asymptomatic shedding is frequent. Recombination between different mutants of HSV in vivo is well established (Javier et al., 1986; Sederati et al., 1988) and has also been reported using mutants of pseudorabies virus (Christensen & Lomniczi, 1993), but complementation is less well studied. Efstathiou et al. (1989) demonstrated that a TK deletion mutant could be complemented by a wild-type virus, a finding which we confirm in this paper, but complementation of a replication-defective virus by a wild-type helper in vivo has, to our knowledge, not been addressed. We show in this paper that HSV-1 SCZgH-pA can be complemented in vivo by wild-type helper virus, but our results are, nevertheless, reassuring in that, despite the use of a mixed inoculum, complementation was never observed at low virus doses and occurred only rarely at high doses of both helper and defective viruses. The most common outcome of mixed infection at high doses was that the presence of defective virus suppressed entry of wild-type virus into the nervous system. We accept that these conclusions must be treated with caution. The data have been obtained from experiments using a limited range of dose combinations, a single route of inoculation and, most importantly, using single times post-infection to assess the virus replication during the acute phase. It is possible that in vivo complementation would be more apparent if a larger number of variables was examined.

HSV-1 SCZgH-pA is a lytic virus that, in normal cells, produces non-infectious virus particles and is, therefore, limited to a single cycle of replication. Disabled single cycle herpes simplex viruses of this type have been proposed as vaccines or vaccine delivery vehicles (McLean et al., 1994) but clearly are not suitable for other applications, such as foreign gene delivery where survival of the infected cell is required. Since CRI cells complement efficiently and SCZgH-pA is helper-
free, the virus can be engineered with the same ease as the wild-type parent. The data presented in this paper show that SCZhH−pA meets the requirements of a safe defective virus/helper cell system and we suggest that it provides a suitable background for engineering viral constructs that might otherwise attract high level containment and for constructing safe replication-defective gene delivery vectors.

This work was supported by the Biological and Biophysical Sciences Research Council, UK and by the Wellcome Trust, UK.

References


Received 22 April 1996; Accepted 5 June 1996