An analysis of the \textit{in vitro} and \textit{in vivo} phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ

Preetha Balan,* Nick Davis-Poynter, Susanne Bell, Helen Atkinson, Helena Browne and Tony Minson

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, U.K.

Mutants of herpes simplex virus type 1 (HSV-1) lacking glycoproteins gG, gE, gI or the putative gJ were constructed by inserting a \textit{lacZ} expression cassette within the US4, US8, US7 and US5 genes respectively. Revertant viruses were then constructed by rescue with a wild-type DNA fragment. Each of these mutant viruses, by comparison with the parental virus HSV-1 SC16, exhibited normal particle to infectivity ratios, and had no discernible phenotypic abnormalities in baby hamster kidney-21 cells following high or low multiplicity infections. Infection of mice by scarification of the ear with these mutant viruses showed the following. (i) Interruption of the US5 (gJ) gene has no effect on the ability of HSV-1 to multiply at the inoculation site or its ability to enter or multiply in the peripheral or central nervous system (CNS). This shows that the US5 gene provides a convenient site for the insertion of foreign genes for both \textit{in vitro} and \textit{in vivo} studies. (ii) Disruption of the US4 (gG) gene results in marginal attenuation in the mouse ear model. (iii) Disruption of the US7 (gI) or US8 (gE) genes results in pronounced attenuation; virus was rapidly cleared from the inoculation site and was barely detectable in sensory ganglia or in the CNS. The failure of gI-negative or gE-negative viruses to replicate efficiently at the inoculation site \textit{in vivo} led to the investigation of virus behaviour in epithelial cells \textit{in vitro}. Viruses lacking gE or gI adsorbed to and entered these cells at normal rates compared with the parental virus, but formed minute plaques. This is consistent with a failure of cell-to-cell spread by the cell contact route. This was confirmed by measurement of the rate of increase in infectious centre numbers following low multiplicity infections. The view that gE and gI influence interactions between cells at the plasma membrane was reinforced by showing that the introduction of disrupted gE or gI genes into a syncytial, but otherwise syngeneic, background resulted in a non-syncytial phenotype. We conclude that the gE–gI complex plays a part, at least in some cell types, in the interactions at the cell surface that allow transmission of the virus from infected to uninfected cells by cell contact. In syncytial strains this leads to uncontrolled membrane fusion. The observation that virions lacking gE or gI enter cells at apparently normal rates reinforces the view that cell-cell fusion is not analogous to the fusion of the virion envelope with the plasma membrane for nucleocapsid entry. It is also apparent that the phenotypes of HSV-1 mutants lacking gI or gE are similar in many respects to those reported for mutants of pseudo-herpes viruses lacking the gE homologue.

Introduction

The short unique (U) region of the herpes simplex virus type 1 (HSV-1) genome includes genes that encode four identified glycoproteins, gG (US4), gD (US6), gI (US7) and gE (US8), and a putative glycoprotein gJ, the predicted product of the US5 gene (McGeoch \textit{et al.}, 1985). Of these, gD is essential for virion infectivity (Ligas & Johnson, 1988) but several studies have demonstrated that the remainder are dispensable for growth \textit{in vitro} (Longnecker & Roizman, 1987; Longnecker \textit{et al.}, 1987; Weber \textit{et al.}, 1987; Neidhardt \textit{et al.}, 1987; Schranz \textit{et al.}, 1989). HSV-1 mutants that lack gG, gE or gI exhibit reduced virulence in experimental animals (Weber \textit{et al.}, 1987; Meignier \textit{et al.}, 1988; Rajcani \textit{et al.}, 1990; Neidhardt \textit{et al.}, 1987) but the mechanisms involved are unclear. No function has been ascribed to gG or to the putative gJ but the complex formed by gE and gI acts as a receptor for the Fc region of IgG (Johnson \textit{et al.}, 1988; Bell \textit{et al.}, 1990). The ability of this activity to protect the virion and the infected cell from antibody-dependent immune effector mechanisms has been demonstrated (Adler \textit{et al.}, 1978; Frank & Friedman, 1989). It is plausible to suppose that mutants lacking gE or gI exhibit reduced virulence by virtue of their reduced ability to evade the immune response. However, several lines of evidence suggest that Fc binding is not the sole function of the gE–gI complex.
Chatterjee et al. (1989) noted that a monoclonal antibody (MAB) directed against gE inhibited cell fusion by syncytial virus strains and that a gE-negative mutant had lost its syncytial phenotype. The view that gE might be involved in membrane fusion is supported by the more detailed studies of the homologous gene in pseudorabies virus (PRV). PRV glycoproteins gp63 and gI are the homologues of HSV-1 gI and gE respectively, and, like their HSV counterparts, they form a complex (Zuckermann et al., 1988). However this complex does not exhibit Fc binding activity (Zuckermann et al., 1988).

Mutants of PRV lacking gI grow to normal yields in vitro and are secreted at elevated levels but, in some cell types, are less fusogenic and exhibit a reduced ability to spread from cell to cell by the direct cell-contact route (Zsak et al., 1992). Other PRV mutants have reduced neurovirulence and altered neurotopism. For example gI deletion mutants infect only a subset of those central nervous system (CNS) neurons that receive retinal projections, suggesting that gI is required for transmission across some, but not all, transneuronal connections (Card et al., 1991, 1992). Taken together these data provide strong evidence that the gp63–gI complex of PRV functions in the cell type-specific intercellular spread of the virus. However it would be unwise to assume a similar function for the gI–gE complex of HSV.

In particular, it is reported that gD of HSV is required for virus entry and for cell-to-cell transmission (Ligas & Johnson, 1988) whereas the PRV homologue, gp50, is required for virion entry but is dispensable for cell-to-cell spread (Peeters et al., 1992). This highlights the dangers of assuming functional identity on the basis of a partial primary sequence similarity. Furthermore, since gp50 does not function in the intercellular spread of the virus it would not be surprising if, in PRV, other proteins performed this function whereas in HSV gD alone was responsible.

Thus there is a considerable body of evidence implicating the non-essential Us-specified glycoproteins of HSV in virulence and there are compelling reasons to believe, particularly by analogy with PRV, that the gI–gE complex might have functions in addition to IgG Fc binding activity. Nevertheless, these accumulated data are difficult to interpret because the various mutants have been made by diverse methods in a variety of genetic backgrounds, the various studies have used different cell types and different animal models and, perhaps most importantly, in many cases the observed phenotypes have not been correlated unambiguously with the engineered alteration in the genome.

In this paper we describe firstly the construction of HSV-1 mutants of the wild-type (wt) strain SC16 in which the US4, US5, US7 or US8 genes have been inactivated by insertion of a lacZ-coding cassette and secondly the construction of revertant viruses in which each gene is rescued. The behaviour of each of the viruses has been investigated in vitro in several cell lines and in vivo using the mouse ear model (Hill et al., 1975). A gB gene containing a syncytial mutation was then introduced into each mutant to determine the influence of the inactivated genes on the fusion phenotype. We report that a mutant lacking the US5 gene has no phenotypic abnormalities in any of these experimental systems, and that a mutant lacking gG exhibited no in vitro abnormalities, but grew to reduced titres in all tissues in vivo.

Mutants lacking gE and gI grew very poorly at all sites in vivo and exhibited several characteristics of PRV gI (the gE homologue) mutants in vitro. These included a loss of fusogenic capacity and reduced cell-to-cell spread in some cell types. We confirm that the gE–gI complex, like its PRV counterpart, contributes to intercellular transmission and to membrane fusion. The phenotype of each mutant virus was shown to be a consequence of the engineered mutation, and reversal of the mutation restored the wt phenotype in each instance.

**Methods**

**Cells and viruses.** Baby hamster kidney (BHK)-21 cells were grown in Glasgow modified Eagle’s medium (GMEM) containing 10% newborn calf serum and 10% tryptose phosphate broth. Camcell-1, a spontaneously transformed human cervical epithelial cell line, was a gift from Dr M. Stanley and was maintained in GMEM supplemented with 10% fetal calf serum (FCS), 5 ng/ml of epidermal growth factor, 0.5 μg/ml of hydrocortisone and 10⁻¹⁰ M-cholera toxin. The African green monkey-derived Vero cell line was grown in GMEM containing 10% FCS.

The parental HSV-1 strain SC16 was used throughout (Hill et al., 1975). HSV-1 SC16 and the mutants derived from it were propagated in BHK-21 cells by infection at an m.o.i. of 0.01 and were titrated by suspension assay in BHK-21 cells (Russell, 1962). Virus particle numbers were estimated by the method of Watson et al. (1963).

**Antibodies.** MABs LP10 specific for HSV-1 gG and LP2 specific for HSV gD have been described previously (Richman et al., 1986; Minson et al., 1986). MABs 3114/109 specific for HSV-1 gE and 3063 specific for HSV-1 gI were gifts from Dr Anne Cross (Institute of Virology, Glasgow, U.K.) and have been described by Cross et al. (1987). Fluorescein isothiocyanate- or peroxidase-conjugated rabbit antiamouse Ig was obtained from Dako.

**Construction of recombinant virus**

(i) **Plasmids.** All HSV-1 sequences are numbered according to McGeech et al. (1988). The following genomic clones were used: pAT-BamJ, containing the BamHI J fragment of strain SC16 (nucleotide positions 136290 to 142747; Richman et al., 1986); pPRWF6, containing the corresponding fragment from strain Patton in pBR322 (Watson et al., 1982); pAT-KpnN, containing the KpnI fragment of strain SC16 (positions 52733 to 57437; Davison & Wilkie, 1983). Plasmid pMV10, containing the lacZ gene flanked by the cytomegalovirus immediate (CMV-IE) core promoter and poly-(A) site, has been described previously (Forrester et al., 1992).

A subclone of the BamHI J fragment containing the Us4 gene was obtained by digesting pAT-BamJ with NsiI, end-repairing with T4 DNA polymerase I and digesting with BamHI. The resulting 1.25 kb
Table 1. Description of viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Description</th>
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<tbody>
<tr>
<td>SC16</td>
<td>Parental wt</td>
</tr>
<tr>
<td>SAUS5- lacZ</td>
<td>lacZ insertion in US4, gG-</td>
</tr>
<tr>
<td>SAUS5- lacZ</td>
<td>lacZ insertion in US5</td>
</tr>
<tr>
<td>SAUS7- lacZ</td>
<td>lacZ insertion in US7, gG-</td>
</tr>
<tr>
<td>SAUS8- lacZ</td>
<td>lacZ insertion in US8, gG-</td>
</tr>
<tr>
<td>SAUS4- lacZ</td>
<td>lacZ insertion in US4, lacZ allele</td>
</tr>
<tr>
<td>SC16 gB ANG</td>
<td>Substitution A(855) → V in SC16 gB, syncytial</td>
</tr>
<tr>
<td>SgB ANG US5- lacZ</td>
<td>Derived by crossing lacZ insertion mutants with SC16 gB ANG</td>
</tr>
</tbody>
</table>

Table 2. Particle to infectivity ratios of HSV-1 SC16 and recombinant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Enveloped particles/p.f.u.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC16</td>
<td>39</td>
</tr>
<tr>
<td>SC16</td>
<td>62</td>
</tr>
<tr>
<td>SgB ANG US4- lacZ</td>
<td>41</td>
</tr>
<tr>
<td>SAUS5- lacZ</td>
<td>57</td>
</tr>
<tr>
<td>SgB ANG US8- lacZ</td>
<td>57</td>
</tr>
</tbody>
</table>

* Each value refers to a different virus stock.

Analysis of HSV-1 non-essential glycoproteins

The rate of cell-to-cell spread of virus was measured in vivo after infection of preformed monolayers with 10 to 100 p.f.u. at various times after infection the monolayers were trypsinized, the cells were dispersed by pipetting and serial dilutions were replated with 2 x 10⁵ BHK-21 cells. Plaques were counted after 2 days.

In vivo characterization of recombinant viruses. The in vitro growth characteristics of the parent or recombinant viruses were assessed using the mouse ear model described by Hill et al. (1975). Female BALB/c mice of 6 weeks of age (Tucks) were infected with 2 x 10⁶ p.f.u. in a 2 μl drop. The inoculum was introduced into the ear pinna tissue by gently scratching through the inoculum droplet with a syringe needle 10 times. At various times after infection groups of four animals were killed and the ear pinna, innervating sensory ganglia (cII, cIII and cIV), and peripheral sensory ganglia (ChI, ChII and ChV), brain stem and spinal cord (0-5 cm from the cervical region) were dissected and frozen at -70 °C. The samples were subsequently thawed, homogenized and assayed. In each experiment one group of animals was maintained for greater than 1 month after infection. The sensory ganglia from these animals were dissected, pooled and immediately inoculated for 5 days in 0.5 ml GMEM containing 1% FCS. The combined tissue and medium were homogenized and assayed for reactivated virus.
Results

Virus construction and preliminary in vitro characterization

Viruses containing a lacZ expression cassette inserted within the gG gene, the US5 ORF, the gI gene or the gE gene are listed in Table 1. The genome structure of each recombinant within the U₅ region was examined by Southern hybridization of BamHI digests with an HSV-1 BamHI J fragment probe or with a lacZ probe. Each recombinant yielded the expected hybridization pattern. The inserted lacZ expression cassette contains translation termination codons in all reading frames within the CMV-IE promoter such that each disrupted gene would encode a truncated product which in each case would lack the predicted transmembrane anchor and a substantial portion of the external domain. The inability of each recombinant to produce an authentic product from the disrupted gene was confirmed using MAbs against gG (LP10), gI (3063) and gE (3114/109). No gene product was detected when each antibody was used against the corresponding mutant either by immunoperoxidase staining of infected cells or by immunoprecipitation of lysates of infected cells labelled with [³⁵S]methionine (data not shown). A similar analysis of mutant SΔUS5–lacZ could not be done because the US5 gene product (the putative gJ) has not been identified. Revertant viruses, in which the engineered mutation was reversed by rescue of the disrupted gene using the wt BamHI J fragment, were subjected to similar Southern hybridization and serological analyses. Each revertant was indistinguishable from the wt parent by these criteria.

A preliminary examination of the growth characteristics of recombinants produced from the first series of transfections revealed that the recombinant in which the US5 gene was disrupted grew to titres approximately 10-fold lower than the parental virus following high multiplicity infections of BHK-21 cells. However this phenotype was retained when the mutation was reversed and we presumed that this recombinant had acquired an unsought mutation during its construction. This recombinant was therefore discarded and a further recombinant virus was constructed, together with its corresponding revertant.

Working stocks of each of the four recombinants were used to calculate particle to infectivity ratios. The first estimations made for recombinant SΔUS4–lacZ suggested that this virus was of a lower specific infectivity than
Analysis of HSV-1 non-essential glycoproteins

The parental virus (Table 2). Analysis of further recombinant and parental virus stocks did not confirm this impression. Given the combined errors inherent in infectivity assays and particle counting, the threefold range of values observed using different stocks of different viruses is probably not significant. All four recombinants exhibited normal plaque size and plaque morphology on BHK-21 cell monolayers and were indistinguishable from the parent virus in one-step growth curves following high multiplicity infection (Fig. 1). In all subsequent in vitro experiments the phenotype of SAUS5-lacZ was not examined since, by this stage, it had become apparent that this recombinant exhibited a normal phenotype in vivo. To assess the ability of the recombinants to spread from cell to cell, BHK-21 cell monolayers were infected at a low m.o.i. (10⁻⁴ to 10⁻⁵ p.f.u./cell) and the number of infectious centres was measured at various times after infection (Fig. 2). The different kinetics of virus spread observed in Fig. 2(a), (b) and (c) reflect the fact that each parent–recombinant comparison was performed on a separate occasion. Nevertheless, it is apparent that each of the recombinants is very similar to the parent virus in its spread through the culture during multiple rounds of virus replication. There is a suggestion that the gE-negative recombinant has a marginally reduced spreading capacity (Fig. 2c), but the observed differences are small (a maximum difference of threefold between numbers of infectious centres formed after 30 h by wt and gE-negative recombinant viruses) and we did not consider this difference to be worthy of further investigation. We conclude that none of the recombinants exhibits a discernibly abnormal phenotype in BHK-21 cells and that neither gG, gI, gE nor the US5 gene product contributes significantly to the infection or growth of the virus in this cell type.

In vivo phenotypes of recombinant viruses

Female BALB/c mice (5 to 6 weeks old) were infected with 2×10⁶ p.f.u. of HSV-1 strain SC16, with the recombinant viruses or with the corresponding revertants by gentle scarification of the left ear pinna after application of the inoculum in a 2 μl droplet. Groups of four mice were killed at various times after infection and virus titres were determined at the inoculation site, the innervating sensory ganglia (cII, cIII, cIV), spinal cord and brain stem. The results are shown in Fig. 3. In this infection model, HSV-1 wt strain SC16 reaches peak titres at the inoculation site 3 to 4 days after infection and in nerve tissue about 5 days after infection (Hill et al., 1975). Ten days after infection all tissues contain little or no infective virus. Recombinant SAUS5-lacZ gave titres similar to the parent virus in all tissues throughout the course of infection, suggesting that the US5 gene product has no function in this infection system and establishing that the expansion of the US region of the genome by insertion of the lacZ gene has
Fig. 3. *In vivo* growth characteristics of HSV-1 SC16 and recombinant viruses. Mice were infected (ear) with 2 x 10^6 p.f.u. of strain SC16 (wt) and in parallel with the same dose of SAUS5-lacZ (a), SAUS4-lacZ (b), SAUS7-lacZ (c) or SAUS8-lacZ (d) or with the
corresponding revertant viruses. Post-infection virus titres in the ear pinna (i), the sensory ganglia (ii), the spinal cord (iii) and the brain stem (iv) were determined. Each point is the geometric mean of the titres from four mice. Bars show the s.d.
no effect on the competence of the virus *in vivo*. Mutant SAUS4–lacZ, lacking gg, exhibited similar growth kinetics to the parental virus but achieved noticeably lower titres in all tissues. The revertant virus was indistinguishable from the wt parent.

Mutants SAUS7–lacZ and SAUS8–lacZ, lacking gI and gE respectively, were dramatically disabled *in vivo*. Both viruses were cleared rapidly from the inoculation site, achieved low and transient titres in sensory ganglia and were virtually undetectable in spinal cord and brain stem. Reversal of the genotype restored the wt phenotype in both instances. It is clear that mutants lacking gE or gI have a reduced ability to grow in the epithelium of the ear pinna. Whether these mutants are also deficient in their ability to grow and spread within the nervous system is not certain since the reduced titres observed could result from the reduced doses of virus delivered to the sensory nerves from the periphery.

One month after infection groups of four animals infected with strain SC16, or with each of the recombinant viruses, were killed and sensory ganglia were tested for the presence of latent virus after incubation in culture medium for 5 days and homogenization. A number of studies have shown that reduction or loss of replication competence during the acute phase of infection does not abolish the ability of HSV-1 to establish latent infection (Meignier *et al.*, 1988) and, as expected, virus could be reactivated from the sensory ganglia of all mice in all groups. No attempt was made to quantify levels of latent infection.

**Phenotypes of recombinant viruses in human epithelial cells**

The gE–gI complex binds the Fc portion of IgG and is presumed to provide the virus with a mechanism of evading humoral immunity *in vivo*. Mutants without gE or gI would lack this mechanism. The attenuated phenotypes of recombinant viruses lacking gE or gI shown in Fig. 3 seem unlikely to result solely from a failure to evade the immune response, since these viruses exhibited a growth restriction soon after infection, before an effective antibody response was mounted. It seemed more likely that these mutants might grow poorly in the epithelial cells at the inoculation site and we therefore examined the mutant phenotype in the Camcell-1 line. In these cells the parent virus and each of the recombinant viruses produced the same number of plaques as on BHK-21 cells. Although the gG-negative virus produced large plaques identical to those produced by the parent virus, recombinant viruses lacking gE or gI produced plaques that were barely visible and consisted of small groups of rounded cells (Fig. 4). Reversal of the mutation restored the wt plaque morphology. This phenotype was not limited to epithelial cells. Mutants lacking gE or gI produced small plaques on Vero cells and on human fetal lung tissue-derived MRC-5 fibroblasts (data not shown), but the phenotype was most pronounced on the Camcell-1 line. It appears that BHK-21 cells are unusual in their ability to support the normal growth and spread of mutants lacking gE or gI.

The growth of recombinants SAUS7–lacZ and SAUS8–lacZ in the Camcell-1 line after high and low multiplicity infection was examined. Fig. 5 shows that after high multiplicity infection the gE-negative recombinant gave a slightly reduced yield in comparison with the wt virus while the yield of the gI-negative recombinant was reduced over 10-fold. The ability of these viruses to spread in a Camcell-1 monolayer was measured following low multiplicity infection (Fig. 6). Recombinants lacking gE or gI exhibited similar phenotypes. At
50 h after infection of monolayers with approximately 50 p.f.u. of strain SC16, most of the cells had become infected. Only around 2% of cells were infected after a similar infection with gI-negative or gE-negative recombinants. The wt phenotype was restored when the mutations were reversed. Thus, in contrast to BHK-21 cells, infection of epithelial cells with gI- or gE-negative recombinants resulted in a marked failure of virus spread (indicated by plaque size or increase in numbers of infectious centres following low multiplicity infection) and in a somewhat reduced virus yield per infected cell. These results are consistent with the restricted growth of these recombinants at the inoculation site in vivo.

The influence of gI and gE on cell fusion

The small plaque phenotype of recombinants SAUS7-lacZ and SAUS8-lacZ in Camcell-1 monolayers and the greatly reduced spread of infectivity in these cells suggested that gE and gI might function in the surface interactions between infected cells and their neighbours that mediate intercellular virus transmission. Since cell-to-cell transmission by the cell contact route presumably involves plasma membrane fusion, this implies that gI and gE might influence the uncontrolled fusion induced by syncytial virus strains. We therefore transferred the disrupted US7 and US8 genes into a syncytial background. In order to maintain an otherwise identical
Fig. 7. Influence of disruption of the US4, US7 or US8 genes on the syncytial phenotype. Vero cell monolayers were infected either at low multiplicity and incubated for 2 days at 37 °C (a), or at an m.o.i. of 10 and incubated for 8 h at 37 °C. They were then fixed and stained using X-gal and 0.1% neutral red. Isolated plaques of the recombinants SgB<sup>ANG</sup> US4-lacZ (i), SgB<sup>ANG</sup> US7-lacZ (ii) and SgB<sup>ANG</sup> US8-lacZ (iii) are shown.

Fig. 8. Adsorption rates of recombinant viruses lacking gE or gI to Vero cell monolayers. Vero cell monolayers were infected with approximately 500 p.f.u. of SC16 (wt), the recombinant virus or the corresponding revertant at 37 °C. The inoculum was removed at various times and the monolayers were washed twice with fresh medium. Following incubation for 2 days at 37 °C the monolayers were fixed and stained and numbers of plaques counted. (a) SΔUS7-lacZ and its revertant; (b) SΔUS8-lacZ and its revertant.
The genetic background we first constructed a syncytial strain of SC16 by introducing the mutation A(855)V into the gB gene, which is responsible for the syncytial phenotype in the HSV-1 ANG strain. The resulting syncytial virus (SC16 gB\textsuperscript{ANG}) was then crossed with SAUS4–lac\textsuperscript{Z}, SAUS7–lac\textsuperscript{Z} or SAUS8–lac\textsuperscript{Z} by co-infection of BHK-21 cells. The progeny were then plated onto Vero cells and lac\textsuperscript{Z}-positive plaques were identified by staining for β-galactosidase. The SC16 gB\textsuperscript{ANG}, SAUS4–lac\textsuperscript{Z} cross yielded β-galactosidase-positive plaques that were unambiguously of the syncytial or non-syncytial phenotypes of the two parent viruses. Lac\textsuperscript{Z}-positive syncytial plaques were picked and the viruses plaque-purified. The genomes of two resulting clones were examined by restriction digestion and Southern hybridization and found to contain the gB\textsuperscript{ANG} nucleotide substitution. The other two crosses yielded lac\textsuperscript{Z}-positive viruses, none of which exhibited a plaque morphology that was clearly syncytial. Two plaque types could be discerned with difficulty. The first showed no evidence of cell fusion and was identical to the lac\textsuperscript{Z}-positive parent. The second scored as non-syncytial upon cursory examination but contained occasional fused cells. Plaques of this type resulting from each cross were picked and the viruses plaque-purified. These clones were found to contain the gB\textsuperscript{ANG} substitution. Examples of the plaque morphology of gG-, gI- and gE-negative recombinants in an SC16 gB\textsuperscript{ANG} background are shown in Fig. 7(a). These viruses were also used to infect Vero cells at a high m.o.i. After 8 h recombinant gB\textsuperscript{ANG} SAUS4–lac\textsuperscript{Z} induced widespread cell fusion whereas the corresponding gE- and gI-negative mutants induced none (Fig. 7b). At later times after infection gE- and gI-negative recombinants induced limited fusion, but this was always much less than that induced by SC16 gB\textsuperscript{ANG} or by the gG-negative recombinant. These data demonstrate that the absence of gI or gE significantly reduces the fusogenic capacity of the syncytial parent.

**Adsorption and penetration are not influenced by gE or gI**

The data presented in the previous section implicate the gE–gI complex in the process of cell–cell fusion and raise the possibility that these proteins might also be involved in interaction of the virion envelope with the plasma membrane during adsorption and penetration. Although the particle to infectivity ratios of gE- or gI-negative virions are not significantly different from that of the wt, these ratios give only a crude indication of the normal adsorption rates and no indication of the penetration rate. The adsorption and penetration rates of strain SC16 and the recombinants SAUS7–lac\textsuperscript{Z} and SAUS8–lac\textsuperscript{Z} were measured at 37 °C on Vero cell monolayers.

The results of the experiments are shown in Fig. 8 and 9. There are no significant differences between the wt parent and the gI- or gE-negative recombinants in their rates of adsorption or penetration.

**Discussion**

This paper describes the construction and characterization of insertion mutants of HSV-1 in which the US4, US5, US7 and US8 genes are disrupted in a single genetic background. During the construction of the four recombinant viruses, the US5 insertion mutant exhibited a phenotype that was not reversed by rescue of the wt gene. We were obliged to construct this virus again. This emphasizes the relatively high risk of acquiring unwanted mutations when engineering HSV-1 and the importance of restoring the wt phenotype by reversal of a mutation before assuming a relationship between the phenotype and an engineered change.

The method used to disrupt these genes was to insert a lac\textsuperscript{Z} expression cassette, which can act as a selectable colour marker. Previous studies have demonstrated that insertion of a similar cassette into the U\textsubscript{e} region of the genome and the expression of β-galactosidase during infection has no adverse effect on the growth of HSV-1.
in vitro or in vivo (MacLean et al., 1991). The phenotype of the US5 insertion mutant described in this paper shows that this is also true when the lacZ cassette is inserted into the US region. Nevertheless, the phenotypes of insertion mutants must be interpreted with care because the insertion may influence not only the ORF in which it lies, but also other genes that form part of the same 3'-coterminal transcription unit. In their studies of the UL10 and UL43 genes MacLean et al. (1991) constructed lacZ insertion recombinants and used these viruses as a means of constructing true deletions. We chose not to use this strategy, partly because of the increased likelihood of acquiring unwanted mutations during two cycles of transfection but also because the presence of the lacZ cassette facilitated the transfer of the disrupted genes into alternative genetic backgrounds.

The US5 ORF was identified by McGeoch et al. (1985), who noted that the predicted translation product contained a potential N-terminal signal peptide, a potential transmembrane anchor and a single N-glycosylation site. The gene product has been named gJ (Roizman & Batterson, 1985) but the gene product has not been identified. Weber et al. (1987) reported that disruption of the US5 gene by insertion of a transposon did not significantly alter virus virulence as measured by intracerebral LD50. Our results show that disruption of the US5 gene has no effect on particle–infectivity ratio, no effect on the growth of the virus in BHK-21 cells and no effect on virus pathogenesis in the mouse ear infection model. Growth kinetics and virus titres were not significantly different from those of the parent virus at the inoculation site, in innervating sensory ganglia or in the CNS. Our data do not identify the function of the US5 gene, but they indicate that foreign genes can be inserted conveniently at this site.

HSV-1 gG, the product of the US4 gene (Richman et al., 1986), is present in the virion membrane and has the predicted structure of a class 1 membrane protein. Mutants lacking gG are viable in culture (Weber et al., 1987; Longnecker et al., 1987) and its function is unknown. Insertion of a lacZ cassette within the US4 gene creates a gG-negative recombinant but may also modify expression of US3, the protein kinase gene (Frame et al., 1987), since the US3 transcript shares its 3' terminus with the transcript of US4. Recombinant SAUS4-lacZ exhibited no abnormal phenotype in vitro but grew to reduced titres in all tissues following infection of mice by scarification of the ear. We do not yet know whether this in vivo phenotype is a consequence of the absence of gG or results from an effect on US3 gene expression. The absence of gG does not alter particle–infectivity ratios, and has no effect on the growth or spread of virus in BHK-21 cells, the efficiency of plaque formation or plaque morphology in Vero, MRC-5 or Camcell-1 cells, or on the fusogenic properties of a syncytial parent.

The glycoproteins gI and gE form a membrane-anchored complex that binds the Fc portion of IgG with higher affinity than gE alone (Bell et al., 1990). This Fc receptor activity has been shown in vitro to be capable of protecting the virus particle from complement-dependent neutralization by antibodies (Frank & Friedman, 1989). The value of this to the virus in vivo has yet to be demonstrated.

Insertion of the lacZ cassette in the US7 gene in SAUS7-lacZ might, in addition to creating a gI-negative phenotype, influence the expression of the US6 gene, since the US6 and US7 transcripts are 3'-coterminal. We have found that recombinant SAUS7-lacZ virus synthesizes reduced amounts of gD compared to the parent virus, and we cannot formally exclude the possibility that the observed phenotype of this virus is due to modification of gD synthesis rather than the absence of gI. However we believe that this is very unlikely because both in vitro and in vivo the phenotypes of SAUS7-lacZ, lacking gI, and SAUS8-lacZ, lacking gE, are virtually identical, even though US7 and US8 lie in different transcription units. Furthermore, we have constructed a mutant of strain SC16 in which the US7 gene is inactivated by deletion rather than by insertion. A preliminary examination of the phenotype of this virus shows that, like SAUS7-lacZ, it forms minute plaques on Vero cells or Camcell-1 cells, but forms normal plaques on BHK-21 cells. The simplest and most convincing interpretation is that the phenotypes of these recombinants are due to the absence of the gE-gI complex rather than to an effect of the deletion on neighbouring genes.

Recombinants lacking gE or gI had similar particle–infectivity ratios to the parent virus and displayed a wt phenotype following high or low multiplicity infection of BHK-21 cells. This is consistent with previous reports (Rajcani et al., 1990; Neidhardt et al., 1987; Meignier et al., 1988), which have found that these viruses were considerably attenuated in vivo, but their growth kinetics are not readily explained by a failure of immune evasion. Infection of a cell line derived from human mucosal epithelium demonstrated that the absence of gE or gI greatly reduces the ability of the virus to spread from cell to cell. Since transmission of virus by cell-to-cell contact is probably the significant route in vivo, we consider that it is this deficiency that accounts for the in vivo phenotype of these recombinants. The view that the gI–gE complex functions in interactions between the plasma membrane of the infected cell and those of its neighbours is supported by the observation that the absence of gE or gI greatly reduces the fusogenic capacity of a syngeneic virus carrying a syncytial mutation in gB, but does not affect the rate of adsorption or penetration of virions.
There is a previous report that construction of a gE-negative mutant resulted in loss of the syncytial phenotype (Chatterjee et al., 1989), but this cannot be interpreted unambiguously because no revertant was made. However it seems that gE cannot be essential for cell fusion in all genetic backgrounds because a gE-negative mutant has been described that has a syncytial phenotype (Neidhardt et al., 1987). Our results indicate that the gE-gI complex is required for full expression of the syncytial phenotype, at least in some genetic backgrounds. We conclude that the gI-gE complex plays no part in adsorption or penetration of virions but is involved in mediating interactions at the plasma membrane that allow transmission of virus from cell to cell by the contact route and which, in a gB<sup>ANC</sup> background, may lead to uncontrolled cell fusion.

The phenotypes of the gE- and gI-negative recombinants reported here are similar to those of a gI (gE homologue)-negative mutant of PRV. This mutant displays reduced virulence, reduced fusogenic capacity and reduced ability to spread in some cell types (Zsak et al., 1992). Whether PRV mutants lacking the gI homologue (gp63) exhibit a similar phenotype is uncertain, but since PRV gp63 and gI, like HSV gI and gE, form a complex it seems likely that these complexes are functionally homologous. Much attention has focused on the Fc receptor activity of the HSV-1 gE-gI complex and its potential role in immune evasion, but the corresponding complex of PRV does not bind IgG. The functional significance of the Fc receptor activity of HSV also remains to be demonstrated in vivo. However it is clear that the gE-gI complex functions in transmission of virus by the cell contact route and that the attenuated phenotype of mutants lacking the complex is most readily explained by the loss of this function.

At the recent 18th International Herpesvirus Workshop, Dingwell et al. from McMaster University, Hamilton, Ontario, Canada, reported that HSV-1 mutants lacking gE or gI were defective in their ability to spread by the cell contact route, a finding similar to that reported in this paper. We are grateful to Dr David Johnson for helpful discussions and for providing us with a copy of his manuscript prior to publication. We thank Dr Margaret Stanley of the University of Cambridge, U.K. for the gift of the Camcell-I line and Dr Anne Cross of the Institute of Virology, Glasgow, U.K. for monoclonal antibodies against gE and gI. Preetha Balan thanks the Cambridge Commonwealth Trust for a Nehru Post-Graduate Scholarship. This work was supported by The Wellcome Trust, U.K.

References


MACLEAN, C. A., ESTATHIOU, S., ELLIOTT, M. L., JAMIESON, F. E. &


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