Journal of General Virology (1999), **80**, 2403–2409. Printed in Great Britain

A study of primary neuronal infection by mutants of herpes simplex virus type 1 lacking dispensable and non-dispensable glycoproteins

N. Babic,[1,2]† G. Rodger,[1]‡ J. Arthur[1] and A. C. Minson[1]

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

[2] Centre National de la Recherche Scientifique, Laboratoire de Genetique des Virus, 91198 Gif-sur-Yvette Cedex, France

Cultures of primary rat dorsal root ganglia neurones were inoculated with various doses of herpes simplex virus mutants deficient in glycoproteins B, D, H, C, G, E, I or J, and the proportion of infected neurones was determined. The behaviour of these mutants on primary neurones was broadly similar to their behaviour on fibroblasts or epithelial cells. Thus, virions lacking the 'non-dispensable' glycoproteins B, D or H were incapable of infecting primary neurones, whereas mutants lacking glycoproteins G, E, I or J infected primary neurones with the same efficiency as wild-type virions. Two independently derived mutants lacking gC displayed a marginal phenotype, infecting neurones with a five- to tenfold reduced efficiency relative to wild-type virus and relative to non-neuronal cells in the same cultures. We conclude that the virion glycoprotein requirements for infection of mammalian neurones are similar to those required for infection of fibroblasts and epithelial cells but that glycoprotein C may enhance infection of neurones.

Introduction

Herpes simplex virus type 1 (HSV-1) exhibits a wide host range *in vitro,* but virus replication *in vivo* is limited primarily to cutaneous or mucosal epithelium and to innervating sensory neurones. This restriction is unlikely to be receptor-mediated because the virus occasionally spreads to the CNS, and infection of neonates can result in a disseminated disease in which a wide variety of cells and tissues is infected. Studies of the functions of HSV-1 surface proteins in virus infection, and of the receptors with which they interact, are restricted largely to fibroblasts and epithelial cells. These studies have established that glycoprotein B (gB), gD and the gH:L complex are essential for infection (Cai *et al.,* 1988; Ligas & Johnson, 1988; Forrester *et al.,* 1992; Roop *et al.,* 1993) and this is reinforced by the observation that expression of these three proteins is both necessary and sufficient to induce cell fusion (Turner *et al.,* 1998). Glycoprotein C is reported to increase the rate of adsorption of HSV-1 to host cells and to be essential for infection of the apical surface of polarized epithelial cells (Fuller & Spear, 1985; Herold *et al.,* 1991; Sears *et al.,* 1991), but gC-negative mutants of some strains of HSV-1 exhibit no discernible adsorption phenotype (Griffiths *et al.,* 1998), and a gC-negative mutant of HSV-2 also adsorbs normally (Gerber *et al.,* 1995).

Our knowledge of the receptors with which these proteins interact remains superficial. Cell surface heparin proteoglycan is required for infection (WuDunn & Spear, 1989) and it is supposed that the interaction of the heparin-binding protein gC with cell surface glycosaminoglycans constitutes an important first step in infection. However, gB also binds heparin (Herold *et al.,* 1994) and this interaction may be of importance in adsorption or penetration. A new member of the immunoglobulin superfamily and a poliovirus receptor homologue have been identified as receptors for gD (Whitbeck *et al.,* 1997; Geraghty *et al.,* 1998). Receptors for the gH:L complex have not been identified.

It is uncertain whether the infection of sensory neurones occurs by the same processes as infection of fibroblasts or epithelial cells. HSV-1 mutants lacking glycoproteins E, I, J or G absorb to and penetrate fibroblasts and epithelial cells normally but, in mouse infections, exhibit reduced neuro-

Author for correspondence: Tony Minson.
Fax +44 1223 336 926. e-mail acm@mole.bio.cam.ac.uk

† **Present address:** Centre de Recherche Pfizer, BP 159, 37400 Amboise, France.

‡ **Present address:** Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, UK.

0001-6400

virulence to varying degrees (Balan *et al.*, 1994; Griffiths *et al.*, 1998). It is impossible to know whether these attenuated phenotypes reflect a specific deficiency for neuronal infection, but more detailed studies indicate that the gE and gI of HSV-1 and pseudorabies virus play an important role in neuroinvasion and, in particular, that mutants lacking these proteins fail to infect second order neurones (Card *et al.*, 1992; Card & Enquist, 1995; Dingwell *et al.*, 1995; Babic *et al.*, 1996). These findings raise the possibility that infection of neurones by HSV-1 might require the participation of viral glycoproteins different from, or in addition to, those required for the infection of fibroblasts or epithelial cells. The objective of the work described here was to examine the ability of a series of HSV-1 mutants, each lacking an individual glycoprotein, to infect primary sensory neurones in culture.

Methods

■ **Viruses.** All virus mutants, other than a gB-negative mutant, were constructed on an HSV-1 strain SC16 background. Mutants lacking gC (SC16-ΔUL44-Z), gE (SC16-ΔUS8-Z), gG (SC16-ΔUS4-Z), gH (SC16-ΔUL22-Z), gI (SC16-ΔUS7-Z) or gJ (SC16-ΔUS5-Z) each contain coding sequences disrupted by insertion of a *lacZ* expression cassette under the control of the CMV IE promoter. The construction of these mutants has been described elsewhere (Forrester *et al.*, 1992; Balan *et al.*, 1994; Griffiths *et al.*, 1998). A mutant lacking gD was constructed as follows: a cloned *Hin*cII fragment corresponding to nucleotides 136449–140533 contains approximately 2 kbp and 0·9 kbp, respectively, 5′ and 3′ of the gD coding sequence. *Eco*RV sites were introduced by *in vitro* mutagenesis at positions 138019 and 139606, corresponding to the 5′ end of the gD promoter (−400 with respect to the 5′ end of the transcript) and the 3′ end of the coding sequence. The resulting *Eco*RV fragment was then excised from the plasmid and replaced with a *lacZ* expression cassette derived from plasmid MV10 (Forrester *et al.*, 1992). The resulting plasmid was co-precipitated with HSV-1 SC16 DNA and transfected into VD60 cells (Ligas & Johnson, 1988), which supply gD *in trans*. Progeny expressing β-galactosidase were purified by plaque picking followed by limiting dilution. The resulting virus was named SC16gDdel-Z. The predicted genome structure of the Us region was confirmed by restriction digestion and hybridization. As predicted from previous work (Johnson & Ligas, 1988; Davis-Poynter *et al.*, 1994) infection of Vero cells with this virus resulted in the production of single β-galactosidase-positive cells which were unable to transmit infectivity to neighbouring cells.

We failed to construct a gB-negative LacZ$^+$ virus on a background of HSV-1 SC16 and resorted to using a mutant based on the HFEM strain. This was constructed using a genomic *Kpn*I fragment (nucleotide 52737–57438) and inserting the *lacZ* expression cassette into the *Sna*B1 site at nucleotide 54269, in the central region of the gB coding sequence. This plasmid was co-transfected with HSV-1 HFEM DNA into the gB helper cell line D6 (Cai *et al.*, 1988) and a LacZ$^+$ recombinant virus was isolated. The resulting isolate, HFEM-ΔUL27-Z, was helper cell-dependent and produced no detectable gB in Vero cells infected at an m.o.i. of 3.

HSV-1 SC16-C3b contains the same *lacZ* expression cassette used in all other mutants inserted within the LAT locus (Lachmann & Efstathiou, 1997). The growth of this virus *in vitro* and during acute infection *in vivo* is indistinguishable from the parent (S. Efstathiou, personal communication). SC16-C3b was used as a β-galactosidase-positive surrogate for wild-type virus in most experiments.

All cells were grown in Glasgow modified Eagle's medium supplemented with 10% bovine foetal serum. Wild-type virus and replication competent mutants were propagated and assayed on BHK-21 cells. The gH-negative and gB-negative mutants were grown on the helper cell lines CR1 and D6 respectively (Cai *et al.*, 1988; Boursnell *et al.*, 1997). The gD-negative mutant was isolated using the VD60 helper cell line (Ligas & Johnson, 1988), but propagation of the mutant on these cells resulted in the generation of contaminating gD$^+$LacZ$^-$ progeny, presumably due to recombination. A gD helper cell line was, therefore, constructed which contained no gD flanking sequences in common with the mutant. The gD promoter and coding sequence (nucleotides 138019–139606) was isolated as an *Eco*RV fragment as described above and ligated to the CMV IE polyadenylation sequence (+2757 to +3053 with respect to the IE transcript start) derived from plasmid MV10. This cassette was inserted into plasmid pSP73 (Promega) and was co-transfected into Vero cells with plasmid pcDNA-3 (Invitrogen). Transfected cells were selected by growth in G418 and individual colonies were tested for their ability to support the growth of a gD-negative virus. A clone of cells was identified and named Vero gD$^+$/19.

Production of virions lacking gB, gD or gH was achieved by infection of BHK cells with the relevant mutant at an m.o.i. of 3. The progeny viruses were quantified by particle counting using the electron microscope.

■ **Preparation of primary rat sensory neurones.** Newborn (day 1–3) Wistar rats were killed by decapitation and the entire spinal column dissected to yield 30–40 dorsal root ganglia from levels C1 to L6. The ganglia were suspended in Ham's F14 medium containing 4% Ultroser-G (Gibco) and treated with 200 μg/ml collagenase types IV and XI (Sigma) for 2 h at 37 °C. The cells were mechanically dissociated by pipetting and the suspension centrifuged at 200 ***g*** for 15 min. The pellet was washed twice in Ham's medium and centrifuged through 15% BSA in Ham's medium to remove debris. The cells were then preplated twice for 15 min on plates coated with 0·5 mg/ml type VII rat tail collagen to reduce the non-neuronal cell population.

Cultures were established on 13 mm glass coverslips which were pretreated for 1 h with 0·5 mg/ml poly-DL-ornithine in water and for 1 h with 10 μg/ml laminin in Ham's F14 medium. Each coverslip received 1000 cells in 100 μl Ham's F14 medium containing 4% Ultroser-G, 2 mM glutamine, 1·25 μg/ml penicillin and streptomycin, 20 ng/ml nerve growth factor (2·5S mouse NGF, Boehringer) and 50 μM 5-fluoro-5′deoxyuridine to prevent growth of mitotic cells. After 3 h a further 200 μl of the same medium was added. Cultures were used after 3 or 4 days after neurite outgrowth had occurred.

■ **Histochemical detection.** Cultures were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min and cells were permeabilized with 0·1% Triton X-100 in PBS at room temperature for 5 min. The cultures were then blocked by incubation for 30 min in 10% horse serum and 15% BSA in PBS. Neurones were identified by staining with mouse anti-human β-tubulin type III (Sigma) followed by Cy3-conjugated donkey anti-mouse IgG or by staining with rabbit anti-mouse neurofilament 200 (Sigma) followed by Cy2-conjugated goat anti-rabbit IgG. Infected cells were detected by staining with a mixture of monoclonal antibodies LP2 and AP2, against gD and VP5 respectively (McLean *et al.*, 1982). Cells infected with viruses expressing β-galactosidase were detected by staining with X-Gal.

Results

Characterization of the cultures

After 4 days the cultures exhibited extensive neurite outgrowth and phase contrast examination suggested that a

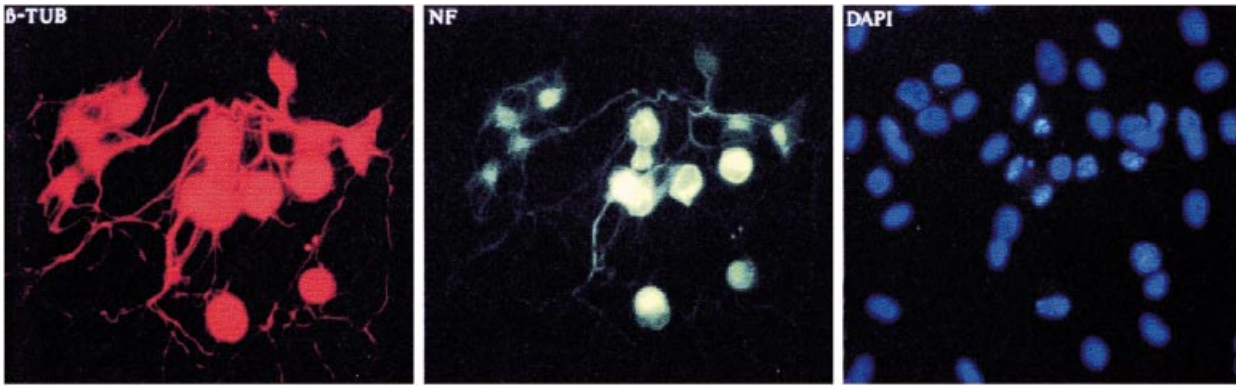

Fig. 1. Primary DRG neuronal cultures. After 4 days, cultures were stained with either β-tubulin type III (β-TUB), antibodies to neurofilament 200 (NF) or propidium iodide (DAPI). In the culture shown, approximately half the cells are positive for neurone-specific antigens.

Table 1. Proportion of neurones infected 7 h after inoculation as a function of virus dose

Infected cells were identified by using antibodies directed against major capsid VP5 and gD of HSV-1 virus (antigen-positive) or by detection of β-Gal expression (β-Gal-positive). Neurones were identified by staining for neurofilament 200 (when infected cells were identified by detection of virus antigen) or by staining for β-tubulin type III (when infected cells were identified by β-Gal staining). The number of neurones counted in each culture is given in brackets and the proportion infected is given as a percentage to two significant figures. Data are from two experiments (expts A and B).

			% of neurones infected (total neurones counted)			
Virus	Expt	Dose (p.f.u.)...	2×10^5	10^5	2×10^4	10^4
Wild-type SC16 (antigen-positive)	A		99 (147)	89 (198)	76 (243)	25 (284)
			97 (98)	89 (130)	81 (247)	21 (141)
			98 (160)	93 (206)	80 (268)	24 (187)
	B		99 (171)	91 (145)	77 (84)	29 (183)
			97 (137)	93 (121)	82 (154)	35 (163)
			99 (104)	88 (138)	76 (101)	25 (168)
SC16-C3b (antigen-positive)	A		99 (231)	90 (255)	81 (174)	30 (202)
	B		98 (98)	92 (91)	80 (137)	31 (149)
			99 (142)	89 (168)	83 (126)	24 (122)
			98 (112)	92 (157)	77 (171)	30 (168)
SC16-C3b (β-Gal-positive)	A		99 (366)	92 (274)	78 (279)	29 (238)
			98 (329)	89 (361)	82 (276)	31 (263)

majority of cells were neurones. Staining with anti-neurofilament 200 or anti-β-tubulin type III established that this was not the case and that in different cultures the proportion of neurones varied from 30 to 50% (Fig. 1). Analysis of infected cultures, therefore, required double staining for virus antigens (or β-galactosidase) and neurone-specific antigens.

Staining for β-tubulin gave a stronger signal and was used in all instances where infection was detected by staining for β-galactosidase. This method could not be used in conjunction with virus antigen detection because the anti-viral antibodies were also monoclonal mouse reagents. Preliminary experiments showed that after infection, virus antigens and β-galactosidase could first be detected after about 3 h, and that the proportion of total cells, and of neurones, scoring positive for infection reached a maximum after 7 h. After 24 h the cells were rounded and had begun to detach. An input of more than 10^5 p.f.u. was required to approach 100% infection, corresponding to an m.o.i. of greater than 100. Multiplicity of

Table 2. Proportion of neurones infected 7 h after inoculation with virus mutants

Infected cells were identified by staining for β-galactosidase. Neurones were identified by β-tubulin staining. ND, Not done.

Virus	Dose (p.f.u.)...	% of neurones infected (total neurones counted)					
		10^6	2×10^5	10^5	2×10^4	10^4	2×10^3
SC16-C3b		ND	99 (387) 96 (289) 99 (294)	91 (274) 89 (274) 92 (169) 91 (201)	80 (313) 83 (341) 82 (354) 80 (324)	21 (288) 27 (172) 32 (289) 27 (403) 19 (243)	5 (347) 3 (368) 5 (268)
SC16-Δ-U_S5-Z (gJ^-)		ND	97 (281) 99 (304)	93 (343) 91 (198)	88 (197) 79 (265)	29 (255) 16 (392) 20 (325) 23 (287) 16 (302)	4 (314)
SC16-Δ-U_S4-Z (gG^-)		ND	96 (449) 98 (334)	93 (311) 89 (267)	80 (247) 77 (308)	33 (232) 32 (321) 20 (268) 23 (336) 26 (182)	5 (293)
SC16-Δ-U_S8-Z (gE^-)		ND	98 (249) 99 (278)	90 (165) 92 (314)	79 (232) 80 (292)	24 (270) 20 (361) 17 (303) 29 (293)	4 (347)
SC16-Δ-U_S7-Z (gI^-)		ND	98 (171) 99 (285)	90 (333) 91 (254)	70 (274) 82 (278)	23 (357) 35 (291) 25 (388) 31 (278)	6 (278)
SC16-Δ-U_L44-Z (gC^-)		93 (318)	87 (159) 88 (294) 85 (268)	69 (201) 80 (181) 55 (212) 73 (287)	19 (368) 8 (206) 13 (281) 16 (198)	13 (321) 8 (417) 9 (244) 5 (325) 9 (164)	< 1 (323)

infection values are, however, of doubtful significance because the cells were very sparsely plated and the majority of the coverslip surface was available for non-specific binding of virions.

To establish that mutant SC16-C3b could be used as a $LacZ^+$ surrogate for wild-type virus, parallel cultures were infected with various doses of strain SC16 or SC16-C3b. After 7 h cultures infected with SC16 were stained for virus antigens and cultures infected with SC16-C3b were stained for virus antigens or β-galactosidase. Neurones were identified by staining for neurofilament 200 (in cultures stained for virus antigens) or β-tubulin (in cultures stained for β-galactosidase). The proportion of neurones infected was then determined in each culture. Table 1 shows the combined results of two experiments and establishes that SC16-C3b and the parent virus, SC16, infect neurones with equal efficiency. In all subsequent experiments SC16-C3b was used as a surrogate for wild-type virus.

Attempts to infect with mutants lacking 'non-dispensable' glycoproteins

Virions lacking gB, gD or the gH:L complex are unable to infect fibroblasts or epithelial cells in culture but, to our knowledge, it has not been established that these proteins are essential for the infection of neurones. Virions lacking each of these proteins were prepared by growth of the relevant mutant in BHK cells and the yield of progeny measured by particle counting. Duplicate neuronal cultures were inoculated with 10^7 enveloped particles lacking gB, gD or gH (a dose which would correspond to approximately 2×10^5 p.f.u. of wild-type virus) or with 5×10^4 p.f.u. SC16-C3b, and the cultures were stained for β-galactosidase after 13 h. No β-galactosidase-

Table 3. Proportion of neurones infected 13 h after inoculation with gC-negative virions

Infected cells were identified by staining for β-galactosidase. Neurones were identified by β-tubulin staining.

Virus	Dose (p.f.u.)…	% of neurones infected (total neurones counted)			
		2×10^5	10^5	2×10^4	10^4
SC16-C3b		97 (155)	87 (198)	84 (317)	34 (256)
		98 (128)	86 (264)	80 (232)	27 (277)
SC16-Δ-U_L44-Z (gC$^-$)		92 (247)	62 (283)	24 (292)	11 (288)
		86 (272)	59 (321)	21 (265)	7 (257)

Table 4. Infection of neurones by gC-negative virions

Infected cells and neurones were identified as described in the legend to Table 2. Cultures were fixed and stained 7 h after inoculation. ND, Not done.

Virus	Dose (p.f.u.)…	% of neurones infected (total neurones counted)					
		10^6	2×10^5	10^5	2×10^4	10^4	2×10^3
SC16-C3b		ND	99 (312)	90 (204)	77 (98)	27 (174)	6 (124)
			99 (284)	84 (253)	92 (197)	29 (297)	6 (246)
			99 (265)	90 (198)	78 (142)	29 (243)	3 (189)
SC16-Δ-U_L44-Z		90 (225)	82 (270)	71 (171)	11 (298)	11 (293)	ND
		98 (204)	82 (168)	79 (319)	12 (156)	9 (325)	
		87 (271)	90 (233)	69 (232)	9 (276)	9 (321)	
SC16-Δ-U_L44-Z (B)		92 (211)	79 (201)	40 (207)	10 (343)	5 (340)	ND
		88 (394)	79 (175)	37 (329)	5 (148)	3 (265)	
		93 (278)	68 (249)	33 (236)	9 (294)	5 (294)	

positive cells were found in any of the cultures inoculated with mutant virions, whereas the cultures infected with SC16-C3b exhibited extensive staining. Glycoproteins B, D and H are, therefore, required for the infection of neurones.

Infection with virions lacking dispensable glycoproteins

Virions lacking glycoproteins C, E, G, I or J are infectious and previous studies with strain SC16 mutants lacking these glycoproteins showed that their particle to infectivity ratios were indistinguishable from that of the wild-type parent, whether infectivity was measured on BHK cells or on polarized epithelial cells (Balan *et al.*, 1994; Griffiths *et al.*, 1998). To determine whether any of the proteins play a significant role in the infection of neurones, cultures were infected with increasing doses of each mutant or with SC16-C3b, and after 7 h the cultures were stained for β-galactosidase expression and for β-tubulin. In these experiments the 'input' inoculum is based on p.f.u. measured in BHK cells, but since the particle to infectivity ratio (as measured on BHK cells) of each mutant is similar, the inocula contain equivalent numbers of virions. Table 2 gives the outcome of an experiment in which multiple cultures were infected with each mutant and the proportion of neurones staining positive for β-galactosidase was determined 7 h after infection. The results show that replicate cultures give reasonably reproducible results and that virions lacking gE, gG, gI or gJ infect neurones with similar efficiency to SC16-C3b. Virions lacking gC infected neurones with somewhat reduced efficiency. It is difficult to place a value on this reduction in efficiency, but it appears that five to ten times as many gC-negative virions are required to achieve a comparable proportion of infected neurones. Infection of neurones by gC-negative viruses and SC16-C3b was compared in a second set of cultures, but the outcome was scored 13 h after infection. Table 3 shows that similar results were obtained. To verify that this phenotype was due to the engineered mutation and not to a fortuitous mutation elsewhere in the genome a second independent gC-negative mutant was constructed using the

Table 5. Infection of non-neuronal cells by gC-negative virions

Infected cells were identified by X-Gal staining. Neurones were identified by β-tubulin staining. Non-neuronal cells were visualized by phloxine counterstaining. ND, Not done.

	% of non-neuronal cells infected (total non-neuronal cells counted)					
Virus Dose (p.f.u.)...	10^6	2×10^5	10^5	2×10^4	10^4	2×10^3
SC16-C3b	ND	95 (152)	72 (204)	42 (167)	38 (168)	23 (218)
		96 (164)	71 (144)	42 (106)	31 (264)	18 (125)
		96 (106)	68 (181)	41 (227)	34 (160)	14 (197)
		96 (147)	72 (141)	46 (226)	30 (92)	12 (183)
		99 (104)	74 (174)	48 (202)	31 (323)	12 (196)
		98 (103)	78 (128)	49 (167)	23 (302)	16 (189)
		99 (140)	76 (152)	46 (254)	22 (250)	13 (192)
		99 (123)	74 (163)	42 (123)	35 (365)	12 (196)
SC16-Δ-U_L44-Z (gC⁻)	100 (198)	95 (225)	55 (272)	42 (201)	17 (224)	ND
	99 (104)	92 (255)	51 (230)	52 (291)	15 (222)	
	99 (106)	92 (223)	54 (245)	47 (261)	17 (198)	
SC16-Δ-U_L44-Z (B) (gC⁻)	99 (135)	90 (209)	53 (430)	38 (177)	19 (159)	ND
	99 (113)	88 (281)	49 (295)	46 (211)	18 (194)	
	99 (127)	92 (236)	45 (217)	41 (181)	17 (196)	

same method (SC16-ΔUL44-ZB). This mutant was then used to infect further cultures and also exhibited a reduced ability to infect neurones (Table 4). We thought it possible that the culture conditions, rather than neurone susceptibility, might be responsible for the reduced infectivity of gC-negative virions. For example, gC-negative virions might absorb more efficiently to the poly-DL-ornithine/laminin-coated coverslips. The cultures used to obtain the data in Table 4 were therefore re-examined and scored for the proportion of β-galactosidase-positive non-neuronal cells. The results (Table 5) were ambiguous. Firstly, the proportion of infected cells in relation to virus dose is not consistent with a Poissonian distribution, implying that all cells are not equally infectible. This is hardly surprising given the mixture of cell types likely to be present. Secondly, the gC-negative virions appear to infect slightly less efficiently than SC16-C3b virions, though the difference is not as great as that observed when infection of neurones was scored.

Discussion

The purpose of this study was to find whether infection of neurones by HSV might involve the function of viral membrane proteins other than those required for the infection of epithelial cells or fibroblasts. Given the number of proteins in the viral envelope and the fact that several have no known function, it seemed possible that some of the proteins might play a role in infection of neurones, and we therefore examined the ability of mutant virions, lacking individual viral glycoproteins, to infect primary neuronal cultures. It should be noted that with the exception of SC16gDdel-Z, which contains no gD coding sequences, each of the mutants contains glycoprotein coding sequences disrupted by a β-galactosidase expression cassette such that partial N-terminal translation products can be produced which are terminated by stop codons in all three reading frames within the expression cassette. It is impossible to be certain that these partial gene products are devoid of biological function, but in each instance they lack the sequence responsible for membrane anchoring and we therefore assume that these products cannot be incorporated into the envelopes of progeny virions. The main conclusion of this paper is that infection of neurones appears to require the same subset of glycoproteins that has been identified as essential for infection of other cell types. Thus, virions lacking gD, gB or gH were non-infectious for neurones whereas virions lacking gI, gE, gG or gJ infected neurones with the same efficiency as wild-type virus, an outcome similar to that observed on fibroblasts or epithelial cells.

We observed that two independent mutants lacking gC infected neurones with a lower efficiency (of about five- to tenfold) than wild-type virus, whereas these mutants have specific infectivities which are indistinguishable from wild-type on BHK, Vero and epithelial cells (Griffiths *et al.*, 1998). We regard this reduced efficiency as marginal, particularly since the same mutants exhibited a slightly lower efficiency of infection of non-neuronal cells in the same culture. Nevertheless, our results support those reported recently by Immergluck *et al.* (1998) who found that gC-negative mutants exhibited an entry defect on primary chick neurones.

It is worth noting that our results do not rule out a role for

gG, gE, gI or gJ in infection of neurones *in vivo*. In these circumstances viruses must enter sensory nerve endings from epithelium at the site of infection, whereas, in culture, the entire neuronal body and neurite surface is available for infection. Nevertheless, the results reported here, together with the reported phenotype of the mutants in mice (Balan *et al*., 1994), argue against a role for these glycoproteins in infection of first order neurones.

This work was supported by grants from The Wellcome Trust, Medical Research Council, UK and the EC (no. BMH4-CT97-2573). N. B. thanks the European Molecular Biology Organization for a Travelling Fellowship. We thank Dr S. Efstathiou for helpful discussion and advice.

References

Babic, N., Klupp, B., Brack, A., Mettenleiter, T., Ugolini, G. & Flamand, A. (1996). Deletion of glycoprotein gE reduces the propagation of pseudorabies virus in the nervous system of mice after intranasal inoculation. *Virology* **219**, 279–289.

Balan, P., Davis-Poynter, N., Bell, S., Atkinson, H., Browne, H. & Minson, T. (1994). An analysis of the *in vitro* and *in vivo* phenotypes of mutants of herpes simplex virus type 1 lacking glycoprotein gG, gE, gI or the putative gJ. *Journal of General Virology* **75**, 1245–1258.

Boursnell, M. E. G., Entwistle, C., Blakeley, D., Roberts, C., Duncan, I. A., Chisholm, S. E., Martin, G. M., Jennings, R., Ni Challanain, D., Sobek, I., Inglis, S. C. & McLean, C. S. (1997). A genetically inactivated herpes simplex virus type 2 (HSV-2) vaccine provides effective protection against primary and recurrent HSV-2 disease. *Journal of Infectious Diseases* **175**, 16–25.

Cai, W., Gu, B. & Person, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *Journal of Virology* **62**, 2596–2604.

Card, J. & Enquist, L. (1995). Neurovirulence of pseudorabies virus. *Critical Reviews in Neurobiology* **9**, 137–162.

Card, J. P., Whealy, M. E., Robbins, A. K. & Enquist, L. W. (1992). Pseudorabies virus envelope glycoprotein gI influences both neurotropism and virulence during infection of the rat visual system. *Journal of Virology* **66**, 3032–3041.

Davis-Poynter, N., Bell, S., Minson, T. & Browne, H. (1994). Analysis of the contributions of herpes simplex virus type 1 membrane proteins to the induction of cell–cell fusion. *Journal of Virology* **68**, 7586–7590.

Dingwell, K. S., Doering, L. C. & Johnson, D. C. (1995). Glycoproteins E and I facilitate neuron-to-neuron spread of herpes simplex virus. *Journal of Virology* **69**, 7087–7098.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N. & Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *Journal of Virology* **66**, 341–348.

Fuller, A. O. & Spear, P. G. (1985). Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralizing antibodies. *Journal of Virology* **55**, 475–482.

Geraghty, R. J., Krummenacher, C., Cohen, C. H., Eisenberg, R. J. & Spear, P. G. (1998). Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280**, 1618–1620.

Gerber, S. I., Belval, B. J. & Herold, B. C. (1995). Differences in the role of glycoprotein C of HSV-1 and HSV-2 in viral binding may contribute to serotype differences in cell tropism. *Virology* **214**, 29–39.

Griffiths, A., Renfrey, S. & Minson, T. (1998). Glycoprotein C-deficient mutants of two strains of herpes simplex virus type 1 exhibit unaltered adsorption characteristics on polarized or non-polarized cells. *Journal of General Virology* **79**, 807–812.

Herold, B. C., WuDunn, D., Soltys, N. & Spear, P. G. (1991). Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *Journal of Virology* **65**, 1090–1098.

Herold, B. C., Visalli, R. J., Susmarski, N., Brandt, C. R. & Spear, P. G. (1994). Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. *Journal of Virology* **75**, 1211–1222.

Immergluck, L. C., Domowicz, M. S., Schwartz, N. B. & Herold, B. C. (1998). Viral and cellular requirements for entry of herpes simplex virus type 1 into primary neuronal cells. *Journal of General Virology* **79**, 549–559.

Johnson, D. C. & Ligas, M. W. (1988). Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. *Journal of Virology* **62**, 4605–4612.

Lachmann, R. H. & Efstathiou, S. (1997). Utilisation of the herpes simplex virus type 1 latency-associated regulatory region to drive stable reporter gene expression in the nervous system. *Journal of Virology* **71**, 3197–3207.

Ligas, M. W. & Johnson, D. C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. *Journal of Virology* **62**, 1486–1494.

McLean, C., Buckmaster, A., Hancock, D., Buchan, A., Fuller, A. & Minson, A. (1982). Monoclonal antibodies to three non-glycosylated antigens of herpes simplex virus type 2. *Journal of General Virology* **63**, 297–305.

Roop, C., Hutchinson, L. & Johnson, D. C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. *Journal of Virology* **67**, 2285–2297.

Sears, A. E., McGwire, B. S. & Roizman, B. (1991). Infection of polarized MDCK cells with herpes simplex virus 1: two asymmetrically distributed cell receptors interact with different viral proteins. *Proceedings of the National Academy of Sciences, USA* **88**, 5087–5091.

Turner, A., Bruun, B., Minson, A. & Browne, H. (1998). Glycoproteins gB, gD and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a COS cell transfection system. *Journal of Virology* **72**, 873–875.

Whitbeck, J. C., Peng, C., Lou, H., Xu, R., Willis, S. H., Ponce de Leon, M., Peng, T., Nicola, A. V., Montogomery, R. I., Warner, M. S., Soulika, A. M., Spruce, L. A., Moore, W. T., Lambris, J. D., Spear, P. G., Cohen, G. H. & Eisenberg, R. I. (1997). Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the TNF/NGF receptor superfamily and a mediator of HSV entry. *Journal of Virology* **71**, 6083–6093.

WuDunn, D. & Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *Journal of Virology* **63**, 52–58.

Received 21 April 1999; Accepted 26 May 1999