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Mapping neuroinvasiveness of the herpes simplex virus type 1 encephalitis-inducing strain 2762 by the use of monoclonal antibodies

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Monoclonal antibodies (MAbs) directed against herpes simplex virus (HSV)-coded glycoproteins gB, gC, gD and gE were employed in an *in vitro* model of neuroinvasiveness using sensory neurons from rat dorsal root ganglion (DRG) cells. The neurons were cultured in a two-chamber system allowing infection via the neuritic extensions exclusively. The effects of 30 MAbs on viral replication of the encephalitis-derived HSV-1 strain 2762 and its less neuroinvasive variant 2762p11 were assayed in this model. One MAb reactive with gD gave a nine-fold reduction of the virus yields of both strains. One MAb directed against gB gave an enhanced virus yield of strain 2762, but not of the 2762p11 variant. Another gB-reactive MAb decreased the virus yield of strain 2762p11, but not of 2762 after neuritic infection. The findings indicate that an alteration of gB has occurred during the passage of the strain 2762. Mutants of the same strain were derived by infecting hybridomas producing MAb reactive with gB, gC, gD and gE, respectively. The gB hybridoma mutant showed a significantly lower neuroinvasiveness in the DRG model, and was non-virulent after snout infection of mice. We suggest that the structure of gB of the strain 2762 is of importance for the neuroinvasiveness of this strain.

KEYWORDS: HSV-1, encephalitis, neuroinvasiveness, monoclonal antibodies, hybridoma mutants.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) may sometimes cause an acute, focal encephalitis; a disease which is frequently lethal and with lasting sequele in spite of adequate antiviral treatment.[1,2] Although HSV-1 is a common cause of acute encephalitis, the disease might still be considered a rare complication among those infected regarding the ubiquitary character of this virus.[1] The reason why HSV-1 sporadically enters the central nervous system to cause devastation is not known. Host factors, such as immunosuppression, have not appeared to be of importance in typical cases.[3]

Efforts have been made to define genes responsible for the neurovirulence of HSV-1 by either deletion of regions of interest[4,5] with resulting avirulence of the deletion mutants, or by using an avirulent HSV-1 × HSV-2 intertypic recombinant in which virulence was restored after rescue with cloned HSV-1 fragments.[6,7] Both these approaches have identified a region close to the LS junction and comprising the gene product ICP 34·5 as essential for infection of mouse neuronal cells.

Clinical oral isolates of HSV-1 are as a rule neurovirulent in animal models,[8–10] yet have not induced encephalitis in their human hosts. Hence, the role of HSV-1 neurovirulence in the pathogenesis of HSE is still unknown. We have approached this question by comparing brain-derived HSV-1 isolates from patients with herpes simplex encephalitis (HSE) with HSV-1 strains isolated from oral cutaneous lesions (OCL) in patients lacking neurological symptoms. When we employed a two-chamber cell culture

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system of rat dorsal root ganglion neurons as an *in vitro* model of neuroinvasiveness, the replication after infection of the neuritic extensions of the HSE-derived strains was significantly greater as compared with the OCL isolates.[10] These findings probably reflected an enhanced neuritic uptake of virus, including fusion of the viral envelope with the neuritic extensions.[10] Likewise, the HSE-derived isolates were significantly more virulent by a peripheral, snout inoculation in mice, but not after intracranial (i.c.) infection. These results indicated a difference in neurovirulence.[11]

In the present study, we have used monoclonal antibodies (MAbs) to map regions of importance for the high degree of neuroinvasiveness of the HSE-derived HSV-1 strain 2762.[10,11] The panel of MAbs used was reactive against HSV-coded glycoproteins gB, gC, gD and gE, which have been reported to be involved in the early virus–cell interactions such as attachment and fusion.[12–16] Also, the neuroinvasiveness of different hybridoma-derived mutants of the strain 2762 was studied.

MATERIALS AND METHODS

HSV strains

Strain 2762 was isolated from a brain biopsy of a 58-year-old man with a focal encephalitis that later proved to be fatal. The isolate was typed as HSV-1 by an EIA using type-specific MAbs.[17] As earlier described, this strain showed a high degree of neurovirulence and neuroinvasiveness *in vivo* and *in vitro*, respectively.[11,10] Low passage material (maximum 3) of the strain was used, maintaining the original neuroinvasiveness. A variant of the strain (2762p11) was obtained after passage 11 times on green monkey kidney (GMK-AH1) cells. This variant of the virus demonstrated as will be shown reduced capacity of neuroinvasion. Virus stocks were prepared on GMK-AH1 cells, and stored at −70°C. Infectivity titres were assayed by conventional techniques, and were expressed as plaque-forming units (pfu) ml^{-1}. Briefly, GMK-AH1 cells were grown in 5 cm plastic dishes with 1% methyl cellulose in Eagle's MEM as the overlaying medium. HSV-1 strains F and HSV-2 strain B4327UR were used for production of immunization and EIA antigens. For both the 2762 strain and the 2762p11 variant the particle/pfu ratio was one.

Production and screening of MAbs

Hybridoma cells secreting MAbs were produced according to the method of Fazekas & Scheidegger.[18] Briefly, female BALB/c mice were immunized by subcutaneous injection of a deoxycholate-solubilized membrane fraction of HSV-1 strain F infected BALB/c embryonic mice cells, or a Triton X-100 solubilized membrane fraction of HSV-2 strain B4327UR infected T_3T cells.[19] Spleen cells from immunized BALB/c mice were fused with FO cells[18] by using polyethylene glycol. The hybrids were selected in HAT (hypoxanthine, aminopterine and thymidine) culture medium and subcloned by limiting dilution. The antibody reactivity was tested in EIA by means of HSV type-specific and type-common antigens in EIA. Subclass specificity was determined by peroxidase-conjugated class-specific anti-mouse reagens (Nordic Immunol. Lab., Tilburg, The Netherlands). The immunoglobulin concentration in the culture supernatants were determined by the Mancini technique.[20] Large quantities of MAbs were produced by growing hybridoma cells in dialysis tubing as described previously.[21,22]

Radioimmunoprecipitation, SDS-polyacrylamide gel electrophoresis, Western blotting and EIA

MAbs were characterized by radioimmunoprecipitation (RIP) and subsequent SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting (WB) and indirect EIA against type-specific and type-common HSV-1 and HSV-2 antigens. RIP was performed by allowing MAbs or monospecific rabbit antisera to react with [^{3}H]glucosamine-labelled Triton X-100 solubilized membrane fractions of HSV-1 and HSV-2 infected GMK-AH1 cells as described earlier.[23] Antigens were separated on SDS-PAGE according to Morse *et al.*,[24] using 9·25% polyacrylamide gels linked with N,N_1-diallyltartardiamide. The gels were dried and autoradiographies were developed on Kodak XRP-1-Omat film. [^{14}C]methylated proteins (14·3–200 kDa, Amersham) were used as molecular weight standards.

HSV-1 and HSV-2 antigens for WB were prepared by infecting HEp-2 cells, and after 24 h incubation, cells were pelleted, solubilized in sample buffer (0·07 M Tris-Cl, pH 6·8, 10% glycerol, 2% SDS, 5% mercaptoethanol and bromophenol blue dye) at 100°C for 8 min and ultrasonicated. Antigens were separated on SDS-PAGE as described above, and transferred to a nitrocellulose paper using a trans-blot apparatus (Bio-Rad). Strips were washed and incubated with monoclonal antibodies overnight at room temperature. Peroxidase-labelled rabbit anti-mouse antibodies (Code no. P260, Dakopatts a/s, Copenhagen, Denmark) was used as conjugate and orthodianisidine as substrate.

For indirect EIA, type-specific, immunosorbent-purified antigens were prepared as described pre-

viously.[23] Briefly, Triton X-100 solubilized membrane fractions from HSV-1 and HSV-2 infected BHK-21 cells were subjected to affinity chromatography using HSV glycoprotein (gB-1, gC-1, gD-1, gE-1 and gG-2) specific monoclonal antibodies coupled to CNBr-activated Sepharose. For HSV-1 and HSV-2 type-common antigens, deoxycholate-solubilized membrane fractions of HSV infected BHK-21 cells were used.[19] For coating of EIA plates all antigens were diluted in sodium carbonate buffer pH 9·6. After incubation with peroxidase-conjugated goat anti-mouse IgG (Code no. 115–036–062, Jackson Immunores. Lab. Inc., West Grove, PA), ortho-phenylenediamine (Abbot Lab., Chicago) was used as substrate and the plates were read at 490 nm.

Hybridoma mutants of HSV-1 strain 2762

Hybridoma cells producing MAbs to HSV glycoproteins gB-1, gC-1, gD-1 and gE-1 were infected with HSV-1 strain 2762 in order to generate viral mutants as described earlier for coronavirus JHM by Wege *et al.*[25] Virus inocula of 0·1 to 0·2 pfu cell^{-1} were used, and the adsorption (1 h, 37°C) was terminated by rinsing the cells with culture medium. Hybridoma cells were kept in culture for 3 weeks before virus strains were harvested. The strains were titrated and plaque-purified in the presence of the respective MAb. Virus stocks were prepared as described above. Antigens were prepared from the hybridoma mutants for characterization of the strains by RIP[23] and EIA.[19]

Nerve cell cultures

To study the interaction between HSV and nerve extensions, the two-chamber technique according to Campenot[26] was applied as described previously.[27] Briefly, dissociated embryonic dorsal root ganglion (DRG) cells of Sprague–Dawley rats were cultured in Eagle's MEM supplemented with 10% foetal calf serum and antibiotics on a collagen coat inside a glass cylinder placed in the middle of a plastic dish. The use of mitosis inhibitors prevented outgrowth of fibroblasts and other dividing cells, and the addition of nerve growth factor stimulated the neuronal cells to differentiate. Neuritic extensions penetrated the methylcellulose and silicone grease, which kept the central glass cylinder in place and acted as a diffusion-tight barrier sealing off the inner culture chamber (the cylinder) from the outer chamber (the dish). The two-chamber system allows the infection of neurites in the outer chamber without simultaneous exposure to HSV of a neuronal cell soma in the inner culture compartment.

HSV replication assay

The effects of the different MAbs on HSV replication were investigated in DRG cells, FR cells (a foetal rat skin-derived cell line, ATCC no. CRL 1213) and GMK-AH1 cells. All three cell types were infected with the strain 2762 (dilution $5{\cdot}0 \times 10^6$ pfu ml^{-1}) pre-incubated with the different MAbs (dilution 1 μg ml^{-1}) for 2 h at 4°C. For DRG cells, 1 ml of each MAb–virus mixture was added to the outer chamber of the cultures in quadruplicates, and adsorption was allowed for 1 h at 37°C. After rinsing, cultures were incubated at 37°C for 20 h and frozen at −70°C. In each experiment, three to four MAbs were tested and results were compared with controls infected with strain 2762 alone. For MAbs of interest, the experiments were repeated in sixtuplicates using both strain 2762 and its variant 2762p11 in the same assay.

For replication assays on GMK-AH1 and FR cells, the procedure was repeated with the exception that a virus inoculum of approximately 0·1 pfu cell^{-1} was used. Furthermore, cells were grown in 24-well plates, and each MAb was tested in sixtuplicates.

The hybridoma mutants of the strain 2762, as well as its variant 2762p11, were assayed for viral replication after outer chamber infection of DRG cultures and in GMK-AH1 cells. The concentration and volume of virus were those described above, but the virus was not pre-incubated with MAbs.

HSV virulence in mice

Groups of five Swiss albino mice (3–4 weeks of age) were infected with 10 log dilutions of the viruses. The virus was inoculated i.c. in the left temporal region (0·025 ml) and onto the snout after superficial scraping (0·1 ml). The concentration of the inoculum per animal were for i.c. infection 10^0–10^5 pfu and for snout inoculation 10^2–10^6 pfu. All groups of mice were coded and observed twice daily for 2 weeks after i.c. infection and for 3 weeks after nose inoculation. For estimation of virulence, log (pfu/LD_{50}) was calculated for each strain.

RESULTS

Yields of infectious virus (strain 2762 compared with its passage-modified variant 2762p11), after infection of neurites in the outer chamber of the DRG cell cultures, were $5{\cdot}76 \pm 0{\cdot}24$ and $4{\cdot}30 \pm 0{\cdot}19$, respectively (mean log pfu ml$^{-1} \pm$ SD, $t = 11{\cdot}2$, $P < 0{\cdot}001$, Student's t-test). When the experiment was repeated using inner chamber infection, i.e. allowing a direct infection of the neuronal cell soma, surpassing the

neuritic uptake and transport, the yield was for strain 2762: $6{\cdot}28 \pm 0{\cdot}18$ and for the 2762p11 variant: $5{\cdot}92 \pm 0{\cdot}16$ log pfu ml^{-1} ($t = 3{\cdot}7$, $P < 0{\cdot}005$). Thus, compared with the original virus the 2762p11 variant demonstrated a highly reduced capacity of neuroinvasion.

Thirty MAbs directed against HSV glycoproteins are characterized in Table 1. HSV-1 type-specific MAbs were predominantly reactive with glycoproteins gC and gE. All the MAbs were now assayed for influence on viral replication of strain 2762 in neuritic infection of DRG cells, and in FR cells and GMK-AH1 cells. As shown in Table 2, only two MAbs fulfilled the following two criteria.

1. Interfering in a statistically significant way with the production of infectious virus in DRG cell cultures after pre-incubation with the respective MAb and evaluating the amounts of virus produced in presence and absence of the MAb.
2. Not significantly influencing on viral replication in non-neuronal cells.

With the two MAbs (1B11D8 reactive against gB and C4D5G2 reactive against gD), the experiments were repeated using strain 2762 and, in addition, the variant 2762p11. Also included were MAbs B3G11E8 reactive against gB, found to significantly decrease replication of 2762p11 after neuritic infection in the DRG system, and 4G2G5 reactive against gE.

As shown in Table 3, pre-incubation of strain 2762 with anti-gB MAb 1B11D8 resulted in an enhanced virus production while no enhancement was seen with the 2762p11 variant of the virus. Although MAb

Table 1. Characterization of MAbs directed against HSV glycoproteins by reactivity in EIA using purified glycoproteins and HSV types -1 and -2 DOC-solubilized membrane antigens, Western blotting (WB) and radioimmunoprecipitation (RIP)

	EIA reactivity				
MAb	Glycoprotein	HSV type	WB	RIP	Ig class
B3G11E8	gB	1+2	gB	gB	G1
4A9	gB	1	neg	neg	G1
1F7	gB	1	neg	gB	G1
E3E9	gB	1+2	neg	neg	G1
1B11C7	gB	1+2	neg	gB	G1
1B11D8	gB	1+2	neg	gB	G1
H2G4B1*	gB	1+2	gB	gB	G1
H2E1C2*	gB	1+2	gB	gB	G1
H2G5B11*	gB	1+2	neg	gB	G1
K4C7*	gB	1+2	gB	gB	G1
H3D3E11*	gB	1+2	neg	gB	G1
2D9	gC	1	neg	gC	M
C2H12H5	gC	1+2	gC	neg	G1
B1C1B4	gC	1	neg	gC	G1
1D9	gC	1	neg	gC	G1
3C2	gC	1	neg	gC	G1
C4H11B6	gC	1	neg	gC	G1
C3F6F3	gD	1	gD	gD	G2a
C4D5G2	gD	1+2	gD	gD	G1
E5G3G9	gD	1	gD	gD	G1
3D11	gD	1+2	neg	n.d.	G1
2D7	gD	1+2	neg	gD	G1
1F5	gD	1	neg	n.d.	n.d.
C4B6A6	gD	1+2	neg	gD	G1
H3E12H6*	gD	1+2	gD	neg	G1
K3G9F7*	gD	1+2	gD	gD	G1
4G2G5	gE	1	gE	gE	G1
4G3	gE	1	gE	gE	G1
B1E6A5	gE	1+2	neg	gE	G1
C7H2	gE	1	neg	gE	G1

* MAbs produced after immunization of mice with HSV-2 (B4327UR) derived antigens. All other MAbs were produced with HSV-1 (F) antigens as the immunogen.
n.d. = not done.

Table 2. Effects of MAbs on replication of HSV-1 strain 2762 in different cell types: infection of neuritic extensions in dual-chamber cultures of sensory neurons derived from rat dorsal root ganglia (DRG), a cell line of rat skin epithelial cells (FR) and GMK-AH1 cells

MAb	Specificity	Replication quotients*		
		DRG cells	FR cells	GMK cells
B3G11E8	gB	0·7	0·8	0·4
4A9	gB	0·5	0·5	0·5
1F7	gB	0·4	0·2	0·5
E3E9	gB	0·2	0·2	0·3
1B11C7	gB	1·2	0·4	0·4
1B11D8	gB	3·4†	0·4	0·9
H2G4B1	gB	0·3	0·3	0·9
H2E1C2	gB	1·5	2·0	0·5
H2G5B11	gB	0·6	0·6	0·5
K4C7	gB	0·4	0·6	0·7
H3D3E11	gB	0·4	0·5	0·5
2D9	gC	0·4	0·2	0·9
C2H12H5	gC	0·9	0·3	0·8
B1C1B4	gC	0·8	0·2	0·6
1D9	gC	1·0	0·4	0·4
3C2	gC	0·3	0·2	0·6
C4H11B6	gC	0·6	1·1	1·8
C3F6F3	gD	0·6	0·4	0·3
C4D5G2	gD	0·1†	1·0	0·4
E5G3G9	gD	0·4	0·8	0·6
3D11	gD	0·4	0·3	0·7
2D7	gD	0·6	0·3	0·6
1F5	gD	1·0	0·7	0·8
C4B6A6	gD	0·9	0·4	0·7
H3E12H6	gD	0·6	1·0	0·6
K3G9F7	gD	0·7	0·3	0·8
4G2G5	gE	1·9	0·9	0·8
4G3	gE	1·1	1·2	1·1
B1E6A5	gE	1·3	0·6	1·1
C7H2	gE	0·7	1·3	1·0

* The yield after pre-incubation of virus with MAb divided with yield in absence of MAb. Means of four assays.
† Significant ($P < 0·05$, Student's *t*-test) alteration (yield of virus + MAb compared with that of virus alone), but non-significant influence of MAbs on virus production in FR- and GMK-AH1 cells.

Table 3. Yield of infectious virus in inner chamber (nerve cell soma) after infection of outer chamber (neuritic extensions) of DRG cultures using strain 2762 and its variant 2762p11, with and without pre-incubation of virus with MAb. Student's *t*-test was used as the statistical method

Antibody		Virus	
MAb	Specificity	Strain	Yield (log pfu ml^{-1}) with/without MAb (mean ± SD, (p))
1B11D8	gB	2762	5·35 ± 0·25/4·81 ± 0·45 ($t = 2·65$)
		2762p11	3·60 ± 0·22/3·51 ± 0·36 n.s.
B3G11E8	gB	2762	5·35 ± 0·57/5·50 ± 0·42 n.s.
		2762p11	2·52 ± 0·37/3·96 ± 0·25 ($t = 7·09$)
C4D5G2	gD	2762	4·16 ± 0·08/5·13 ± 0·08 ($t = 6·83$)
		2762p11	3·36 ± 0·35/4·10 ± 0·07 ($t = 5·14$)
4G2G5	gE	2762	5·00 ± 0·56/4·74 ± 0·14 n.s.
		2762p11	3·53 ± 0·35/3·96 ± 0·25 n.s.

n.s. = not significant.

B3G11E8, also reacting with a gB-epitope, did not influence on replication of strain 2762, it reduced the production of the passage virus 2762p11, compatible with a gB-mediated effect on the nerve cell infection. MAb C4D5G2 (anti-gD) reduced replication of both viruses and MAb 4G2G5 (anti-gE) did not affect the rate of infection significantly.

In another set of experiments the 2762 virus was used for infecting hybridoma cell lines (see the MAbs listed in Table 3, and in addition the anti-gC MAbs B1C1B4 and C2H12H5). In spite of repeated attempts, cell line 1B11D8 did not produce a viable virus. With all the other hybridomas tested, viable mutants were obtained. EIA antigens were now produced, and the reactivity to all the different MAbs was assayed. The pattern observed suggested alteration of the one epitope only corresponding to the MAb of the hybridoma cell line tested. An illustrative example (B1C1B4) is presented in Fig. 1.

RIP of the hybridoma mutants with polyclonal sera to HSV glycoproteins did not suggest any gross differences in molecular weight between the mutated glycoproteins and the parental strain 2762 (three representative examples are shown in Fig. 2).

We next assayed the capacity of the mutants to

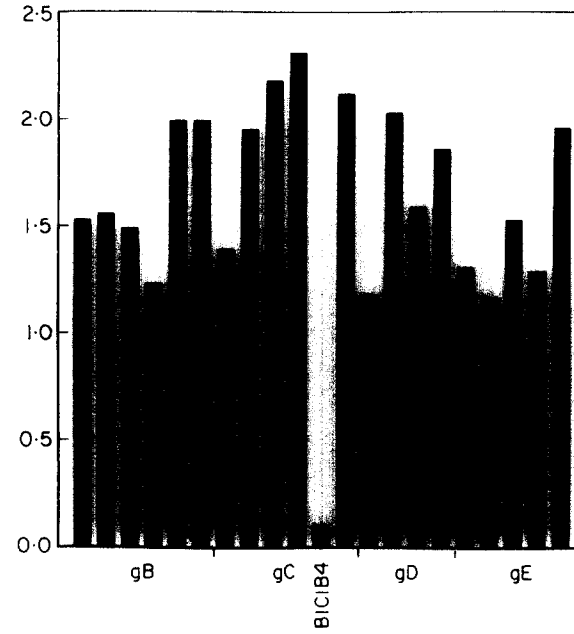

Fig. 1. EIA reactivity of the hybridoma mutant B1C1B4, derived after infecting a cell line producing a monoclonal antibody directed against gC with HSV-1 strain 2762. A panel of MAbs were all reactive against the mutant with the exception of the MAb produced by the infected hybridoma.

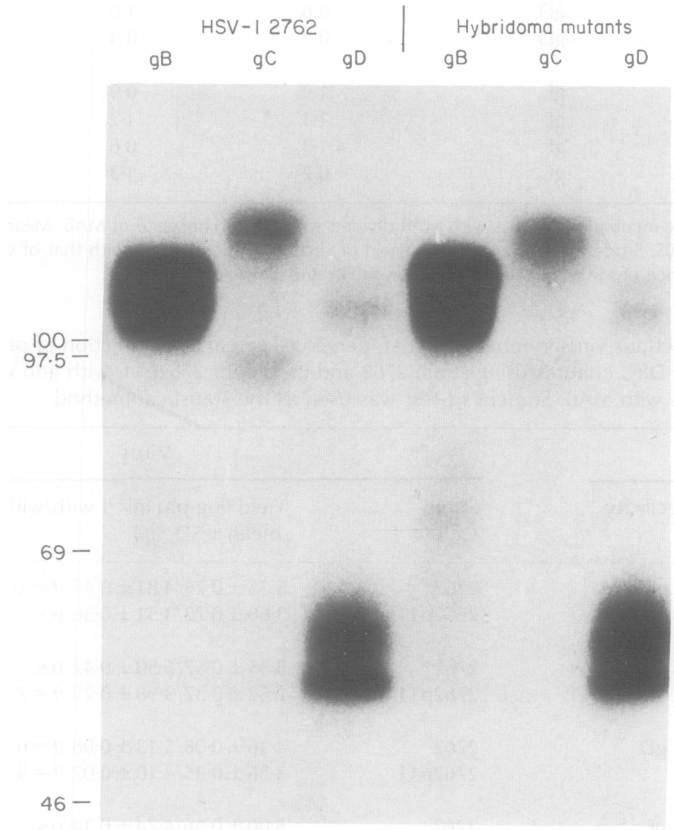

Fig. 2. Radioimmunoprecipitation (RIP) of the hybridoma mutants B3G11E8 (gB), B1C1B4 (gC) and C4D5G2 (gD) using glucosamine-labelled antigen precipitated with rabbit hyperimmune sera achieved by immunizing with purified glycoproteins. Molecular weight markers in kDa are indicated to the left.

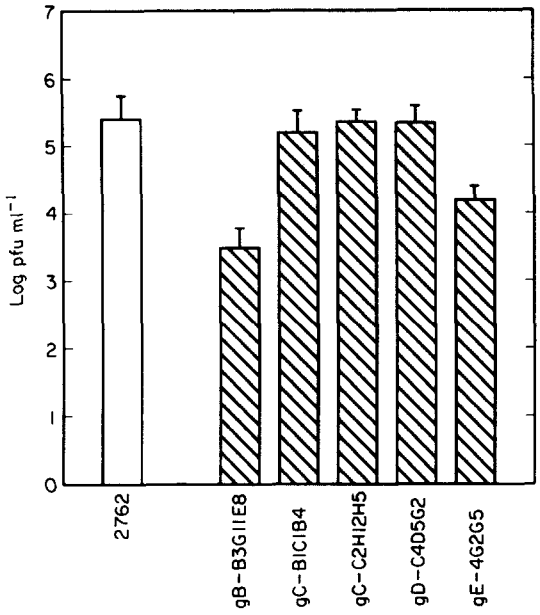

Fig. 3. Viral production in a two-chamber culture system of sensory neurons derived from rat dorsal root ganglion cells, allowing infection of the neuritic extensions exclusively. Yields in log pfu ml^{-1} from the inner chamber are shown for HSV-1 strain 2762 and the hybridoma cell line-derived mutants, respectively.

produce virus infectious for neuritic extensions (outer chamber inoculation) of DRG cultures. Two mutants, B3G11B8 (gB) and 4G2G5 (gE), then demonstrated a reduced capacity to infect and replicate in DRG cells (Fig. 3). In contrast, the yields using the GMK-AH1 cells were identical or only insignificantly reduced.

Finally, we have investigated *in vivo* neurovirulence after inoculation of mice with strain 2762 and the 2762p11 variant (Table 4). After peripheral inoculation of mice, the strain 2762p11 showed reduced neurovirulence not demonstrable by i.c. inoculation. The hybridoma-derived mutants, B3G11E8 and 4G2G5, were both associated with reduced virulence after i.c. inoculation, and were non-lethal with the peripheral inoculation route (Table 4).

DISCUSSION

HSV-1 strains isolated from brains of patients with encephalitis displayed both an enhanced neuroinvasiveness in an *in vitro* assay using cultured rat dorsal root ganglion neurons[10] and increased neurovirulence in a mouse model with peripherally (snout-) inoculated animals,[11] as compared with strains of oral or cutaneous origin. In the present study, we have selected one of the strains (2762) with proven encephalitogenic properties in man, to define the viral gene region of importance for neuroinvasiveness. This property, as demonstrated by our *in vitro* assay, was not genetically stable, since multiple passage of the virus strain yielded a variant, 2762p11, which disclosed markedly reduced neuroinvasiveness.

Since all wild-type HSV-1 strains we have investigated have shown the ability to infect almost any neuronal cell type, an *in vitro* model of neuroinvasiveness will at best reveal relative differences between isolates. Utilizing cell cultures of neuroblastoma cells, astrocytes, as well as with non-neuronal cells such as fibroblasts and epithelial cells, no correlation was found between viral replication and *in vivo* neuroinvasiveness[10,11] (T. Bergström *et al.*, submitted). One reason why the dual-chamber model of rat sensory neurons could discriminate encephalitis-causing HSV-1 strains from ordinary oral isolates could be that this model focuses on early interactions (i.e. attachment and/or fusion) between virus and neuritic extensions.[10]

These early interactions are dependent on functions of viral envelope glycoproteins such as gB, gC, gD and gE,[12–16] which is why we screened MAbs for effects on the infection of the neuritic extensions in

Table 4. Virulence in mice of the HSV-1 strain 2762, the 2762p11 variant and the hybridoma-derived mutants of strain 2762 after intracranial and peripheral inoculation. Cell culture produced suspensions (1×10^7 pfu ml^{-1}) were titrated in 10-fold dilutions in mice and the log (pfu/LD50) was calculated

	Log (pfu/LD50) in mice after:	
Viral strain	intracranial	snout inoculation
2762	−0·35	3·5
2762p11	−0·10	5·3
2762/B3G11E8 (gB)	2·7	nl*
/B1C1B4 (gC)	−0·10	4·3
/C4D5G2 (gD)	0·001	4·8
/4G2G5 (gE)	2·5	nl*

* nl = non-lethal with the inocula used.

the *in vitro* model. We found two MAbs of interest (one reactive against gB and one against gD), that showed a neuronal-specific effect on replication of strain 2762, and one anti-gB MAb influencing neuronal infection with the 2762p11 variant. When the effects of these MAbs were compared the results suggested that by passaging the virus a gB-associated alteration had occurred, concomittantly with a reduction of the neuroinvasiveness of the strain.

Derivation of hybridoma mutants has earlier been used in the study of the neurovirulence of coronavirus JHM.[25] When we applied this technique on the strain 2762, a mutant derived from the hybridoma-producing MAb cell line, 4G2G5 (gE), showed a reduced neuroinvasiveness in the DRG model. This mutant was also non-lethal after snout inoculation of mice. As reported by Rajcáni *et al.*,[28] a gE minus mutant of the encephalitogenic strain ANGpath was not taken up by neurons nor axonally transported to the trigeminal ganglia after peripheral inoculation into mice.

We observed an even stronger reduction of the neuroinvasiveness with the gB hybridoma mutant B3G11E8. The observed $>$one ^{10}log reduction of *in vitro* neuroinvasiveness was in parity with the earlier described difference between encephalitis-causing and oral HSV-1 strains, using the same model.[10] Furthermore, this mutant was also non-lethal after peripheral inoculation in our mouse model.

Although sequencing has as yet not been carried out, the characterization by EIA and RIP indicated that probably only minor genetic changes had occurred. With a similar technique employed for tick-borne encephalitis virus, a strongly reduced virulence after peripheral inoculation in mice was encountered and found to be dependent on the substitution of one amino-acid only of the E envelope glycoprotein.[29]

Recent studies on gB HSV-1 escape mutants have indicated that glycoprotein B is essential for virus penetration, and that minor variations will result in greatly altered functions such as prevention of cell-to-cell spread.[12] It is noteworthy that the hybridoma producing the gB-reactive MAb 1B11D8 in our study was unpermissive to infection with strain 2762. It has earlier been reported that transfer of a gB fragment from an avirulent strain was associated with loss of pathogenicity after peripheral infection with the neuroinvasive strain ANGpath in transfection experiments.[30] A more detailed analysis of gB of the strain 2762 and its 2762p11 variant, and an epitope mapping of the gB-reactive MAbs 1B11D8 and B3G11E8 may provide information relevant for the genetic mapping of HSV neuroinvasiveness.

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