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Differences in resistance to herpes simplex virus type 1 (HSV-1) among oligodendroglia derived from different strains of mice are determined after viral adsorption but prior to the expression of immediate early (IE) genes

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The nature of an innate cellular resistance to HSV-1 of cultured murine oligodendrocytes (OLs) in three strains of mice (C57BL/6J, Balb/cByJ and A/J) was investigated. The expression of immediate early (ICP4), early (ICP8) and late (gC) antigens in primary OL cultures was studied using an indirect immunofluorescence (IF) technique. HSV-1 infected OLs from C57BL/6J mice showed no viral antigens at 24 h post infection (p.i.) but rather a marked delay in antigen expression beginning at 60 h p.i. In contrast all three proteins were expressed in A/J OLs at 24 h p.i. while Balb/cByJ OLs showed an intermediate protein expression pattern. These results suggest that the innate cellular resistance to HSV-1 is determined prior to the expression of immediate early viral antigens. To further study these differences, the adsorption capacity between the three mouse strains was compared using dextran purified, [^{3}H]thymidine labelled virus. No differences in HSV-1 adsorption were identified. Results from viral penetration studies approached statistical significance suggesting that penetration may be impaired in C57BL/6J and Balb/cByJ OLs when compared to A/J OLs and is likely fusion independent. The selective differences in HSV-1 resistance mediated by OLs, reflect differences in virus host cell interactions, that likely contribute to differences in mortality, viral spread, and the ability of virus to induce central nervous system (CNS) demyelination.

Keywords: herpes simplex virus (HSV); oligodendrocytes; viral resistance; intrinsic resistance

Introduction

Herpes Simplex virus type 1 (HSV-1), a neurotropic virus, is known to gain access to the central nervous system (CNS) via retrograde axonal transport (Cook and Stevens, 1973). The restriction of virus to defined neuroanatomic pathways in the CNS (Ugolini *et al*, 1989) suggests that it travels by transsynaptic transfer but may at times spread to invade glial cells similar to that observed with pseudorabies virus (Rinaman *et al*, 1993a, b). The ability of glia to restrict HSV-1 infection may therefore influence the ability of the virus to induce severe destruction as seen in human herpes encephalitis (HSE) (McKendall, 1994) or demyelination in animal models (Kristensson *et al*, 1982; Kastrukoff *et al*, 1992). Mechanisms mediating resistance to virus at the cellular level are likely an important but poorly understood aspect of HSV-1 pathogenesis. The host response to HSV-1 CNS infection varies between individuals, both in humans (McKendall, 1994) and in experimental animals (Kastrukoff *et al*, 1982, 1986, 1992). The reason for this is likely multifactorial and dependent on viral factors (Stevens, 1993; Yuhash and Stevens, 1993), the nature of the host immune response (Fraser and Valai-Nagy, 1993; Mester *et al*, 1992) and virus-cell interactions. Innate HSV-1

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resistance in non CNS cells was recently reviewed (Wu and Morahan, 1992). Less is known about innate celluar resistance to HSV-1 within the CNS.

We have previously described genetically determined resistance to HSV-1 infection in C57BL/6J mice (resistant), Balb/cByJ mice (intermediate) and A/J mice (susceptible) (Kastrukoff *et al*, 1986). Lip inoculation of HSV-1 in these strains will result in a spectrum of CNS pathology ranging from focal collections of inflammatory cells at the trigeminal root entry zone in resistant mice (C57BL/6J), to unifocal demyelinating lesions in moderately resistant strains (Balb/cByJ), and multifocal demyelinating lesions throughout the brain in susceptible strains (A/J) (Kastrukoff *et al*, 1986, 1987, 1992). Furthermore, primary oligodendrocyte cultures (OL) cultures from the three mouse strains showed differences in resistance to HSV-1 infection which correlated with the previously described clinical outcome and extent of CNS infection (A/J OLs susceptible; Balb/cByJ, OLs moderately resistant; C57BL/6J OLs, resistant) (Thomas *et al*, 1991).

Based on these results, we hypothesize that genetically determined, innate cellular resistance of glia may play an important role in determining the outcome of HSV-1 CNS infection. This study investigates the nature of the HSV-1 resistance *in vitro* of cultured, murine OLs by assessing viral adsorption, fusion, penetration and the expression of viral antigens over time.

Results

HSV-1 antigen synthesis (ICP4, ICP8 and gC) in primary OL cultures

Glial cell cultures (Figure 1a), consisting primarily of oligodendrocytes (Figure 1b) and occasional astrocytes (Figure 1c) were infected with HSV-1 14 days after the cultures were established. The combined results of three experiments between 6 and 24 p.i. h are given in Tables 1 and 2. To illustrate further the trend of antigen expression, extended time points up to 72 h p.i. are depicted in Table 2. Two of the three experiments included all time points. One experiment only included 6, 24 and 48 h p.i. This resulted in a total of six coverslips at 6 and 24 h p.i. but only a total of four coverslips at 12 and 18 h p.i. (Table 1). HSV-1 infected OLs from C57BL/6J mice were negative for all three viral antigens 24 h post infection (p.i.). In contrast 33.3% of A/J OLs were ICP4 positive, 10% ICP8 positive and 7.4% gC positive at 24 h p.i. Balb/cByJ OLs showed an intermediate protein expression pattern at 24 h p.i. with 7.5% of cells ICP4 positive, 0.4% ICP8 positive but none gC positive (Table 1). The expression of immediate early (ICP4), early (ICP8) and late (gC) antigens in A/J strain derived cultures and their corresponding phase contrast photographs are shown in Figure 2. Table 2 includes pooled data from time points through 72 h p.i. and demonstrate a trend of antigen expression. All OLs from A/J mice express all three proteins by 72 h p.i. Limited numbers of OLs from C57BL/6J mice express ICP4 and ICP8 (10% of cells) but no gC at 72 h p.i. Balb/cByJ show 'intermediate' antigen expression at 72 h p.i. (Table 2). The IF pattern differs in this strain from the other two murine strains and is characterized by a more focal pattern of fluoresence. Areas of intense fluorescence are

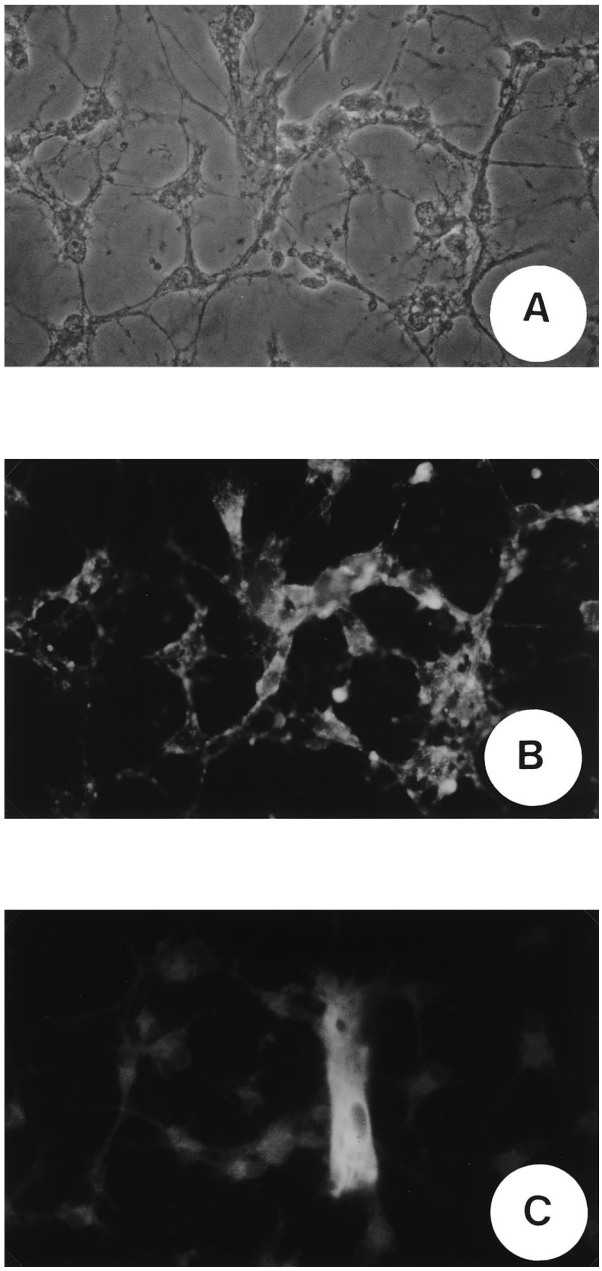

Figure 1 Primary glial cultures from A/J mice, 14 days after being established. (a) Phase contrast photomicrograph (×400). (b) Majority of cells are stained with anti-Gal-C (×400). (c) Occasional cells are anti-GFAP positive (×400)

Table 1 Sequential expression of HSV-1 ICP4, ICP8 and gC expression in A/J, Balb/cByJ and C57BL/6J OLs from 6–24 h p.i.

Mouse strain	*Time p.i. (h)*	n	*ICP4 Mean (%)*	*s.d. (%)*	n	*ICP8 Mean (%)*	*s.d. (%)*	n	*gC Mean (%)*	*s.d. (%)*
A/J	6	6	0.0	0.0	6	0.0	0.0	6	0.0	0.0
	12	4	7.5	2.9	4	4.4	4.9	4	0.0	0.0
	18	4	13.5	5.1	4	7.3	3.2	4	0.0	0.0
	24	6	33.3	16.3	6	10.0	0.0	6	7.4	4.3
Balb/cByJ[a]										
	6	6	0.0	0.0	6	0.0	0.0	6	0.0	0.0
	12	4	0.0	0.0	4	0.0	0.0	4	0.0	0.0
	18	4	5.0	3.5	4	0.3	0.3	4	0.0	0.0
	24	6	7.5	3.9	6	0.4	0.2	6	0.0	0.0
C57BL/6J										
	6	6	0.0	0.0	6	0.0	0.0	6	0.0	0.0
	12	4	0.0	0.0	4	0.0	0.0	4	0.0	0.0
	18	4	0.0	0.0	4	0.0	0.0	4	0.0	0.0
	24	6	0.0	0.0	6	0.0	0.0	6	0.0	0.0

The combined results of both replications of three experiments are shown. The ICP4 and ICP8 proteins were detected in the nucleus and the gC protein displayed a membrane fluorescence. All cells on each slide were evaluated microscopically, counted and the proportion of fluorescing cells on each slide was recorded. The standard deviation (s.d.) refers to the variation between the means (%) of the four or six determinations for the stated combination of strain, time and antigen.[a]Focal fluorescence=Areas of strong fluorescence among non fluorescing cells

interspersed with large areas of apparently non infected cells which is maintained at 72 h p.i. In contrast, fluorescence is evenly spread throughout the cultures of infected A/J OLs.

Adsorption of HSV-1 in primary OL cultures
The combined results of two experiments are given in Figure 3. Net adsorption is expressed as the mean of log adsorbed c.p.m./3000 cells. The analysis of variance shows no significant difference between the three strains (P=0.725).

HSV-1 fusion in primary OL cultures
C57BL/6J and Balb/cByJ OLs were treated with PEG in order to enhance virus-cell membrane fusion of dextran purified non radiolabelled HSV-1. Data from two experiments (two determinations per experiment) are shown in Table 3. PEG treated C57BL/6J OLs produce a mean of 2.63 $\log_{10}$ p.f.u./ml per coverslip while Balb/cByJ OLs produce a mean of 2.79 $\log_{10}$ p.f.u./ml per coverslip. Untreated A/J OLs produce a mean of 3.21 $\log_{10}$ p.f.u./ml per coverslip. A two way analysis of variance shows a significant difference (P=0.005) between the HSV-1 susceptible A/J, resistant Balb/cByJ and C57BL/6J OLs, suggesting that PEG does not increase virus yield from the resistant OLs. We could not identify PEG toxicity immediately after or at 48 h of PEG treatment using trypan blue staining of OLs derived from all three murine strains. Analysis of variance, however showed a significant decrease in virus produced from all PEG treated coverslips (P=0.001).

HSV-1 penetration in primary OL cultures
The combined results of three experiments are given in Figure 4. Radiolabelled HSV-1 (c.p.m./3000 cells) penetrating Balb/cByJ and C57BL/6J OLs is expressed as a percentage of the [^{3}H]thymidine labelled HSV-1 (c.p.m./3000 cells) penetrating A/J OLs. The results suggest an overall reduction of the penetration capacity in Balb/cByJ and C57BL/6J when compared with the A/J OLs at 6, 16 and 24 h post adsorption. Statistical analysis comparing A/J with the two other strains indicate that the results approach significance (P=0.053).

Discussion

Using IFA, we have identified the sequential expression of the transcriptional protein ICP4 (IE) along with ICP8(E) and gC (L) (Honess and Roizman, 1974, Zhu *et al*, 1994) in OLs derived from three different murine strains. These results would suggest that a replicative block exists prior to the expression of IE genes in both C57BL/6J and Balb/cByJ OLs when compared to the A/J OLs (Figure 2, Tables 1 and 2). Furthermore, the IF appearance of the viral antigens in OLs derived from Balb/cByJ mice is focal when compared with the diffuse appearance of viral antigens observed in OLs derived from A/J and C57BL/6J mice. There could be several possible explanations. First, the Balb/cByJ cultures may have contained subgroups of OLs, some of which are more permissive than others. This phenomenon has been reported for HSV replication in rat glial cells (Bergström, 1994). Second, the focal infection may have been initiated from a few infected permissive astrocytes. This would provide a 'focal increase' of viral m.o.i. and subsequently an increased m.o.i. of adjacent OLs. This explanation, although unlikely because very

Table 2 Sequential expression of HSV-1 immediate early, early and late antigen expression in A/J, Balb/cByJ and C57BL/6J OLs from 6 to 72 h p.i.

Mouse strain	*Time p.i. (h)*	*ICP4*	*ICP8*	*gC*
A/J	6	–	–	–
	12	+	–	–
	18	+	+	–
	24	3+	+	+
	30	4+	2+	+
	36	6+	3+	+
	42	6+	4+	3+
	48	8+	8+	6+
	54	10+	10+	9+
	60	10+	10+	10+
	66	10+	10+	10+
	72	10+	10+	10+
Balb/cByJ[a]	6	–	–	–
	12	–	–	–
	18	+	–	–
	24	+	–	–
	30	+	–	–
	36	+	+	–
	42	3+	2+	–
	48	5+	2+	–
	54	5+	3+	+
	60	5+	3+	+
	66	5+	4+	2+
	72	5+	4+	2+
C57BL/6J	6	–	–	–
	12	–	–	–
	18	–	–	–
	24	–	–	–
	30	–	–	–
	36	–	–	–
	42	–	–	–
	48	–	–	–
	54	–	–	–
	60	1 cell	–	–
	66	+	–	–
	72	+	+	–

The pooled results of both replications of three experiments are shown. The ICP4 and ICP8 proteins were detected in the nucleus and the gC protein displayed a membrane fluorescence. All cells on each slide were evaluated microscopically, counted and the percentage of fluorescing cells in relation to the total number of cells was recorded. A scoring system was employed to summarize the microscopical findings as follows: +=10%; 2+=20%; 3+=30%; 4+=40%; 5+=50%; 6+=60%; 7+=70%; 8+=80%; 9+=90%; 10+=100% infected cells respectively. [a]Focal fluorescence= Areas of strong fluorescence among non fluorescing cells

few astrocytes (<1%) were present in the cultures, cannot be entirely excluded. In the case of C57BL/6J OLs, no antigen expression was observed by 24 h p.i. whereas restricted ICP4 and ICP8 expression was present at 72 h p.i. (Figure 2, Table 2), suggesting that the replicative block is delayed in this case and extends throughout the entire C57BL/6J OL population. The delay of antigen expression in C57/BL6J OLs is in concordance with HSV 1 replication results in our previous report (Thomas *et al*, 1991). In that study we were unable to demonstrate any difference in adsorption between

Table 3 OLs grown on four coverslips per mouse strain were exposed to 1 p.f.u. per cell of non radiolabelled, dextran purified HSV-1

Treatment	*Mouse strain* *A/J*	*Balb/cByJ*	*C57BL/6*
PEG	2.98 ± 0.04[a]	2.79 ± 0.001	2.63 ± 0.03
No PEG	3.21 ± 0.07	3.08 ± 0.01	3.07 ± 0.06

Two coverslips were treated with PEG following viral adsorption and two remained untreated. Progeny virus was assayed by plaque titration on CV 1 cells. The results represent 2 experiments with two determinations per experiment and OL strain. [a]Log_{10} p.f.u. (Mean $\pm$ s.e.m.)

the three OL strains. We have now confirmed the lack of adsorption differences, using a more sensitive methodology which employs enriched OLs grown on coverslips. This approach confirms culture purity by using OL specific antibodies (Kim *et al*, 1983). A dextran gradient centrifugation for virus purification (Spear and Roizman., 1972; Cassai *et al*, 1987) replaces the previous Sepharose CL-2B exclusion chromatography technique (Thomas *et al*, 1991). This method results in an increased yield of radioactive virus. When bulk cultures of OLs were used (Thomas *et al*, 1991), we were able to perform the experiments at 4°C. When the experiments were repeated with OLs grown on coverslips the cells were found to dislodge. This made it necessary to perform the new set of adsorption experiments at room temperature, where non specific endocytosis is minimal (Lycke *et al*, 1991). Using the modified protocol, we were not able to demonstrate any significant difference in adsorption capacity between the OLs, thus confirming our previous observations.

Two major pathways of viral entry, endocytosis and fusion, are used by viruses (Spear *et al*, 1989). HSV-1 likely only uses virion envelope – plasma membrane fusion for infective entry (Spear *et al*, 1992; Wittels and Spear, 1991; Shieh and Spear, 1994; Herold *et al*, 1994). In an attempt to facilitate virus entry in Balb/cByJ and C57BL/6J OLs we enhanced fusion by PEG treatment. If the replicative block were to exist at the virion envelope – OL plasma membrane fusion stage, an increase in HSV-1 progeny, approaching the amount of progeny virus produced in nontreated A/J OLs, would have been expected. Trypan blue staining did not detect a direct toxic effect of PEG in the OLs, but did detect toxicity of non – OLs 48 h after treatment. This may have contributed to the decrease of progeny virus from all three OL cultures following PEG treatment. Within these limits, PEG treatment failed to increase the virus yield in both Balb/cByJ and C57BL/6J OLs when compared to untreated A/J OLs. The results suggest that differences at the fusion stage do not explain the replicative block in the OLs derived from different inbred strains of mice.

Subsequent to these studies we were able to demonstrate an overall reduction in the uptake of radioactively labelled HSV-1 in Balb/cByJ and C57BL/6J OLs when compared to A/J OLs. These results approached statistical significance suggesting that viral penetration may be impaired in C57BL/6J and Balb/cByJ mice compared to A/J mice. The assay however, did not include synchronization of penetration by a primary adsorption step at 4°C. This was for two reasons. The OLs grown on coverslips are sensitive to temperature change, and any manipulation of these cells at 4°C tends to dislodge the cells from the coverslips if further incubation is required. The second reason is that temperature shift-up has been reported to induce endocytosis (Anderson *et al*, 1977; Fuller and Spear, 1987). Furthermore, the viral penetration assay employs OLs grown on coverslips and measures overall cellular uptake but does not allow the distinction between uptake in the cytoplasmic and nuclear fractions. To further define this, we attempted additional studies employing an experimental technique to assess viral transport to the nucleus as outlined by Svennerholm *et al* (1982).

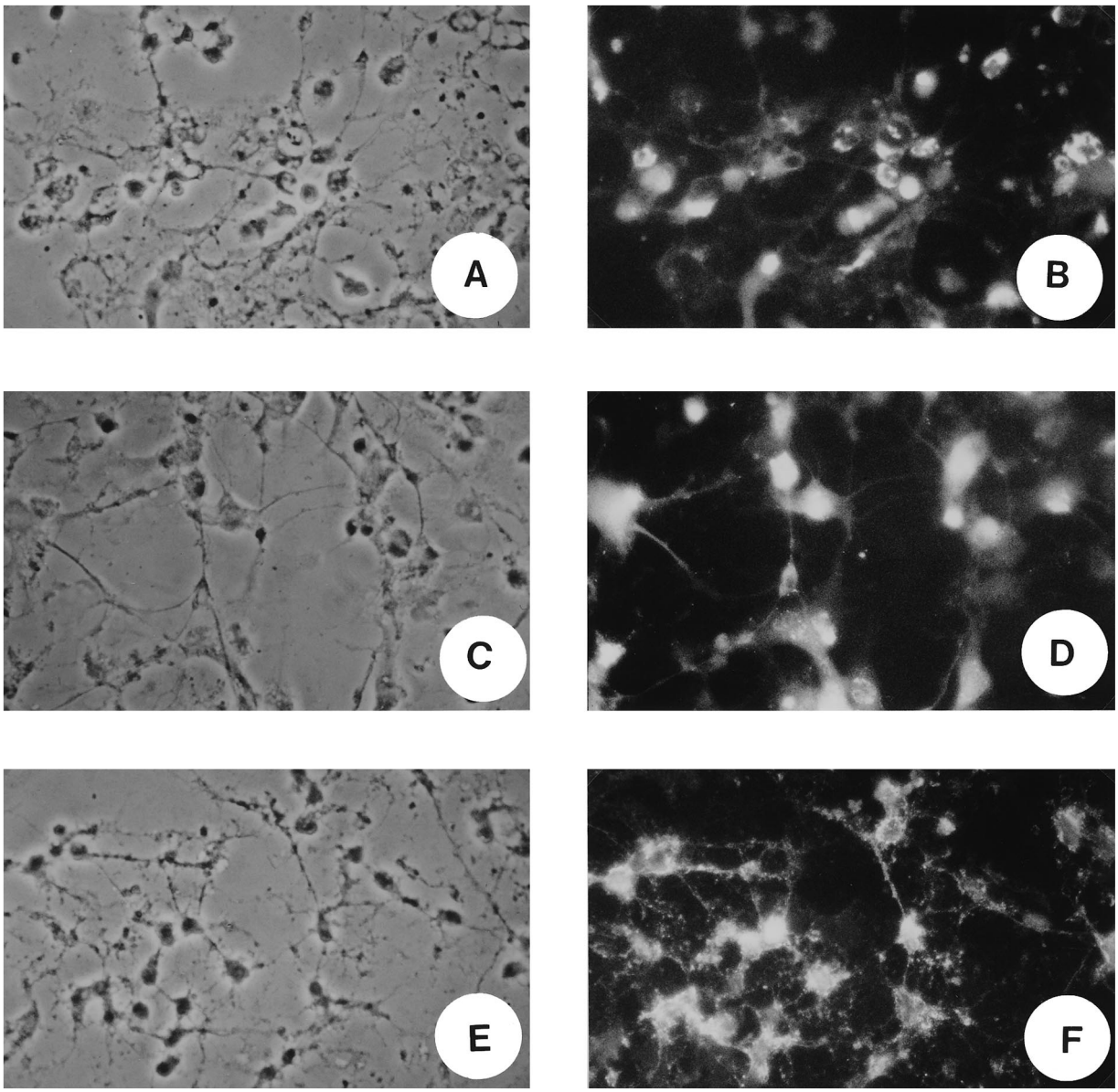

Figure 2 Primary cultures of OLs from A/J mice, infected with HSV-1 at an m.o.i. of one. (a) Phase contrast photomicrograph of OLs infected with HSV-1 30 h p.i. (×400). (b) 40% of OL in (a) have positive staining with anti-ICP4 (×400). (c) Phase contrast photomicrograph of OLs infected with HSV-1 48 h p.i. (×400). (d) 80% of OLs in (c) have positive staining with anti ICP8 (×400). (e) Phase contrast photomicrograph of OLs infected with HSV-1, 72 h p.i. (×400). (f) 100% of OLs in (e) have membrane staining with anti-gC (×400)

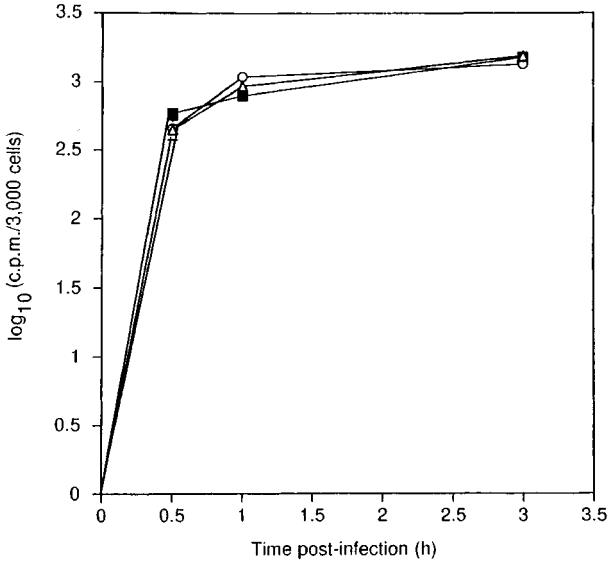

Figure 3 Adsorption of HSV-1 to primary OL cultures from A/J (△), Balb/cByJ (■) and C57BL/6J (○) mice, using [^{3}H]thymidine labelled, dextrane gradient purified HSV-1 at an m.o.i. of 1 p.f.u./cell. Each point represents two experiments with three determinations each. The standard error of the mean (s.e.m) were so small that it was not possible to represent them graphically

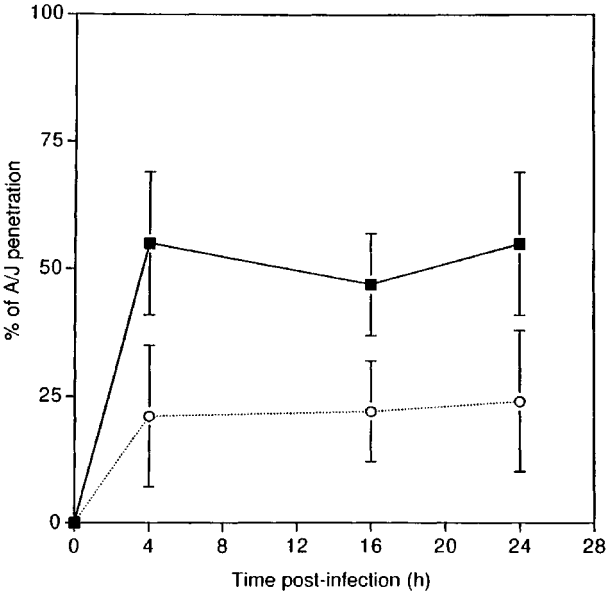

Figure 4 Penetration of [^{3}H]thymidine labelled, dextran gradient purified HSV-1 at an m.o.i. of 1 p.f.u./cell in Balb/cByJ (■) and C57BL/6J (○) OLs, expressed as a percentage of c.p.m./3000 cells penetrated into A/J OLs. At each time point two coverslips were rinsed four times with citrate buffer, pooled and lysed in PBS (1% SDS and 1% Triton X-100) prior to determination of radioactivity in a scintillation counter. Each point represents the mean of three experiments with three determinations per experiment. Each bar represents the standard error of the mean (s.e.m)

These studies identified a decrease in nuclear uptake in Balb/cByJ and C57BL/6J OLs when compared to A/J OLs (unpublished observations), but were not of sufficient sensitivity to permit a formal, statistical evaluation. Quantitative *in situ* polymerase chain reaction (PCR) assays would be required to address this question.

In conclusion, our studies to date suggest that the HSV-1 replicative blocks in primary OL cultures from Balb/cByJ and C57/BL6J mice, although not identical, are determined prior to the expression of IE gene products. Within some experimental limitations, the difference in HSV-1 susceptibility does not appear to exist at the level of adsorption or fusion, while transport of virus from the cell surface appears to be impaired in both C57BL/6J and Balb/cByJ OLs and may represent the most likely explanation.

The innate differences in HSV-1 resistance detailed in this paper, reflect differences in virus host cell interactions which likely contribute to the differences in mortality, viral spread and pathological differences following HSV-1 infection of the CNS in the different mouse strains.

Materials and methods

Animals

Three inbred strains of mice (C57BL/6J, Balb/cByJ, A/J) were obtained from Jackson Laboratories, Bar Harbour, Me. They were maintained for 2 weeks prior to use at 10 to 12 weeks of age. Fifteen male mice of each strain were used for each experiment.

Virus and cells

HSV-1 laboratory strain 2 was used; virus was propagated and plaque assayed on CV-1 cells as described previously (Kastrukoff *et al*, 1987).

Isolation of murine OLs

Primary cultures of uninfected murine OLs were established according to the method of Kim *et al* (1983). Briefly, CNS tissue is removed from 15 mice of each strain, cut into 3 mm pieces, and incubated in 0.25% trypsin (Gibco, Burlington, Ontario) and 0.2 mg/ml DNase (Sigma, Mississauga, Ontario) for 45 min at 37°C. After incubation the dissociated tissue is passed through a 150 μm nylon mesh filter and centrifuged at 400 g in a Beckman J-6B centrifuge with a JS-4 rotor at 4°C for 10 min. The tissue is rewashed and centrifuged in a Percoll gradient (Pharmacia Biotech, Quebec) at 31 000 g (Beckman J2-21 centrifuge with a JA-17 rotor) at 4°C for 25 min. The OL layer is removed, washed repeatedly in Hanks balanced salt solution (BSS), and the cells are either plated on poly-L-lysine-coated Aclar (Allied-Signal) coverslips or grown in suspension using Eagle's MEM supplemented with glutamine, 0.5% glucose, 500 U/mL penicillin,

500 μg/mL streptomycin, 25 μg/mL gentamicin, 1.25 μg/mL fungizone and 5% foetal bovine serum (FBS). Plated cells are allowed to settle for 24 h, and non attached cells are subcultured in a similar fashion. OL cultures grown on coverslips, are used in virus protein expression analysis 14 days after they were established. OLs were actively extending processes at this time. Approximately $4-5\times10^3$ OLs will grow on each coverslip, but, as this is somewhat variable after 14 days, cells on each coverslip had to be counted and the virus inoculum adjusted to ensure that the correct m.o.i. was achieved. OLs grown in suspension are used after 24 h. This allows a larger number of cells (1×10^7) to be used, which is required to determine the cytoplasmic/nucleic ratio of penetrated virus. As the number of OLs in each suspension culture is corrected for inter-batch differences, the efficiency of virus penetration is not affected by variation in cell numbers. The cultures were 99% pure OLs, as determined by double labelling with mouse-anti-galactocerebroside (anti-Gal-C) and rabbit-anti-glial fibrillary acidic protein (anti-GFAP) antibodies (Kim *et al*, 1983).

Preparation of [^{3}H]thymidine labelled virions
This was performed by the method of Spear and Roizman (1972) and modified by Cassai *et al* (1987). Briefly, viral DNA is radiolabelled by infecting 20 175 cm^2 flasks growing confluent CV-1 cells at a multiplicity of three. Following removal of the inoculum, 1 mCi [^{3}H]thymidine (Amersham; TRK 120) is added to the growth medium 2 h postinfection (p.i.) and the flasks incubated at 37°C for 48 h. Cells are then removed directly into the medium and collected by low speed centrifugation (800 g for 10 min) at 4°C. Cytoplasmic extracts are prepared by suspending 4×10^8 cells in phosphate buffered saline (PBS) pH 7.4 for 10 min followed by disruption of cells in a Dounce homogenizer. Nuclear pellets are obtained by centrifugation for 10 min at 800 g, 4°C. This pellet is washed twice with PBS. The combined nuclear and cytoplasmic fraction is then layered onto a dextran gradient (Dextran T10; Pharmacia Biotech, Quebec) in PBS and centrifuged for 1 h at 52 000 g in a 60 Ti rotor using a Beckman L8-55 ultra centrifuge. The enveloped virus, is aspirated and the purity confirmed by electronmicroscopy. The preparation was diluted in PBS and the virions pelleted at 115 000 g for 2 h in a 60 Ti rotor. The pellet is collected and stored at −70°C until used. This procedure results in preparations with a viral titer varying between 1×10^7 to 1×10^8 p.f.u./ml and radioactivity between 1×10^7 to 1×10^8 c.p.m./ml.

Serial viral antigen expression by indirect immunofluorescence assay (IFA)
HSV-1 immediate early (IE), early (E) and late protein expression, was assayed by IFA. The method has been previously reported (Kastrukoff *et al*, 1988). In brief OLs on coverslips are infected with HSV-1 at an m.o.i. of one p.f.u./cell, incubated at 37°C and immuno stained every 6 h from 6 to 72 h p.i. At each time of harvest six coverslips are rinsed three times with PBS, fixed with 2% paraformaldehyde for 30 min, and rinsed twice in PBS. Two of the 4–6 coverslips per time point from each strain are incubated for 1 h with monoclonal antibodes: anti ICP4 (immediate early), anti ICP8 (early) and anti gC (late) (kindly supplied by Drs F Tufaro, D Knipe and Dr L Pereira respectively). The monoclonal antibodies are diluted in PBS – 1% BSA as follows: anti ICP4 (1 : 850); anti ICP8 (1 : 25); anti gC (1 : 100). The coverslips are then incubated for 30 min in PBS – 1% BSA. A 1 : 1000 dilution of a fluorescein isothiocyanate (FITC) conjugated anti mouse IgG antibody (Jackson Research Labs, Mississauga, Ontario) in PBS 1% – BSA is then added to the coverslip and incubated for 1 h at 37°C. The coverslips are rinsed in distilled H_2O and mouted. All immunostained cultures from one experiment were examined in a blinded fashion by the same observer using a Zeiss Universal microscope equipped with fluorescence and phase optics. All cells were microscopically evaluated and counted and the percentage of fluorescing cells in relation to the total number of cells was recorded. A scoring system was employed to summarize the microscopical findings as follows: +=10%; 2+=20%; 4+=40%; 6+=60%; 8+=80%; 10+=100% infected cells respectively.

Viral adsorption
In situ adsorption on coverslips was performed by the method of WuDunn and Spear (1989). Briefly, coverslips with OLs are pretreated for 15 min at 37°C with PBS containing 1% FCS, 0.1% glucose and 5% bovine serum albumin (BSA). All adsorption experiments are carried out at 20°C to avoid nonspecific endocytosis (Lycke *et al*, 1991). HSV-1 is added at an m.o.i. of 1 p.f.u./cell and the adsorption is assayed up to 6 h p.i. Three coverslips are removed at 0, 1, 3 and 6 h p.i. respectively. Coverslips were washed repeatedly in PBS, lysed in PBS – 1% sodium dodecyl sulphate (SDS) – 1% Triton X-100 and radioactivity is determined in a Beckman LS-4000 scintillation counter. For each time point, nonspecific adherence on cell free control coverslips was subtracted from the corresponding OL coverslips prior to presentation of the data in a graph (Wu Dunn and Spear, 1989).

Viral penetration
Viral penetration was determined using the method of Huang and Wagner (1964) and modified by Highlander *et al* (1987). Briefly, ten coverslips with OLs are placed in a 60 mm petridish, treated for 15 min at 37°C with PBS+1% FBS, 1% glucose and 5% bovine serum albumin (BSA). Cell free cover-

slips treated in the same fashion served as controls. Supernatant is removed and 1 p.f.u./cell of [^{3}H]thymidine radiolabelled, dextran purified HSV-1 is added and the coverslips incubated at 37°C in a CO_2 incubator. Penetration proceeds for 6, 16 or 24 h. At each time point two slides are washed repeatedly with citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3) to remove non penetrated virus. The OLs from two coverslips are pooled and lysed in PBS – 1% SDS – 1% Triton X-100. Radioactivity is determined in a Beckman LS-4000 scintillation counter. Non specific adherence of virus is subtracted from the cellular penetration values prior to presentation of the data (Wu Dunn and Spear, 1989).

Viral fusion

Viral entry into C57BL/6J and Balb/cByJ OLs was facilitated by polyethylene glycol (PEG) enhancement using the method of Sarmiento *et al* (1979). Briefly, four coverslips with OLs are exposed to 1 p.f.u./cell of non radiolabelled dextran purified HSV-1 (Spear and Roizman, 1972; Cassai *et al*, 1987). After adsorption for 4 h at room temperature (Lycke *et al*, 1991), the inoculum is removed, cells are rinsed repeatedly with PBS – 0.1% glucose and 1% FBS. Two coverslips are exposed to 40 g of melted PEG 8000 (Sigma, Mississauga, Ontario) diluted in 36.4 ml of Eagle's MEM. The remaining two coverslips serve as controls and are processed in the same fashion but without PEG. Exposure of cells to PEG solution was minimized according to Sarmiento *et al* (1979) and the procedure completed with three final washes with MEM+10% FBS. Cultures are then incubated with MEM+10% FCS for 2.5 h at 37°C to allow PEG induced fusion to proceed. Cells are rinsed repeatedly with citrate buffer to remove any remaining extracellular virus, followed by repeated washes in MEM+5% FBS. Cells are incubated in FBS free MEM for 48 h and frozen at −70°C. Total virus titers were determined by plaque titration using CV 1 cells. Potential PEG toxicity was excluded by trypan blue staining of non infected PEG treated OLs.

Statistical analysis

Analyses of variance were carried out on results from the virus adsorption, penetration and fusion experiments. Three factor analyses were performed on the logarithms ($\log_{10}$) of the measurements. For viral adsorption and penetration, the factors were experiment, strain and post infection time. With the fusion experiment the factors were experiment, strain and whether or not the cells had been treated with PEG. Two factor analyses (experiment and strain) were also performed on each of the two levels of PEG, since it appeared that the additivity assumptions might not be met with the incorporation of PEG into the model. In the penetration analysis, two outlying observations were removed, one being 2.35 and the other being 2.57 standard deviations from the mean. Means and their standard errors were calculated for each combination of factors.

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