Variation in resistance to herpes simplex virus type 1 of oligodendrocytes derived from inbred strains of mice

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Primary oligodendrocyte (OL) cultures from three inbred strains of mice with known differences in resistance to herpes simplex virus type 1 (HSV-1) infection in vivo (A/J, susceptible; BALB/cByJ, moderately resistant; C57BL/6J, resistant), also display a similar pattern of resistance in vitro. The nature of the in vitro resistance at the cellular level was investigated. Virus production at different m.o.i.s indicated that the differences in HSV-1 replication are m.o.i.-dependent. Overall, virus yield from the OL cultures infected at a multiplicity of 1 increased 48 h post-infection (p.i.); no additional enhancement occurred 72 h p.i. However, the difference in the replication capacity of the three OL cultures observed at 24 h p.i. persisted at 48 and 72 h p.i. Serial electron microscopy studies on infected OL cultures derived from the different murine strains suggested that the resistance to HSV-1 infection occurs at different stages during the replicative cycle. Virus was detected at the nuclear membrane 5 min p.i. in A/J cells, but was not observed until 120 min p.i. in BALB/cByJ cells, whereas virus could not be detected at the nuclear membrane of C57BL/6J cells, even at 24 h p.i. Virus adsorption, determined by assay of residual non-adsorbed virus infectivity and cell-associated, radiolabelled HSV-1, did not differ in the OL cultures. The cumulative data suggest that A/J cells display the same replication pattern as permissive CV-1 cells, whereas the major replicative blocks in the other two murine strains occur at the level of the cytoplasmic membrane in C57BL/6J OLs, and at the level of the nuclear membrane in BALB/cByJ cells.

Introduction

Genetically determined resistance to herpes simplex virus (HSV) infection in mice has been reported previously (Lopez, 1975; Kastrukoff et al., 1986). Lopez has suggested that resistance is dominant and that two non-H-2 loci are responsible (Lopez, 1980). Furthermore, he has interpreted these results as indicating an important role for the immune system. Such immune-mediated resistance is likely to be multifactorial and involve both humoral and cellular mechanisms (Stevens, 1986). An HSV footpad inoculation model in susceptible and resistant mouse strains (A/J and C57BL/6J) has shown that virus replication is restricted initially at the level of the spinal ganglia, and that this restriction is immune-mediated (Cook, 1983). Recent studies suggest that an even greater degree of complexity may exist for variation in resistance. Macrophages derived from resistant mice (C57BL/6J) show variations in intrinsic cellular resistance to HSV in vitro, with the extent of replicative resistance being determined by the site of origin of these cells (Sarmiento & Kleinerman, 1990).

Other investigators suggest that natural resistance to HSV infection may also be mediated by non-immune mechanisms expressed at the level of the infected cell (Collier et al., 1983; Vahlne et al., 1981; Tenney & Morahan, 1987). Since the nervous system is the target of both acute and latent HSV infections (Roizman & Sears, 1987; Rawls, 1985), differences in the genetically determined resistance of central nervous system (CNS) cells could be important in determining the outcome of HSV infection. We have reported previously a correlation between resistance to HSV in primary cultures of oligodendrocytes (OL) derived from different inbred strains of mice in vitro and mortality in vivo following virus inoculation into the lip (Kastrukoff et al., 1987). A similar correlation has been made between resistance in vitro and the extent of pathological involvement of the CNS in vivo in a limited number of inbred strains (Kastrukoff et al., 1987). In this study, we have further characterized the replicative capacity of HSV in primary cultures of OLs derived from three different inbred strains of mice with known differences in HSV resistance.
Methods

Animals. Three inbred strains of mice (C57B/6J, BALB/cByJ, A/J) were obtained from Jackson Laboratories, Bar Harbor, Me., U.S.A. They were maintained for 2 weeks prior to use at 10 to 12 weeks of age.

Virus and cells. HSV-1 laboratory strain 2 was used; virus was propagated on BHK-21 cells and plaque assayed on CV-1 cells, as described previously (Kastrukoff et al., 1987).

Isolation of primary murine OLs. Primary in vitro cultures of uninfected murine OLs were established according to the method of Kim et al. (1983). Briefly, CNS tissue was removed from 15 mice of each strain, cut into 3 mm pieces, and incubated in 0.25% trypsin and 0.2 mg/ml DNase for 45 min at 37 °C. After incubation the dissociated tissue was passed through a 150 lam nylon mesh filter and centrifuged at 400 g in a Beckman J-6B centrifuge with a JS-4.2 rotor at 4 °C for 10 min. The tissue was resuspended and centrifuged in a Percoll gradient (Pharmacia) at 31000 g (Beckman J-21 centrifuge with a JA-17 rotor) at 4 °C for 20 min. The OL layer was washed, resuspended repeatedly, and cells were either plated on polylysine-coated Aclar (Allied Chemical) coverslips or grown in suspension using MEM with Earle's salts supplemented with glutamine, 0.5% glucose, 50000 units penicillin, 50 mg streptomycin, 25 mg gentamicin, 125 µg fungizone and 5% foetal calf serum. Cultures grown on coverslips were used in virus production and electron microscopy (EM) studies 14 days after they were established. As these cells divide in culture, confluent monolayers are not obtained, but they are actively extending processes at this time. Approximately 4000 to 5000 OLs will grow on each coverslip, but, as this is somewhat variable after 14 days, cells on each coverslip have to be counted and the inoculum adjusted to ensure that the correct m.o.i. is achieved. OLs grown in suspension were used after 48 h of culture for virus adsorption studies, including assay of residual non-adsorbed virus infectivity and determination of cell-associated radiolabelled HSV. As the number of OLs in each suspension culture was corrected for inter-batch differences, the efficiency of virus adsorption was not affected by variation in cell numbers. The cultures were 95% to 98% pure OLs, as determined by double labelling with anti-galactocerebroside (anti-Gal-C) and anti-glia fibrillary acidic protein antibodies (Kim et al., 1983).

Virus production in OLs. OLs grown on coverslips were counted and infected with HSV-1 (0.5 ml at the appropriate m.o.i.) Following adsorption for 1 h, cells were washed four times with Hank's balanced salt solution (BSS) and incubated with 1 ml of maintenance medium in 5% CO₂ at 37 °C. Twenty-four hours post-infection (p.i.) or at timed intervals thereafter (growth curve experiments), the cells and supernatant were removed, frozen and thawed three times, and total virus produced was titrated by plaque assay.

Electron microscopy. Primary murine OLs derived from the three inbred strains of mice were grown on coverslips and infected at a multiplicity of 0-1 at 37 °C for 5, 15, 30 and 120 min, as well as for 24 h. The cells were then washed three times with Hank's BSS, fixed in 3% glutaraldehyde in PBS, post-fixed in 1% osmium tetroxide for 60 min and dehydrated in alcohol. Cells were infiltrated with EM Bed 812 over 36 h. sectioned on an ultramicrotome and examined with a Philips 300 electron microscope. Three separate sets of experiments, each consisting of 50 OLs derived from each of the three different strains of mice, were performed at each time.

Radiolabelling of HSV DNA. Radiolabelled viral DNA was obtained by infecting confluent CV-1 cells in flasks with HSV at a multiplicity of 5 to 10 and adding 1 mcCi [³H]thymidine (Amersham; TRK120) 3 h p.i. Cultures were then incubated at 37 °C for a further 18 to 24 h.

Preparation of virions. Cells infected with HSV-1 and labelled with [³H]thymidine as above were removed from culture and homogenized in Ten Broeck glass tissue grinders. After low speed centrifugation (800 g for 5 min) the pelleted cells were resuspended in 1 mM phosphate buffer pH 7.4 and further disrupted. Nuclei were removed by an additional low speed centrifugation. The supernatants were pooled, centrifuged at 15000 g (Beckman J-21 centrifuge with a JA-17 rotor) for 5 min and the supernatant obtained was passed through a Sepharose CL-2B (Pharmacia) column. The void volume, containing all virus infectivity (5 × 10⁶ to 3 × 10⁷ p.f.u./ml), was used as the virion preparation. The radioactivity of the preparations ranged from 5 × 10⁶ to 2 × 10⁷ c.p.m./ml.

Virus adsorption. Virus adsorption was determined by two separate methods, an assay of residual non-adsorbed virus infectivity and determination of cell-associated, radiolabelled HSV (Vahlne et al., 1978, 1980). OLs (1 × 10⁷ cells/ml) in suspension were used throughout. Briefly, for residual infectivity studies cells were suspended in 0.9 ml of Eagle's MEM, agitated in a water bath at 4 °C or 37 °C and 0.1 ml HSV was added at a multiplicity of 1:10. Samples of culture media (100 µl) were removed at various times after the addition of virus, added to 9.9 ml of ice-cold medium and centrifuged for 10 min at 1000 g. Residual infectivity in the supernatant was assayed by plaque titration. Adsorption curves were constructed by plotting the percentage of infectivity remaining against the time of adsorption, after correcting for the amount of loss of infectivity in cell-free controls. Attachment of [³H]-labelled virions was studied in a similar fashion. A purified [³H]-thyminine-labelled HSV suspension containing 2 × 10⁴ c.p.m. and 1 × 10⁷ p.f.u./ml was added to suspension cultures (1 × 10⁶ cells), and cell samples were collected at different times. Cells were then washed twice and counted in a Beckman LS-4000 scintillation counter.

Statistical analysis. Statistical analysis was performed by an independent statistician (Dr Jonathan Berkowitz, Department of Statistics, University of British Columbia, Canada) using a two-factor analysis of variance (ANOVA) on log transformed data.

Results

Determination of HSV-1 production in infected primary OL cultures

HSV-1 growth curves at different m.o.i.s in primary OL cultures from A/J, BALB/cByJ and C57BL/6J are shown in Fig. 1. For each of the three experiments a two-factor ANOVA was undertaken, using log transformed data to stabilize the variance across factor levels. For each experiment, the results of the analysis indicated that there is a highly significant group effect resulting from the difference between A/J mice, (P < 0.001) and BALB/cByJ and C57BL/6J mice, as well as a highly significant difference between the different m.o.i.s (P < 0.001). The interaction effect between groups and m.o.i.s was also statistically significant (P < 0.001). The cumulative results presented in Fig. 1 show that the virus yield was greatest at an m.o.i. of 100, whereas the greatest difference in virus production by A/J, BALB/cByJ and C57BL/6J OL occurred at an m.o.i. of 0.01.

To determine whether the lower virus yield in BALB/cByJ and C57BL/6J cells was due to delayed HSV
Resistance of oligodendrocytes to HSV-1

In the cytoplasm (Fig. 3). In BALB/cByJ OLs, virus was observed at the cytoplasmic membrane but not in the cytoplasm immediately after infection. At 24 h p.i., non-enveloped virus was observed in the cytoplasm, but virus was not present in the nucleus (Fig. 3). In contrast, the majority of OLs derived from C57BL/6J mice showed no evidence of infection, even 24 h p.i. (Fig. 3). Up to 5% of OLs of the latter strain did have occasional non-enveloped virus in the cytoplasm and/or nucleus.

Although the cultures were 95 to 98% pure anti-Gal-C antibody-positive cells, occasional cells with the morphological appearance of astrocytes were observed. In OL cultures derived from C57BL/6J mice, many virus particles were observed in both the nucleus and cytoplasm of cells with this appearance 24 h p.i. (data not shown).

Adsorption of HSV-1 to primary OL cultures

Adsorption of HSV-1 to OLs at 37 °C, as determined by assay of residual non-adsorbed virus infectivity using CV-1 cells as controls, is shown in Fig. 4; the adsorption profiles have been corrected for loss of infectivity. The overall adsorption was slightly higher in the CV-1 cells than in any of the OL cultures, but there was no apparent difference in net adsorption at 60 min between OL cultures, although there is a suggestion of higher adsorption in A/J cells 15 min p.i. Adsorption experi-
Fig. 3. EM photomicrographs of HSV-1-infected OLs derived from three strains of mice. (a) OLs derived from A/J mice contain virions in the nucleus as well as enveloped and non-enveloped viruses in the cytoplasm 24 h p.i. Bar marker represents 500 nm. (b) OLs derived from BALB/cByJ mice contain non-enveloped viruses in the cytoplasm as well as adjacent to the nuclear membrane 24 h p.i. Bar marker represents 500 nm.

Fig. 4. Adsorption of HSV-1 to primary OL cultures from A/J (●), BALB/cByJ (▲) and C57BL/6J (●) mice, as measured by disappearance of virus from the medium. CV-1 cells (■) were used as a control. An m.o.i. of 1 p.f.u. virus/cell was used.

Fig. 5. Adsorption of HSV-1 to primary OL cultures from A/J (●), BALB/cByJ (▲) and C57BL/6J (●) mice, as measured using [3H]thymidine-labelled HSV-1. CV-1 cells (■) were used as a control. An m.o.i. of 1 p.f.u. virus/cell was used.
Table 1. EM studies of HSV-1 infection in primary OL cultures

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<th>OL culture</th>
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* - , No virus observed in any cell; +, few viruses (1 or 2), 10% of cells; + +, > 10 viruses, 10 to 50% of cells; + + +, many viruses, 50 to 75% of cells; + + + +, many viruses, 75 to 100% of cells.

ments were also performed at 4 °C, but no difference in net adsorption between the cell lines was observed. To exclude the possibility that proteolytic enzymes released from the cells might have caused degradation of virus with loss of infectivity, and to increase the sensitivity of the adsorption experiments, virus attachment at 37 °C and 4 °C was also studied using purified, radiolabelled HSV virions. The results at 37 °C are presented in Fig. 5. Significant adsorption, although less than in CV-1 cells, was observed 5 min p.i. in all OL cultures, but no difference in net adsorption at 60 min could be detected at either temperature.

Discussion

The nature and extent of infection with HSV-1 is likely to be the result of multiple factors, including those determined by the virus and the host, and expressed as an interaction between the two. In mice, natural resistance to HSV-1 appears to be genetically determined and there is good evidence to support the theory that it is immune-mediated (Lopez, 1980; Cook & Stevens, 1983). Less clearly understood is the possible contribution of non-immune cells to resistance and their relative contribution to the final outcome of infection.

Following reports that differences in the resistance of structural cells to HSV-1 may exist (Collier et al., 1983), preliminary studies were conducted with primary OL cultures, as the interaction of HSV with nervous system cells was believed to be relevant to the pathological outcome of infection with a neurotropic virus. An immunofluorescence assay (IFA) of HSV-infected OLs from A/J, BALB/cByJ and C57BL/6J mice cultured in vitro using an m.o.i. of 5 p.f.u./cell, and staining with an anti-gC monoclonal antibody (MAb) at different times p.i. showed that the infectious cycle of HSV was markedly delayed in BALB/cByJ and C57BL/6J OLs compared to that in A/J OLs (Kastrukoff et al., 1986). These studies suggest that differences in resistance to HSV-1 do exist in OL cultures derived from different inbred strains of mice. We have now extended these studies of HSV-1 resistance in primary cultures of mouse OLs derived from three inbred mouse strains.

The results indicate that the pattern of resistance of the three OL cultures to HSV in vitro is influenced by the initial m.o.i., with the most pronounced replicative differences occurring at low m.o.i. This phenomenon has been observed with cultured neuroblastoma cells (Vahlne et al., 1981). In this case, replication inhibition is dependent on a non-interferon host cell protein of 15K which actively inhibits virus-encoded functions (Thomas, 1985). This multiplicity-dependent outcome of HSV infection has also been shown in mice during acute primary infection (Thomas et al., 1985). We speculate that our results in vitro and in vivo might reflect a similar mechanism of resistance; that is, cell-regulated suppression of HSV replication which can be overcome by an increase in infectious dose. We have shown that this proposed innate, cell-regulated replication block can be overcome by a high input of virus (Fig. 1), and partly overcome by allowing prolonged replication (Fig. 2). One explanation for this is that our cultures contain approximately 5% astrocytes (all of which are permissive), thus a 'focal increase' in m.o.i. will occur in the vicinity of permissively infected astrocytes after 24 h. This will in turn allow adjacent OLs to be infected with a higher m.o.i. than that used initially and the proposed intracellular replicative block will be overcome in these cells.

Following the observation that net virus production does differ among the three strains at low m.o.i., EM studies were performed which show qualitative as well as quantitative replication differences, indicating that the kinetics of early HSV-1 replication differ. Cellular protein synthesis is shut off very early in the productive HSV replication cycle (Roizman & Sears, 1987). To be efficient, any cellular interference with virus replication
would probably have to affect an early replicative step. The initial events during HSV infection of permissive cells include attachment to specific receptors, fusion of the envelope to the plasma membrane (Roizman & Sears, 1987; Cai et al., 1988) and transportation of virion components, including DNA-containing nucleocapsids and tegument proteins, to the nuclear pores (Roizman & Sears, 1987). In our in vitro system, HSV replication in OLs derived from A/J mice appears to be comparable with that observed in permissive cells (Roizman & Sears, 1987; Table 1). Virus can be seen in the cytoplasm of A/J OLs almost immediately after infection, with a full replicative cycle occurring within 24 h. In contrast, the moderately resistant BALB/cByJ OLs appear to block virus transport at the nuclear membrane. Virions are observed in the cytoplasm shortly after infection, but no virus can be identified in the nucleus, even 24 h p.i. The C57BL/6J OLs either impair virus adsorption or block virus transport at the level of the cytoplasmic membrane.

No virus is observed in these cells until 24 h p.i., and at that time only a few cells are infected with a small number of virions.

To investigate replication restriction further, virus adsorption was studied using two different assay systems. The sensitivity of these methods did not allow them to be applied to small numbers (2 x 10^3) of OLs grown on coverslips. Virus adsorption studies therefore were performed on OLs grown in suspension; these cells differ morphologically from those grown on coverslips, primarily in that they lack cytoplasmic processes. As a result, it is possible that they lack virus receptors that may be present on cells in older and more established cultures. However, we could not detect a difference in virus production with the two culture techniques, suggesting that the expression of virus receptors is not altered.

The adsorption curve for OLs indicates that many virus particles are attached initially; this is most marked for A/J cells (Fig. 4 and 5). Approximately 30 min p.i., some of the adsorbed virus appears to detach. Virus adsorption curves in permissive cells usually progress to a maximum without peaks or troughs prior to the saturation level (Vahlne & Lycke, 1978), a pattern also displayed by our control CV-1 cells (Fig. 4 and 5). One possible explanation for initial reversible attachment is that immediate adsorption occurs with low affinity, but complete adsorption requires attachment to more than one site on the cells so that the adsorption observed initially is followed by early dissociation of some receptor–ligand complexes (Kaner et al., 1990). It is also possible that our system is not sensitive enough to delineate adsorption kinetics reliably during the first 15 min of infection. The overall adsorption in CV-1 cells is greater than in any OL culture using both assays. However, CV-1 cells are approximately three- to fourfold larger than OLs and may potentially express a greater number of receptors. However, there is no difference in the net adsorption of virus 60 min p.i. in the OL cultures examined and therefore it does not appear to be the rate-limiting step for the difference in replicative capacity observed. Within the limitations of the assay systems employed, the adsorption studies suggest that the replicative block occurs after virus has adsorbed to the cytoplasmic membrane of BALB/cByJ- and C57BL/6J-derived cells. Our cumulative data suggest that the major HSV-1 replicative block occurs at the level of the cytoplasmic membrane in C57BL/6J OLs, and at the level of the nuclear membrane in BALB/cByJ cells.

Ultimately, many factors are involved in determining the outcome of HSV-1 infection in vitro. Kastrukoff et al. (1987) have demonstrated that transport of HSV-1 to the trigeminal root entry zone occurs in A/J, BALB/c and C57 BL/6 mice, but the further spread of virus in the CNS and the pathological appearance of cells differ among the three strains. Other investigators have reported that peripherally 'non-neurovirulent' HSV strains are transported to the CNS but do not replicate there (Dix et al., 1983). Such inhibition could be the result of immunological factors, but our studies also suggest a role for the structural cells of the CNS in mediating resistance. We propose that infection of glial cells may play a role in replication and spread of HSV-1 within the CNS. Differences in the resistance of glial cells to virus could be of importance in determining susceptibility to acute diseases, such as necrotizing encephalitis, or to the development of latency.

Further studies of mechanisms mediating the difference in resistance to HSV-1 in oligodendrocytes using IFA, and MAbs directed to immediate early, early and late HSV proteins are in progress.

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References


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