Persistent infection of a glioma cell line generates a Theiler's virus variant which fails to induce demyelinating disease in SJL/J mice

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Theiler's murine encephalomyelitis virus (TMEV) induces demyelinating disease which is associated with persistent virus infection of the central nervous system. To study the interaction between TMEV and host cells, we infected the G26-20 glioma cell line in vitro, and this resulted in a lytic infection in which most, but not all, cells were killed. Surviving cells divided and formed a viable monolayer in which a small proportion of cells displayed viral cytopathic effects. Levels of virus produced by these cultures over a 6 month period fluctuated between 6 and 8 log_{10} p.f.u./ml as measured by viral plaque assay. Similarly, the percentage of cells producing both viral antigen and viral RNA, as measured by a simultaneous immunoperoxidase in situ hybridization technique, varied between 5 and 30%. Although persistently infected cultures were susceptible to challenge by both vesicular stomatitis virus and herpes simplex virus, they were resistant to infection by homologous viruses. Interferon activity was not identified. TMEV isolated from passage 12 produced smaller plaques than wild-type Daniels strain virus (wt-DAV) on L-2 cell monolayers. In contrast to demyelination induced in SJL/J mice after intracerebral inoculation with wt-DAV, mice infected with the small plaque variant virus failed to develop viral persistence or chronic demyelination. However, following immunosuppression by total body irradiation, SJL/J mice infected with the small plaque variant developed viral persistence but no demyelination. Characterization of the biochemical and molecular determinants of the variant will lead to a better understanding of determinants important in viral persistence.

Introduction

Theiler's murine encephalomyelitis virus (TMEV) is a naturally occurring picornavirus which induces a characteristic biphasic disease when inoculated intracerebrally into susceptible strains of mice (Lipton, 1975; Theiler, 1937). The early phase of the disease is characterized by acute polioencephalomyelitis in which the virus lytically infects and actively replicates in neurons of grey matter. All surviving mice with a susceptible genotype develop chronic demyelination in association with mononuclear cell infiltrates. Data suggest that demyelination is both a consequence of direct lytic infection of myelin-producing cells (oligodendrocytes) and an immune-mediated response to infected cells (Rodriguez et al., 1983a; 1986; Rodriguez & Quddus, 1986; Roos et al., 1982a). Chronic demyelination is associated with persistent infection in which small amounts of infectious virus are recovered from the central nervous system (CNS) despite an active immune response. Pathological and immunological properties of TMEV-induced demyelination are similar to those seen in human multiple sclerosis, thus providing a model for the study of aspects of this disease.

The mechanisms by which this potentially lytic virus establishes persistent infection are unknown. Although viral antigen and RNA are detected in oligodendrocytes, astrocytes and macrophages at sites of demyelinating lesions (Aubert et al., 1987; Rodriguez et al., 1983a), the specific nature of these interactions are inferred from in vitro studies which suggest that TMEV lytically infects oligodendrocytes and neurons but persists in astrocytes and macrophages (Graves et al., 1986). Persistently infected cell systems involving picornaviruses have provided useful models for the study of features of virus-host interactions (Roos et al., 1982b; Schnurr & Schmidt, 1984; Takemoto & Habel, 1959; Vallbracht et al., 1984). For some virus-cell systems, establishment and maintenance of persistent infection has been attributed to genetic changes in the virus, including generation of defective-interfering particles, temperature-sensitive (ts) mutants or plaque size variants (De la Torre et al., 1985; Holland et al., 1980). Evidence for a role of the host cell has been provided in other cases. These include direct mechanisms such as production of interferon (Roos et al., 1982b), and more subtle changes including alteration of viral receptors (Kaplan et al., 1989), presence of
intracellular blocks to viral replication (Kaplan et al., 1989) and altered capability for cell-to-cell fusion (Mizzen et al., 1983).

We established a persistent infection in a cell line of glial origin. This persistently infected line demonstrated moderate viral cytopathic effect, fluctuation in levels of infectious virus and reduced rates of protein synthesis. Viral replication was not restricted in persistently infecting virus and reduced rates of protein synthesis. Persistent infection of glial cells resulted in a variant virus which was capable of infecting and replicating in cell cultures in vitro but persisted in the CNS of susceptible SJL/J mice only after immunosuppression, even though it was not capable of inducing demyelination.

**Methods**

**Virus and cells.** The wild-type Daniels strain of TMEV (Daniels et al., 1952) (wt-DAV) was used for all experiments. This strain, originally obtained from J. P. Lehrich and associates at the University of Chicago (Lehrich et al., 1976) was grown to 5 × 10^6 p.f.u./ml in baby hamster kidney-21 cells (Lipton & Dal Canto, 1979). The glial cell line was isolated from a carcinogen-induced glioma in a C57BL/6 mouse (Sundarraj et al., 1975). The infected cell line, designated glioma-P, was propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum. Viruses isolated from these cultures is designated DAV-P. Herpes simplex virus (KOS strain) (HSV) was kindly provided by A. R. Hayward (University of Colorado Health Sciences Center, Denver, Co., U.S.A.) and was passaged in rabbit skin cells (RSC). Vascular stomatitis virus (VSV), Indiana, was obtained from the American Type Culture Collection and propagated in RSC.

**Mice.** Four- to 5-week-old female SJL/J mice were purchased from Jackson Laboratories and used for experiments after resting for 1 week. Animals were injected intracerebrally with 2 × 10^5 p.f.u. of virus in a 10 μl volume. In some experiments, mice received total body radiation, 300 rads) from Isomedic, and incubaedostted in situ with a polyclonal antisem to purified DAV antigens (Rodriguez et al., 1983) with the avidin-biotin immunoperoxidase technique. Slides were developed with a solution of 75 μg of Hanks-Yates reagent (poly-phenylenediamine dihydrochloride and catechol). After acetylation, slides were dehydrated in 70% and 95% ethanol and hybridized with 2 ng/μl of a 35S-labell ed cDNA probe corresponding to the 3' end of TMEV RNA, strain GD VII (Ozden et al., 1986). The cDNA probe was radio labelled to 2 × 10⁸ c.p.m.μg DNA with [35S]ATP by nick translation (Amersham). Hybridization was carried out overnight at 37 °C in sodium citrate containing 50% formamide, 20 μM-Tris–HCl pH 7.4, 1 mM-EDTA, 0.5% polyethylene glycol, 100 μM-aurotricarboxylic acid, 0.6 μM-NaCl, 0.2 M-HEPES pH 7.2, 1 × Denhardt's solution (Maniatis et al., 1982), 10 μg/ml yeast tRNA, 1 mg/ml poly(A) and 10 mM-dithiothreitol. At the end of the hybridization period, slides were washed for 5 min in 50% formamide, 10 mM-EDTA and 0.6 M-NaCl (HWM), then 5 min in 2 × SSC at 22 °C, 1 h in 2 × SSC at 70 °C and finally 1 to 3 days in HWM at 22 °C. Washed slides were dehydrated in a graded series of alcohols containing 300 mM-ammonium acetate, air-dried and immersed in NTB-2 emulsion (Eastman Kodak). After 1 to 3 days of exposure at 4 °C, slides were developed with Kodak D-19 and washed with Kodak fixer. Slides were counterstained with haematoxylin.

**Fluorescence-activated cell sorting (FACS) analysis.** Cells were trypsinized from culture flasks, washed twice in FACS buffer (PBS with 1% BSA and 0.02% sodium azide) and resuspended to 1 × 10^6 cells/0.1 ml well of a round bottom non-tissue culture treated plate. Cells were fixed for 2 min in 67% alcohol in PBS, and incubated with rabbit anti-DAV serum diluted in FACS buffer for 1 h. Cells were washed three times and incubated with goat anti-rabbit secondary biotin-conjugated antibody (Vector Laboratories) and incubated for 45 min. Cells were washed three times and incubated with streptavidin–fluorescein isothiocyanate (Amersham) diluted 1:500. After 15 min incubation, cells were washed three times, resuspended in FACS buffer and analysed immediately using a FACS IV flow cytometer (Becton Dickinson FACS Systems).

**Radiolabelling, immunoprecipitation and SDS-PAGE.** Cell cultures were radiolabelled as described (Patrick & Hinne, 1984). Cultures were rinsed with methionine-free DMEM and replenished with labelling medium containing [35S]methionine (37-5 μCi/ml, sp. act. 1298 Ci/mmol; Amersham) and 1/25th the concentration of methionine. At the end of the labelling period cells were rinsed twice with ice-cold PBS and incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma; 1:1000 dilution). Plates were reacted with Sigma 104 phosphatase substrate and the reaction was terminated with 50 μl of 1 M-NaOH. The absorbance (410 nm) was read with an MR 600 Microplate reader (Dynatech) and plotted against reciprocal serum dilution.

**Immunocytochemistry and in situ hybridization.** In situ hybridization was performed on cell preparations on Denhardt's solution-treated acetylated slides (Brachic et al., 1984; Uhl, 1987). Cells from cultures were harvested by trypsinization and deposited onto treated slides by cyto centrifugation. After air drying, cells were fixed for 20 min in ice cold fixative containing 0.5% formaldehyde, 0.5% glutaraldehyde, 0.1 M-phosphate buffer pH 6.9, 1.6% glucose, 0.002% CaCl₂ and 1% dimethyl sulphoxide (PFG). After fixation, cells were quenched with ice-cold 0.15 M-ethanolamine (pH 7.5) for 20 min and washed twice in PBS for 5 min. Prior to immunocytochemistry, cells were permeated by 4 min incubation in PBS with 0.1% Triton X-100, and immunostained with a polyclonal antisem to purified DAV virions (Rodriguez et al., 1983) with the avidin-biotin immunoperoxidase technique. Slides were developed with a solution of 75 μg of Hanks-Yates reagent (poly-phenylenediamine dihydrochloride and catechol). After acetylation, slides were dehydrated in 70% and 95% ethanol and hybridized with 2 ng/μl of a 35S-labeled cDNA probe corresponding to the 3' end of TMEV RNA, strain GD VII (Ozden et al., 1986). The cDNA probe was radio labeled to 2 × 10⁸ c.p.m. μg DNA with [35S]ATP by nick translation (Amersham). Hybridization was carried out overnight at 37 °C in sodium citrate containing 50% formamide, 20 mM-Tris–HCl pH 7.4, 1 mM-EDTA, 0.5% polyethylene glycol, 100 mM-aurotricarboxylic acid, 0.6 μM-NaCl, 0.2 M-HEPES pH 7.2, 1 × Denhardt's solution (Maniatis et al., 1982), 10 μg/ml yeast tRNA, 1 mg/ml poly(A) and 10 mM-dithiothreitol. At the end of the hybridization period, slides were washed for 5 min in 50% formamide, 10 mM-EDTA and 0.6 M-NaCl (HWM), then 5 min in 2 × SSC at 22 °C, 1 h in 2 × SSC at 70 °C and finally 1 to 3 days in HWM at 22 °C. Washed slides were dehydrated in a graded series of alcohols containing 300 mM-ammonium acetate, air-dried and immersed in NTB-2 emulsion (Eastman Kodak). After 1 to 3 days of exposure at 4 °C, slides were developed with Kodak D-19 and fixed with Kodak fixer. Slides were counterstained with haematoxylin.
and solubilized in Tris–saline buffer containing 0.5% Triton X-100, 1% aprotinin (Sigma) and 1 mM-phenylmethylsulphonyl fluoride. Equal amounts of protein from lysates were analysed immediately by SDS-PAGE, or following immunoprecipitation.

For immunoprecipitation, lysates were pre-cleared with Protein A-Sepharose CL-4B (Sigma) by incubation at 4 °C for 30 min. Appropriate dilutions of primary antibody were added to the cleared lysates and incubated overnight at 4 °C. Immune complexes were isolated by incubation with Protein A-Sepharose for 30 min at 4 °C, followed by centrifugation. Pellets were washed sequentially with Tris–Triton buffer pH 7.4 (0.1 M-Tris-HCl, 0.15 M-NaCl, 0.2% Triton X-100), Tris–Triton buffer pH 8.0 (0.1 M-Tris–HCl, 0.5 M-NaCl, 0.5% Triton X-100) and HEPES–Triton buffer pH 7.4 (0.2 mM-HEPES, 0.2% Triton X-100). Samples were denatured and solubilized by heating at 100 °C in sample buffer consisting of 2.3% SDS, 5-0% 2-mercaptoethanol, 10% glycerol and 0.0625 M-Tris–HCl pH 6.8. Antigen was separated from Protein A-Sepharose by centrifugation and analysed in 12% polyacrylamide SDS gels following a modification of the discontinuous buffer system of Laemmli (1970). After electrophoresis, gels were stained with 0-125% Coomassie blue in 50% methanol–10% acetic acid and destained with 5% methanol–7.5% glacial acetic acid. Dried gels were exposed on Kodak XAR-5 film with Kodak X-Omatic regular intensifying screens at −70 °C.

Preparation of tissue for light microscopy and quantitative analysis.
Mice were anaesthetized with 0.2 ml pentobarbital intraperitoneally (i.p.) and perfused by intracardiac puncture with Trump's fixative (phosphate-buffered 4% formaldehyde with 1-0% glutaraldehyde pH 7.2). Spinal cords were removed, sectioned coronally and serially into 15 to 20 blocks, osmicated and embedded in 2-hydroxyethyl methacrylate (JB-4 system from Polysciences). Using a modified erichrome method with cresyl violet (Pierce & Rodriguez, 1989), 2 μm sections were stained to detect demyelination. Detailed morphological analysis was performed by examining each quadrant from 15 to 20 spinal cord coronal sections from each mouse for the presence or absence of demyelination and/or inflammation in grey matter, white matter and meninges (Rodriguez et al., 1986). A maximum score of 100 represented the presence of abnormalities in every quadrant of every spinal cord section examined. Chi-square Fisher's exact test was used to evaluate significance of differences in pathological scores between mice infected with different viruses.

Results

Establishment of persistently infected cell cultures
A confluent monolayer of G26-20 glioma cells was infected with DAV at an m.o.i. of 1.0. Viral c.p.e. was first observed 12 h after infection. In contrast to the complete cell destruction observed in L-2 cell cultures infected in parallel, a small population of infected glioma cells continued to survive 24 h later. These cells formed confluent monolayers which were subsequently passaged and maintained for 8 months. Persistently infected glioma cell cultures, designated glioma-P, showed evidence of viral c.p.e. (Fig. 1b).

To determine the levels of infectious virus released from glioma-P cultures over time, media were removed during the course of infection and assayed for virus on L-2 cells. Initially, there were cyclical fluctuations in the amount of virus produced; virus titles ranged from greater than 7 to 8 log_{10} p.f.u./ml of virus to less than 6 log_{10} p.f.u./ml (Fig. 2). Increased amounts of virus produced early were reflected in periods of cell crisis in which most cells were lysed. Later, levels of virus stabilized at approximately 6.5 log_{10} p.f.u./ml.
Viral antigen and RNA expression in persistently infected cultures

To determine the percentage of cells producing viral antigen and RNA, cells were removed from cultures at various times after infection, cytocentrifuged onto glass slides and immunoreacted with polyclonal antiserum to purified DAV virions or hybridized in situ with a 35S-labelled cDNA probe to TMEV. Not all cells were infected; the percentage of cells expressing viral antigen and RNA fluctuated between less than 5% to greater than 30% (Fig. 3). The percentage of cells producing viral antigen and RNA did not correlate with either periods of cell crisis or yields of infectious virus. Cells producing viral antigen and RNA may have been lysed and, therefore, unavailable for analysis.

The demonstration in vivo that many cells in white matter contain viral RNA but not viral antigen led Cash et al. (1985) to propose that a block in viral replication plays a role in the mechanism of persistent infection. In experiments in which viral RNA and viral antigen were measured on separate slides, the percentage of cells expressing viral RNA as measured by in situ hybridization was greater than the number of cells producing viral antigen for most time points examined (Fig. 3). To determine whether this represented the existence of a population that expressed viral RNA but not antigen, a double labelling immunocytochemistry/in situ hybridization was performed on cytocentrifuge cell preparations prepared at various times after infection. Of the cells which expressed viral antigen, 98.5% to 100% also expressed RNA at all time points examined; cells that displayed autoradiographic grains also displayed immunocytochemical substrate (Fig. 4).

Synthesis of DAV-induced polypeptides

Synthesis of DAV-induced polypeptides in glioma-P cultures was studied by radiolabelling cells with [35S]methionine for 6 h periods. Acutely infected and uninfected glioma cultures were labelled in parallel. Cell extracts were prepared and analysed directly, or following immunoprecipitation with polyclonal serum to purified DAV virions, by SDS-PAGE. Virion structural proteins (VP0, VP1, VP2 and VP3) were synthesized in glioma cells infected acutely with DAV for 24 h (Fig. 5). In contrast, although the percentage of cells producing viral antigen did not differ between acutely infected and persistently infected cultures (29.7 ± 1.9 and 25.7 ± 2.3, respectively), DAV structural polypeptides were not detected in cell extracts prepared from [35S]methionine-labelled glioma-P cultures, and could be detected only in small amounts after immunoprecipitation. Labelling for
Fig. 5. SDS-PAGE analysis of [35S]methionine-labelled polypeptides in persistently infected, acutely infected and mock-infected glioma cell cultures. At the end of the labelling period (6 h), cell cultures were either solubilized (extracts) or immunoprecipitated, with rabbit anti-DAV serum or normal rabbit serum, and then analysed by SDS PAGE. Lanes 1 and 8, mock infected; lanes 2, 4 and 7, persistently infected; lanes 3, 5 and 6, acutely infected. Lanes 1 to 3, anti-DAV serum; lanes 4 and 5, normal serum; lanes 6 to 8, extracts. Figures at left indicate Mr.

longer periods of time or addition of higher concentrations of radioisotope did not increase the intensity of bands. In a separate experiment using FACS analysis, the percentage of cells containing viral antigen (19.5%) from glioma-P cultures was similar to that of cells from acutely infected cultures (17%). In addition, persistently infected glioma cells stained as intensely with a polyclonal antiserum to DAV virions as cells from acutely infected cultures. The capability of glioma-P cultures to produce polypeptides was confirmed by immunoperoxidase studies; monospecific antisera (Ozden et al., 1988; Cash et al., 1986) which recognized VP1, VP2, VP3, viral protease (3C) and viral polymerase (3D) in acutely infected glioma cells also reacted with cells from glioma-P cultures (data not shown).

Table 1. Resistance of glioma-P cultures to challenge with homologous viruses

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Virus</th>
<th>Cytopathic effect*</th>
<th>Viral titre (log_{10} p.f.u./ml)$\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-2 (uninfected)</td>
<td>HSV</td>
<td>+ ++ + +</td>
<td>ND$\ddagger$</td>
</tr>
<tr>
<td></td>
<td>VSV</td>
<td>++ ++ +</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>wt-DAV</td>
<td>++ ++ +</td>
<td>8.6 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>DAV-P</td>
<td>++ ++ +</td>
<td>7.5 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>Mock$\S$</td>
<td>—</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Glioma (uninfected)</td>
<td>HSV</td>
<td>+ ++ + +</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VSV</td>
<td>++ ++ +</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>wt-DAV</td>
<td>++ ++ +</td>
<td>8.9 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>DAV-P</td>
<td>++ ++ +</td>
<td>8.2 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Mock</td>
<td>—</td>
<td>&lt;1.7</td>
</tr>
<tr>
<td>Glioma-P</td>
<td>HSV</td>
<td>++ ++ + +</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>VSV</td>
<td>++ ++ +</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>wt-DAV</td>
<td>+</td>
<td>&lt;3.7</td>
</tr>
<tr>
<td></td>
<td>DAV-P$\II$</td>
<td>+</td>
<td>6.1 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>Mock$\S$</td>
<td>+</td>
<td>6.0 ± 0.03</td>
</tr>
</tbody>
</table>

* Cultures were infected with an m.o.i. of 5 and graded for c.p.e. 36 h after infection; — signifies no c.p.e. and + + + + signifies complete cell destruction.

$\dagger$ Confluent monolayers were infected with viruses at an m.o.i. of 1 or mock-infected with diluent only. Viral titres were determined 18 h after infection (means ± s.d.).

$\ddagger$ ND, Not determined.

$\S$ Mock, Diluent only.

$\II$ Levels of infectious virus measured in these infections were similar to those obtained from mock-infected Glioma-P cultures. It was possible to differentiate the yield of DAV but not DAV-P by plaque size.

Resistance of glioma-P cultures to homologous viral infection

To determine whether glioma-P cultures were resistant to superinfection by unrelated or by homologous viruses, VSV, HSV wt-DAV or DAV-P were inoculated onto either uninfected glioma, L-2 or glioma-P cell cultures. The amount of infectious virus released into the culture medium was determined 18 h after infection and cultures were qualitatively scored for the presence of viral c.p.e. 36 h after infection. Infection with each virus resulted in complete cell destruction in glioma and L-2 cultures (Table 1). Although glioma-P cultures were lytically destroyed after exposure to either VSV or HSV, no c.p.e., other than that observed in mock-challenged glioma-P cultures, was evident after infection with wt-DAV or DAV-P viruses. Resistance of glioma-P cultures to challenge with these viruses was confirmed by viral plaque assay in which no plaques characteristic of wt-DAV were detected in glioma-P cultures. Although similar titres of DAV-P and wt-DAV were obtained from glioma cell cultures, the final yield of DAV-P was 10-fold less than that of wt-DAV when grown in L-2 cells. The lower yield of progeny virus correlated with the smaller plaque size phenotype of DAV-P when assayed on L-2 cell monolayers (Fig. 6).

To determine whether resistance was due to the
presence of interferon in glioma-P cultures, media were removed from cultures at several passage intervals and tested by a c.p.e. reduction technique (Rubinstein et al., 1981) for their ability to protect L-2 cells against subsequent challenge with VSV. Interferon was not present because media from glioma-P cultures did not protect L-2 cells from VSV-induced cell death. Furthermore, addition of anti-mouse interferon at a concentration sufficient to neutralize $10^3$ i.u./ml did not result in additional c.p.e. to glioma-P cultures nor were levels of infectious virus significantly increased ($6.32 \pm 0.17 \log_{10}$ p.f.u./ml from anti-interferon treated cultures compared to $6.08 \pm 0.03 \log_{10}$ p.f.u./ml from control treated cultures).

To determine whether other interfering factors were present in glioma-P cultures, mixtures of wt-DAV from glioma-P cultures from several different passage intervals were diluted in culture medium and used to infect L-2 cells. No reduction in virus yield was observed when compared to infection by wt-DAV alone. Thus, resistance of glioma-P cells to superinfection by homologous virus does not appear to be mediated by components in the culture medium.

Alteration of virus during persistent infection

To determine whether ts mutants were generated, viruses from different passage intervals were tested for their capability to form plaques on L-2 cells at 33 °C and 39-5 °C. Virus derived from glioma-P cultures was not temperature-sensitive as virus plaqued equally well at all temperatures with efficiency of plating ($33 \, ^\circ \text{C}/39.5 \, ^\circ \text{C}$) values ranging from 0.98 to 1.1. However, virus did change during persistent infection since virus isolated from glioma-P cultures at passage 12 produced smaller plaques than wt-DAV on L-2 cell monolayers (Fig. 6).

Pathogenesis of small plaque variant

To determine whether the biological properties of this small plaque variant were altered, irradiated or non-irradiated SJL/J mice were infected intracerebrally with $2 \times 10^3$ p.f.u. of either DAV-P or wt-DAV. Forty-five days after infection, mice were perfused with fixative, brain and spinal cords removed and processed and quantitative analysis was performed. SJL/J mice infected with wt-DAV had prominent meningeal inflammation and demyelination (Table 2). These lesions were
Table 2. Extent of pathology in spinal cords of SJL/J mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dose</th>
<th>n*</th>
<th>Grey matter inflammation</th>
<th>Meningeal inflammation</th>
<th>Demyelination</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-DAV</td>
<td>–</td>
<td>9</td>
<td>0.9 ± 1.4†</td>
<td>27.9 ± 15.4</td>
<td>37.5 ± 17.2</td>
</tr>
<tr>
<td>DAV-P</td>
<td>–</td>
<td>9</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>DAV-P</td>
<td>300 rads</td>
<td>5</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

* n, Number of mice.
† Values expressed as mean ± S.D. Statistical analysis by Chi-square Fisher's exact test comparing pathological scores of irradiated or non-irradiated mice inoculated with DAV-P versus non-irradiated mice inoculated with wt-DAV (P < 0.0001).

Table 3. Infectious virus in CNS of irradiated and non-irradiated SJL/J mice (45 days post-infection)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dose</th>
<th>Virus isolation*</th>
<th>Viral titre (log10 p.f.u./g CNS)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-DAV</td>
<td>–</td>
<td>4/4</td>
<td>3.2 ± 0.23</td>
</tr>
<tr>
<td>DAV-P</td>
<td>–</td>
<td>0/5</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>DAV-P</td>
<td>300 rads</td>
<td>3/5</td>
<td>2.0 ± 1.9</td>
</tr>
</tbody>
</table>

* Number of mice positive for virus/number tested.
† Mean ± s.d.

characterized by extensive inflammation and prominent myelin destruction with preservation of axons (Fig. 7a). In contrast, white matter of irradiated or non-irradiated mice infected with DAV-P had no demyelination or inflammatory infiltrates (Table 2, Fig. 7b). Previous results (Rodriguez et al., 1990) have shown that the majority of mice of the susceptible genotype infected with wt-DAV die following total body irradiation and infection; thus this group was not included in this analysis.

To determine levels of infectious virus in irradiated or non-irradiated mice infected with either virus, brains and spinal cords were removed 45 days post-infection and analysed for infectious virus by plaque assay. Virus persisted in all mice infected with wt-DAV (mean of 3.2 log10 p.f.u./g CNS) as well as three of the five irradiated mice (mean of 2.0 log10 p.f.u./g CNS) infected with DAV-P. In contrast, virus was not detected in the CNS of non-irradiated mice infected with DAV-P. To determine whether non-irradiated mice exposed to DAV-P had mounted an immune response, serum was collected and examined for the presence of anti-DAV antibodies by ELISA (Fig. 8). Although mice infected with wt-DAV produced higher levels of virus-specific antibody than mice infected with DAV-P, DAV-P-infected mice produced statistically significantly higher levels of antibody than uninfected mice. This indicates that DAV-P replicated sufficiently in non-irradiated mice to induce an antibody response.

Discussion

Attributes of both the virus and the host interact to determine the outcome of infection, i.e. lytic infection or persistence. Mechanisms by which potentially lytic viruses are able to establish persistent infections in vitro and in vivo are paradoxical and poorly understood. In this study we demonstrate the establishment and characterization of a mouse glioma cell line persistently infected with the DA strain of TMEV. In addition, we describe the isolation of a novel virus variant which was able to establish persistence only after immunosuppression but was not able to induce demyelination.

Restricted c.p.e., consistent isolation of infectious virus from culture media and infection of a small percentage of cells suggests that the glioma-P cell line is a carrier-state culture similar to that which has been described for other picornaviruses (Takekoto & Habel, 1959; Schnurr & Schmidt, 1984; Roos et al., 1982b; Ackermann & Kurtz, 1955). A variety of mechanisms involving changes in host, virus or both have been proposed to explain viral persistence. Direct alterations in the host cell resulting in the inability of cells to undergo virus-induced fusion (Mizzen et al., 1983) or to bind virus (Chinami et al., 1986) have been postulated to account for viral persistence in some systems. Although not clearly defined, the apparent resistance of some persistently infected cell cultures to viral infection has been attributed to an intracellular block resulting in decreased viral RNA production (Kaplan et al., 1989; De la Torre et al., 1988).

Alterations in the virus have been described in other systems (Holland et al., 1980). DAV-P was capable of forming plaques with equal efficiency at 33 °C or 39 °C and thus did not appear to be ts. However, the virus population did evolve, as evidenced by the decrease in plaque size of DAV-P on L-2 cell monolayers. The generation of small plaque variants appears to be a common feature of many persistent infections (Bernstein et al., 1985; De la Torre et al., 1985; Kaplan et al., 1989;
a poliovirus mutant that produced smaller plaques on HeLa cells and replicated to lower titres when compared to wild-type virus. The mutant also failed to inhibit host cell translation which resulted in little viral protein synthesis. Further data suggested that this may have been due to a viral protease which failed to cleave the p220 protein component of the host translational machinery. Kaplan et al. (1989) isolated a small plaque variant of poliovirus from persistently infected HeLa cells. Radiolabelling of these cultures with $^{35}$S methionine followed by immunoprecipitation revealed accumulation of the viral precursor polypeptide 3CD. Based on this observation they suggested that the limited proteolysis of this viral precursor may be important in maintaining persistent infection. In our system, although DAV-P replicated to the same level as wt-DAV in glioma cells, the yield of DAV-P in L-2 cells was significantly decreased. In addition, analysis of autoradiograms of immunoprecipitates of glioma-P cultures resolved by SDS-PAGE suggested a decreased rate of viral protein synthesis. This reduction in viral protein synthesis could not be explained by differences in either the percentage of cells containing viral antigen or in levels of viral antigen per cell as measured by FACS. These data are consistent with the observation that glioma cells infected with wt-DAV release greater amounts of infectious virus than cells from glioma-P cultures (Table 1). The mechanism accounting for the reduced rate of protein synthesis, the apparent accumulation of viral protein and decreased release of infectious virus remains to be determined.

It has been proposed that persistent infections are maintained by a co-evolution process involving combinations of both host and viral genetic changes (Ron & Tal, 1985; De la Torre et al., 1988; Ahmed et al., 1981). In addition to a change in the plaque size phenotype of DAV-P, we found that host cells from glioma-P cultures were resistant to superinfection by wt-DAV and DAV-P. Although the mechanism for this phenomenon is unknown, it was not due to a general metabolic defect or a gross membrane alteration of cells because glioma-P cultures were susceptible to infection with VSV and HSV. Also, production of interferon, which was shown to be responsible for maintenance of TMEV persistent infection in L-929 cells (Roos et al., 1982b), was not detected here. The presence of other anti-viral cytokines, including tumour necrosis factor, was similarly not detected (data not shown). The mechanism for this resistance appeared to operate at the cellular level since culture fluid from glioma-P cells did not interfere with the ability of wt-DAV to replicate in permissive cell lines. Since 70 to 95% of glioma-P cells did not contain detectable viral antigen or RNA but were resistant to infection, this further implies a co-evolutionary process in which host cells with increased resistance to DAV survived.

Cash et al. (1985) have proposed that persistent TMEV infection in vivo is maintained by restriction at the RNA level. This observation was suggested by the demonstration that cells within mouse CNS contained viral RNA but not viral antigen. This mechanism did not appear to contribute to the maintenance of persistent infection in glioma-P cultures in vitro as the majority of cells were positive for both viral antigen and viral RNA, and only a few cells expressed viral RNA alone.

We injected DAV-P into susceptible SJL/J mice to determine whether the biological properties of the virus had been altered. Mice that were infected with wt-DAV developed a characteristic low level persistent infection and chronic demyelinating disease. Although DAV-P was able to replicate and lyse permissive cells in vitro, those alterations which resulted in persistence in vivo also attenuated virus growth in vivo since mice inoculated with DAV-P were clear of virus by 45 days post-infection and did not develop demyelination. However, DAV-P does induce early grey matter disease comparable to that observed in mice infected with wt-DAV (preliminary data). In addition, DAV-P is able to persist in mice late after infection following immunosuppression, but does not induce demyelination. These studies indicate that DAV-P does infect cells of the CNS but fails to induce demyelinating disease. Whether this failure derives from the ameliorative effect of immunosuppression on disease (Roos et al., 1982a; Rodriguez et al., 1986; Rodriguez & Quddus, 1986) or results from the inability of DAV-P to infect the appropriate target cell, e.g. the oligodendrocyte, remains to be determined.

Theiler's virus variants with altered patterns of pathogenesis have been isolated by a variety of methods, including selection for neutralization-resistant escape mutants (Roos et al., 1989; Zurbriggen & Fujinami, 1989) and selection for different plaque size phenotypic variants (Oleszak et al., 1988). Our study is the first to demonstrate that selecting for a Theiler's virus from a persistently infected glial cell line can produce a variant with altered pathogenic capability. With the determination of the complete nucleotide sequences of strains of TMEV (DA and GD VII) that differ in pathogenicity (Ozden et al., 1986; Ohara et al., 1988), it will be possible to analyse the molecular differences that may correlate with disease. Specific base changes in the poliovirus genome have been shown to be major determinants in attenuating certain viral strains (Racaniello, 1988). Furthermore, Monica & Racaniello (1989) found that those poliovirus strains containing mutations which resulted in attenuation in vivo replicated to lower titres in vitro than neurovirulent viruses. This defect appeared to
result from a reduction in viral RNA translation. Identification and characterization of the specific biochemical and molecular defects that are responsible for the differential pathogenesis of DAV-P and other variants in vivo will lead to better understanding of determinants leading to viral neuropathogenesis and persistence.

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