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Murine gammaherpesvirus-68 infection of and persistence in the central nervous system

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Murine gammaherpesvirus-68 (MHV-68) was originally isolated from a bank vole by passage through mouse brain. Given its ability to replicate in mouse brain and its subsequent reisolation from trigeminal ganglia, it was originally considered to be an alphaherpesvirus. Molecular studies have now firmly established MHV-68 to be a gammaherpesvirus. Other gammaherpesviruses have been suggested to cause and in some cases shown to cause neurological disease. Given the isolation history of MHV-68, we have studied the ability of this virus to gain access to, to replicate in and to persist in the mouse CNS. Following intranasal inoculation the virus was not generally neuroinvasive. However, in mice with a deletion of the type-I interferon receptor gene, peripheral virus titres are higher and perivascular CNS infection was observed. There was no evidence of virus spread via olfactory routes. Direct intracerebral inoculation of virus was fatal with widespread infection and destruction predominantly of meningeal and ependymal cells. Hippocampal pyramidal neurons, oligodendrocytes, Bergmann glia cells in the cerebellar cortex and neural progenitor cells in the rostral migratory stream were also infected. A similar infection was observed in younger mice. CNS infection following virus reactivation was investigated by implantation of infected glial cells. Implantation into a brain ventricle led to widespread fatal infection, principally involving ependymal and meningeal cells. Implantation into the striatum resulted in a predominantly neuronal infection. Implantation of cells into mice transiently treated with the antiviral thionucleoside analogue 2′-deoxy-5-ethyl-β-4′-thiouridine resulted in survival with detection of virus-infected cells in the brain 1 year later.

Introduction

Murine gammaherpesvirus (MHV-68) is genetically related to the human gammaherpesviruses Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) (Efstathiou *et al.*, 1990; Virgin *et al.*, 1997). Immunopathological and molecular studies have shown that MHV-68 infection of mice offers a small animal model relevant to aspects of EBV and KSHV infection (for review see Stewart, 1999). In particular, MHV-68 infection of laboratory mice permits precise dissection of viral and host factors associated with pathological events common to this group of viruses. Following intranasal inoculation with MHV-68, an ordered and reproducible infection occurs (Sunil-Chandra *et al.*, 1992*a*). The virus replicates transiently in lungs followed by spread to B cells in the spleen. A splenomegaly then ensues in concert with a peak in latently infected B cells approximately 14 days post-infection. Splenomegaly resolves around 21 days and the spleen then becomes a primary reservoir of latently infected B cells (Sunil-Chandra *et al.*, 1992*b*). The virus also establishes long-term latency in other tissue sites, primarily lung epithelial cells (Stewart *et al.*, 1998), as well as populations of B cells and macrophages within the peritoneum (Weck *et al.*, 1999). A consequence of long-term infection is the development of lymphomas in a proportion of mice (Sunil-Chandra *et al.*, 1994). Characterization of immunological and viral factors important to pathogenesis and latency *in vivo* has been possible in this system (Nash *et al.*, 1996; Stewart, 1999).

Intriguingly, MHV-68 was originally isolated from the tissues of a bank vole, *Clethrionomys glareolus*, in Slovakia by passage through neonatal mouse brain (Blaskovic *et al.*, 1980) and was subsequently reisolated from the trigeminal ganglia of naturally and experimentally infected mice (Blaskovic *et al.*, 1984; Rajcani *et al.*, 1985). This and the virus growth

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characteristics led to a preliminary and incorrect classification of this virus as an alphaherpesvirus (Svobodova *et al.*, 1982). The virus is now known to be a gammaherpesvirus. By analogy with other gammaherpesviruses, the natural route of infection is likely to be oral or respiratory. How this virus gains access to the CNS and how frequently this occurs remain unknown. Other gammaherpesviruses have been associated with neurological disease. There is evidence to suggest that EBV is an important factor in the increased incidence of primary CNS lymphomas in both immunocompetent and immunosuppressed populations (Grant & Isaacson, 1992; Itoyama *et al.*, 1994). There has also been a long-standing association of EBV with diverse neurological disorders including meningoencephalitis, multiple sclerosis, Guillain–Barre syndrome, chronic fatigue syndrome and facial palsy (Archard & Bowles, 1988; Hotchin *et al.*, 1989; Bray *et al.*, 1992; Roberg *et al.*, 1991; Martyn *et al.*, 1993; Imai *et al.*, 1993; Haahr *et al.*, 1994). In this study, we examine the ability of MHV-68 to gain access to, and to spread and persist within, the CNS.

Methods

■ **Mice.** CBA, BALB/c and 129 mice were purchased from Bantin and Kingman or Harlan UK. Interferon-α/β receptor gene-deficient mice (IFN-α/β-R$^{o/o}$), which were derived from 129 mice, were purchased from Bantin and Kingman with kind permission of Michel Aguet (Muller *et al.*, 1994). All mice were maintained under UK regulatory authorities-approved conditions at the Laboratory for Clinical and Molecular Virology, University of Edinburgh. Mice were female and 4–5 weeks of age unless otherwise stated. 2′-Deoxy-5-ethyl-β-4′-thiouridine (4′-S-EtdU) is an antiviral thionucleoside analogue which has been shown to be a potent inhibitor of gammaherpesvirus, including MHV-68, infection (Barnes *et al.*, 1999). This compound was kindly supplied by Glaxo-Wellcome (Stevenage, UK) and was administered to mice in the drinking water at a concentration of 0·33 mg/ml (Barnes *et al.*, 1999).

■ **Cells.** A CNS-derived temperature-sensitive SV40 large T-antigen-transformed cell line, MGC7 (Terry *et al.*, 1997), was infected with MHV-68 (m.o.i. 5 p.f.u.) and maintained in culture for several weeks in the presence of 4′-S-EtdU at a concentration of 1 μg/ml. Prior to inoculation, cells were removed from the flask using EDTA and washed in PBS at 4 °C.

■ **Virus and virus administration.** MHV-68 was prepared by infection of BHK-21 cells at low multiplicity (0·001 p.f.u. per cell) as described previously (Sunil-Chandra *et al.*, 1992*a*; Usherwood *et al.*, 1996) and stored at −80 °C. Intranasal inoculations were performed under light halothane anaesthesia; 2×10^4 p.f.u. virus in 50 μl PBS was placed ont—o the nares and the mice were allowed to inhale the inoculum. For intracerebral inoculation, 2×10^4 p.f.u. of virus in a total volume of 20 μl was injected close to the midline using a 27 gauge needle. Cellular implantation of infected or control (uninfected) MGC7 cells was performed using a stereotaxic frame with co-ordinates for striatal and ventricular inoculation and confirmed by histological inspection post-mortem. Mice were anaesthetized using Hypnorm (Janssen Pharmaceuticals) and Hypnovel (Roche) injected into the peritoneum. Using a small drill, a hole was made in the skull and over 2–3 minutes the cell suspension (2×10^4) in 2 μl PBS was injected using a Hamilton syringe. The syringe was withdrawn slowly and the skin sutured. Mice were checked daily. No adverse reactions to the surgery were observed. Virus plaque assays were performed on BHK cell monolayers as previously described (Sunil-Chandra *et al.*, 1992*a*).

■ **Immunocytochemistry.** Unless stated otherwise, animals were sacrificed under anaesthesia by extensive perfusion with PBS alone or PBS followed by 2% paraformaldehyde–lysine–periodate (PLP) solution. Brains perfused with PBS only were removed and bisected down the midline. One half was immersion-fixed in 4% phosphate-buffered formal saline, processed and embedded in paraffin. The remaining half was either processed for DNA extraction or immersed in 20% sucrose solution, frozen in isopentane and then stored at −80 °C. PBS- and PLP-perfused brains were immersed in sucrose and cryopreserved as above. Paraffin-embedded sections were cut 5 μm thick and cryostat sections were cut 10–15 μm thick, both sagittally, and mounted on Biobond- (British Biocell) coated slides, air-dried overnight at room temperature and stored at either 4 °C (paraffin-sections) or at −20 °C (cryosections). Immunostaining of paraffin-embedded sections for MHV-68 was performed as described previously (Sunil-Chandra *et al.*, 1994). Briefly, sections were incubated for 2 h at room temperature with rabbit hyperimmune serum against MHV-68 (Sunil-Chandra *et al.*, 1992*a*) followed by incubation for 1 h with a secondary biotinylated goat anti-rabbit IgG (Vector laboratories). Signal was amplified using the Vector laboratories ABC kit and visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma fast). Non-specific staining was blocked with 5% normal goat serum prior to application of the primary antibody and all washes were done with PBS. No staining was observed on sections from the brains of uninfected control mice or the brains of mice infected with Semliki Forest virus. Immunostaining for lymphocyte subpopulations was carried out on cryosections cut from unfixed brains, perfused with PBS only. The sections were fixed in 100% ethanol at 4 °C for 10 min and rinsed immediately in PBS before incubation with antibodies to CD3 (KT3.1, a gift from S. Cobbold, Oxford, UK), CD4 (YTS 179.1) or CD8 (YTS 169.4) for 2 h following immersion in 0·3% hydrogen peroxide to block endogenous peroxidase activity. After washing in TBS–0·1% Tween 20, cryosections were incubated for 1 h with biotinylated rabbit anti-rat antibody (Vector). All subsequent steps were as described above. Sections were counterstained in haematoxylin. Dual-colour fluorescent immunostaining to identify oligodendrocytes in the brain was performed on cryosections from PLP-fixed brains using rabbit polyclonal anti-bovine 2′,3′-cyclic nucleotide-3′-phosphohydrolase (CNPase, a gift from F. A. McMorris, Wistar Institute, USA; Raible & McMorris, 1989) as primary antibody following the protocol described above. Sections were then incubated with FITC-labelled goat anti-rabbit (Serotec) for 1 h and remaining sites were blocked using 10% rabbit serum. Biotinylated MHV-68 rabbit serum diluted in PBS containing 10% normal rabbit serum was applied to slides for 1 h prior to washing and incubation with rhodamine-labelled streptavidin for 30 min. Confocal microscopy was done out using a Leica microscope attached to a Silicon Graphics work station.

■ ***In situ* hybridization.** *In situ* hybridization on formalin-fixed, paraffin-embedded sections was done using either a DNA probe to the viral repeat region or an RNA probe to the virus-encoded tRNAs (Sunil-Chandra *et al.*, 1994; Stewart *et al.*, 1998). Briefly, following pretreatment including microwaving in citrate buffer, hybridization was done with digoxigenin-labelled probes. Probes were detected with either an alkaline phosphatase-labelled or a biotin-labelled sheep anti-digoxigenin antibody and visualized using BCIP–NBT substrate (Sigma) or following amplification with the Vector ABC system with DAB, as described previously (Fazakerley *et al.*, 1993; Stewart *et al.*, 1998). To control for specificity, the

probes were hybridized to sections of Semliki Forest virus-infected mouse brain; no signal was detected.

■ **DNA preparation and PCR analysis.** DNA from PBS-perfused brains frozen at −80 °C was prepared using Qiamp tissue kits (Qiagen). PCR analysis for MHV-68 DNA was performed using two sets of nested primers specific for the gp150 gene, as described previously (Stewart *et al.*, 1998). The sensitivity of this nested PCR was found to be one copy of viral DNA in a background of 1 μg of cellular DNA.

Results

MHV-68 is not generally neuroinvasive

To determine whether MHV-68 is neuroinvasive and generally infects the CNS following extraneural inoculation, virus infection of brain and lung tissues was determined in three different mouse strains following intranasal inoculation. Intranasal inoculation is most likely to mimic the natural route of infection. Groups of ten, 4–5-week-old BALB/c, CBA and 129 mice were inoculated intranasally under light halothane anaesthesia with 2×10^4 p.f.u. of virus in 50 μl PBS. Mice were monitored daily for clinical signs for 3 weeks. At days 4, 7 and 10 post-infection, two mice from each group were sampled and the lungs and brains removed for study. Prior to removal of these tissues the mice were perfused via the left cardiac ventricle with PBS, followed by PLP fixative. The brains were bisected down the midline and each half brain and the lungs were immersion-fixed for a further 2 days before being processed for histological studies. Paraffin sections from at least five randomly chosen brain areas were each screened for virus infection by both *in situ* hybridization and immunostaining. Viral nucleic acids were detected by *in situ* hybridization using a DNA probe to the viral repeat region and viral proteins were detected using a polyclonal rabbit antiserum raised against whole virions (Sunil-Chandra *et al.*, 1994). In all three mouse strains, the animals looked slghtly unwell with ruffled fur at around 7 days post-infection, which lasted for 2 or 3 days; thereafter the mice showed no signs of infection for the remaining period of observation. Histological study of all three mouse strains at days 4, 7 and 10 demonstrated that virus DNA-positive and protein-positive cells were readily observed in the lungs but never in the brains (Table 1). No major differences between these three mouse strains were obvious in the extent or distribution of the lung infection or the clinical outcome of the infection. We conclude that in the first 10 days of infection MHV-68 is not normally neuroinvasive.

Table 1. Extent of lung and brain infection

The extent of infection was monitored by determining the relative numbers of virus RNA-and protein-positive cells in tissue sections. Three adjacent sections from each of five different tissue areas were screened for viral DNA by *in situ* hybridization using a DNA probe hybridizing to the viral repeat regions. Three adjacent sections from each of five different tissue areas were also screened for viral proteins by immunostaining. Three mice were examined by each technique at each time-point. No major differences were observed between the results obtained by *in situ* hybridization, which detects genomic DNA in both productively infected and latently infected cells, and immunostaining, which detects productively infected cells only. Lungs were not examined in the mice sampled at day 10. 0, No virus-positive cells observed; +, single scattered virus-positive cells; ++, small foci of virus-positive cells; +++, large foci or small confluent areas of virus-positive cells; ++++, widespread infection of large numbers of adjacent cells or large foci of infection in many areas.

Mouse strain	Extent of lung infection		Extent of brain infection		
	Day 4	Day 7	Day 4	Day 7	Day 10
BALB/c	++	++++	0	0	0
CBA	++	+++	0	0	0
129	+	+++	0	0	0

MHV-68 can infect several CNS cell types

Many neurological diseases caused by virus infections of the CNS are relatively rare in comparison to the number of individuals infected. Subacute sclerosing panencephalitis is a rare complication of measles virus in children. Visna is a relatively rare neurological disease of sheep compared to the more common lung disease maedi, both of which result from infection with the same lentivirus, maedi–visna virus. It is likely that in many viral infections of the CNS, the low incidence of neurological disease results from inefficient spread of virus to the CNS. To determine whether MHV-68 can infect CNS cells, a group of ten, 4–5-week-old BALB/c mice was inoculated intracerebrally with 2×10^4 p.f.u. of virus. Three mice were sampled on each of days 3 and 6 post-infection. Between 5 and 7 days, the mice became hunched and lethargic, lost weight and were euthanized when moribund. Examination of the brains demonstrated infection of meningeal and ependymal cells. This was more extensive at day 6 than at day 3 (Fig. 1A, B). A predominantly mononuclear cell inflammatory infiltrate was present in the meninges and adjacent to infected ependymal cells by day 3. Many of these cells were also positive for viral DNA and viral proteins (Fig. 1C). In some areas the infection had clearly spread to cells underlying the meninges and ependyma (Fig. 1D). Infected cells were positive for viral DNA in the nucleus (Fig. 1A, B, C) and expressed viral proteins in the cytoplasm (Fig. 1D, E). It was notable that the majority of choroid plexus cells were uninfected, despite close proximity to productively infected ependymal cells (Fig. 1B). At day 6 there was infection and destruction of white matter tracts adjacent to the ventricular system (Fig. 1E). This was most clearly apparent in the corpus callosum overlying the lateral ventricles and the internal capsule. Double-labelling studies with anti-CNPase antibodies, a marker of oligodendrocytes, confirmed infection of these cells (Fig. 1G). A

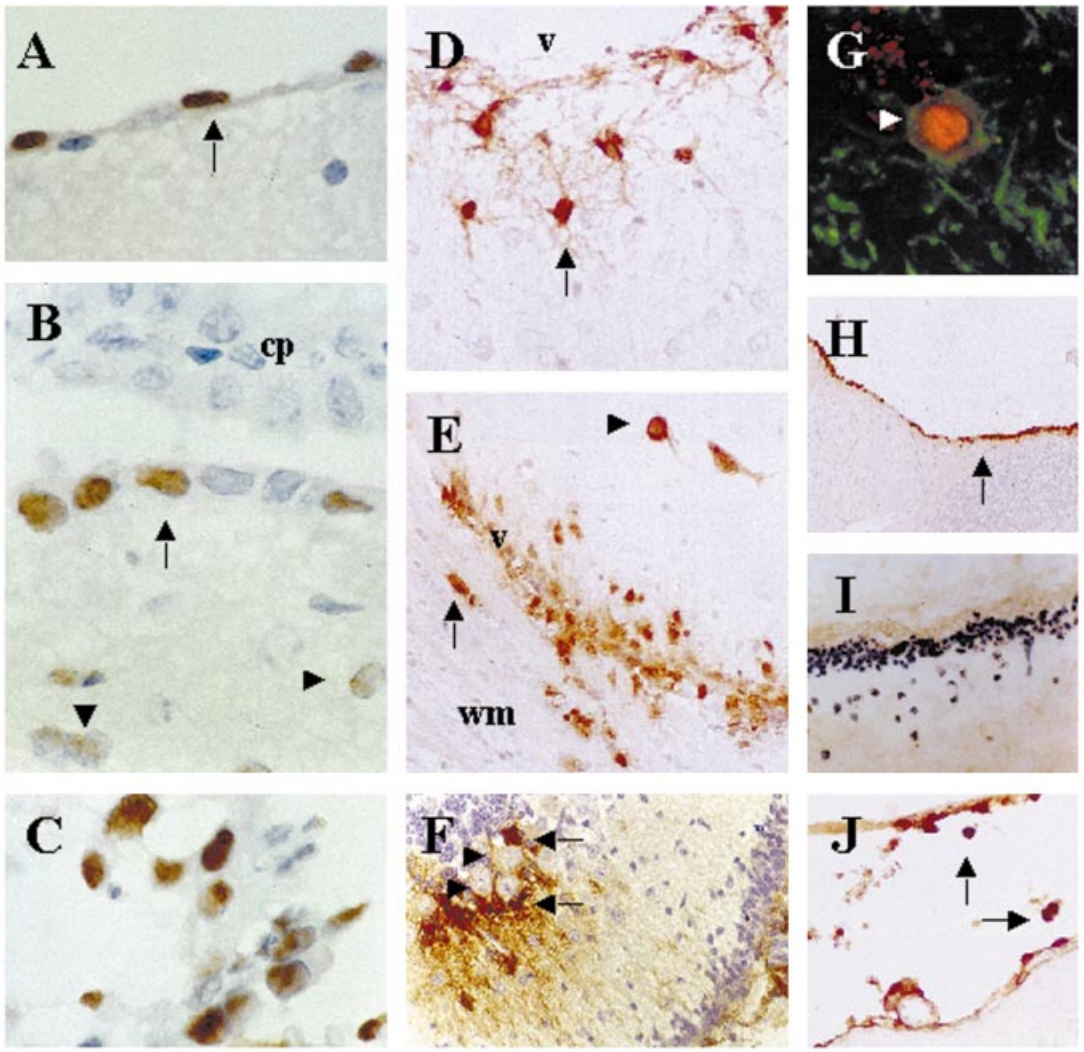

Fig. 1. Viral nucleic acids were detected by *in situ* hybridization using a DNA probe to the viral repeat region. Viral protein was detected using a polyclonal rabbit antiserum raised against whole virions (Sunil-Chandra *et al*., 1994). (A)–(E) Paraffin-embedded brains 6 days after intracerebral infection of 4–5-week old BALB/c mice. (A) Viral DNA-positive meningeal cells (e.g. arrow), × 600. (B) Viral DNA-positive ependymal cells (brown, e.g. arrow) and cells underlying the ventricle (arrowheads), × 800. The choroid plexus cells (cp) are uninfected. (C) Viral DNA-positive inflammatory cells in an area of vacuolation adjacent to a ventricle, × 800. Note in (A), (B) and (C) that, as expected, the *in situ* hybridization (brown) detects viral DNA only in the nuclei of infected cells. (D) Virus protein-positive glial cells (brown, e.g. arrow) underlying the ventricle (v), × 400. (E) Virus protein-positive cells surrounding collapsed ventricle (v), × 200. The infection includes cells in the white matter tract (wm) (e.g. arrow) and cells of a neuronal morphology (e.g. arrowhead). (F) Virus protein-positive Bergmann glia cells (brown, arrows) adjacent to Purkinje cells (unstained, arrowheads) in the molecular layer of the cerebellar cortex, × 250. Two days after intracerebral infection of a 7-day-old BALB/c mouse. Note in (D), (E) and (F) that the immunostaining for viral proteins detects these in both the cytoplasm and the nucleus. (G) Confocal microscopy and double-fluorescence labelling demonstrating (arrowhead) a virus DNA-positive (red, nucleus) oligodendrocyte (green, $CNPase^{+}$), 4–5-week-old BALB/c mouse, × 1200. (H, I) Cells positive for viral protein (brown) in the meninges (H), × 40, and for viral tRNA (blue) in the rostral migratory stream (I), × 200, of a BALB/c mouse inoculated intracerebrally at 14 days of age, 3 days post-infection. (J) Virus protein-positive cells (brown) in the pia and arachnoid meninges and in the subarachnoid space (arrows), 6 days after intranasal infection, 4–5-week-old 129–IFN-α/β-$R^{o/o}$ mouse, × 200.

band of cells, presumed to be the rostral migratory stream, extending from the periventricular layer of the frontal cortex to the olfactory bulb, was clearly infected. Most of the cells in this pathway were virus DNA- and protein-positive and cell debris, pyknotic and karyorrhexic nuclei were apparent throughout the length of this system. At day 6, infection of hippocampal pyramidal neurons was frequently observed, as was infection of Bergmann glia cells adjacent to generally uninfected Purkinje cells in the molecular layer of the cerebellar cortex.

Effect of age on spread of MHV-68 to and within the CNS

To determine the effect of age on the ability of virus to enter the CNS and on the course of the CNS infection, groups of seven BALB/c mice aged 7, 14, 21 and 28 days were inoculated either intranasally or intracerebrally with 2×10^4 p.f.u. virus. For the mice inoculated intranasally, three mice from each age group were studied at each of days 4 and 6 and for the mice inoculated intracerebrally three mice from each age group were studied at day 3 or when moribund. Brains were removed and screened by histology for viral DNA and proteins. Following intranasal inoculation, for mice of each age group, no virally infected cells were detected in the brains of the three mice studied at day 4, by either *in situ* hybridization or immunostaining. The remaining four mice were observed over a 3 week period. Mice inoculated at 7 and 14 days of age all died within 7 days of infection; most of the mice inoculated at 21 days of age became unwell at around 6 days but survived; some of the mice inoculated at 28 days of age displayed mild clinical signs between days 5 and 7 and thereafter remained healthy. Following intracerebral inoculation, mice of all ages rapidly became unwell, moribund and were destroyed. There was a direct correlation between age at inoculation and time of onset of clinical signs. We did not use large groups of mice to quantify this but all of the mice inoculated at 7 and 14 days of age were dead or had been sampled when moribund by day 3; by day 5 all of the mice inoculated at 21 days of age were dead or had been sampled; by day 7 all of the mice inoculated at 28 days of age had died or been sampled. In mice of all age groups studied at 3 days there was infection and destruction of meningeal and ependymal cells (Fig. 1H). The extent of infection at this time was inversely proportional to age at inoculation. As observed in the older mice, there was also infection of cells in the rostral migratory stream (Fig. 1I), hippocampal neurons, Bergmann glia cells in the cerebellum (Fig. 1F) and cells in the white matter tracts, which were often adjacent to infected ependymal cells.

MHV-68 can generally spread to the CNS in the absence of type-I interferon responses

The cells observed to be infected by any virus are those in which the virus can replicate to levels detectable by the assay system in the presence of host responses. In the absence of the type-I interferon system some RNA viruses have been shown to infect a wider range of cell types than observed in the presence of interferon (Ryman *et al.*, 2000). The type-I interferon system has been shown to be functionally important in controlling MHV-68 infection. Infection of mice deficient in the common type-I interferon receptor (IFN-α/β-R$^{o/o}$) results in a 100- to 1000-fold increase in lung virus titres and dissemination of the infection to other organs including the adrenal glands. The CNS was not examined in these studies (Dutia *et al.*, 1999). To determine whether these high virus titres and the absence of a functional interferon system result in spread of infection to the brain, parallel groups of six 4–5-week old 129 and 129–IFN-α/β-R$^{o/o}$ mice were infected intranasally with 2×10^4 p.f.u. of MHV-68 and studied for the presence of virus-infected cells in the brain at 6 days post-infection. All of the 129–IFN-α/β-R$^{o/o}$ mice demonstrated extensive infection of meningeal cells (Fig. 1J) and more rarely infection of cells underlying the meninges. In addition there were perivascular foci of infection, indicative of virus spread from the blood. No virus-positive cells were observed in the main olfactory bulb, indicating that at least by day 6 following intranasal inoculation, even in the absence of interferon, virus was unable to enter the CNS along the olfactory nerve. Consistent with our first study on 129 mice (Table 1), no virus-infected cells were observed in the brains of these mice following intranasal infection. We conclude that, although MHV-68 does not generally infect the CNS within 10 days of an intranasal inoculation, it has the ability to do so in the absence of a type-I interferon response and possibly therefore in other cases where high blood virus titres are established.

Infection of CNS cells following reactivation from a non-productive infection

Another route by which virus may gain access to the CNS during the natural course of an MHV-68 infection could be within infected leukocytes. MHV-68 infects B-lymphocytes and can establish a latent infection of these cells (Sunil-Chandra *et al.*, 1992*a*). To study the course of CNS infection following initiation from non-productively infected cells a transplantation model was adopted. We previously generated a temperature-sensitive SV40 large T-antigen-transformed glial cell line MGC7, from the brains of CBA mice (Terry *et al.*, 1997). MHV-68 infection of these cells *in vitro* is permissive and destructive (data not shown). 4′-S-EtdU is a compound which has been shown to inhibit replication of MHV-68 and other gamma-herpesviruses (Barnes *et al.*, 1999). Continual treatment of MHV-68-infected MGC7 cells with 4′-S-EtdU results in their survival with persistence of virus in a non-productive state (Barnes *et al.*, 1999). MHV-68-infected MGC7 cells could be maintained and passaged in the presence of 4′-S-EtdU for several weeks. Following drug withdrawal, productive virus replication resumed with peak infectious virus titre on day 4 and death of all cells in the culture by day 7.

MHV-68-infected or uninfected MGC7 cells, which had been cultured with 4′-S-EtdU for 5 weeks, were stereotaxically implanted into either the striatum or the lateral ventricle of five, 4–5-week-old CBA mice. Mice receiving uninfected MGC7 cells remained healthy for 8 weeks after which time they were euthanized. Both groups of mice receiving MHV-68-infected cells showed signs of morbidity at day 7 and were euthanized. Histological analysis confirmed the site of in-

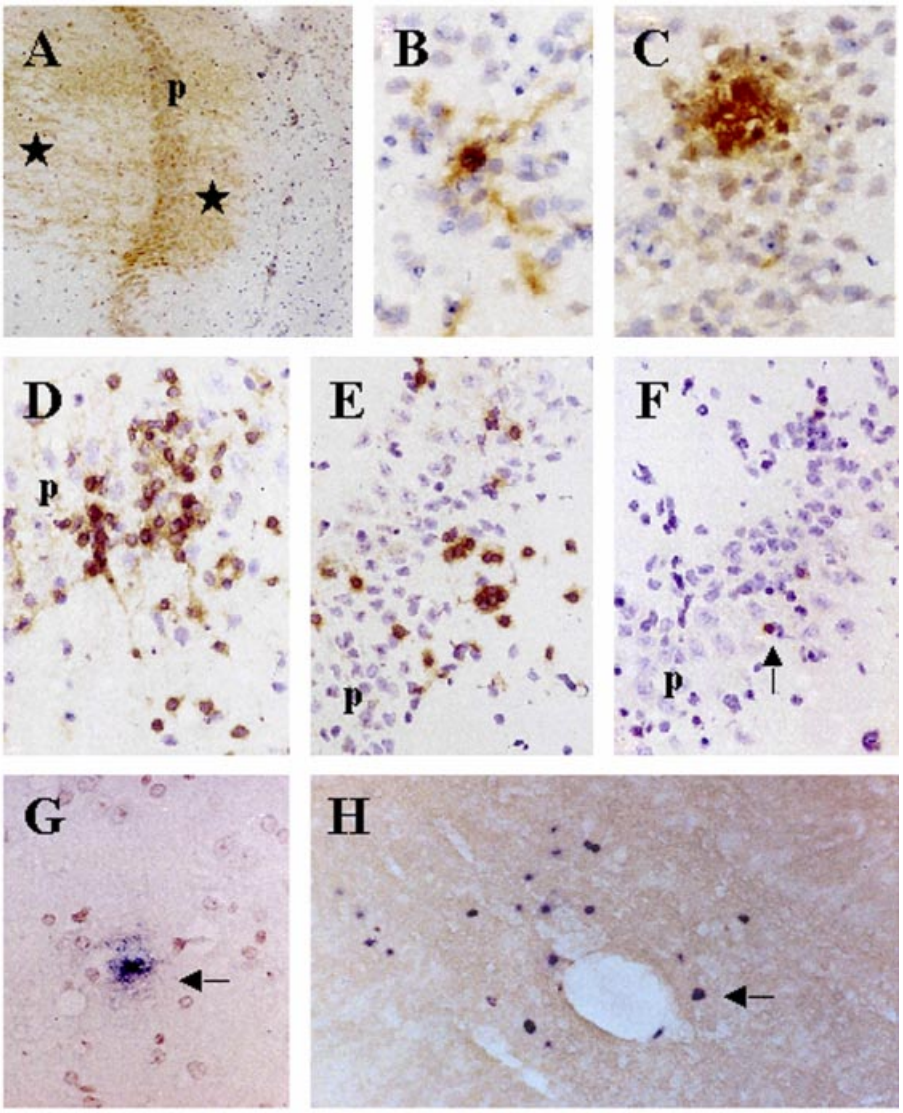

Fig. 2. (A) Cryosection of adult CBA mouse brain (× 100), 7 days after implantation of infected MGC7 cells into the striatum. Widespread virus antigen-positive neurons and their neurites (stars) in the pyramidal layer (p) of the hippocampus. (B, C) MHV-68 protein-positive cells, 30 days after striatal inoculation of infected MGC7 cells into adult mice treated with 4′-S-EtdU. (D)–(F) Inflammatory response in these mice. (D) $CD3^+$ cells in the pyramidal layer (p) of the hippocampus, (E) $CD8^+$ cells and (F) $CD4^+$ cells (e.g. arrow). (G, H) Viral tRNA-positive cell (arrow) (G) and cells (e.g. arrow) (H), 12 months after implantation of infected MGC7 cells into the striatum of a CBA mouse which received 4′-S-EtdU for the first 10 days after inoculation. The positive cells in (H) appear to be adjacent to a blood vessel (mouse brain perfused). Magnification of panels (B)–(H) is × 400.

oculation. Following striatal implantation, neither meningeal nor ependymal cells showed signs of infection but widespread infection of the hippocampal pyramidal neurons was observed (Fig. 2A), as were foci of infected cells within the cortex. Following ventricular implantation the pattern of infection was similar to that observed following direct inoculation of virus with infection predominantly of meningeal, ependymal and underlying cells. We conclude that initiation of infection from a non-productive source within the CNS can result in a widespread CNS infection.

MHV-68 can persist in the CNS

Mice inoculated directly into the CNS either with free virus or with infected cells became moribund and died within 7 days. Whether virus can establish a persistent state in the CNS cannot therefore be addressed using these systems. To determine whether MHV-68 can establish persistence in the CNS, MHV-68-infected MGC7 cells, which had been cultured for 5 weeks in the presence of 4′-S-EtdU, were implanted into the striatum of ten CBA mice. In order to inhibit virus

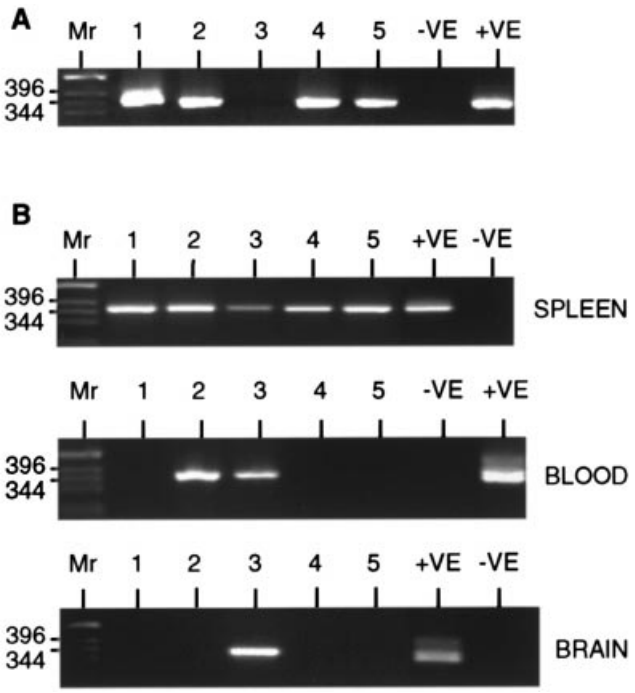

Fig. 3. Detection of MHV-68 in mice by nested PCR. DNA was extracted from the organs of mice and analysed for the presence of MHV-68 by nested PCR using primers specific for the gp150 gene (Stewart *et al*., 1998). Products were analysed on 2% agarose gels containing ethidium bromide and visualized using a transilluminator. Molecular size determinations were made relative to a 1 kb DNA ladder (Mr), and the sizes of the pertinent bands are shown at the left. In all panels, DNA from the MHV-68-positive cell line S11 was used as a positive control (+VE) and produced the expected product of 368 bp. DNA from the MHV-68-negative tumour line S31 was used as a negative control (-VE). (A) Analysis of the brains of five CBA mice that were infected by implantation with infected MGC7 cells 12 months previously. (B) Analysis of spleens, blood and brains of five 129 mice that had been infected intranasally 12 months previously with MHV-68.

replication, these mice also received 4′-S-EtdU ad libitum in their drinking water. This regimen has previously been shown to be effective in inhibiting MHV-68 replication in adult mice (Barnes *et al*., 1999). Animals were monitored daily and showed no clinical signs of infection. At 10 days post-challenge the drug was withdrawn and the mice remained healthy. Half the mice were sampled at 30 days; the other half were continually monitored and after a year without showing any signs of illness were euthanized for study. In the five animals studied at 30 days, small foci or individual virus protein-positive cells were observed scattered throughout the parenchyma (Fig. 2B, C). Attempts to identify the phenotype of these cells were unsuccessful. Positive cells were surrounded by infiltrates of leukocytes (Fig. 2D), the majority of which were $CD8^+$ T lymphocytes (Fig. 2E). Occasional $CD4^+$ lymphocytes were also observed at infected sites (Fig. 2F), although the majority were associated with perivascular cuffs (not shown). The five mice studied at 12 months were extensively perfused with PBS before removal of the brains. One half-brain was processed for DNA extraction and PCR analysis and the other for histological analysis. Since we anticipated that persistence in the CNS could be a rare event, brains were initially screened for MHV-68 DNA by PCR. Surprisingly, MHV-68-specific bands were observed in four of the five brains (Fig. 3A). For each of these brains, the histologically preserved half was completely sectioned and screened by *in situ* hybridization for virus-infected cells using a probe to the MHV-68 tRNAs. This probe recognizes both productively and latently infected cells (Bowden *et al*., 1997). Positive cells of unknown phenotype were observed on occasional sections either as isolated cells (Fig. 2G) or as foci of infected cells (Fig. 2H).

To determine whether MHV-68 could be detected in the CNS several months after an intranasal inoculation, a group of five 129 mice which had been inoculated intranasally with 2×10^4 p.f.u. MHV-68 a year previously were sampled. Following exsanguination and extensive perfusion, brains and spleens were removed. DNA was prepared from blood, brains and spleens and analysed by PCR for MHV-68 sequences (Fig. 3B). All five mice had viral DNA in the spleen. In one mouse viral DNA was also detected in the brain sample. It should be noted that this same mouse and one other also had viral DNA in the blood.

Discussion

The results presented here demonstrate that MHV-68 does not establish a CNS infection during the acute phase of infection in immunocompetent adult mice. However, on gaining access to the CNS, MHV-68 infects principally meningeal and ependymal cells and can also infect neurons and oligodendrocytes.

As a result of the relative inaccessibility of the CNS, many neurological diseases of viral aetiology are rare complications of relatively common systemic infections. However, viruses may gain access to the CNS by two principle routes, either by neuronal transport following infection of the peripheral nervous system or via the blood. The latter may provide entry either as cell-free virus in plasma or cell-associated virus in leukocytes. The most direct nerve entry route is the olfactory nerve, which provides a direct single cell pathway from the olfactory mucosa to the olfactory bulb within the CNS. In the studies presented here all inoculations were intranasal but no evidence of virus entry via the olfactory bulb was observed. The trigeminal nerve, which partly innervates the nasal epithelium, could also be involved in translocation of virus to the CNS via the trigeminal ganglia. Although not investigated in this study the trigeminal ganglia have been reported to be a reservoir of MHV-68 in the natural host (Blaskovic *et al*., 1984; Rajcani *et al*., 1985). The second putative route of entry via the blood was investigated in mice lacking a functional type-I interferon system. MHV-68 is present at high titre in the blood (Dutia *et al*., 1999) under these conditions and virus was

able to enter the CNS resulting in widespread infection of the meninges and perivascular foci of infection.

Many viral encephalitides are highly neuroinvasive: alphaviruses such as the equine encephalitis viruses, Sindbis virus and Semliki Forest virus efficiently gain access to the CNS early in infection when virus titres in the blood are high and before they are brought under control by immune responses. The studies reported here indicate that MHV-68 does not generally establish a CNS infection during the first 10 days of infection. MHV-68 establishes a lifelong latent infection in B-lymphocytes (Sunil-Chandra *et al.*, 1992*b*; Weck *et al.*, 1999). This provides a long period of time over which rare events that give rise to CNS infection could occur and accumulate. Activated but not resting lymphocytes are able to enter the CNS (Knopf *et al.*, 1998; Hickey, 1999) and spread of infection following entry of an MHV-68-infected B-lymphocyte would seem a likely scenario for seeding a CNS infection. Though we were unable to parallel this experimentally using infected B-lymphocytes, widespread infection of the CNS was demonstrated following reactivation of MHV-68 from implanted non-productively infected glial cells. Perhaps with time, there is occasional spread of the infection to the CNS, or perhaps with time the cumulative effect of rare events giving rise to CNS infection establish this in most animals. Our limited study of five mice, 1 year after intranasal inoculation, suggests that CNS infection is not generally established. Even the finding of viral DNA by PCR in one of five brains at 1 year post-infection must be interpreted with caution since the blood from this mouse was also PCR-positive and although the mice were extensively perfused it cannot be ruled out that residual blood remained, perhaps in a blocked vessel.

Whereas these studies provide no clear evidence that MHV-68 generally establishes a CNS infection at early time-points or over the long-term, it is clear from our studies on the IFN-$R^{0/0}$ mice that this virus can under some circumstances gain access to the CNS and our implantation studies demonstrate that MHV-68 can persist in the brain for at least 12 months. These mice in which long-term persistence was observed had received implants of non-productively infected glial cells and were treated with an antiviral drug for 10 days after implantation. Mice that received implanted cells and no drug developed a widespread infection and died. The survival of the drug-treated mice after drug withdrawal could well reflect priming of immune responses by low levels of virus. This is also likely to be the case in any leukocyte-mediated spread of MHV-68 to the CNS from persistently infected cells at any time after establishment of antiviral immune responses.

DNA of other gammaherpesviruses including equine herpesvirus-2 (EHV-2) and bovine herpesvirus-4 (BHV-4) has been demonstrated by PCR in the olfactory bulbs and other regions of the CNS of ponies, calves and mice following experimental intranasal infections (Borchers *et al.*, 1998; Egyed & Bartha, 1998; Rizvi *et al.*, 1997*a*, *b*). In HIV-infected human individuals, EBV-positive primary CNS lymphomas are a major and growing problem (Flinn & Ambinder, 1996) and there has been a long-standing association of EBV with a number of diverse neurological disorders (Archard & Bowles, 1988; Hotchin *et al.*, 1989; Bray *et al.*, 1992; Roberg *et al.*, 1991; Martyn *et al.*, 1993; Imai *et al.*, 1993; Haahr *et al.*, 1994). A recent PCR-based survey has indicated a high incidence (63%) of KHSV nucleic acid in post-mortem brains of normal healthy individuals in the Chinese population in Hong Kong, suggesting that this virus is neuroinvasive and has the ability to persist in the human CNS (Chan *et al.*, 2000). The development of MHV-68 infection in the mouse provides a good small-animal model to study gammaherpesvirus biology, particularly human EBV and KSHV infections where studies have been restricted by the limited host range of these viruses and where direct studies on humans are complicated or in many cases not possible. So far the MHV-68 model has been used to understand the pathology and immunology of this virus with respect to its peripheral tropism and transforming characteristics. The study presented here extends the model by characterizing events in the CNS.

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