Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Microbial Pathogenesis 1992; **13**: 1–15

SJL/J resistance to mouse hepatitis virus-JHM-induced neurologic disease can be partially overcome by viral variants of S and host immunosuppression

John M. M. Pasick, Greame A. R. Wilson,* Vincent L. Morris and Samuel Dales

Cytobiology Group, Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada N6A 5C1

(Received March 23, 1992; accepted March 24, 1992)

Pasick, J. M. M. (Cytobiology Group, Dept of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada N6A 5C1), G. A. R. Wilson, V. L. Morris and S. Dales. SJL/J resistance to mouse hepatitis virus-JHM-induced neurologic disease can be partially overcome by viral variants of S and host immunosuppression. *Microbial Pathogenesis* 1992; **13**: 1–15.

The basis of the resistance of SJL/J mice to various strains of mouse hepatitis virus (MHV) has been the subject of some debate, especially as it relates to the number and nature of the determinants involved. Our previous work demonstrated that resistance by primary SJL/J glial cultures may involve events subsequent to viral gene expression, possibly due to a defect in cell-to-cell spread of the infection. Since S, the virion's major spike glycoprotein, is known to facilitate the spread of infection due to its syncytiogenic properties, we decided to investigate the role of this viral structural protein in resistance by primary SJL/J glial cells. Variants possessing deletions within the S coding region were able to grow in SJL/J glial cells 10–100 times easier and fuse five-times more efficiently than wt virus. Induction of neurologic disease in SJL/J mice following intracranial inoculation with either wt JHMV or the S deletion variant, AT11f cord, was age-dependent, occurring only in animals inoculated under 4 weeks of age. Resistance in older animals to wt and variant viruses could be abrogated by immunosuppression with cyclosporin A. However, both disease incidence and viral brain titers were higher in animals receiving the JHM variant AT11f cord virus, suggesting that SJL/J resistance to neurologic disease may manifest itself through interactions between inefficient cell-to-cell spread of the infection and protective aspects of the immune response.

Key words: SJL/J mice; mouse hepatitis virus; resistance; S deletion variants.

Introduction

Neurotropic strains of MHV are capable of inducing fatal encephalitic disease following intracranial (i.c.) inoculation in the majority of strains of inbred mice that have been tested.[1,2] The genetic basis for the relative resistance of SJL/J mice to the neurotropic strains MHV-JHM (JHMV), MHV-4 and MHV-A59, has been attributed to a single recessive gene,[2–4] or to at least two genes, one dominant and one recessive.[1] The functional basis of SJL/J resistance has been correlated with the following: (1) failure

* Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA, U.S.A.

0882-4010/92/070001+15 $08.00/0

to productively infect primary spinal cord neuronal and peritoneal macrophage cultures;[2,4,5] (2) age-related changes of an adherent macrophagic cell type from spleen or peritoneal exudates capable of adoptively transferring protection from naive, resistant, 12-week-old mice to susceptible 6-week-old animals;[6] (3) failure of JHMV spread from primary replication sites within the nasal cavity to secondary targets such as brain, intestine and liver;[7] and (4) the absence of virus-binding activity by hepatocytes and enterocytes, attributed to a deletion in, or mutation of, the virus-binding domain of a 110–120 kDa glycoprotein.[8,9] Previous studies from our laboratory have demonstrated, however, that resistance of primary SJL/J glial cultures to JHMV could not be attributed to any deficiency in virus attachment, penetration, or early expression of viral genes, but was possibly due to a failure in cell-to-cell dissemination of the infection.[10] Consequently, the exact nature of resistance has been the subject of some dispute, particularly as it relates to whether single or multiple determinants are involved and to the characteristics of the virus-target cell interactions responsible for mediating the resistant phenotype.

Our earlier work[10] indicated that resistance of primary SJL/J glial cultures may be associated with a defect in the dissemination of infection, potentially involving S, the virion's major spike glycoprotein. In this regard, S has been shown to function in virion attachment to target cells, mediation of syncytiogenesis and elicitation of a neutralizing antibody response.[11–13] Certain strains of MHV also express a second surface glycoprotein that is found in lesser abundance than S and referred to as HE (hemagglutinin esterase). HE possesses esterase activity and shares sequence homology with the influenza C hemagglutinin.[14] In JHMV, HE expression is variable, suggesting that it may be an accessory protein not absolutely necessary for viral replication.[15] This is in contrast to the situation in bovine coronavirus where the HE protein has been reported to contribute to infectivity.[16] Thus, the exact role HE plays in MHV infections has yet to be determined.

The present study was undertaken to ascertain whether variant viruses possessing deletions within the coding sequences of the two surface glycoproteins, S and HE, had growth characteristics different from those of wt JHMV in SJL/J primary glial cultures and the central nervous system (CNS) *in vivo*.

Results

Comparison of the replication of wt JHMV and S1 deletion variants in primary glial cultures from SJL/J and CD.1 mice

Growth of wt JHMV and the AT11f cord variant was assessed on primary glial cultures derived from resistant SJL/J or susceptible CD.1 mice. Our wt JHMV was originally obtained from the ATCC and shown to contain no deletions in either S or HE when propagated under the culture conditions we employed.[17] The AT11f cord variant of JHMV[18] has been shown to possess a 441 nucleotide deletion in the middle third of the S1 portion of S in addition to a 738 nucleotide deletion in the coding region of HE.[17] This variant was shown to be more fusogenic and capable of replicating to higher titers in G26–24 oligodendroglioma cells than wt JHMV.[18]

Primary glial cultures from resistant SJL/J and susceptible CD.1 mice were inoculated at between 7 and 12 days *in vitro* (D.I.V.) at a multiplicity of infection (m.o.i.) of 1. At the time of inoculation, cultures were of mixed phenotype consisting of a bedlayer formed predominantly by astrocytes, on top of which were cells of the oligodendrocytic lineage. The AT11f cord variant consistently grew to higher titers in SJL/J glial cultures than did wt JHMV. However, the magnitude of this difference was dependent upon the method by which the virus inoculum was prepared. When

Table 1 Replication of wt-JHMV and variant AT11f cord virus in mixed glial cultures derived from resistant and susceptible strains of mice

Strain of mouse	Days p.i.	Titer of culture supernatant[a] wt-JHMV	AT11f cord
SJL/J	1	4.6±0.15 (5)	825±110 (5)
	2	3.9±2.1 (5)	1080±350 (5)
	3	10.4±4.3 (5)	1320±640 (5)
CD.1	1	375[b]	9000
	2	16000	39000
	3	25000	390[c]

[a]Cultures were derived from postnatal day 1 animals and seeded at a density equivalent to 3.6 cerebral hemispheres per 35 mm culture dish. At 7 days *in vitro*, the resultant stratified cultures comprised predominantly of macroglia, were inoculated with either wt-JHMV or AT11f cord variant at a m.o.i. of 1 in an inoculum volume of 200 μl. The values are means with standard deviations expressed as $\times 10^2$ pfu/ml. The number of cultures tested is shown in parentheses.

[b]Values are means of duplicate samples expressed as $\times 10^2$ pfu/ml. Standard deviations were not calculated due to the obvious difference from growth observed in SJL/J cultures.

[c]Titer drop was associated with massive cell killing.

the inoculum consisted of concentrated, extracellularly released virus,[10] a 100-fold difference in growth between wt and variant virus was observed (Table 1). This difference was usually only 10-fold when the inoculum consisted of membrane-associated virus preparations derived from disrupted cells as described in Materials and methods (Table 2). In spite of the enhanced replication observed with variant virus, production was still 1–2 orders of magnitude less in SJL/J than in CD.1 glial cultures (Table 1). The foci of fusion observed in SJL/J cultures inoculated with wt JHMV were significantly smaller than those produced in AT11f cord infections at equivalent time points post-inoculation (p.i.). Polykaryons evident at 3 days p.i. in AT11f cord infected cultures contained 697±245 (SD) nuclei versus 142±118 nuclei in those induced by wt JHMV ($P < 0.001$, t-test) (Fig. 1 A and B). The syncytia contained predominantly $GFAP^+$ astrocytes while among the overlying oligodendrocytes there was usually some evidence of lysis (Fig. 1 C, D, E and F). More rapid replication in CD.1 cells was accompanied by accelerated and more extensive

Table 2 Comparison of replication of wt JHMV and selected variants in SJL/J glial cultures

Days p.i.	Titer of culture supernatant[a] wt-JHMV	AT11f brain	AT11f cord	V5A13 (88)
2	22.3±6.8	21.1±14.3	402.0±28.3	476.0±14.1
3	30.0±19.3	22.8±11.7	311.0±177.4	278.7±5.5
4	12.5±7.6	26.6±15.7	150.5±61.5	127.3±26.4
6	10.1±2.7	10.2±4.4	22.8±6.0	20.8±3.3

[a]Cultures were derived from postnatal day 1 animals and established in 24 well dishes. At 7 D.I.V. the mixed glial cultures were inoculated at a m.o.i. of approximately 1 pfu/cell. The wt-JHMV and variant viruses employed are described in the text. The titers are means with standard deviations from triplicate cultures, expressed as $\times 10^2$ pfu/ml.

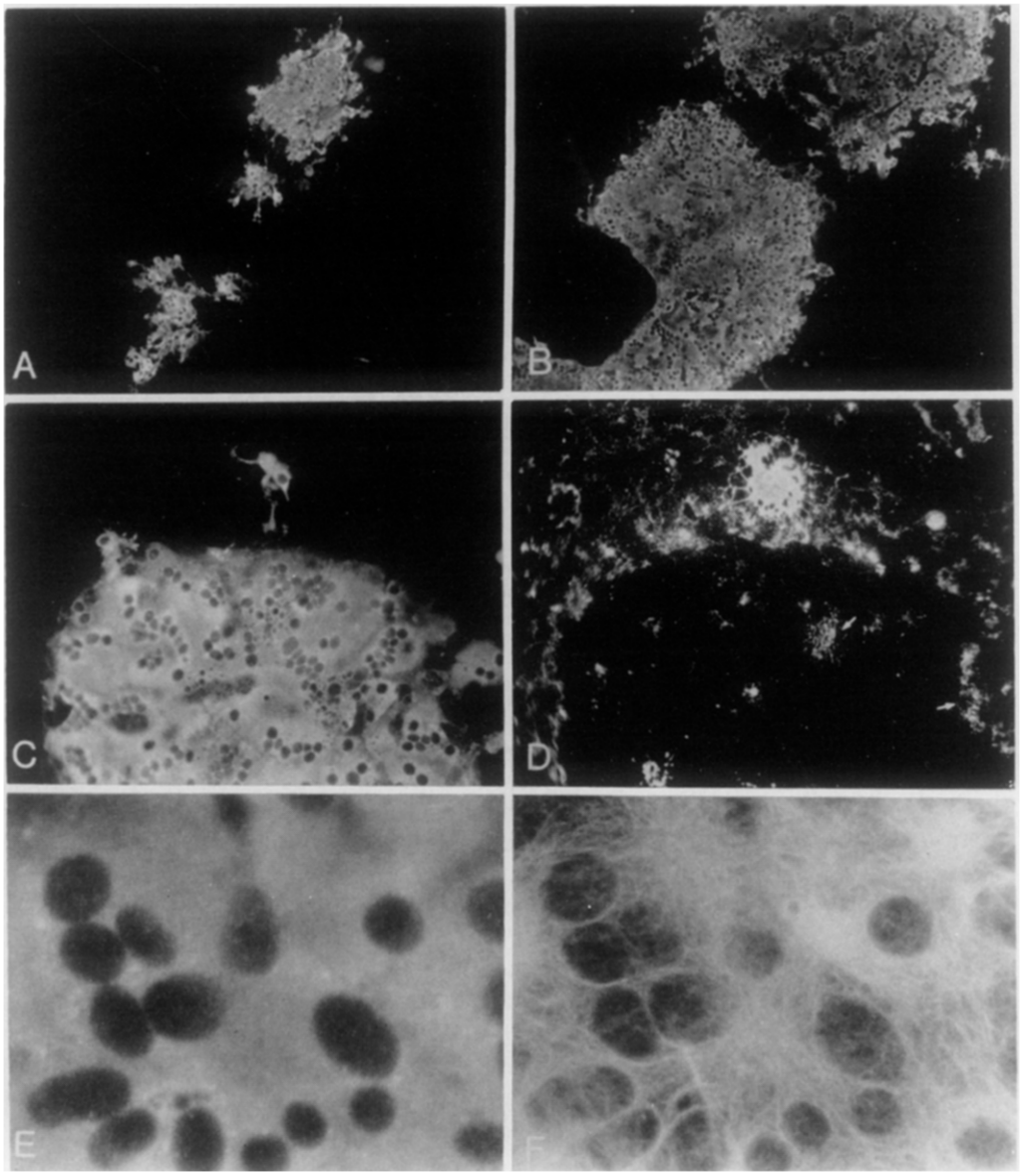

Fig. 1. Comparison of infection at 8 D.I.V. and P8 of SJL/J primary glial cultures with wt JHMV or AT11f cord variant. (A) Foci of wt JHMV infected cells at 3 days p.i. identified by reaction with mAb against JHMV nucleocapsid (N) protein and FITC-conjugated goat anti-mouse IgG. (B) Foci of AT11f cord infected cells sampled and reacted as in (A). Note that the foci are about five times larger in (B) than in (A). (C) Inoculated with AT11f cord and reacted for N as in (A). (D) Same field as in (C) reacted with rabbit anti-galactosyl cerebroside (GC) and Texas Red-conjugated goat anti-rabbit IgG to demonstrate oligodendrocytes. Arrows point to GC^+ disrupted cells. (E) Appearance of culture infected and reacted for N as in (B) at higher magnification. Note the absence of antigen from the nuclei. (F) Same field as in (E) demonstrating the co-localization of glial fibrillary acidic protein (GFAP) in astrocytes by reaction with rabbit anti-GFAP and TR-conjugated goat anti-rabbit IgG. Magnifications: (A) and (B) ×227; (C) and (D) ×273; (E) and (F) ×1625.

syncytia formation, culminating in massive cell killing by 3 days p.i. (Table 1). By contrast, even at 10 day p.i. the cytopathic effect due to AT11f cord virus involved no more than 30–40% of the area occupied by SJL/J glial cultures.

In order to determine which deletions in the AT11f cord variant were responsible for the enhanced efficiency of replication in SJL/J glial cultures, we compared growth of AT11f cord, V5A13 (88), AT11f brain and wt JHMV viruses. V5A13 (88) which was selected *in vitro* from wt MHV-4 infected SAC−-cells on the basis of resistance to neutralization with monoclonal antibody (mAb) 5A13.5,[13] possesses a 447 nucleotide deletion in the S coding region[19] that overlaps that present within AT11f cord (Fig. 2). In addition, MHV-4 apparently does not express detectable levels of HE (M. Buchmeier, pers. comm.). The AT11f brain variant was an independent isolate from the same animal that the AT11f cord virus was derived[18] and possesses the identical deletion in the HE gene as AT11f cord but has no deletion within S.[17] AT11f brain variant was therefore used as a control to assess the defect in HE expression observed in both AT11f cord and V5A13 (88). It is evident from the data in Table 2, that AT11f cord and V5A13 (88) variants grew to 10-fold higher titers than both AT11f brain virus and wt JHMV. In some experiments however, AT11f brain virus grew about twice as efficiently as wt JHMV (data not shown). These results indicated that the enhanced growth of the AT11f cord variant could most likely be attributed to the alteration associated with the S coding region. Nevertheless, the coincidental defect involving the HE envelope glycoprotein may also play a contributory role. Interestingly, infection of SJL/J glial cells never progressed to the point where total destruction of the culture was observed. In fact, a gradual but progressive decrease in titer was observed with all viral strains assayed as a function of time p.i.

When viral gene expression of wt JHM, AT11f brain and AT11f cord virus infected SJL/J glial cultures was assayed at 24 and 48 h p.i., the magnitude of expression of viral RNA paralleled the differences observed in progeny virus release and fusogenic potential (Fig. 3). Thus, virus-specific RNA synthesis of AT11f cord virus was greater than AT11f brain virus, which in turn was greater than wt JHMV. These data, however,

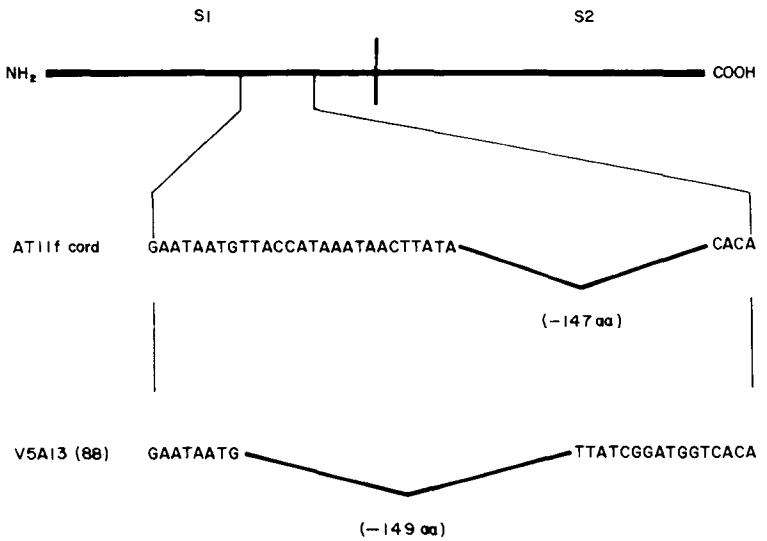

Fig. 2. Diagrammatic representation of the overlapping deletions in the S1 component of AT11f cord and V5A13 (88) variants of MHV-JHM and MHV-4, respectively. Deleted segments involve amino acids (aa) approximately between 430–590, located within the distal third of S1. Alignment of the deleted sequences was based on published sequence data.[17,19]

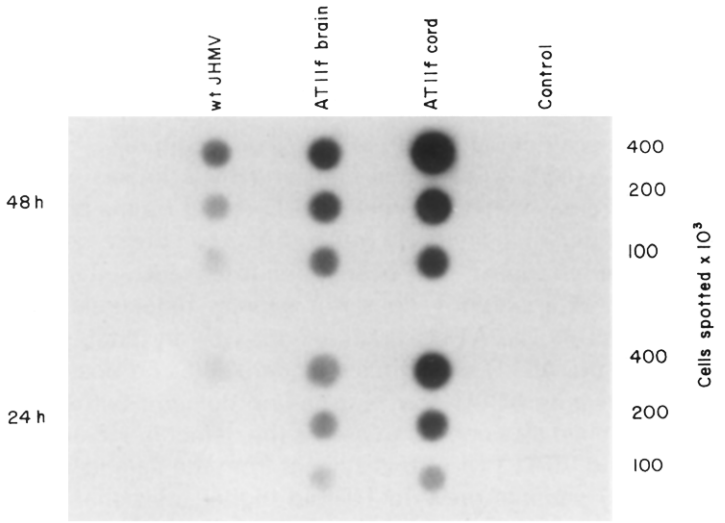

Fig. 3. *In situ* dot-blot analysis of MHV RNA present in infected SJL/J glial cultures. Primary SJL/J glial cultures were inoculated at 7 D.I.V. with JHMV, AT11f cord or AT11f brain virus using a m.o.i. of 1. At 24 and 48 h post-inoculation, cells were washed and harvested by scraping into PBS. After resuspending the cells and adjusting to a final concentration of 1×10^6 cells/ml, 400, 200 and 100×10^3 cells per sample were spotted onto nitrocellulose and probed for MHV-specific RNA. Equivalent numbers of uninoculated cells were used as a negative control.

do not enable us to distinguish with certainty which step(s) in the interaction between virus and host is responsible for the advantage observed with the S deletion variant.

Comparison of replication efficiency and the disease process in resistant SJL/J and susceptible CD.1 mice inoculated with wt JHMV and variant AT11f cord virus

In view of the enhanced replication obtained by AT11f cord virus in primary glial cultures, we next determined whether the CNS disease process was also affected. SJL/J mice were inoculated i.c. with varying doses of either wt JHMV or AT11f cord variant at 1, 2, 4, 5, 6 and 11 weeks of age. Comparison was made with age-matched, susceptible CD.1 mice as controls. The data, summarized in Table 3, demonstrate that in the case of SJL/J mice an age-related resistance developed. Thus, at 1 and 2 weeks of age, SJL/J sucklings were completely susceptible and by 6 weeks uniformly resistant to both viruses. Some differences were evident between the wt and variant at 4–5 weeks of age when SJL/J mice demonstrated full resistance to wt JHMV and partial resistance to AT11f cord variant. This age dependent development of resistance, although similar to that previously reported,[6] differs with respect to the exact ages at which the shift from susceptibility to resistance was observed. However, our data were virtually in complete agreement with those reported by another group[7] where encephalitis was only observed in 1-week-old SJL/J animals following intranasal inoculation with JHMV. As anticipated, CD.1 mice remained fully susceptible at all ages tested and their mean time to death ranged from 1.5–5.3 days p.i., depending on the age at inoculation, size of inoculum and virus used. Generally, AT11f cord virus produced acute encephalitis in CD.1 mice in half the time required by wt JHMV at equivalent doses. The rapidity of the disease process in CD.1 mice corresponded with the efficiency of virus replication in primary glial cultures (Table 1). Interestingly, it has been previously reported[13] that S deletion variants of MHV-4 were neuroattenuated in BALB/c mice based on increased LD_{50}s and the propensity to induce chronic,

Table 3 Cumulative data obtained with varying doses of wt-JHMV and AT11f cord following *in vivo* challenge of resistant and susceptible strains of mice

Strain of animal	Age at time of i.c. challenge (weeks)	Size of inoculum (pfu)	No. of animals dead or sacrificed *in extremis*/No. of animals inoculated	
			wt-JHMV	AT11f cord
SJL/J	1	1×10^5	4/4	5/5
	2	1×10^5	4/4	4/4
	4	1×10^5	0/2	0/3
		1×10^6	0/4	1/5
	5	1×10^6	0/8	2/8
	6	1×10^3	ND	0/3
		1×10^4	ND	0/3
		1×10^5	0/2	0/2
		1×10^6	0/2	0/2
	11	1×10^6	0/4	0/4
CD.1[a]	3	1×10^4	3/3	3/3
		1×10^6	3/3	2/2
	4	1×10^5	5/5	5/5
		1×10^6	2/2	2/2
	6	1×10^6	2/2	2/2

[a]It was established (G. A. R. Wilson, unpublished data) that 2000 pfu of wt-JHMV were lethal for 100% of CD.1 mice challenged at 3 weeks of age.
ND = Not done.

demyelinating, rather than acute, encephalitic, disease. This, however, has not been our experience with CD.1 mice and the doses of inoculum we have employed.

Since even the more efficent S deletion variants of JHMV replicated in SJL/J glial cultures to titers that were at least one order of magnitude lower than those observed with CD.1 cells, we hypothesized that some additional factor such as the host immune response may be involved in controlling the acuity of a potentially slow, progressive CNS disease process. The role of the host cellular immune response was therefore studied by administration of the immunosuppressant Cyclosporin A (CsA).

As Table 4 illustrates, CsA treatment of animals following i.c. inoculation with either

Table 4 Effect of cyclosporin A on disease and mortality caused by wt-JHMV and AT11f cord virus in SJL/J mice

Age at time of i.c. challenge (weeks)	Size of inoculum (pfu)	No. of animals dead or sacrificed *in extremis*/No. of animals inoculated[a]	
		wt-JHMV	AT11f cord
4	1×10^6	9/14	14/16
5	1×10^6	0/7	4/7
6	1×10^3	ND	0/3
	1×10^4	ND	0/3
	1×10^6	0/2	0/2
11	1×10^6	0/4	1/4

[a]Cumulative data were obtained following i.c. inoculation at the times and virus concentrations indicated. Animals were treated with 25 mg/kg body weight CsA beginning either the day before or the day of inoculation with virus and continued for an additional 5–10 days following inoculation.
ND = Not done.
Note that uninoculated control mice from both 4 and 11 week old age groups showed no observable toxic effects to cyclosporin administration (6 consecutive days for 4 week old animals and 10 consecutive days for 11 week old animals).

wt JHMV or AT11f cord variant led to a significant breakthrough of resistance in the 4-week-old age group. Clinical signs consisted of lethargy and piloerection which progressed to irritability and seizures. The mean days to advanced disease was 8.1 ± 1.8 and 8.0 ± 2.6 days p.i. for wt and variant viruses, respectively. This was longer than the development of advanced disease observed for age-matched CD.1 mice or 1- and 2-week-old SJL/J mice, the latter of which succumbed to advanced disease in half this time. In the 5-week-old age group, the AT11f cord variant produced a significantly higher incidence of encephalitis as compared with wt JHMV inoculants ($P = 0.035$, Fisher's exact test). Again, an age-related shift in resistance was observed in spite of CsA treatment, though not as pronounced as that observed in the non-CsA-treated group. Consistent with the observed growth of wt and variant virus in tissue culture, AT11f cord virus appeared to demonstrate a growth advantage over wt JHMV *in vivo* as well (Table 5). Histopathology included perivascular cuffing and mononuclear cell infiltration within the forebrain, loss of neurons within Ammon's horn of the hippocampus and vacuolation of the neuropil within the midbrain (Fig. 4). Viral antigen was localized primarily to cells possessing glial morphology, as well as to fiber tracts surrounding the hippocampal formation (Fig. 4). Interestingly, those animals not developing acute encephalitis did not subsequently go on to develop chronic demyelinating disease as assessed both clinically and histologically during the 60 day period following inoculation. Infectious virus was never recovered from the livers of mice that had developed signs of clinical disease. This, combined with the preceding data, strongly suggests that these animals were succumbing to an encephalitic disease.

Discussion

This study suggests that the resistance to JHMV by the SJL/J strain of mouse is complex and likely contingent on more than one virus or host determinant. Our previous work demonstrated that the genetic resistance of SJL/J mice to JHMV was already inherent in primary glial cultures derived from neonates.[10] Glia appear to be targets for JHMV in susceptible C57BL/6 mice during both acute encephalomyelitis

Table 5 Comparison of wt JHMV and At11f cord virus growth in the brains of SJL/J mice challenged at different ages

Age at time of i.c. inoculation (weeks)	Days p.i.	Viral brain titers[a] — pfu/g of brain tissue			
		wt-JHMV		AT11f cord	
		CsA⁻	CsA⁺	CsA⁻	CsA⁺
1	4	1.6×10^7 (2)	ND	2.1×10^8 (2)	ND
2	4	3.8×10^6 (1)	ND	3.0×10^8 (2)	ND
	5	7.0×10^7 (2)	ND	5.8×10^8 (1)	ND
4	4	ND	ND	2.6×10^6 (2)[b]	3.0×10^6 (2)[b]
	5	ND	ND	ND	6.4×10^7 (2)
	6	ND	4.7×10^7 (2)	ND	7.8×10^8 (3)
	8	ND	3.3×10^6 (2)	9.0×10^5 (1)	2.9×10^7 (2)
	13	ND	ND	2.9×10^4 (1)[b]	2.5×10^3 (1)
5	4	ND	ND	ND	6.2×10^5 (2)
11	6	ND	ND	ND	2.6×10^4 (1)

[a] Animals were sacrificed *in extremis* and either all or half the brain was homogenized in serum supplemented medium for virus titer determination. The data are from single animals or the averages of two or three animals, as indicated within parentheses.

[b] Asymptomatic.

ND = Not done.

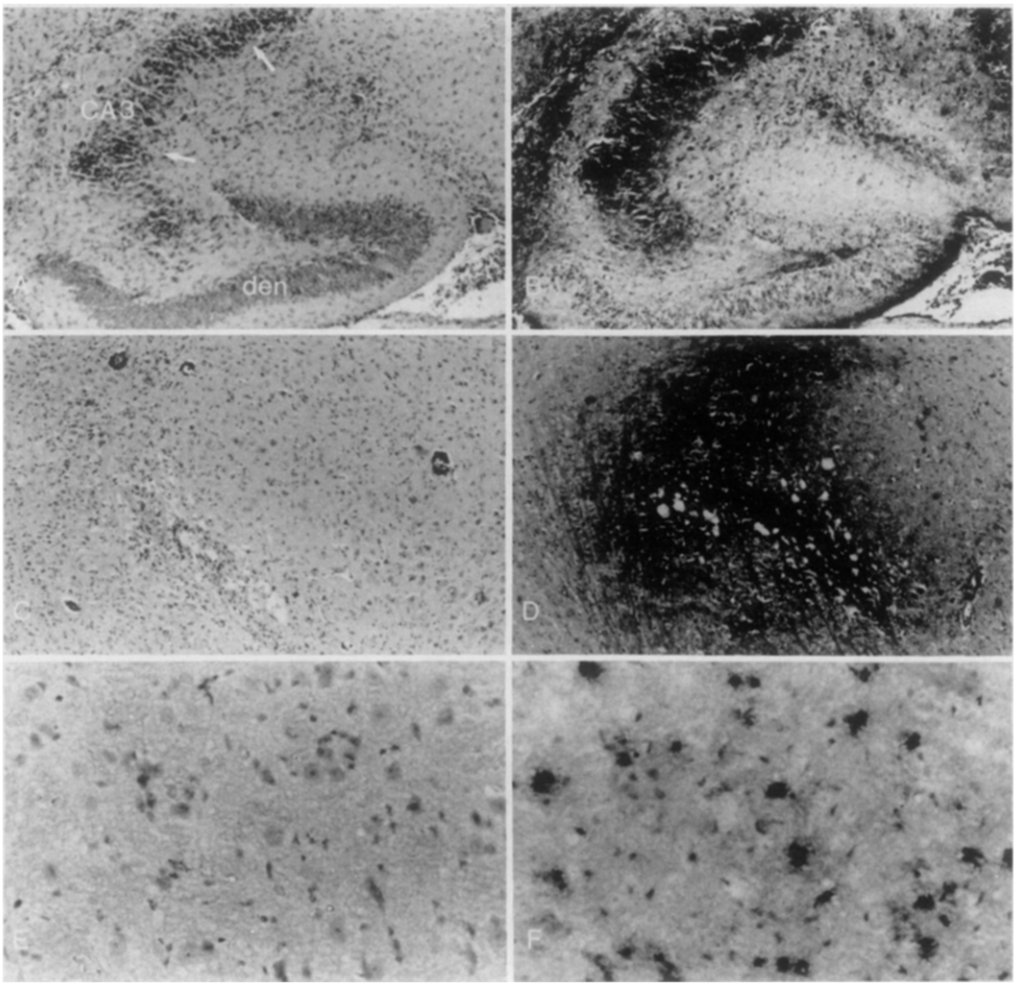

Fig. 4. Histology and immunohistochemistry of various brain regions from SJL/J mice inoculated with AT11f cord variant. (A) H&E stained section of the hippocampus at 13 days p.i. from an animal inoculated at 4 weeks of age and CsA^+. Arrows point to prominent mononuclear infiltrates within Ammon's horn (CA3 region). Note that the dentate gyrus (den) appears normal. (B) Adjacent section to that in (A) demonstrating large amounts of viral antigen in Ammon's horn detected by anti-N immunohistochemistry. (C) H&E stained section of the midbrain at 13 days p.i. from an animal inoculated at 4 weeks of age and CsA^-. Note the perivascular cuffing, mononuclear infiltrates and vacuolation of the neuropil. (D) Adjacent section to that in (C) demonstrating intense viral antigen staining (N) in the area of vacuolation. (E) H&E stained section of the hindbrain at 9 days p.i. from an animal inoculated at 5 weeks of age and CsA^-. Note the relatively normal histologic appearance. (F) Adjacent section to that illustrated in (E) demonstrating N^+ cells that are morphologically typical of glia. Magnifications: A, B, C and D ×65; E and F ×260.

and delayed demyelinating disease.[20] Hence, glial infections may play an important role in the pathogenesis of both forms of neurologic disease, especially when one considers the important supportive role that astrocytes have in maintaining normal neuronal function. Characterization of virus–glial interactions *in vitro* therefore has the potential to provide insight into pathogenesis *in vivo*. The observation that both AT11f cord and V5A13 (88) variants possessed growth advantages over wt JHMV in primary glial cultures was in turn reflected by enhanced growth and ability to induce disease *in vivo*. The enhanced growth of AT11f cord virus correlated with increased viral gene expression and the ability of the variant to incorporate greater numbers of cells in syncytia, a phenomenon that is most likely attributable to the molecular nature of S. Using strain distribution analysis of recombinant-inbred mice, other workers[3] have demonstrated that the susceptibility of peritoneal macrophages for MHV-4 was linked to the *Svp*-2 locus on the proximal end of mouse chromosome 7. Interestingly, these authors alluded to the fact that several loci on this chromosome encode trypsin-like proteases and molecules involved in secretory processing. These correlations fit with our hypothesis that resistance of SJL/J glia to JHMV involves a defect in cell-to-cell spread, which may be intimately dependent upon syncytiogenesis. This in turn may be associated with defective processing of S by SJL/J glia necessary for optimal syncytiogenesis and thus spread of infection.

S is a 180 kDa protein that is co-translationally glycosylated in the endoplasmic reticulum, and reportedly undergoes trimerization prior to assembly into virions.[21] Virion assembly occurs by budding into a smooth membrane compartment between transitional elements of the Golgi stack[22] and the progeny virions thus formed are released from the cell via a constitutive exocytic pathway.[23] Spike glycoprotein not incorporated into virions is transported to the plasma membrane where it can induce cell-to-cell fusion from within.[24] Proteolytic cleavage of S to 90 kD N-terminal S1 and C-terminal S2 products has been shown to be a necessary requirement for virus-induced cell fusion and has been reported to occur on virion associated S just prior to release from infected cells.[12] The degree to which S on virions is cleaved to S1 and S2 subunits appears to be host cell-dependent and is mediated by host proteases.[25] Though the molecular mechanism by which S induces host cell fusion is not known, determinants along both S1 and S2 subunits have been implicated,[26,27] along with conformational changes that take place in S1 which are accompanied by aggregation of S2 subunits.[28,29] The allocation of particular biological functions to either S1 or S2 subunits is complex. In fact, neutralizing epitopes, as well as fusogenic and neurovirulence determinants, appear to be associated with multiple sites along S.[27,30,31]

Previously, an infectious centers assay suggested that the restriction of growth of wt JHMV in primary SJL/J glial cultures was associated with defective cell-to-cell spread from initially infected foci.[10] Both resistant SJL/J and susceptible CD.1 glial cultures adsorbed and sequestered the inoculum with equal efficiency.[10] In the current study, a small but progressive increase in size of infected foci was observed during the initial 3–4 days following inoculation with the AT11f cord variant. Thus, coupled with previous results, this favors the hypothesis that enhanced growth of S deletion variants over wt JHMV in SJL/J glial cultures is likely due to enhanced spread of infection rather than to differences in numbers of cells initially infected or replication rates per cell. However, one cannot rule out the possibility that yield of virus per infected cell may differ between wt and variant viruses. This explanation was advanced to explain enhanced growth of MHV-4 S deletion variants in DBT cells.[32] The higher yields of these variants versus wt MHV-4 in DBT cells were concluded to be the result of a decrease in the specific fusogenicity of the truncated versions of S. This apparently explained the attenuated cytopathic effect and thus prolonged growth cycle of the

variants versus wt MHV-4. In this regard, however, we have observed that V5A13 (88), along with AT11f cord virus, are more fusogenic than wt JHMV in G26-24 cells (unpublished data). Hence, this attribute may be host cell, as well as virus, dependent.[25] In contrast, the basis of resistance implicated for SJL/J enterocytes and hepatocytes is the lack of a functional receptor.[8,9] The 110–120 kDa glycoprotein identified as the virus receptor on hepatocytes and enterocytes is a member of the carcinoembryonic antigen family in the immunoglobulin superfamily.[33] SJL/J hepatocytes and enterocytes express a form of this protein that apparently lacks virus-binding activity.[8,9] The most conclusive proof that this protein is a functional MHV receptor has come from a recent report in which resistant human and hamster cells were made MHV-susceptible following transfection and expression of a cDNA clone for this protein.[34] The monoclonal antireceptor antibody CC1 was used to assess the distribution of this receptor in different tissues of susceptible BALB/c and resistant SJL/J mice. This antibody does not recognize the allelic form of the protein expressed by SJL/J mice. However, in BALB/c mice, the receptor was found to be relatively abundant in liver, small intestine and colon, but absent in brain and spinal cord.[33] This may suggest that an alternative receptor(s) may be used by this virus to infect cells within the CNS. Some support for this notion comes from previous work[7] which demonstrated that JHMV replicated equally well in BALB/c and SJL/J brain following intranasal inoculation of 1-week-old animals, but not in intestine and liver of these two strains of mice. Other viruses have been reported to utilize more than one receptor for attachment and internalization of target cells. Recently, it has been reported that herpes simplex virus 1 is capable of utilizing two asymmetrically distributed receptors on polarized Madin-Darby canine kidney cells for attachment and subsequent internalization events.[35] It is therefore conceivable that the basis for resistance of SJL/J mice to MHV-JHM, MHV-4 and MHV-A59 may to some extent be tissue-dependent.

The fact that CsA immunosuppression was able to accentuate CNS disease production by both wt and variant viruses suggests that resistance may in fact be due to a dynamic interaction between the kinetics of virus growth and spread and the immune response to the infection. CsA is known to inhibit the activation of T lymphocytes by antigen,[36] apparently by specifically inhibiting the function of nuclear proteins important for the transcriptional activation of the genes for interleukin-2 (IL-2), its receptor, IL-4 and gamma interferon (γIFN).[37] Though CsA is thought to act primarily through direct effects on T cells, particularly $CD4^+$ cells, secondary indirect as well as direct effects on B cells and macrophages cannot be ruled out. Lethal infection by JHMV in C57BL/6 mice has been reported to be prevented by adoptive transfer of virus-specific T cell clones implicated in a local delayed type hypersensitivity (DTH) response, but this protection apparently did not result in the inhibition of virus replication.[38,39] No difference in viral yields was observed in 4-week-old CsA^- or CsA^+ AT11f cord inoculants at 4 day p.i. In this age group, viral brain yields peaked at 6 day p.i. (Table 5) which preceded the mean days to advanced disease at 8 days p.i. The single untreated control animal which succumbed to encephalitis at 8 days p.i. had a viral brain titer which was 1 $\log_{10}$ lower than CsA-treated animals at the equivalent days p.i. This difference was, however, not apparent between CsA^- and CsA^+ mice at 11 days p.i. This possibly suggests that CsA may interfere with an immune mechanism that protects the host from the lethal effects of JHMV without necessarily interfering with mechanisms involved in inhibiting virus growth.[38,39] Clearance of JHMV from the CNS has been shown to be T cell mediated and likely requires both $CD4^+$ and $CD8^+$ phenotypes.[40–43] The reason CsA immunosuppression was unable to abrogate resistance to either virus in the 11-week-old age group is not known, but may be attributable to age-dependent changes in neural targets. Alternatively, CsA may not interfere with

the function of macrophage-like cells described previously to be protective in older animals,[6] or that of natural killer (NK) cells which may also play a potentially important role in the reduction of virus within the CNS.[42]

In summary, the observed resistance of SJL/J mice to JHMV-induced neurologic disease does not appear to be fully attributable to any single factor, but potentially appears at least bifactorial in nature. This is consistent with the genetic analysis carried out on SJL×B10.S F1 hybrids and F1 backcrosses, which suggested that resistance was not likely due to a simple single gene model but more likely mediated by at least two genes.[1] Our own work supports this latter model and suggests a defect in cell-to-cell spread of the infection, as well as to protective functions of the immune response.

Materials and methods

Viruses. MHV-JHM was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). The isolation and characterization of JHMV variants AT11f cord and AT11f brain, as well as the MHV-4 variant V5A13 (88), have been previously published.[17–19] V5A13 (88) was obtained as a gift from Dr M. Buchmeier of the Scripps Clinic and Research Foundation, La Jolla, California. All viral strains were propagated on L-2 mouse fibroblast monolayers by inoculating at a m.o.i. of approximately 0.1. Inoculum prepared from extracellularly released virus was collected from the culture supernatant when 90–100% of cells were incorporated into syncytia and was concentrated as previously described.[10] Membrane-associated inoculum was prepared from combined culture supernatant and cell-associated virus as previously described.[44] Virus titers were determined as plaque-forming units (pfu) per milliliter on L-2 cell monolayers.

Animals. Inbred SJL/J and CD.1 mice were obtained from the Jackson Laboratory, Bar Harbor, Maine, and bred in-house as reported previously.[45] Animals in our colony have remained MHV free as determined by periodic serological testing.

Primary murine glial cultures. Primary, dissociated, murine glial cultures were prepared as previously described,[46] with minor modifications. Briefly, mixed cultures consisting predominantly of astrocytes and cells of the oligodendrocytic lineage were derived from dissociated cerebral hemispheres of day old mice. Tissue culture plastic or 12 mm diameter, glass coverslips coated with poly-L-lysine were seeded at densities ranging from 0.18–0.36 cerebral hemispheres per cm^2 in Dulbecco's modified Eagle medium (DMEM-high glucose) supplemented with 10% fetal bovine serum (FBS) (Bocknek), 1.2 g/l $NaHCO_3$, 25 mM Hepes, pH 7.3 (Sigma) and 10 g/ml gentamycin. A half medium change was made at day 4 *in vitro* and every third day thereafter until inoculated with virus.

Infection of animals and primary glial cultures. Mice were lightly anesthetized with methoxyflurane and then inoculated i.c. with a membrane-associated virus suspension in either a 30 or 40 μl volume using a 31 gauge needle. Uninfected L-2 cell lysate was used as a control. Mixed glial cultures were inoculated with either a concentrated extracellular or crude, membrane-associated virus preparation at a m.o.i. of approximately 1 pfu per cell. The inoculum was absorbed for 60 min at 37°C, after which, the inoculum was removed, cultures washed twice with warm phosphate buffered saline (PBS), pH 7.2, and after addition of fresh medium, incubated in a humidified atmosphere containing 5% CO_2.

Immunosuppression with cyclosporin A. Cyclosporin A was obtained as Sandimmune (Sandoz Canada, Inc.) as a 50 mg/ml solution and diluted to a 2 mg/ml working concentration with PBS. Mice were weighed and injected i.p. to deliver 25 mg/kg body weight per day. Animals were begun on CsA either the day of, or the day before, i.c. inoculation with virus. CsA treatment was continued on a daily basis for 5–10 days post-inoculation, depending on the age of the animal at the time of inoculation. Animals inoculated at 11 weeks of age were treated with CsA for 10 consecutive days post-inoculation. Control animals were injected i.p. with PBS.

Virus titration of brain tissue. Aseptically removed brains were weighed, placed into ice-cold tissue culture medium supplemented with 10% FBS, and 10% w/v tissue homogenates prepared.

The resulting suspensions were clarified by centrifugation and the supernatant used to make serial 10-fold dilutions that were inoculated onto L-2 cell monolayers. After virus adsorption for 60 min at 20°C with continuous rocking, the cultures were overlayed with medium containing carboxymethylcellulose and incubated for 18–24 h before counting plaques.

Immunostaining infected primary glial cultures. At 3 days post-inoculation, infected coverslips were washed with PBS and fixed in 4% paraformaldehyde in PBS for 5 min at 20°C. Where indicated, cells were permeabilized by exposure for 30 s to acetone, then thoroughly rinsed in PBS. Coverslips were incubated with primary antibodies diluted in 3% bovine serum albumin (BSA) in PBS for 20–30 min as follows: mAb against JHMV nucleocapsid protein (N) as tissue culture supernatant (a generous gift from M. J. Buchmeier, Scripps Clinic and Research Foundation, La Jolla, California) 1:8; rabbit anti-bovine glial fibrillary acidic protein (GFAP) (Dakopatts, Denmark) 1:50; and rabbit anti-galactosyl cerebroside (GC)[44] 1:10. Secondary conjugated antibodies diluted in PBS were applied at 1:30 for goat anti-mouse IgG-fluorescein isothiocyanate (FITC) (Sigma) and 1:50 for goat anti-rabbit IgG-Texas Red (TR) (Jackson Immunoresearch Laboratories, Inc.).

Histology and immunohistochemistry. Animals were sacrificed *in extremis* and the CNS immediately removed and fixed by submersion into 10% neutral buffered formalin in preparation for paraffin embedding. 10 μm sections were stained with hematoxylin and eosin for routine histopathological examination. Viral N antigen was detected in 10 μm paraffin sections using a biotin–anti-biotin amplification system conjugated to alkaline phosphatase (AS/AP Plus immunostaining kit, BIO/CAN SCIENTIFIC). Following deparaffinization, sections were treated in a 0.5% pepsin solution for 20 min at 37°C. Monoclonal antibody against JHMV N protein as tissue culture supernatant was then incubated overnight at room temperature at a 1:18 dilution prior to the amplification and chromogenic reactions. Sections were then briefly counterstained with hematoxylin.

In situ *dot-blot analysis of MHV RNA.* At 7 D.I.V. primary SJL/J glial cultures situated in 35 mm culture dishes were inoculated with wt JHMV, AT11f cord virus or AT11f brain virus at a m.o.i. of 1. At 24 and 48 h post-inoculation, the culture supernatants were removed and the cultures thoroughly washed with ice-cold PBS. Cells were then gently scraped from the dishes and resuspended in PBS by gentle passage through fire-polished Pasteur pipettes to a final concentration of 1×10^6 cells/ml. Whole cells were spotted onto nitrocellulose using a Schleicher and Schuell 'minifold' filtration apparatus and processed for *in situ* hybridization using previously described methods.[47] The blot was probed at 42°C with nick-translated pg344[48] isotopically labelled with ^{32}P. Blots were exposed to film for 24–48 h at −70°C.

We are grateful to Dr M. Buchmeier for providing the MHV-4 variant V5A13 (88). This work was supported by a grant from the Multiple Sclerosis Society of Canada to V.L.M., and grants from the Medical Research Council of Canada and the Multiple Sclerosis Society of Canada to S.D. J.M.M.P. and G.A.R.W. were the recipients of a Multiple Sclerosis Society of Canada post-doctoral fellowship and studentship, respectively.

References

1. Stohlman SA, Frelinger JA. Resistance to fatal central nervous system disease by mouse hepatitis virus. Immunogenet 1978; 6: 277–81.
2. Knobler RL, Haspel MV, Oldstone MBA. Mouse hepatitis virus type 4 (JHM strain)-induced fatal central nervous system disease I. Genetic control and the murine neuron as the susceptible site of disease. J Exp Med 1981; 153: 832–43.
3. Knobler RL, Taylor BA, Woodel MK, Beamer WG, Oldstone MBA. Host genetic control of mouse hepatitis virus type-4 (JHM strain) replication. Exp Clin Immunogenet 1984; 1: 217–22.
4. Smith MS, Click RE, Plagemann PGW. Control of mouse hepatitis virus replication in macrophages by a recessive gene on chromosome 7. J Immunol 1984; 133: 428–32.
5. Knobler RL, Tunison LA, Oldstone MBA. Host genetic control of mouse hepatitis virus type 4 (JHM strain) replication. I. Restriction of virus amplification and spread in macrophages from resistant mice. J Gen Virol 1984; 65: 1543–8.
6. Stohlman SA, Frelinger JA, Weiner LP. Resistance to fatal central nervous system disease by mouse hepatitis virus, strain JHM. J Immunol 1980; 124: 1733–9.

7. Barthold SW, Smith AL. Response of genetically susceptible and resistant mice to intranasal inoculation with mouse hepatitis virus JHM. Virus Res 1987; 7: 225–39.
8. Boyle JF, Wesmiller DG, Holmes KV. Genetic resistance to mouse hepatitis virus correlates with absence of virus-binding activity on target tissues. J Virol 1987; 61: 185–9.
9. Williams RK, Jiang G-S, Snyder SW, Frana MF, Holmes KV. Purification of the 110-kilodalton glycoprotein receptor for mouse hepatitis virus (MHV)-A59 from mouse liver and identification of a nonfunctional, homologous protein in MHV-resistant SJL/J mice. J Virol 1990; 64: 3817–23.
10. Wilson GAR, Dales S. *In vivo* and *in vitro* models of demyelinating disease: efficiency of virus spread and formation of infectious centers among glial cells is genetically determined by the murine host. J Virol 1988; 62: 3371–7.
11. Collins AR, Knobler RL, Powell H, Buchmeier MJ. Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. Virology 1982; 119: 358–71.
12. Sturman LS, Ricard CS, Holmes KV. Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. J Virol 1985; 56: 904–11.
13. Dalziel RG, Lampert PW, Talbot PJ, Buchmeier MJ. Site-specific alteration of murine hepatitis virus type 4 peplomer glycoprotein E2 results in reduced neurovirulence. J Virol 1986; 59: 463–71.
14. Vlasak R, Luytejes W, Leider J, Spaan W, Palese P. The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetylesterase activity. J Virol 1988; 62: 4686–90.
15. Yokomori K, Banner LR, Lai MC. Heterogeneity of gene expression of the hemagglutinin-esterase (HE) protein of murine coronaviruses. Virology 1991; 183: 647–57.
16. Deregt D, Babiuk LA. Monoclonal antibodies to bovine coronavirus: characteristics and toplogical mapping of neutralizing epitopes on the E2 and E3 glycoproteins. Virology 1987; 161: 410–20.
17. La Monica N, Banner LR, Morris VL, Lai MC. Localization of extensive deletions in the structural genes of two neurotropic variants of murine coronavirus JHM. Virology 1991; 182: 883–8.
18. Morris VL, Tieszer C, MacKinnon J, Percy D. Characterization of coronavirus JHM variants isolated from wistar furth rats with a viral-induced demyelinating disease. Virology 1989; 169: 127–36.
19. Parker SE, Gallagher TM, Buchmeier MJ. Sequence analysis reveals extensive polymorphism and evidence of deletions within the E2 glycoprotein gene of several strains of murine hepatitis virus. Virology 1989; 173: 664–73.
20. Perlman S, Schelper R, Bolger E, Ries D. Late onset, symptomatic encephalomyelitis in mice infected with MHV-JHM in the presence of maternal antibody. Microb Pathogen 1987; 2: 185–94.
21. Delmas B, Laude H. Assembly of coronavirus spike protein into trimers and its role in epitope expression. J Virol 1990; 64: 5367–75.
22. Tooze SA, Tooze J, Warren G. Site of addition of N-acetyl-galactosamine to the E1 glycoprotein of mouse hepatitis virus-A59. J Cell Biol 1988; 106: 1475–87.
23. Tooze J, Tooze SA, Fuller SD. Sorting of progeny coronavirus from condensed secretory proteins at the exit from the trans-Golgi network of AtT20 cells. J Cell Biol 1987; 105: 1215–26.
24. Vennema H, Heijnen L, Zijderveld A, Horzinek MC, Spaan WJM. Intracellular transport of recombinant coronavirus spike proteins: implications of virus assembly. J Virol 1990; 64: 339–46.
25. Frana MF, Behnke JN, Sturman LS, Holmes KV. Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: host-dependent differences in proteolytic cleavage and cell fusion. J Virol 1985; 56: 912–20.
26. Makino S, Fleming JO, Keck JG, Stohlman SA, Lai MC. RNA recombination of coronaviruses: localization of neutralizing epitopes and neuropathogenic determinants on the carboxyl terminus of peplomers. Proc Natl Acad Sci USA 1987; 84: 6567–71.
27. Routledge E, Stauber R, Pfleiderer M, Siddell SG. Analysis of murine coronavirus surface glycoprotein functions by using monoclonal antibodies. J Virol 1991; 65: 254–62.
28. Sturman LS, Ricard CS, Holmes KV. Conformational change of the coronavirus peplomer glycoprotein at pH 8.0 and 37°C correlates with virus aggregation and virus-induced cell fusion. J Virol 1990; 64: 3042–50.
29. Weismiller DG, Sturman LS, Buchmeier MJ, Fleming JO, Holmes KV. Monoclonal antibodies to the peplomer glycoprotein of coronavirus mouse hepatitis virus identify two subunits and detect a conformational change in the subunit released under mild alkaline conditions. J Virol 1990; 64: 3051–5.
30. Takase-Yoden S, Kikuchi T, Siddell SG, Taguchi F. Localization of major neutralizing epitopes on the S1 polypeptide of the murine coronavirus peplomer glycoprotein. Virus Res 1990; 18: 99–108.
31. Wang F-I, Fleming JO, Lai MC. Sequence analysis of the spike protein gene of murine coronavirus variants: study of genetic sites affecting neuropathogenicity. Virology 1992; 186: 742–9.
32. Gallagher TM, Parker SE, Buchmeier MJ. Neutralization-resistant variants of a neurotropic coronavirus are generated within the amino-terminal half of the spike glycoprotein. J Virol 1990; 64: 731–41.
33. Williams RK, Jiang G-S, Holmes KV. Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. Proc Natl Acad Sci USA 1991; 88: 5533–6.
34. Dveksler GS, Pensiero MN, Cardellichio CB *et al.* Cloning of the mouse hepatitis (MHV) receptor: expression in human and hamster cell lines confers susceptibility to MHV. J Virol 1991; 65: 6881–91.
35. Sears AE, McGuire BS, Roizman B. Infection of polarized MDCK cells with herpes simplex virus 1: two asymmetrically distributed cell receptors interact with different viral proteins. Proc Natl Acad Sci USA 1991; 88: 5087–91.

36. Shevach EM. The effects of cyclosporin on the immune system. Ann Rev Immunol 1985; 3: 397–423.
37. Emmel EA, Verweij CL, Durand DB, Higgins KM, Lacy E, Crabtree GR. Cyclosporin A specifically inhibits the function of nuclear proteins involved in T cell activation. Science 1989; 246: 1617–20.
38. Stohlman SA, Matsushima GK, Casteel N, Weiner LP. *In vivo* effects of coronavirus-specific T cell clones: DTH inducer cells prevent a lethal infection but do not inhibit virus replication. J Immunol 1986; 136: 3052–6.
39. Stohlman SA, Sussman MA, Matsushima GK, Shubin RA, Erlich SS. Delayed-type hypersensitivity response in the central nervous system during JHM virus infection requires viral specificity for protection. J Neuroimmunol 1988; 19: 255–68.
40. Sussman MA, Shubin RA, Kyuwa S, Stohlman SA. T-cell-mediated clearance of mouse hepatitis virus strain JHM from the central nervous system. J Virol 1989; 63: 3051–6.
41. Williamson JSP, Stohlman SA. Effective clearance of mouse hepatitis virus from the central nervous system requires both $CD4^+$ and $CD8^+$ T cells. J Virol 1990; 64: 4589–92.
42. Williamson JSP, Sykes KC, Stohlman SA. Characterization of brain-infiltrating mononuclear cells during infection with mouse hepatitis virus strain JHM. J Neuroimmunol 1991; 32: 199–207.
43. Yamaguchi K, Goto N, Kyuwa S, Hayami M, Toyoda Y. Protection of mice from a lethal coronavirus infection in the central nervous system by adoptive transfer of virus-specific T cell clones. J Neuroimmunol 1991; 32: 1–9.
44. Beushausen S, Dales S. *In vivo* and *in vitro* models of demyelinating disease. XI. Tropism and differentiation regulate the infectious process of coronavirus in primary explants of the rat CNS. Virology 1985; 141: 89–101.
45. Sorensen O, Dugre R, Percy D, Dales S. *In vivo* and *in vitro* models of demyelinating disease: endogenous factors influencing demyelinating disease caused by mouse hepatitis virus in rats and mice. Infect Immun 1982; 37: 1248–60.
46. Wilson GAR, Beushausen S, Dales S. *In vivo* and *in vitro* models of demyelinating diseases. XV. Differentiation influences the regulation of coronavirus infection in primary explants of mouse CNS. Virology 1986; 151: 253–64.
47. Paeratakul U, De Stasio PR, Taylor MW. A fast and sensitive method for detecting specific viral RNA in mammalian cells. J Virol 1988; 62: 1132–5.
48. Budzilowicz CJ, Wilczynski SP, Weiss SR. Three intergenic regions of coronavirus mouse hepatitis virus strain A59 genome RNA contain a common nucleotide sequence that is homologous to the 3′ end of the viral mRNA leader sequence. J Virol 1985; 53: 834–40.