



# Dissociation of demyelination and viral clearance in congenitally immunodeficient mice infected with murine coronavirus JHM

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Infection of rodents with murine coronavirus JHM results in a subacute or chronic demyelinating disease which serves as a model for the human disease multiple sclerosis. Previous studies with JHMV have established a role for the immune system in both viral clearance and demyelination. To further clarify the role of the immune system in JHMV pathogenesis, several strains of congenitally immunodeficient mice were studied. Infection of immunocompetent C57BL/6 mice with JHMV resulted in severe paralysis and demyelination and complete clearance of infectious virus from the brain (C<sup>+</sup>D<sup>+</sup> phenotype). In contrast, infected SCID mice showed little or no paralysis or demyelination and were unable to clear infectious virus (C<sup>-</sup>D<sup>-</sup> phenotype). Athymic nude mice and a proportion of mice lacking MHC Class I or II expression exhibited robust demyelination but did not completely clear infectious virus from the brain (C<sup>-</sup>D<sup>+</sup> phenotype). These results are consistent with an immune-mediated mechanism for JHMV-induced demyelination, but indicate that the immune mechanisms which participate in demyelination and viral clearance are distinct. It may thus be possible to experimentally alter immunopathological responses without impairing antimicrobial immunity.

**Keywords:** multiple sclerosis; immunopathology; mouse hepatitis virus

## Introduction

Murine coronaviruses (mouse hepatitis virus or MHV) are enveloped positive-stranded RNA viruses with a variety of tissue tropisms. JHM virus (JHMV, MHV-4) is a neurotropic murine coronavirus. Depending on experimental conditions, JHMV and related coronaviruses produce a number of neurological diseases in rodents, including acute encephalitis, subacute and chronic demyelination and persistent, inapparent infection (Compton *et al*, 1993; Kyuwa and Stohlman, 1990; Lavi and Weiss, 1989). These diseases share many characteristics with human diseases and therefore have been used as experimental models of human conditions. JHMV infection of mice has frequently been studied as a model of the human disease multiple sclerosis (Dal Canto, 1990; Dal Canto and Rabinowitz, 1981; Fazakerley and Buchmeier, 1993; Martin and Nathanson, 1979).

The immune response to JHMV plays a critical

role in the associated pathogenesis. Central nervous system (CNS) infection with JHMV results in intense infiltration of tissues by lymphocytes and macrophages (Dörries *et al*, 1991; Wang *et al*, 1992b; Williamson *et al*, 1991). Furthermore, cellular and humoral immune responses influence the outcome of JHMV infection. Virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, as well as antiviral antibodies, can each protect mice from a lethal challenge with JHMV (Buchmeier *et al*, 1984; Fleming *et al*, 1989; Jacobsen and Perlman, 1990; Perlman *et al*, 1987; Stohlman *et al*, 1986, 1988, 1995; Yamaguchi *et al*, 1991; Yokomori *et al*, 1992). Both CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes, however, are required for effective clearance of infectious virus from the CNS (Pearce *et al*, 1994; Sussman *et al*, 1989; Williamson and Stohlman, 1990). In contrast to these protective mechanisms, recent evidence points to an immunopathological mechanism for primary demyelination induced by JHMV. Immunosuppressive doses of irradiation up to 6 days after JHMV inoculation can prevent the onset of demyelination in mice, and adoptive transfer of JHMV-immune splenocytes restores demyelination to infected irradiated recipients (Wang *et al*, 1990). In addition, Thy-1<sup>+</sup> cells

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are essential for restoration of demyelination, suggesting a role for T lymphocytes in demyelination (Fleming *et al*, 1993). An immunopathological mechanism for JHMV-induced demyelination has also been demonstrated in rats (Schwender *et al*, 1994). Despite these reports, however, the precise role of the immune system in demyelination caused by JHMV and the relationship between viral clearance and demyelination remain poorly understood.

To further clarify the role of the immune system in JHMV pathogenesis, we performed a systematic evaluation of both viral clearance and demyelination in several strains of congenitally immunodeficient mice infected with JHMV. Nude mice are athymic and thus lack conventional, thymically educated T lymphocytes. They do, however, possess B lymphocytes, natural killer (NK) cells, and cells of the monocyte/macrophage line, as well as a population of extrathymically educated T lymphocytes (Kennedy *et al*, 1992; Kindred, 1981; Rocha *et al*, 1992). Mice with the severe combined immunodeficiency (SCID) mutation possess NK cells and monocytes/macrophages, but lack functional B and T lymphocytes because they are unable to rearrange the genes for immunoglobulins and T cell receptors (Dorshkind *et al*, 1984; Kirchgessner *et al*, 1995). Mice in which the  $\beta_2$ -microglobulin gene has been disrupted by homologous recombination in embryonic stem cells ( $\beta_2m^{-/-}$ ) lack stable cell surface expression of MHC class I molecules and are deficient in CD8<sup>+</sup> T lymphocytes (Raulet, 1994). Mice of the  $A_\beta^{-/-}$  strain lack the I-E gene and have had the I-A gene disrupted by homologous recombination in embryonic stem cells. These mice lack conventional expression of MHC class II molecules and are deficient in CD4<sup>+</sup> T lymphocytes (Cardell *et al*, 1994).

Congenitally immunodeficient mice were infected with a strain of JHMV which consistently causes robust demyelination with little clinical encephalitis in immunocompetent mice. The results reported here are consistent with an immune-mediated mechanism for demyelination. Surprisingly, however, we found a dissociation between clearance of infectious virus and the induction of subacute demyelinating immunopathology in different mouse strains. These findings indicate that distinct immune mechanisms may be responsible for viral clearance and demyelination in this model system. This dissociation of immunopathology and antimicrobial immunity may have implications for the treatment of immunopathological disorders.

## Results

Mice were infected with  $10^3$  plaque-forming units (PFU), of variant 2.2-V-1 of JHMV (Fleming *et al*, 1986). Since previous studies have shown that mice which will undergo demyelination will have done so by 10–12 days PI (Fleming *et al*, 1986; 1993), and since SCID mice began to show signs of severe encephalitis by 12–14 days PI, all mice were sacrificed at 12 days PI. Mortality in immunodeficient mice was no higher than that in immunocompetent mice during the first 12 days PI. Clinical signs of encephalitis were observed in 1 of 10 immunocompetent C57BL/6 mice, in 2 of 9 nude mice and in 5 of 12 SCID mice by 12 days PI. Clinical encephalitis was not observed in  $\beta_2m^{-/-}$  or  $A_\beta^{-/-}$  mice or in adoptive transfer recipients. On sacrifice, there was no evidence of hepatitis or other systemic disease.

**Table 1** Serum immunoglobulins and anti-JHMV antibody responses in immunodeficient and immunocompetent C57BL/6 mice infected with JHMV

Group	Experiment <sup>a</sup>	n	Serum immunoglobulins <sup>b</sup>		Antiviral Antibody <sup>c</sup>	
			IgM	IgG	IgM	IgG
1	C57BL/6	10	97 ± 37	3422 ± 1680	299 ± 257	340 ± 219
2	Nude	9	131 ± 41	117 ± 69	151 ± 60	— <sup>d</sup>
3	SCID	12	—	—	—	—
4	$\beta_2m^{-/-}$	9	455 ± 203	1573 ± 937	380 ± 148	252 ± 192
5	$A_\beta^{-/-}$	7	169 ± 60	309 ± 168	177 ± 62	—
6	C57BL/6 to SCID <sup>e</sup>	4	24 ± 7	42 ± 20	69 ± 25	—
7	Nude to SCID <sup>e</sup>	11	15 ± 5	9 ± 12	43 ± 33	—

<sup>a</sup>Mice were infected i.c. with  $10^3$  PFU of JHMV variant 2.2-V-1

<sup>b</sup>Immunoglobulins are expressed as  $\mu$ g/ml (mean ± SD) and were determined on serum taken 12 days PI by capture ELISA as described in Materials and methods

<sup>c</sup>Antiviral antibodies were determined on serum taken 12 days PI by ELISA as described in Materials and methods and are expressed as endpoint dilutions (mean ± SD) corresponding to an absorbance of 0.25

<sup>d</sup>Not detectable

<sup>e</sup>Adoptive transfers of immune splenocytes from immunocompetent C57BL/6 (group 6) or nude mice (group 7) to infected SCID mice were performed as described in Materials and methods

### Serology

At 12 days PI, all SCID mice showed a complete lack of serum IgG and IgM and produced no detectable antiviral antibodies (Table 1). All nude and  $A_{\beta}^{-/-}$  were deficient in serum IgG and produced only IgM in response to virus.  $\beta_2m^{-/-}$  mice had somewhat reduced levels of serum IgG, but produced antiviral IgG and IgM antibodies comparable to C57BL/6 controls. Thus, serum immunoglobulin levels in these mice were consistent with published findings, and antiviral responses of these mice were comparable to those reported for other viral systems (Bloemmen and Eyssen, 1973; Bosma *et al*, 1983; Burns *et al*, 1975; Cardell *et al*, 1994; Cosgrove *et al*, 1991; Klein-Schneegans *et al*, 1990; Lehmann-Grube *et al*, 1993; Nonoyama *et al*, 1993; Raulet, 1994; Spriggs *et al*, 1992).

### Viral clearance

Whereas immunocompetent C57BL/6 mice were able to completely clear infectious virus from the brain by day 12 PI, both nude and SCID mice had high titers of infectious virus remaining in the brain at day 12 (Table 2).  $\beta_2m^{-/-}$  and  $A_{\beta}^{-/-}$  mice had moderate titers of infectious virus in the brain by day 12, indicating an impaired ability to clear virus.

### Disease

JHMV produced marked paralysis in immunocompetent C57BL/6 and nude mice (Table 2), and robust demyelination was demonstrable in the spinal cords of these mice (Figures 1A, 1C). In marked contrast, SCID mice showed only minimal effects, with a single animal out of 12 showing severe paralysis and demyelination (Table 2). With the exception of this mouse, no demyelination was evident in SCID mouse spinal cords (Figure 1E). The

paralyzed mouse had no detectable serum immunoglobulins or antiviral antibody (Table 1), and mice at this young age (6 weeks) are typically not 'leaky' (Nonoyama *et al*, 1993; Bosma *et al*, 1988). Analysis of SCID mice also showed that histopathological scores were a more reliable indication of disease than clinical observations, possibly because of confounding features such as the high viral load, severe encephalitis, and non-specific weakness of these animals, which rarely survived beyond 14–18 days PI (data not shown).

Responses in  $\beta_2m^{-/-}$  and  $A_{\beta}^{-/-}$  mice were quite variable; demyelination was seen in about half of these mice (4 of 8  $\beta_2m^{-/-}$  and 3 of 7  $A_{\beta}^{-/-}$ ), with the other half showing little or no demyelination (Table 2, Figures 1G and 1I). As a result of this bimodal distribution, there was no statistically significant difference between histopathological scores of these mice and immunocompetent C57BL/6 controls, despite numerical reduction in histopathological scores and statistically significant reduction in clinical scores. The presence or absence of demyelination in individual mice did not, however, correlate with serum immunoglobulin levels or antiviral antibody titers (data not shown). The bimodal distribution of demyelination in  $\beta_2m^{-/-}$  and  $A_{\beta}^{-/-}$  mice may be due to genetic heterogeneity in these mice (Cosgrove *et al*, 1991; Koller and Smithies, 1989), and suggests that genes other than MHC Class I and II may play a role in demyelination.

### Dissociation of viral clearance and demyelination

The relationship between viral clearance and demyelination can be assessed by examining these parameters in individual mice. We sought to determine if individual mice of a given strain would

**Table 2** Clinical and histopathological scores and virus isolation for immunodeficient and immunocompetent C57BL/6 mice infected with JHMV

Group	Experiment <sup>a</sup>	Number examined clinically <sup>b</sup>	Clinical score <sup>c</sup>	Number examined histologically <sup>b</sup>	Histological score <sup>c</sup>	Viral titer <sup>d</sup>
1	C57BL/6 <sup>d</sup>	10	3.7 ± 0.4	8	3.2 ± 1.4	<2.0
2	Nude	9	3.5 ± 0.9	7	3.2 ± 1.3	4.9 ± 0.8
3	SCID	12	2.2 ± 1.0**	10	1.4 ± 1.2**	5.7 ± 0.6
4	$\beta_2m^{-/-}$	9	2.7 ± 0.6**	8	2.1 ± 1.3	3.7 ± 1.7
5	$A_{\beta}^{-/-}$	7	2.6 ± 1.1**	7	1.7 ± 1.9	3.6 ± 1.4
6	C57BL/6 to SCID <sup>e</sup>	4	3.5 ± 0.2**	4	3.3 ± 0.7**	<2.0
7	Nude to SCID <sup>e</sup>	11	2.6 ± 0.8	10	1.4 ± 1.3	5.3 ± 0.4

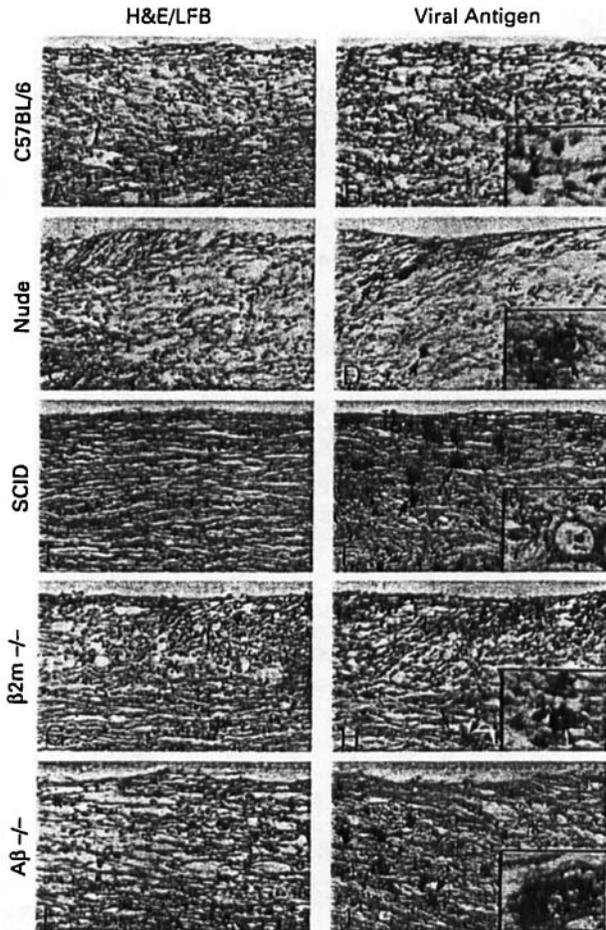
<sup>a</sup>Mice were infected i.c. with  $10^3$  PFU of JHMV variant 2.2-V-1. For statistical analysis, clinical and histological scores of immunodeficient mice (groups 2–5) were compared to those of immunocompetent C57BL/6 mice (group 1). \*\* $P < 0.05$

<sup>b</sup>Number of mice examined 12 days PI. For technical reasons, the number of mice examined clinically did not always equal the number examined histologically. The number of mice assayed for infectious virus in each group equalled the number examined histologically

<sup>c</sup>Mean ± SD. Scores were assigned on a scale from 0 to 4 on day 12 PI as described in Materials and methods

<sup>d</sup> $\log_{10}$  PFU/g of brain homogenate from mice sacrificed 12 days PI

<sup>e</sup>Adoptive transfers of immune splenocytes from immunocompetent C57BL/6 (group 6) or nude mice (group 7) to infected SCID mice were performed as described in Materials and methods. For statistical analysis, clinical and histological scores of SCID recipients (groups 6 and 7) were compared to those of infected SCID mice which did not receive splenocytes (group 3). \*\* $P < 0.05$



**Figure 1** Photomicrographs of spinal cords from mice inoculated i.c. with JHMV 2.2-V-1 and examined at day 12 PI. (A) H&E/LFB stained longitudinal section from an immunocompetent C57BL/6 mouse showing a focus of demyelination (\*) in the white matter ( $\times 100$ ). (B) Immunohistochemical stain for viral antigen of a section from an immunocompetent C57BL/6 mouse. Note the minimal staining ( $\times 100$ , inset  $\times 400$ ). (C) H&E/LFB stained section from a nude mouse showing a focus of demyelination (\*) in the white matter ( $\times 100$ ). (D) Immunohistochemical stain for viral antigen of a section from a nude mouse. Viral antigen can be detected in cells in normal white matter (arrows), but not within the lesion itself (\*) ( $\times 100$ ). At higher magnification, glial cells show a cytoplasmic pattern of staining for viral antigen ( $\times 400$ , H&E counterstain). (E) H&E/LFB stained section from a SCID mouse, showing normal white matter ( $\times 100$ ). (F) Immunohistochemical stain for viral antigen of a section from a SCID mouse. Note dense staining of glia in both the white and grey matter ( $\times 100$ ). Higher magnification (inset) shows staining of cells adjacent to neurons in the gray matter ( $\times 400$ , H&E counterstain). (G) H&E/LFB stained section from a  $\beta_2m^{-/-}$  mouse which underwent demyelination (\*) ( $\times 100$ ). (H) Immunohistochemical stain for viral antigen of a section from a  $\beta_2m^{-/-}$  mouse which underwent demyelination (\*). Viral antigen can be detected in the normal white matter (arrows) but not within the lesion ( $\times 100$ , H&E counterstain). Higher magnification (inset) shows cytoplasmic staining of infected cells (I) H&E/LFB stained section from an  $A\beta^{-/-}$  mouse which underwent demyelination (\*) ( $\times 100$ ). (J) Immunohistochemical stain for viral antigen of a section from an  $A\beta^{-/-}$  mouse which underwent demyelination (\*), showing staining (arrows) only in white matter outside of the lesion ( $\times 100$ ). Higher magnification (inset) reveals viral antigen in infected cells. ( $\times 400$ , H&E counterstain).

respond to JHMV by successfully clearing infectious virus ( $C^+$  phenotype) and/or by demonstrating paralysis and demyelination ( $D^+$  phenotype). In confirmation of previous studies (Wang *et al*, 1990; Fleming *et al*, 1987, 1993), we found that immunocompetent C57BL/6 mice consistently cleared virus and experienced demyelination, with 6 of 8 mice showing the  $C^+ D^+$  phenotype. The phenotypes of individual mice are depicted graphically in Figure 2A; most C57BL/6 mice fall within the lower right quadrant, indicating that they exhibited both complete viral clearance and demyelination. In contrast, the most severely immunodeficient animals, that is, SCID mice, consistently demonstrated the  $C^- D^-$  phenotype, and points representing these mice fall into the upper left quadrant of Figure 2A.

Dissociation of clearance and demyelination was evident in nude,  $\beta_2m^{-/-}$  and  $A\beta^{-/-}$  mice. Six of 7 nude mice showed a  $C^- D^+$  phenotype, as represented graphically in the upper right quadrant of Figure 2A. Of the  $\beta_2m^{-/-}$  and  $A\beta^{-/-}$  mice, about half of each group demonstrated the  $C^- D^+$  phenotype (Figure 2B). The  $C^- D^+$  phenotype seen in nude mice and approximately half of  $\beta_2m^{-/-}$  and  $A\beta^{-/-}$  mice indicates that clearance and demyelination could be dissociated in individual animals, and were not necessarily linked, as they were in immunocompetent C57BL/6 mice with the  $C^+ D^+$  phenotype or SCID mice with the  $C^- D^-$  phenotype.

#### Immunohistochemistry

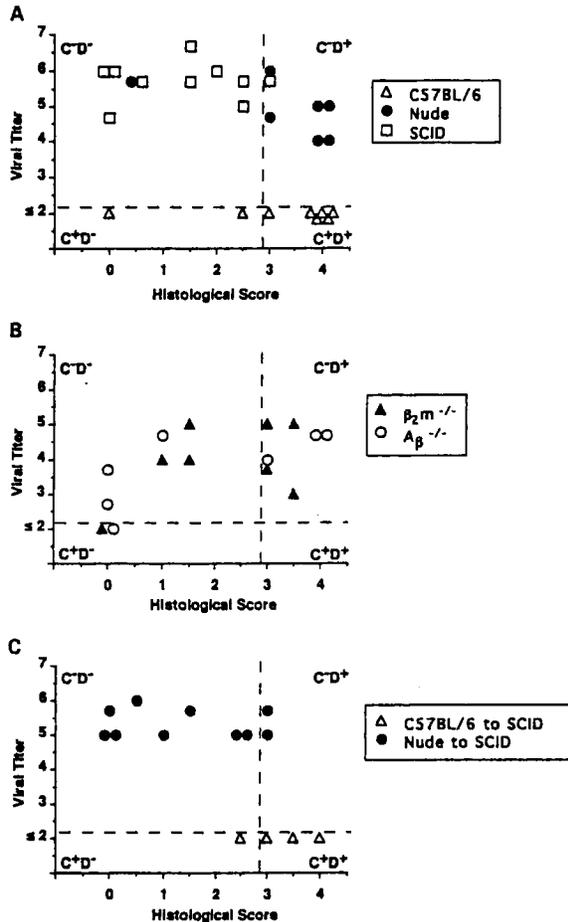
Cells positive for viral antigen were observed infrequently in the white matter of immunocompetent C57BL/6J mice at 12 days PI (Figure 1B). In contrast, antigen-positive cells were more numerous in the CNS of all immunodeficient mice, with positive cells frequently observed in the gray matter of SCID mice (Figure 1F), corresponding with increased incidence of clinical encephalitis in these mice. In nude,  $\beta_2m^{-/-}$  and  $A\beta^{-/-}$  mice which underwent demyelination but did not completely clear virus ( $C^- D^+$ ), viral antigen-positive cells were dense in the normal white matter, but the lesions themselves were free of viral antigen (Figure 1D, 1H, 1J). Immunohistochemical staining for the T-200 antigen revealed dense infiltration of cells of bone marrow origin in demyelinated lesions with sparser staining in the unaffected white matter. Numerous cells staining positive for the T-200 antigen were also seen in perivascular areas of the white matter (data not shown).

#### Adoptive transfers

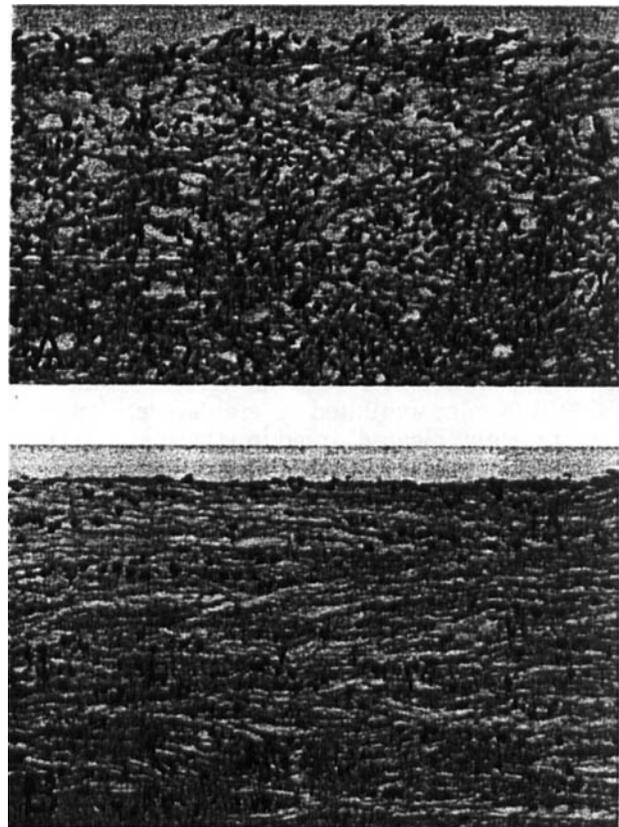
The above results suggested that a cell population present in nude and immunocompetent C57BL/6 mice but absent in SCID mice was essential for JHMV-induced demyelination. Therefore, immune splenocytes from nude and immunocompetent C57BL/6 mice were transferred into infected SCID

mice. Three of 4 SCID recipients of donor cells from immunocompetent mice developed clinical disease and demyelination comparable to that seen in immunocompetent C57BL/6 controls and were able to completely clear infectious virus from the brain (Table 2, Figure 3). Thus, adoptive transfer of

immune splenocytes from immunocompetent C57BL/6 mice converted SCID mice from a C<sup>-</sup> D<sup>-</sup> to a C<sup>+</sup> D<sup>+</sup> phenotype (Figure 2C). In contrast, SCID recipients of nude mouse splenocytes showed only a small, statistically insignificant increase in demyelination, compared to SCID mice which did not receive immune splenocytes. Unlike SCID recipients of splenocytes from immunocompetent mice, SCID recipients of splenocytes from nude mice were unable to completely clear infectious virus by 12 days PI. Thus, 8 of 10 SCID recipients of immune splenocytes from nude mice retained their C<sup>-</sup> D<sup>-</sup> phenotypes (Figure 2c). Serum immunoglobulin and antiviral IgG levels in SCID recipients of splenocytes from both nude and immunocompetent C57BL/6 mice were minimal, and antiviral IgG was undetectable in both groups (Table 1). The unexpected inability of immune splenocytes from nude mice to transfer demyelination (despite the marked demyelination observed in infected nude



**Figure 2** Dissociation of viral clearance and demyelination in immunodeficient strains of mice. Viral titers ( $\log_{10}$  PFU/g) at 12 days PI are depicted on the vertical axes with horizontal dashed lines indicating the lower detection limit of the assay ( $10^2$  PFU/g). Histological scores at 12 days PI as described in Materials and methods are depicted on the horizontal axes, with vertical dashed lines representing the threshold for marked demyelination (as evidenced by loss of LFB staining). Thus, points which fall within the upper left quadrant indicate those mice which did not undergo demyelination and were unable to completely clear infectious virus (C<sup>-</sup> D<sup>-</sup>), points which fall within the upper right quadrant indicate those mice which underwent demyelination and did not clear infectious virus (C<sup>-</sup> D<sup>+</sup>), points which fall within the lower left quadrant indicate those mice which did not undergo demyelination but completely cleared infectious virus (C<sup>+</sup> D<sup>-</sup>), and points within the lower right quadrant indicate those mice which underwent demyelination and completely cleared infectious virus (C<sup>+</sup> D<sup>+</sup>). (A) Scores for individual immunocompetent C57BL/6, nude and SCID mice. (B) Scores for individual  $\beta_2m^{-/-}$  and  $A\beta^{-/-}$  mice. (C) Scores for individual SCID recipients of immune splenocytes from nude or immunocompetent C57BL/6 mice.



**Figure 3** Adoptive transfer of demyelination into infected SCID mice by immune splenocytes from immunocompetent C57BL/6 mice, but not from nude mice. (A) H&E/LFB stained section from a SCID mouse which received splenocytes from immunocompetent C57BL/6 mice shows demyelination (\*)( $\times 100$ ). (B) H&E/LFB stained section from a SCID mouse which received splenocytes from nude mice shows no demyelination ( $\times 100$ ).

mice) may be due to insufficient cell numbers, differences in homing patterns or interactions within the CNS microenvironment of recipient mice.

## Discussion

Previous studies have shown that the activity of the immune system is a critical determinant of JHMV pathogenesis; in fact, there is evidence that some outcomes of JHMV infection, such as paralysis and demyelination, may be immunopathologically mediated (Wang *et al*, 1990; Fleming *et al*, 1993; Schwender *et al*, 1994). Despite intensive investigations, however, our understanding of the relationship between viral clearance and demyelination in the immune response to JHMV remains incomplete in many key respects. In order to better understand the role of the immune system in this model of CNS disease, we employed a genetic approach, in which mice with a variety of congenital immunodeficiencies were infected with JHMV and monitored for both viral clearance and demyelination. These findings have provided further support for an immune-mediated mechanism for demyelination and confirm the necessity of both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes for effective viral clearance from the CNS (Pearce *et al*, 1994; Sussman *et al*, 1989; Williamson and Stohlman, 1990; Wang *et al*, 1990; Fleming *et al*, 1993). Moreover, these observations provide evidence for a dissociation of immune mechanisms in JHMV pathogenesis; those elements which are necessary for immune-mediated demyelination are distinct from the CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes which are essential for viral clearance from the CNS.

In the work reported here, 6 of 8 immunocompetent C57BL/6 mice exhibited severe demyelination and completely cleared infectious virus (C<sup>+</sup> D<sup>+</sup> phenotype). This is consistent with previous reports (Wang *et al*, 1990; Fleming *et al*, 1993). In contrast, SCID mice showed minimal demyelination and failed to clear virus (C<sup>-</sup> D<sup>-</sup> phenotype). Published studies have shown that irradiated mice, like SCID mice, show a C<sup>-</sup> D<sup>-</sup> phenotype (Wang *et al*, 1990; Fleming *et al*, 1993). These findings support immune mediated mechanisms for both viral clearance and demyelination, since neither occurred in these severely immunodeficient mice.

By examining both demyelination and viral clearance in nude mice, we provided dramatic evidence of dissociation of viral clearance and demyelination, with 6 of 7 nude mice exhibiting a C<sup>-</sup> D<sup>+</sup> phenotype. Although our report is the first to address both parameters, our results are consistent with prior reports which indicate that nude mice are unable to completely clear MHV variants V5A13.1 or OB V60 from the CNS (Pearce *et al*, 1994; Fazakerley *et al*, 1992) and that nude mice and rats

inoculated with JHMV develop demyelination (Sorensen *et al*, 1982; 1987). Except for a finding of increased levels of viral RNA in paralyzed nude rats compared to heterozygotes (Sorensen *et al*, 1987), these earlier studies did not assess viral clearance and demyelination simultaneously, and thus could not demonstrate dissociation of these phenomena. Our findings of striking dissociation of viral clearance and demyelination in nude mice suggests that the elements of the immune system required for demyelination are distinct from those required for viral clearance. Furthermore, nude mice must by inference possess those components of the immune system required for demyelination, but lack those required for viral clearance.

Dissociation of viral clearance and demyelination was also observed in approximately half of the JHMV-infected  $\beta_2m^{-/-}$  and  $A\beta^{-/-}$  mice, which showed a C<sup>-</sup> D<sup>+</sup> phenotype. Although these mice were able to limit viral replication to some extent, they were unable to completely clear infectious virus (Table 2). These findings are consistent with studies which show that  $\beta_2m^{-/-}$  mice infected with the A59 strain of MHV can undergo demyelination and show delayed clearance of MHV strain A59 (Gombold *et al*, 1995). Since both CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes are required for effective clearance of MHV from the CNS (Pearce *et al*, 1994; Sussman *et al*, 1989; Williamson and Stohlman, 1990), intermediate viral titers in  $\beta_2m^{-/-}$  and  $A\beta^{-/-}$  mice may reflect the presence of one of the two cell populations required for optimal viral clearance.

This is the first study in which demyelination and viral clearance have been systematically examined in a panel of mice with defined deficiencies in cellular immunity. Published reports, however, support our findings and provide additional evidence for dissociation of viral clearance and demyelination in JHMV-infected mice. Mice protected from lethal encephalitis by passive immunization with anti-JHMV monoclonal antibodies go on to develop robust demyelination, whether or not a reduction in viral replication is demonstrated (Buchmeier *et al*, 1984; Fleming *et al*, 1989; Yokomori *et al*, 1992). In addition, mice protected from lethal JHMV challenge by transfer of cytotoxic T lymphocyte clones show a reduction in viral titer, but do not show chronic demyelination (Stohlman *et al*, 1995). Conversely, suckling mice protected from fatal JHMV encephalitis by maternal antibodies can go on to develop demyelination, and infectious virus can be isolated from these paralyzed mice as late as 60 days PI (Perlman *et al*, 1987).

Previously published findings of demyelination in immunodeficient or immunosuppressed rodents have been interpreted as evidence against an immune-mediated mechanism for demyelination (Sorensen *et al*, 1982, 1987; Weiner, 1973; Zimmer and Dales, 1989). The findings presented here, however, illustrate the complexity of the immune

system and the importance of considering those components which remain functional in partially immunodeficient mice as possible mediators of immunopathology. In this regard, the demyelination exhibited by nude,  $\beta_2m^{-/-}$ , and  $A_\beta^{-/-}$  mice in the present study appears to be immune-mediated because all mice with a  $D^+$  phenotype, regardless of level of immunocompetence, showed similar kinetics of JHMV-induced paralysis (data not shown), with histological findings of inflammatory infiltrates of hematogenous cells and a lack of viral antigen within demyelinated lesions. In addition, SCID mice and irradiated mice (Wang *et al*, 1990; Fleming *et al*, 1993) are more profoundly immunodeficient than nude,  $\beta_2m^{-/-}$  and  $A_\beta^{-/-}$  mice and allowed comparable or greater viral replication in the CNS, yet underwent little or no demyelination, suggesting that an immune component crucial for demyelination is absent in SCID and irradiated mice. Thus the present study supports the immunopathological model for demyelination even in partially immunodeficient mice. Furthermore, the immune-mediated demyelination observed in immunocompetent, nude,  $\beta_2m^{-/-}$  and  $A_\beta^{-/-}$  mice appears to be mediated by some component common to these mice.

Previous adoptive transfer studies have shown that a population of cells essential for JHMV-induced demyelination bear the Thy-1 marker, and thus are presumably T lymphocytes (Fleming *et al*, 1993). Paradoxically, in the present study most of the nude mice and half of the  $\beta_2m^{-/-}$  and  $A_\beta^{-/-}$  mice exhibited severe demyelination. Since nude mice lack thymically educated T lymphocytes, and  $\beta_2m^{-/-}$  and  $A_\beta^{-/-}$  mice are deficient in  $CD8^+$  and  $CD4^+$  T lymphocytes respectively, this suggests that conventional thymically educated T cells are not essential for demyelination. Common immunopathogenic components, thus, may include unconventional T lymphocytes (eg,  $\gamma\delta$  T lymphocytes), elements of innate immunity (eg, NK cells or macrophages), B lymphocytes or cytokines. Alternatively, compensatory immune mechanisms may perform the required functions for demyelination in the immunodeficient mice. If this were the case, however, dissociation would still be evident, since these compensatory mechanisms would be unable to perform the viral clearance functions of  $CD4^+$  and  $CD8^+$  T lymphocytes. Demyelination is most likely a complex phenomenon involving multiple, and possibly redundant cell types and cytokines. While  $CD4^+$  and  $CD8^+$  T lymphocytes may influence the process or may participate when they are present, the above results indicate that they are not indispensable elements. Furthermore, expression of MHC class I and II molecules does not appear to be essential for JHMV-induced demyelination, since these molecules are not expressed stably in  $\beta_2m^{-/-}$  and  $A_\beta^{-/-}$  mice, respectively.

In conclusion, we have shown that nude,  $\beta_2m^{-/-}$  and  $A_\beta^{-/-}$  mice, like immunocompetent mice, could undergo severe demyelination when infected with JHMV. In contrast, SCID mice, like irradiated mice (Wang *et al*, 1990; Fleming *et al*, 1993), showed minimal demyelination. These findings support an immune-mediated mechanism for demyelination and suggest that an immune component present in immunocompetent, nude,  $\beta_2m^{-/-}$  and  $A_\beta^{-/-}$  mice, but deficient in SCID and irradiated mice, is essential for JHMV-induced demyelination. Furthermore, this as yet unidentified immunopathological component appears to be distinct from the  $CD4^+$  and  $CD8^+$  T lymphocytes which are required for viral clearance. Our findings that viral clearance and demyelinating immunopathology may be dissociated have implications for the treatment of this and other immunopathological disorders, raising the possibility of selective suppression of immunopathological responses while leaving antimicrobial immunity intact.

## Materials and methods

### Mice

Male C57BL/6J, C57BL/6J-*nu* (nude) and C57BL/6J-*scid*/SzJ (SCID) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).  $A_\beta^{-/-}$  mice were developed by Dr Diane Mathis (Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Strasbourg, France) (Cosgrove *et al*, 1991) and kindly provided by Drs Robert Auerbach (Department of Zoology, University of Wisconsin-Madison) and William P Weidanz (Department of Medical Microbiology and Immunology, University of Wisconsin-Madison).  $\beta_2M^{-/-}$  mice were generously provided by Dr Daniel Muller (Department of Medicine, University of Wisconsin-Madison) (Koller and Smithies, 1989). Selected mice were tested prior to inoculation and found to be negative for serum antibodies to MHV. Mice were inoculated at 5–7 weeks of age. Mice were housed in microisolator cages, handled in a biosafety cabinet, and provided with autoclaved feed, water, and bedding.

### Virus

The JHMV antigenic variant 2.2-V-1 has been described previously (Fleming *et al*, 1986). This virus has a point mutation in the spike gene (Wang *et al*, 1992a) and produces demyelination with little clinical encephalitis (Fleming *et al*, 1987). Mice were infected with  $10^3$  PFU of virus in 30  $\mu$ l of DMEM by the intracerebral (i.c.) route. Donor mice for adoptive transfers were immunized with  $10^6$  PFU i.p. 6 days prior to transfer.

### Animal evaluations

Mice were monitored for signs of paralytic disease until 12 days PI, using a scale from 0 (normal) to 4 (paraplegia) (Fleming *et al*, 1993). At 12 days PI,

mice were bled and sacrificed. Brains were assayed in duplicate for infectious virus on DBT cells (Stohlman *et al*, 1986). Pilot studies on split samples showed no difference in viral titers between tissue which was assayed immediately and that which was frozen at  $-70^{\circ}\text{C}$  for assay at a later date (data not shown). Thereafter, assays were performed on either fresh or previously frozen tissues.

Spinal cords were fixed in Clarke's solution and embedded in paraffin for histopathological evaluation (Wang *et al*, 1992b). A combined hematoxylin and eosin/luxol fast blue stain (H&E/LFB) was used to stain myelin. Histopathological scores were assigned on a scale modified from that of Wang *et al*. (1992b). A score of 0 indicated normal appearance. Scores of 1 and 2 corresponded to mild or moderate inflammation, respectively, without disruption of white matter architecture and with relative preservation of luxol fast blue staining. A score of 3 indicated one or two foci of intense hypercellularity and white matter rarefaction with loss of luxol fast blue staining, and thus a loss of myelin. A score of 4 indicated numerous (at least three) or confluent areas of such pathology. Previous studies have shown that this type of lesion with loss of luxol fast blue staining at the light microscopic level correlates with primary demyelination at the ultrastructural level (Wang *et al*, 1992b; Fleming *et al*, 1986). Scores were assigned by two independent observers without knowledge of the experimental groups. In cases of one-point discrepancy ( $n=14/55$  observations), the mean of the two scores was used. In cases of two-point discrepancy ( $n=4/55$  observations), the sections were re-evaluated without revealing experimental groups, and mean values of the new scores were used. Statistically significant differences between experimental groups were determined for clinical and histopathological scores using the two-tailed Mann-Whitney test for nonparametric samples (Statsoft Statistical Programs, Tulsa OK).

Viral antigen and inflammatory cells infiltrating the spinal cord were stained by immunohistochemistry with an avidin-biotin immunoperoxidase procedure (Vectastain, Vector Laboratories, Burlingame, CA). Viral antigen was detected using a monoclonal antibody (J.3.3) directed at the JHMV nucleocapsid protein (Wang *et al*, 1990). Infiltrating inflammatory cells were detected using a monoclonal antibody directed against the murine leukocyte common antigen (CD45 or T-200; hybridoma

M1/HL.2; TIB 122 American Type Culture Collection, Rockville, MD). Hybridoma culture supernatants were used at 1:100.

#### Serology

Anti-JHMV IgM and IgG antibody titers were determined by ELISA as previously described (Fleming *et al*, 1993). Results are expressed as endpoint dilutions corresponding to an absorbance of 0.25 and were determined by extrapolation from two sets of duplicate dilutions (usually  $10^{-1}$  and  $10^{-2}$ ). Serum IgM and IgG levels were determined by ELISA (Fleming and Pen, 1988) using an affinity-purified goat anti-mouse IgA + IgG + IgM (H + L) antibody as a capture antibody and horseradish peroxidase-conjugated goat anti-mouse IgM ( $\mu$ ) or IgG ( $\gamma$ ) antibody for detection (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Mouse myeloma IgG2b, $\kappa$  (MOPC 195) and IgM, $\kappa$  (ABPC 22) were used as immunoglobulin standards (Sigma Chemical Company, St. Louis, MO). The absorbance values of duplicate tenfold dilutions of serum were compared to a standard curve on each plate and immunoglobulin concentrations were calculated by linear regression. A dilution of  $10^{-3}$  was used to calculate the immunoglobulin concentration, unless its absorbance did not fall within the linear range of the standard curve. If this occurred, the closest dilution to  $10^{-3}$  to fall within the linear range was used.

#### Adoptive transfers

Donor nude or immunocompetent C57BL/6 mice were immunized i.p. 6 days prior to transfer with  $10^6$  PFU of 2.2-V-1. Recipient SCID mice were infected i.c. 3 days prior to transfer with  $10^3$  PFU of 2.2-V-1. Donor splenocytes were purified by passage over Histopaque 1083 (Sigma) and  $4 \times 10^6$  cells in DMEM were transferred i.v.

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