Short Communication

In Vivo Expression of Major Histocompatibility Complex Molecules on Oligodendrocytes and Neurons during Viral Infection

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Demyelination in multiple sclerosis and in animal models is associated with infiltrating CD8+ and CD4+ T cells. Although oligodendrocytes and axons are damaged in these diseases, the roles T cells play in the demyelination process are not completely understood. Antigen-specific CD8+ T cell lysis of target cells is dependent on interactions between the T cell receptor and major histocompatibility complex (MHC) class I-peptide complexes on the target cell. In the normal central nervous system, expression of MHC molecules is very low but often increases during inflammation. We set out to precisely define which central nervous system cells express MHC molecules *in vivo* during infection with a strain of murine hepatitis virus that causes a chronic, inflammatory demyelinating disease. Using double immunofluorescence labeling, we show that during acute infection with murine hepatitis virus, MHC class I is expressed *in vivo* by oligodendrocytes, neurons, microglia, and endothelia, and MHC class II is expressed only by microglia. These data indicate that oligodendrocytes and neurons have the potential to present antigen to T cells and thus be damaged by direct antigen-specific interactions with CD8+ T lymphocytes. (*Am J Pathol* 2001, 159:1219–1224)

Cell death associated with a local inflammatory response within the central nervous system (CNS) occurs in many neurological diseases, including multiple sclerosis (MS) and progressive multifocal leukoencephalopathy (PML) caused by JC virus.^{1–7} Identifying which cell types express MHC, and therefore which cells can directly interact with and be damaged by T cells, is crucial to understanding the pathogenesis of neurological diseases involving inflammation in animal models and in humans.

In animal models of virus-induced demyelination, oligodendrocyte and neuronal loss occur following CNS infection by murine hepatitis virus (MHV) and Theiler's murine encephalomyelitis virus (TMEV).^{2,8} There is evidence that T cells contribute to cell death, leading to increased tissue damage and clinical impairment.^{3,9,10} Specific mechanisms of cell death associated with inflammation are not completely understood and need to be further explored. One possible mechanism is T cell lysis of target cells via interactions between the CD8+ T cell receptor and a MHC class I peptide complex on the target cell.^{11,12}

Antigen-specific T cell lysis of target cells is MHC restricted, and dependent on expression of MHC on the target cell. In most of the normal CNS, MHC expression at the protein level does not occur or is below detection levels. During an inflammatory response or following viral infection, MHC expression in the CNS can be detected. However reports of specific cell types within the CNS that express MHC have often been conflicting, and the number of studies using double labeling techniques to clearly identify cell types expressing MHC *in vivo* has been limited.⁴

In the present study, we used an established murine model of CNS inflammation and demyelination to determine definitively which cells of the CNS express MHC molecules during an acute viral infection. We used an attenuated variant of murine hepatitis virus (MHV)-JHM (MHV V5A13.1)⁵ that infects neurons and glial cells and induces chronic demyelination. Following an intracranial (i.c.) injection into mice, MHV V5A13.1 spreads through-

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out the brain and spinal cord causing demyelination and an inflammatory response.^{3,5,6} Infectious MHV V5A13.1 is cleared from the CNS of mice by 12 days post infection (dpi),^{5,6} although viral RNA persists.¹³ During acute demyelination and inflammation we identified the spinal cord cell types expressing MHC class I and class II molecules *in vivo* by using double label immunofluorescence imaged by confocal microscopy to colocalize MHC expression with specific cell type markers.

Materials and Methods

MHV Injections

Six- to eight-week-old BALB/c female and male mice were anesthetized with methoxyfluorane and intracranially injected with 30 μ l of phosphate-buffered saline (PBS) ($n = 8$) or 10–100 plaque forming units (PFU) of MHV V5A13.1. Following MHV infection, mice were perfused at 10 dpi ($n = 6$), 14 dpi ($n = 3$), 24 dpi ($n = 3$) and 49 dpi ($n = 5$).

Tissue Preparation

For all double immunolabeling studies, mice were anesthetized with chloral hydrate and transcardially perfused with saline, and spinal cords were removed and stored at -20°C . For immunostaining to identify demyelinated lesions with cyclic nucleotide phosphodiesterase (CNP), mice were anesthetized and transcardially perfused with saline followed by 4% paraformaldehyde.

Immunohistochemistry

Sagittal spinal cord sections were fixed in 95% ethanol at 4°C (15 minutes), blocked with avidin and biotin, and incubated with antibodies to CD4 (clone RM4-5 1:150, Pharmingen, San Diego, CA), CD8 (clone 53-6.7, 1:200 Pharmingen), MHC class I (clone ER-HR-52 1:5000; Bachem, Torrance, CA), or MHC class II (Clone ER-TR-3 1:200; Bachem) at 4°C overnight. Slides were then incubated with a biotinylated anti-rat secondary (Vector Labs, Burlingame, CA), followed by biotin/avidin/horseradish peroxidase (HRP) reagents (Vector Labs), and color was developed with diaminobenzidine (Zymed Labs, San Francisco, CA) for approximately 8 minutes. The reaction was stopped with water, the slides were counterstained with hematoxylin, and slides were mounted with Aquamount (Polysciences, Inc., Warrington, PA).

Immunofluorescence

To identify demyelinated lesions, coronal sections from paraformaldehyde perfused mice were pretreated with 100% methanol (15 minutes at -20°C), blocked with 25% donor calf serum, and incubated with anti-CNP 1:100 (clone 11-5B, Sigma, St. Louis, MO). Slides were then incubated with an anti-mouse IgG F(ab)₂ conjugated to fluorescein isothiocyanate (FITC) (1 hour at room temper-

ature) and mounted with Vectashield (Vector Labs, used in all immunofluorescence experiments). To identify viral antigen, sagittal spinal cord sections were blocked with donor calf serum, incubated with purified monoclonal antibody 4B62⁵ (that recognizes MHV nucleocapsid), rinsed, incubated with anti-mouse secondary antibody conjugated to FITC, rinsed, and mounted. For all double label experiments involving immunofluorescence labeling of MHC class I or class II and a second antigen, sections were serially stained with anti-MHC antibodies followed by antibodies to the second antigen. As a control, the primary antibodies were omitted to ensure fluorescence was specific for antibody binding. To verify specificity of the MHC class I antibody clone ER-HR-52, the antibody was incubated on spinal cords from $\beta_2\text{-microglobulin}$ knockout mice that lack cell surface MHC class I expression. No MHC class I expression was detected on the blood vessels of $\beta_2\text{m}^{-/-}$ mice, compared to clear detection of MHC class I staining on blood vessels from normal mouse spinal cord. For labeling of MHC antigens, slides were fixed in cold 95% ethanol for 15 minutes, blocked with serum/PBS/3% bovine serum albumin (BSA), and incubated with anti-MHC class I (clone ER-HR-52 1:2000) or MHC class II (clone M5/114, Boehringer Mannheim Biochemicals, Indianapolis, IN, or clone ER-TR-3, Bachem) overnight at 4°C . Slides were then incubated with an anti-rat secondary IgG F(ab)₂ antibody conjugated to a fluorochrome (FITC or Texas Red) absorbed for minimal cross-reactivity with other species (Jackson ImmunoResearch, West Grove, PA). For anti-MHC class I antibody clone M1/42 (data not shown, Boehringer Mannheim Biochemicals), slides were incubated with a biotinylated anti-mouse antibody, followed by avidin/biotin/HRP reagents (Vector Labs), followed by incubation with an anti-HRP antibody conjugated to either FITC or Texas Red (Jackson ImmunoResearch).

For double immunofluorescence of MHC antigens with CNP, slides were then fixed in 4% paraformaldehyde for 30 minutes followed by cold methanol for 15 minutes at -20°C . They were then blocked and incubated with anti-CNP as described above. Finally, they were incubated with a secondary anti-mouse antibody conjugated to FITC or Texas Red and mounted. For double immunofluorescence of MHC antigens with glial fibrillary acidic protein (GFAP), slides were then fixed in 4% paraformaldehyde and incubated with polyclonal rabbit anti-cow GFAP (1:100, DAKO, Carpinteria, CA) in PBS/3% BSA/0.1% Triton X-100 for 2 hours at room temperature. They were then incubated with an anti-rabbit secondary conjugated to FITC and mounted. For double immunofluorescence of MHC antigens with mac-1 (CD11b) after MHC staining, the slides were incubated with anti-mac-1 directly conjugated to FITC (Pharmingen) for 2 hours at room temperature, rinsed, and mounted. For double immunofluorescence of MHC antigens with CD31, slides were then fixed in 4% paraformaldehyde for 30 minutes at room temperature, and incubated with hamster anti-CD31 (1:100; Chemicon International, Temecula, CA) for 2 hours at room temperature. They were then incubated with an anti-hamster secondary conjugated to FITC (1:200; Jackson ImmunoResearch) and mounted. For dou-

Figure 1. MHV V5A13.1 infection induces an inflammatory response and demyelination in mouse spinal cord. Mouse spinal cords taken 10 to 14 days after intracranial infection with MHV have inflammation characterized by expression of MHC class I (**A1**), MHC class II (**A2**), and infiltration of CD8⁺ T cells (**A3**) compared to PBS-injected mouse spinal cords (**B1–B3**). In addition, MHV V5A13.1 induces demyelination in ventral-lateral white matter as early as 10 dpi indicated by reduced CNP stain and vacuolization seen in **A4**, compared to normal white matter seen in PBS injected mice (**B4**). Viral antigen can be detected at 10 dpi in spinal cord white matter (**C1**) and gray matter (**C2**). Original magnifications: **A1–A3**, **B1–B3**, 125 $\times$; **A4**, **B4**, 500 $\times$; scale bar in **C1**, **C2**, 50 μ m.

ble immunofluorescence of MHC antigens with neuron specific enolase (NSE), slides were then fixed in 4% paraformaldehyde for 30 minutes at room temperature, incubated with polyclonal anti-mouse NSE (1:50; Polysciences, Warrington, PA) overnight at 4°C, and incubated with a biotinylated anti-mouse antibody (1:400). They were then incubated with an avidin/biotin/HRP complex (Vector Labs) followed by an anti-HRP conjugated to FITC (1:400, Jackson ImmunoResearch) and mounted.

Confocal Microscopy

Single plane confocal microscope images were collected sequentially using a Bio-Rad MRC-1024 unit attached to a Zeiss Axiovert S100TV microscope with 40 $\times$ or 63 $\times$ objective lenses. A krypton/argon mixed gas laser produced excitation wavelengths at 488 nm and 568 nm for FITC or Texas Red, respectively. In some cases, z-series images from 3 to 5 planes 0.2 μ m apart were collected for reconstruction. Individual fluorophore images were merged and pseudocolored using Adobe Photoshop (version 5.5).

Results

In agreement with previous reports,^{3,13} in spinal cords we identified increased expression of MHC class I and class II molecules in association with infiltrating T lymphocytes and demyelinated lesions as early as 10–14 days dpi (Figure 1, panels A1–A4). CD8⁺ T cells (Figure 1, panel A3) as well as CD4⁺ T cells (not shown) were present. Virally-infected cells were identified in areas of inflammation by immunohistochemical staining with an antibody to the MHV nucleoprotein (Figure 1, panels C1 and C2). In sham-infected mice, infiltrating lymphocytes and demyelination were not detected, and MHC class I was never detected in the parenchyma, but could be detected on some vessels (Figure 1, panel B1). At 10 dpi, MHC class

I was clearly expressed on many cells identified as oligodendrocytes by staining with an antibody to the oligodendrocyte/myelin-specific protein 2'3'-CNP (Figure 2, rows A and B). MHC class I was detected around the cell body of oligodendrocytes as well as along myelinated processes. Colocalization of MHC class I with CNP was confirmed with a second anti-MHC class I antibody (clone M1–42, not shown). At 3–7 weeks postinfection, MHC class I expression by oligodendrocytes was much less common than during the acute viral infection (not shown). This corresponds to a well-documented period in which remyelination is observed during MHV infection.⁵ Although MHC class II was also expressed in white matter, its expression was distinct from cell bodies and processes labeled with CNP (Figure 3, panels A1–A3).

At 10 dpi, we detected rare spinal cord neurons that clearly expressed MHC class I *in vivo* by double labeling with antibodies to MHC class I and the neuron-specific protein (NSE) (Figure 2, rows C and D). The neurons expressing MHC class I were medium or small, and not the much larger α motor neurons in the ventral horn. No neurons in sham infected mice expressed MHC class I. In contrast to the findings with MHC class I, NSE positive neurons were not found to express MHC class II (data not shown).

To identify astrocytes, sections were stained with a polyclonal antibody to the astrocyte-specific protein, GFAP. Unlike oligodendrocytes and neurons, astrocytes did not express MHC molecules during MHV V5A13.1-induced demyelination (Figure 2, panels E1–E3; Figure 3, panels B1–B3). Figures 2 and 3 show staining of astrocytic processes with GFAP that do not colocalize with MHC. Astrocyte cell bodies also did not costain with MHC class I or II (not shown).

Microglia/macrophage lineage cells were identified with an antibody to CD11b, and most expressed MHC class I following MHV infection (Figure 2, panels F1–F3). CD11b-positive cells expressing MHC class I had a ram-

Figure 2. MHC class I expression in mouse spinal cords following MHV V5A13.1 infection. Double immunofluorescence imaged with confocal microscopy was used to identify cells expressing MHC class I (shown in red) and cell type specific markers (green). Colocalization of MHC class I and cell type markers is shown as yellow in the merged panels in the third column. Within or near the edge of a demyelinated lesion at 10–14 dpi, oligodendrocytes (**A1**) and myelinated processes (**B1**) labeled with CNP express MHC class I (**A2**, **B2**), indicated by yellow structures in **A3** and **B3**. In **B3**, the **arrow** indicates a yellow process colocalizing CNP and MHC class I. The **arrowhead** indicates a green process labeled with CNP only. Spinal cord neurons labeled with NSE (**C1**, **D1**) also expressed MHC class I (**C2**, **D2**), indicated by the yellow cell in **C3** and two yellow cells in **D3**. Astrocytes labeled with GFAP (**E1**) did not express MHC class I (**E2**; merge in **E3**). Microglia expressed MHC class I as indicated by yellow colocalization (**arrow** in **F3**) of Mac-1 (**F1**) and MHC class I (**F2**). The **arrowhead** in **F3** indicates a blood vessel labeled with MHC class I. MHC class I expression by endothelial cells was confirmed by colocalization of CD31 (**G1**) and MHC class I (**G2**) shown with **arrow** in **G3**. Perivascular cells were often seen expressing MHC class I (**arrowhead** in **G3**). Rows **A** and **F** are three-dimensional reconstructions from 3 to 4 planes 0.2 μm apart. Scale bars, 10 μm .

ified (or resting) morphology (Figure 2, panels F1, F3) and a reactive morphology with shorter, broader processes. Microglia/macrophage lineage cells expressing CD11b also expressed MHC class II following MHV infection (Figure 3, panels C1–C3), and in fact were the only resident cell type in the spinal cord to express MHC class II. Reactive microglia/macrophages with short broad processes expressed MHC class II, while ramified or resting

Figure 3. MHC class II expression in mouse spinal cords following MHV V5A13.1 infection. Double immunofluorescence imaged with confocal microscopy was used to identify cells expressing MHC class II (red) and cell type specific markers (green) at 10 to 14 days after i.c. infection with MHV. Colocalization of MHC class II and cell type markers is shown as yellow in the merged panels in the third column. CNP staining (**A1**) was distinct from MHC class II staining (**A2**). The **arrow** in **A3** indicates a myelinated process near a non-oligodendrocyte lineage cell expressing MHC class II but not CNP (**arrowhead**). Astrocyte processes (**B1**) did not express MHC class II (**B2**) indicated by lack of colocalization of GFAP (**arrow** in **B3**) with MHC class II (**arrowhead** in **B3**). Microglia/macrophage lineage cells labeled with Mac-1 (**C1**) clearly expressed MHC class II (**C2**) as indicated by yellow colocalization seen in **C3**. Endothelial cells expressing CD31 (**D1**) did not express MHC class II (**D2**), however perivascular cells were often seen that did express MHC class II (**arrowhead** in **D3**) adjacent to CD31 expressing endothelial cells (**arrow** in **D3**). All panels are single plane images. Scale bars, 10 μm .

microglia/macrophages typically did not express MHC class II. MHC class II-positive/CD11b-positive cells could be detected throughout white matter and gray matter, but were most abundant in scattered focal regions in white matter.

Vascular endothelial cells expressed MHC class I at low levels in sham-infected mice, and at much higher levels following MHV infection (Figure 2, panels G1–G3). Endothelial cells did not express MHC class II (Figure 3, panels D1–D3), although MHC class II expressing cells were often adjacent to endothelial cells in the perivascular region (as in Figure 3, panel D3). These cells were probably perivascular microglia, infiltrating macrophages, and/or pericytes.

Discussion

In these studies we have demonstrated that acute infection with MHV-induced MHC class I expression on oligodendrocytes, neurons, microglia, and endothelia. Since MHV is known to infect all of these cell types,¹⁴ and

CD8+ T cells have been implicated in viral clearance, one mechanism of cell damage may be by virus-specific CD8+ T cell lysis. MHC class I was observed on oligodendrocyte cell bodies and processes around demyelinated lesions 10–14 days post infection. There are several reports of MHC class I and class II expression by oligodendrocytes *in vitro* in response to viral infection or inflammatory cytokines.^{15–17} However, there are very few reports of MHC expression by oligodendrocytes *in vivo*. MHC class II was detected by immunoelectron microscopy during Theiler's virus infection,¹⁸ and MHC class I and class II were induced on oligodendrocytes by the transgenic expression of IFN γ as identified by double immunofluorescence labeling.¹⁹ Detection of MHC class I expression by oligodendrocytes in MHV infection provides direct evidence that oligodendrocytes are capable of presenting antigen to CD8+ T cells *in vivo*.

Neurons and the complex pattern of connections each neuron maintains are extremely difficult or impossible to replace. To prevent cytotoxic T lymphocyte (CTL)-mediated killing, neurons tightly regulate the peptide presentation process. For example, neurons have been shown to express only very low levels of the class I heavy chain and the peptide transporters TAP1 and TAP2.²⁰ MHC class I expression following MHV infection was previously observed in neuron-rich gray matter regions,²¹ although a neuronal phenotype was not confirmed. There are currently very few reports of neurons that express MHC class I in an adult rodent CNS identified by *in situ* double labeling as shown here. Interestingly, recent reports have identified MHC class I expression in a subset of neurons in the developing and mature cat and rodent CNS.^{22,23} This expression appears to be required for activity dependent remodeling and synaptic plasticity, thus indicating that MHC molecules have functions that extend beyond their roles in immune responses.

In vitro studies have demonstrated that electrically silent neurons treated with IFN γ express MHC class I proteins on the cell surface.^{24–26} MHV infection of neurons *in vivo* may disrupt normal neuronal electrical functions. IFN γ secreted by infiltrating T cells could then induce MHC class I expression, leading to recognition and lysis by virus-specific CTL. Neurons that were double labeled with MHC class I and NSE in MHV-infected mice were rare. However a low number was anticipated since it was likely that only damaged neurons would express MHC class I. Triple immunofluorescence labeling studies to identify MHV-infected neurons expressing MHC class I were done, but these technically difficult experiments were not successful since the viral antigen staining pattern was often observed as small blebs that resembled dead or dying cells. Although *in vitro* and *in vivo* evidence for cell surface expression of MHC class I has been reported,^{22–25,27} our experiments do not specifically localize MHC class I to the neuronal cell surface since it is very difficult to distinguish surface staining from cytoplasmic staining in tissue sections. MHC class I staining did not appear to be restricted to the endoplasmic reticulum, but was found throughout the neuronal cytoplasm although it did not extend into axons or dendrites.

Since no MHC proteins were detected on astrocytes at any time point examined, the role of astrocytes *in vivo* in the inflammatory response following MHV V5A13.1 does not appear to involve antigen presentation. This lack of MHC expression by astrocytes in an inflamed CNS is consistent with other *in vivo* observations during MHV infection²⁸ and with our own studies of CNS inflammation in transgenic mice¹⁹ (J Redwine, L Shriver, and C Evans, manuscript in preparation). *In vitro*, MHC class I and class II expression can be induced on astrocytes in response to interferon- γ or viral infection.^{29,30} However, MHC expression by astrocytes *in vitro* can be inhibited by neuronal contact or brain-derived gangliosides,^{31,32} so these factors may actively suppress MHC expression by astrocytes *in vivo*.

Microglia expressed MHC class I and were the only cell type found to express MHC class II at all time points examined following MHV infection. Therefore these cells are the only cell type capable of presenting antigen to both CD8+ and CD4+ T cells. Resident microglia and infiltrating macrophages likely play major roles in regulating chronic inflammation by presenting antigen and expressing chemokines that attract T cells and/or macrophages.^{3,13} Microglia can also express B7 costimulatory molecules^{33,34} which increases the ability of these cells to activate T cells.

CNS endothelial cells were found to express MHC class I but not MHC class II following MHV infection. Although some studies of different models have reported MHC class II expression on endothelial cells,³⁵ others have reported MHC class II expression on perivascular cells but not endothelial cells.³⁶ The disparate findings may be due to the different models studied, or to the resolution of the various detection methods. Vessel endothelial cells and perivascular cells are likely important in antigen presentation to lymphocytes being recruited into the CNS. In addition, endothelial cells express the receptor for MHV,^{37,38} so they may play a role in virus dissemination throughout the CNS.

Clearly, the mechanisms of MHV-induced pathology are multifactorial, since some demyelination can occur even in the absence of functional MHC class I or CD8+ T cells.³⁹ We found that at early time points post-MHV infection, oligodendrocytes expressed MHC class I, but expression decreased during the chronic phase of disease. Initial damage to CNS cells by virus-specific CD8+ T cells and by lytic viral infection may initiate a cascade of immune responses resulting in chronic demyelination that would not require continuing MHC class I expression. For example, macrophages may subsequently present viral antigen to CD4+ T cells in the context of MHC class II, thus continuing a local cycle of cytokine and chemokine production, T cell activation, and tissue damage.³ In light of these findings with MHV, it is possible that in humans, a CNS viral infection could result in the up-regulation of MHC class I molecules on oligodendrocytes or neurons during acute disease, thus rendering them susceptible to attack by CD8+ T cells. Chronic disease could then result due to a cascade of immune responses involving mechanisms such as epitope spreading.⁴⁰ Our findings of *in vivo* expression of MHC class I by oligodendrocytes and neurons are novel and have broad implications for the pathogenesis of neurological diseases that involve CD8+ T cell infiltration, such as MS.

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