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Viruses can silently prime for and trigger central nervous system autoimmune disease

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Although many viruses have been isolated from patients with multiple sclerosis (MS), as yet, no one agent has been demonstrated to cause MS. In contrast, epidemiological data indicate that viral infections are associated with exacerbations of MS. Here, we present data showing that virus infections can subclinically prime animals for central nervous system (CNS) autoimmune disease; long after the original infection has been eradicated, a nonspecific challenge/infection can trigger an exacerbation. The priming infectious agent must show molecular mimicry with self-CNS antigens such as glial fibrillary acidic protein (GFAP), myelin associated glycoprotein (MAG) or myelin proteolipid protein (PLP). The subsequent challenge, however, may be nonspecific; complete Freund's adjuvant (CFA), or infection with a recombinant vaccinia virus encoding an irrelevant protein, could trigger CNS disease. In the CNS, we could detect a mononuclear cell infiltration, but no demyelination was found. However, if the pathogenesis of MS is similar to that of this novel animal model for CNS autoimmune disease, our findings could help explain why exacerbations of MS are often associated with a variety of different viral infections. *Journal of NeuroVirology* (2001) **7,** 220–227.

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Introduction

Multiple sclerosis (MS), thought to be an autoimmune disease of the central nervous system (CNS), affects approximately 1/1000 individuals in the United States (Anderson *et al*, 1992). Despite extensive investigation, the etiology and pathogenesis of multiple sclerosis (MS) are still not known. However, many features important for MS have begun to be elucidated. These include: genetics, environmental factors, and infections playing important roles in susceptibility and triggering or exacerbating the disease.

Individuals of Northern European descent have a higher incidence of MS for at least two reasons. First, genetic susceptibility; these people have a higher prevalence of certain histocompatibility antigens (HLA) – HLA3, B7, and especially DR15, Dw2, and –DQw6 (Weinshenker *et al*, 1998). Second, exposure to an unidentified environmental factor at a young age (Martin, 1997; Kalman and Lublin, 1999); migration studies performed by Dean and Kurtzke (Dean and Kurtzke, 1971) and Alter (Alter *et al*, 1971) have shown that individuals living in a high-risk area who migrate to a low-risk area after the age of 15 retain the high risk for developing MS associated with their original geographic location. In contrast, individuals who migrate prior to the age of 15 acquire the risk of the geographical region into which they have moved. These data have been interpreted to indicate that the types of infections in areas of high risk for MS (Kurtzke, 1993) as well as possibly in low-risk regions for MS affect the likelihood of developing disease (Alter *et al*, 1986).

From studies over the past 50 years, no 1 virus or microbe has been identified as a causative agent for MS. However, several reports indicate that viral or bacterial infections can exacerbate MS (Sibley *et al*, 1985; Edwards *et al*, 1998; James, 1988; Andersen *et al*, 1993; Morrison and Nelson, 1994;

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Panitch, 1994; Rapp *et al*, 1995; Metz *et al*, 1998). In this study, we provide evidence that certain kinds of infection can prime animals for autoimmune CNS disease, and at a later time infection or other nonspecific immunologic stimuli can trigger an attack. We based our model on SJL/J mice, which are highly susceptible to experimental allergic encephalomyelitis (EAE), an animal model for MS (Livingstone *et al*, 1995; Encinas *et al*, 1996; Butterfield *et al*, 1998; Butterfield *et al*, 1999; Charles *et al*, 1999; Teuscher *et al*, 1999). Because the "priming" event for MS takes place before 15 years of age, we primed our mice at 3–4 weeks of age (just prior to puberty); furthermore, because the incidence of MS is higher in women (Smith *et al*, 1999), we used only female mice (Baker *et al*, 1995).

Although, as in many other possible autoimmune diseases, the autoantigens responsible for MS have not been identified, several candidate antigens have been proposed. We selected 3 candidates as potential autoantigens: glial fibrillary acidic protein (GFAP), myelin associated glycoprotein (MAG), and myelin proteolipid protein (PLP) (Yoshimura *et al*, 1985; Sobel *et al*, 1986). Adoptive transfer of T cells specific for GFAP and MAG has been reported to cause CNS disease (Berger *et al*, 1997; Weerth *et al*, 1999), whereas PLP is widely used for induction of EAE in animals (Tsunoda and Fujinami, 1996), and PLP reactive cells have been found in increased numbers in MS patients (Trotter *et al*, 1998; Illés *et al*, 1999). In addition, we previously proposed that molecular mimicry was a viable mechanism for the development of autoimmune CNS disease (Fujinami and Oldstone, 1985). Here, we hypothesized that infection of young females by viruses or microbes having immunologically cross-reactive epitopes could prime for autoimmune disease, and that overt disease would be revealed following a subsequent nonspecific immunostimulation. To mirror a viral infection having molecular mimicry, we employed a cDNA encoding PLP fused to ubiquitin (pUPLP); ubiquitin has been demonstrated to target proteins into proteasomes, allowing degradation and presentation mostly through the major histocompatibility complex (MHC) class I pathway, which in turn enhances the induction of $CD8^+$ T cells (Rodriguez *et al*, 1998). This scenario would approximate what could occur during virus infection having molecular mimicry, when viral proteins are synthesized within the cytoplasm of the infected cell. As an alternative priming method, we used recombinant vaccinia virus encoding CNS proteins.

Materials and Methods

Mice and induction of disease

Three-to 4-week-old female SJL/J and PL/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were injected intramuscularly (i.m.) 3 times at 1-week intervals with 100 μg plasmid, and DNA was dissolved in 1 N saline. On each occasion, 50 μg was injected into each anterior tibial muscle with a 28-gauge needle. Mice were observed and weighed every second day. One week after the last injection, groups of mice received either (i) PBS emulsified in complete Freund's adjuvant (CFA), (ii) 100 nmol of modified $PLP_{139-151}$ (HSLGKWLGHPDKF) emulsified in CFA (Barnett *et al*, 1993), (iii) PBS alone subcutaneously (s.c.), at the base of the tail or (iv) 5×10^6 PFU intraperitoneal (i.p.) of VV_{SC11}, a recombinant vaccinia virus encoding β-galactosidase (Barnett *et al*, 1993). Control mice received no DNA injection, and were "challenged" with CFA. Mice were observed and weighed daily.

In other experiments mice were primed with recombinant vaccinia viruses encoding PLP (VV_{PLP}) (Barnett *et al*, 1993), MAG (VV_{MAG}), or GFAP (VV_{GFAP}) [5×10^6 PFU i.p. of recombinant vaccinia virus], and 5 weeks later were challenged with CFA with or without *Bordetella pertussis* (BP). Recombinant viruses were constructed as described in Barnett et al (Barnett *et al*, 1993).

Clinical signs of EAE were assessed according to the following criteria: 0=no clinical disease; 1=loss of tail tonicity; 2=mild hind leg paresis; 3=moderate hind leg paralysis; 4=complete paraplegia; and 5=quadriplegia, moribund or death.

Plasmid constructs

The PLP gene was fused to ubiquitin-A76 and subcloned into the *Not* I site of plasmid pCMV, which was derived by excision of the β-galactosidase gene from pCMVβ (Clontech, Palo Alto, CA) (Tsunoda *et al*, 1999). The resultant expression vector (pUPLP) contained the widely expressed immediate-early promoter from cytomegalovirus. A plasmid encoding PLP without ubiquitin ($pPLP_{ALL}$) (Tsunoda *et al*, 1998) was used as a control. Plasmid DNA was purified from transformed DH5α *Escherichia coli* with an EndoFree Plasmid Maxi Kit (Qiagen, Chatsworth, CA).

Proliferation assays

The regional lymph nodes and spleens from mice of each group were removed and pooled, and a single cell suspension was prepared. A volume of 0.2 ml containing 2×10^5 cells in RPMI supplemented with 1% glutamine, 1% penicillin/streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal bovine serum was added to each well and modified $PLP_{139-151}$, $PLP_{178-191}$, or MOG_{92-106} (Amor *et al*, 1994) peptides, which are encephalitogenic in SJL/J mice, were added at 50 μg/ml. The cells were cultured 72 h, each well was pulsed with 1 μCi of tritium thymidine, and cells were cultured another 24 h. Cultures were harvested onto filters using a multiwell cell harvester and counted using standard liquid scintillation techniques. All assays were performed in triplicate.

Histology and immunohistochemistry
Histology and immunohistochemistry were performed as described in Tsunoda *et al* (Tsunoda *et al*, 2000). Slides were coded and read blind.

Results

In humans, infections prior to the age of 15 as well as genetic background are believed to contribute to MS. To investigate whether infections early in life in genetically susceptible individuals could prime for autoimmune disease, we inoculated pUPLP 3 times into young SJL/J mice, which are susceptible to PLP-induced EAE. pUPLP encodes ubiquitin in frame with PLP, leading to enhanced degradation through the proteasome pathway. Because intracellular virus infection leads to presentation of fragments of viral protein by MHC class I molecules (Whitton and Oldstone, 1996), our new model mimics infection with a virus that has molecular mimicry with CNS antigens, but that does not cause cytopathic effects in the host.

One week after the last pUPLP injection, the mice were challenged with a nonspecific stimulus (CFA), which has long been used as an adjuvant in many autoimmune disease models such as EAE (Morgan, 1946; Morgan, 1947; Kabat *et al*, 1946; Kabat *et al*, 1947; Freund *et al*, 1947; Kopeloff and Kopeloff, 1947; Morrison, 1947). Ten days after the CFA administration, all animals (5/5) that had been injected with pUPLP showed weight loss, hypomotility and very mild hind limb weakness (Figure 1). Two control groups of mice were followed in parallel.

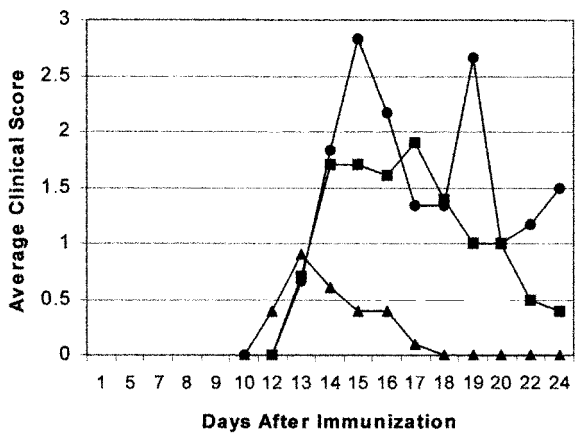

Figure 1 After pUPLP injection, mice were challenged either with CFA (▲) or with $PLP_{139–151}$/CFA (■) on day 0. Control EAE was induced in mice with $PLP_{139–151}$/CFA without pUPLP injection (●). Data are mean clinical scores of 5 mice. Mice were followed for clinical signs of disease according to the following scale: 0 – no clinical disease; 0.5 – loss of tonicity of the distal half of the tail; 1 – complete loss of tail tonicity; 2 – mild hind leg paresis; 3 – moderate hind leg paralysis; and 4 – complete paraplegia. None of the negative control mice that received either pUPLP alone or CFA without pUPLP showed clinical signs.

Table 1 Neuropathology in mice primed with cDNA constructs, followed by CFA challenge[a]

Plasmid[b]	*CFA*	*Pathology/total mice*[c]
pUPLP	+	4/20
pUPLP	–	0/10
—	+	0/10
$pPLP_{ALL}$	+	0/10

[a]SJL/J mice were primed with cDNA encoding PLP 3 times followed by a challenge of CFA.
[b]pUPLP encodes PLP fused with ubiquitin; $pPLP_{ALL}$ encodes PLP alone (Tsunoda *et al*, 1998).
[c]Number of mice that have CNS lesions/total number of mice examined.

One group received PBS instead of the plasmid pUPLP and were sensitized with $PLP_{139–151}$ emulsified in CFA (a stimulus known to induce EAE in SJL/J mice). The other group was injected with pUPLP and then subsequently injected with $PLP_{139–151}$ also emulsified in CFA. As expected, animals in both control groups also developed clinical EAE (Figure 1). Clinical signs did not develop in any mice in 2 negative control groups; the first group had been primed with pUPLP but received no subsequent immunostimulatory challenge; and the second group received CFA alone, without prior priming.

An additional experiment was performed with 4 groups of mice: (i) 20 mice were primed 3 times with pUPLP and challenged with CFA, (ii) 10 mice were primed 3 times with $pPLP_{ALL}$ (PLP construct not encoding ubiquitin) and challenged with CFA, (iii) 10 mice were primed with pUPLP, but no CFA challenge was carried out, and (iv) 10 mice were challenged with CFA, without prior priming (Table 1). A total of 20 mice injected with pUPLP and challenged with CFA were examined for pathologic changes in the CNS. In 4 of these mice, mononuclear cells (MNC) cell infiltrates were found in the leptomeninges (Figure 2a); the lymphocytes present in the perivascular space were identified as being mainly T cells when stained with an anti-CD3 antibody (Figure 2a insert). Although no demyelination was present, occasional areas in the white matter had an edematous appearance. This is similar to the pathology found in acute EAE, which often shows inflammation and edema, but no demyelination. In several models of acute EAE, edema with inflammation, but not demyelination, is believed to cause clinical signs (Levine *et al*, 1966; Simmons *et al*, 1982; Kerlero de Rosbo *et al*, 1985). No lesions or cellular infiltrates were found in mice (30 total) in any of the other 3 groups. The absence of clinical or histological signs in the second group of mice ($pPLP_{ALL}$ priming, CFA challenge) suggests that the initial priming event requires effective MHC class I presentation of the mimicking antigen. MNC from mice injected with pUPLP and further challenged with $PLP_{139–151}$ peptide, when restimulated *in vitro* with the same peptide, had a

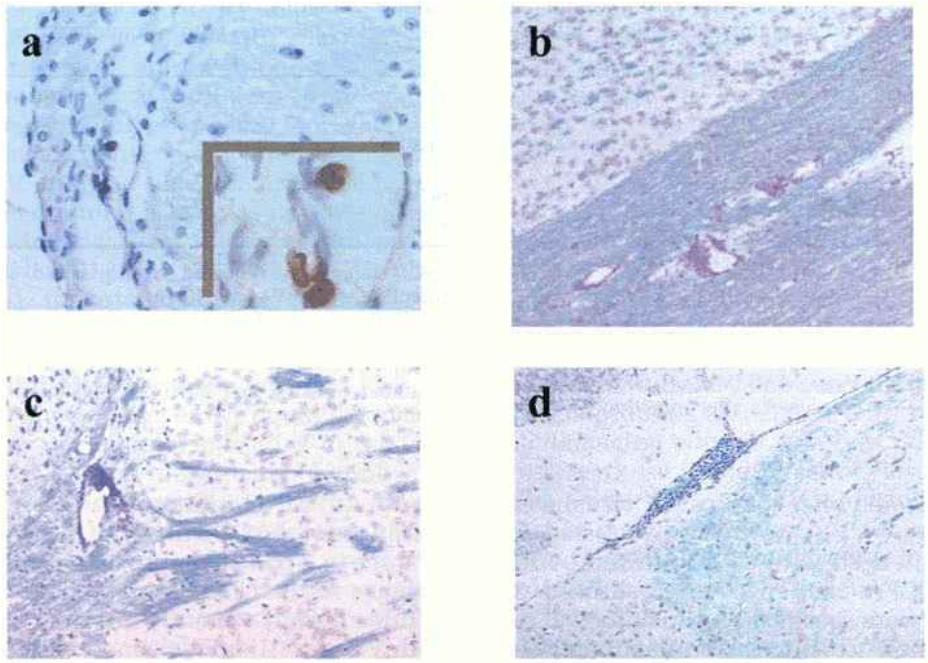

Figure 2 Histopathological changes in the CNS. **a**) Brain section from a mouse primed with pUPLP and challenged with CFA. Mild infiltration of the leptomeninges. $CD3^+$ T cells around the vessel (insert). Immunohistochemistry was performed using anti-CD3 antibody. **b**) Spinal cord section from a mouse infected with VV_{MAG} and immunized with CFA. Perivascular cuffing in the white matter of the lumbar spinal cord. **c**) Brain section from a mouse infected with VV_{GFAP} and challenged with CFA. Perivascular cuff in the white matter. **d**) Brain section from a mouse primed with pUPLP and infected with VV_{SC11}. MNC infiltration in the leptomeninges. **b–d**) Luxol fast blue staining that stains myelin.

lower proliferative response compared to the MNC from EAE mice. The proliferative responses of MNC to PLP, from mice vaccinated with pUPLP and challenged with CFA, were higher than the ones seen in the vaccinated group challenged with CFA/PLP. Lymphocytes from mice vaccinated with pUPLP readily proliferated in response to $PLP_{139–151}$ on days 12 (Figure 3) and 24 post-CFA challenge (not shown).

We next determined whether pUPLP priming could be replaced by priming with a virus having molecular mimicry with self-CNS antigens (Table 2). The 3 recombinant vaccinia viruses used are described in

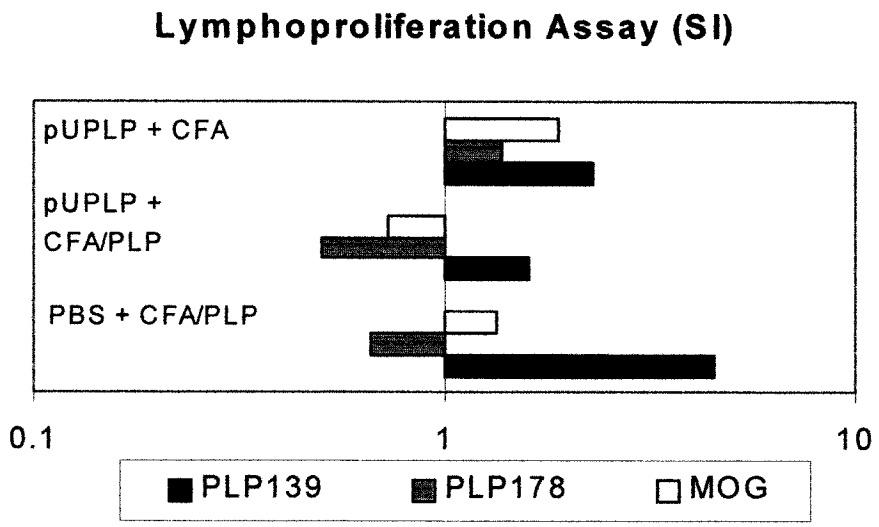

Figure 3 Stimulation indices (SI) of lymphocytes from mice primed with pUPLP and challenged with $PLP_{139–151}$/CFA or CFA or mice with EAE (PBS + CFA/PLP). Pooled lymphocytes were obtained from 2 mice in each group at day 12 after challenge. Lymphocytes (5×10^5 cells/well) were incubated with 10 nmol/ml of different encephalitogenic peptides ($PLP_{139–151}$, $PLP_{178–191}$, and $MOG_{92–106}$). Lymphocytes from the mice vaccinated with pUPLP and immunized with CFA proliferated to all encephalitogenic peptides.

Table 2 Neuropathology in mice infected with recombinant vaccinia virus encoding myelin or nonmyelin antigens followed by CFA challenge

			Pathology/Mice	
Virus	*Challenge*	*Mouse Strain*	*Exp. I*	*Exp. II*
VV_{PLP}	CFA	SJL/J	5/5	5/5
VV_{MAG}	CFA	SJL/J	5/5	2/5
VV_{GFAP}	CFA	SJL/J	5/5	4/5
VV_{SC11}	CFA	SJL/J	0/9	0/5
VV_{PLP}	CFA	PL/J		0/4
VV_{GFAP}	CFA	PL/J		0/5

SJL/J mice or PL/J mice were infected with recombinant vaccinia virus encoding either PLP (VV_{PLP}), MAG (VV_{MAG}), GFAP (VV_{GFAP}), or β-galactosidase (VV_{SC11}). Five weeks after infection, CFA was injected with or without *BP*.

Table 3 Neuropathology in mice primed with cDNA constructs, followed by infection with recombinant vaccinia virus encoding β-galactosidase[a]

Plasmid[b]	*Challenge*	*Pathology/total mice*[c]
pUPLP	VV_{SC11}	1/5
—	VV_{SC11}	0/5
pCMVβ	VV_{SC11}	0/5
$pPLP_{ALL}$	VV_{SC11}	0/5

[a]SJL/J mice were injected with plasmid 3 times, followed by infection with VV_{SC11}.
[b]pUPLP encodes PLP fused with ubiquitin; pCMVβ encodes β-galactosidase, and $pPLP_{ALL}$ encodes PLP alone (Tsunoda *et al*, 1998).
[c]Number of mice that have CNS lesions/total number of mice examined.

Materials and Methods. Mice (5 mice per virus) were infected with VV_{PLP}, VV_{MAG}, or VV_{GFAP}, rested for 5 weeks, then challenged with CFA followed by *BP*. At 1 month post-CFA challenge, mice showed no clinical signs and were sacrificed for histological examination. The majority of mice had inflammatory lesions in the brain or spinal cord (Table 2). As shown in Figures 2b and 2c, MNC infiltrates were evident within the white matter and meninges. When these regions were stained with an anti-CD3 antibody, most of the inflammatory infiltrating cells were identified as T cells. As a negative control, 9 mice were primed with VV_{SC11}, a recombinant vaccinia virus that encodes β-galactosidase but no CNS proteins. β-galactosidase has no known molecular mimicry with PLP or CNS antigens. Following the CFA challenge, no inflammatory changes were seen in the CNS of VV_{SC11} infected mice. Thus, this second experiment showed the encephalitogenicity of VV_{PLP}, VV_{MAG}, and VV_{GFAP} (Table 2).

To demonstrate the genetic influence on the priming event, we infected PL/J mice with two highly encephalitogenic recombinant vaccinia viruses, VV_{PLP} and VV_{GFAP}, and later challenged them with CFA; none of 10 PL/J mice primed with recombinant viruses showed either clinical signs or pathological changes in the CNS. Therefore, genetic background seems to play an important role in this disease model, as in EAE and MS. These data indicate that infections having molecular mimicry can subclinically prime animals for autoimmune CNS disease and at a later time a nonspecific infection or challenge could initiate overt disease.

To determine whether a nonspecific virus infection of primed animals would produce the same outcome as CFA challenge, the following experiment was performed. Five mice were primed 3 times with pUPLP, rested for 1 week, then infected with VV_{SC11}. Mice were followed for 2 weeks and then sacrificed. No mice showed clinical signs during the 2-week observation period. Brain and spinal cords were removed and examined for pathologic changes. As shown in Figure 2d, MNC infiltrates were evident within the meninges of the CNS of one of these mice. None of the 15 mice in 3 control groups showed any pathological changes in the CNS (Table 3). These control groups (5 mice/group) were: (i) unprimed mice challenged with VV_{SC11}, (ii) mice primed with pCMVβ followed by VV_{SC11} challenge, and (iii) mice primed with $pPLP_{ALL}$ and challenged with VV_{SC11}. Therefore, in mice primed with pUPLP, subsequent challenge with either VV_{SC11} (Table 3) or CFA (Table 1) induced CNS inflammation in 20% of mice. However, because nonspecific (CFA) challenge induced autoimmune CNS disease in approximately 90% of mice which had been primed with vaccinia viruses encoding self-CNS antigens (Table 2), the consequences of molecular mimicry appear to be more serious following live virus priming than after priming with plasmid DNA.

Discussion

Our study indicates that, following an initial early priming event by an antigen that can mimic self-epitopes, subsequent immunostimulation (for example, during a nonspecific virus infection) could trigger inflammatory changes within the CNS, or overt disease. Importantly, whereas the original event must prime for an autoimmune response against a self-antigen, the subsequent virus does not need to have molecular mimicry with the target self-CNS protein, nor need it directly infect the CNS. This model mirrors the epidemiologic data supporting MS as being a disease relating to environment, genetics, and age.

Our data are compatible with those of others (reviewed in Goverman, 1999) where transgenic mice were made only expressing the T-cell receptor (TCR) specific for an encephalitogenic peptide of myelin basic protein. When raised under normal conditions, these mice had a high incidence of EAE, but under specific pathogen-free conditions, the mice had a much lower incidence of spontaneous EAE.

In our model, we found that sensitization of young genetically susceptible animals with plasmids encoding self-CNS proteins followed by a nonspecific immunologic challenge was sufficient to induce inflammatory changes within the CNS. Our data suggest that the first encounter with microbes can set the stage for autoimmune disease. In addition, the MHC class I presentation pathway is important. Previously, we reported significant lymphoproliferative responses against $PLP_{139–151}$ following CFA challenge of $pPLP_{ALL}$ inoculated mice (Tsunoda *et al*, 1998). In contrast, control mice that were injected with pCMVβ with or without CFA challenge showed no lymphoproliferative response against PLP. In $pPLP_{ALL}$-primed mice, however, we could detect neither clinical signs nor histologic lesions in the CNS (Tsunoda *et al*, 1998), and these data are confirmed in the present report. In contrast, mice primed with pUPLP, which enhances MHC class I presentation, show clinical signs after CFA challenge, suggesting that ubiquitination or processing and presentation through MHC class I is an important contribution, and perhaps a necessary component.

In our study, we could prime for autoimmune disease not only with a cDNA or a virus encoding the highly encephalitogenic myelin antigen, PLP, but also with the less encephalitogenic myelin antigen, MAG, and a nonmyelin antigen, GFAP. This is the first report that demonstrates MAG and GFAP as potential CNS antigens in mice. Previously, Weerth *et al* demonstrated that a mild inflammatory disease could be induced in Lewis rats after adoptive transfer of MAG specific T cells. However, sensitization with MAG peptides failed to induce acute EAE (Weerth *et al*, 1999). Berger *et al* showed that adoptive transfer of GFAP-specific T cell lines could cause CNS inflammation in Lewis rats, while ovalbumin or purified protein derivative (PPD) specific T cells could not (Berger *et al*, 1997). Our results are in accord with these previous reports; CNS inflammation was induced with recombinant vaccinia virus encoding CNS antigens, VV_{PLP}, VV_{MAG}, and VV_{GFAP}, but not with VV_{SC11}, a vaccinia virus encoding a non-CNS protein, β-galactosidase.

We propose that a virus having molecular mimicry with a self-CNS antigen may be involved in some of the early priming events occurring in young genetically susceptible MS individuals. The first agent need not cause obvious autoimmunity or disease; instead, these are provoked by a subsequent nonspecific challenge. Potentially deleterious priming events were induced not only by recombinant vaccinia viruses encoding proteins found in oligodendrocytes, but also by a virus encoding the astrocytic protein GFAP. Therefore, autoimmunity induced by molecular mimicry can cause harm not only when it affects proteins associated with myelin; it may also be damaging when it involves neighboring proteins such as those found in astrocytes. In addition, using VV_{PLP} and VV_{GFAP}, we could induce CNS inflammation in most SJL/J mice, but not in PL/J mice. Therefore, genetic background seems to play an important role in the pathogenesis of this model similar to that in "classical" EAE and MS.

Our data would explain why no single virus has been demonstrated to have a cause-and-effect relationship with MS. We would also predict that there is a window of susceptibility where animals can be primed and rendered susceptible to subsequent challenge. Outside this window, individuals would be refractory to disease induction. This could help explain why MS is seen mostly in individuals between the age of 20 and 40 and then the incidence decreases.

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