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Sequential Polymicrobial Infections Lead to CNS Inflammatory Disease: Possible Involvement of Bystander Activation in Heterologous Immunity

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Abstract

VV_{PLP} is a recombinant vaccinia virus (VV) encoding myelin proteolipid protein (PLP) that has been used to investigate molecular mimicry and autoimmunity. Since virus infections can cause bystander activation, mice were first infected with VV_{PLP}, and later challenged with wild-type VV, lymphocytic choriomeningitis virus (LCMV), or murine cytomegalovirus (MCMV). Among the VV_{PLP}-primed mice, only MCMV challenge induced significant Ki-67⁺, CD3⁺ T cell infiltration into the central nervous system (CNS) with a mild PLP antibody response. While MCMV alone caused no CNS disease, control VV-infected mice followed with MCMV developed mild CNS inflammation. Thus, heterologous virus infections can induce CNS pathology.

Keywords

Autoimmunity; CNS demyelinating autoimmune diseases; Experimental autoimmune encephalomyelitis; IL-12; Multiple sclerosis; Theiler's murine encephalomyelitis virus

1. Introduction

Polymicrobial infections have been shown to induce distinct disease phenotypes compared to infection with a single agent (Tuft, 2006). Welsh and Selin (2002) proposed that previous exposure to related or, perhaps, unrelated infectious agents alters the host's immune response to subsequent infection and cause a marked deviation in the disease course (heterologous immunity). Experimental models have shown that a history of unrelated viral infections can greatly influence immunopathology. One example of this is dengue shock syndrome, which can arise if a host that has been previously exposed to one of the four dengue virus serotypes is later exposed to a second serotype (Welsh and Selin, 2002).

It is well described that virus infection may initiate or exacerbate organ-specific autoimmune diseases (Grigoriadis and Hadjigeorgiou, 2006). We have proposed that a combination of two mechanisms, molecular mimicry and bystander activation, induced by polymicrobial infection, can lead to autoimmune disease (McCoy et al., 2006; Theil et al., 2001). Molecular mimicry involves the *de novo* activation of autoreactive T cells due to the cross-reactivity between self-

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epitopes and viral epitopes during a virus infection. Bystander activation is a nonspecific activation of pre-existing autoreactive T cells resulting from inflammatory responses elicited by infection with irrelevant viruses. We hypothesized that viral proteins having molecular mimicry with self-proteins can prime genetically susceptible individuals for autoimmune disease (fertile field hypothesis) (von Herrath et al., 2003). Once this priming has occurred, a second virus infection could result in disease through bystander activation by cytokines and/or molecular mimicry (McCoy et al., 2006).

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) (Willenborg and Staykova, 2003). The development of MS is believed to occur in genetically predisposed individuals exposed to a putative trigger(s) in the environment during a period of vulnerability (Banwell, 2004). Several clinical studies have suggested that MS in general, as well as episodes of disease exacerbation, are associated with concomitant viral or microbial infections (Alotaibi et al., 2004; Grigoriadis and Hadjigeorgiou, 2006). For example, upper respiratory tract infections can trigger acute relapses of MS, resulting in an increase in the risk of clinical exacerbations during the weeks that follow the onset of virus infection (Kriesel and Sibley, 2005). This has led to studies of molecular mimicry between infectious agents and the self-antigens associated with MS (Fujinami and Oldstone, 1985). However, despite many claims a single causative infectious agent for MS has not been identified. An alternative possibility is that many infectious agents are capable of nonspecifically enhancing the likelihood of an autoimmune event.

We previously demonstrated that molecular mimicry alone might be incapable of causing clinical disease, and can do so only in conjunction with other factors, which could be provided by a nonspecific immunostimulation or by virally induced immune stimulation (Theil et al., 2001; von Herrath et al., 2003). When genetically susceptible SJL/J mice were infected with a plasmid DNA or recombinant vaccinia virus (VV) encoding a self CNS protein (model for molecular mimicry), these mice did not develop autoimmune disease clinically or histologically (Theil et al., 2001). However, these mice were primed for disease; if they receive a nonspecific immunostimulation elicited by complete Freund's adjuvant (CFA), most mice developed CNS inflammatory disease. These results indicate that antigen-specific cells induced by molecular mimicry can be activated by a nonspecific immune stimulus (bystander activation).

VV_{PLP} is a recombinant VV encoding myelin proteolipid protein (PLP) that has been used to investigate molecular mimicry and autoimmunity (Theil et al., 2001). Since VV_{PLP} has molecular identity with host PLP, infection with VV_{PLP} can cause a similar outcome to what happens in infection with virus that has molecular mimicry with PLP. Previously, we evaluated how various immunologic stimuli contribute to induction of CNS inflammatory disease in mice that were primed by infection with VV_{PLP}, compared with infection with control VV_{SC11}, a recombinant VV encoding β -galactosidase (Barnett et al., 1993). We found that VV_{PLP}-primed mice developed an experimental allergic (autoimmune) encephalomyelitis (EAE)-like disease in the CNS when mice were later challenged with CFA and *Bordetella pertussis* (BP) (Theil et al., 2001). Our data could be relevant to the findings in Tg4 mice, in which over 90% of T cells expressed a T cell receptor for the encephalitogenic Ac1-9 epitope of myelin basic protein (MBP) (Kissler et al., 2001). In Tg4 mice, spontaneous disease did not occur, while non-specific stimuli, including CFA, incomplete Freund's adjuvant (IFA) and pertussis toxin were capable of inducing EAE, in the absence of the encephalitogenic peptide. The authors postulated that an infection by a range of possible infectious agents will be capable of initiating autoimmunity in susceptible individuals without the need for molecular mimicry between virus and host (Kissler et al., 2001).

Here, we asked whether different types of second viral infections could replace CFA/BP, inducing CNS disease in VV_{PLP}-primed mice. Mice were first infected intraperitoneally (i.p.) with VV_{PLP} and then challenged 5 weeks later by i.p. infection with wild-type VV, lymphocytic choriomeningitis virus (LCMV), or murine cytomegalovirus (MCMV), or by intravenous (i.v.) infection with Theiler's murine encephalomyelitis virus (TMEV). We found that the majority of VV_{PLP}-primed mice challenged with MCMV developed CNS lesions, and a small percentage of TMEV-challenged mice had inflammation in the CNS. In contrast, no VV_{PLP}-primed mice developed substantial CNS inflammation when later challenged with VV or LCMV. MCMV-challenged mice had Ki-67⁺, CD3⁺ T cell infiltration in the CNS with a mild anti-PLP immune response. These results suggest that polymicrobial infections can induce heterologous immune responses that lead to CNS inflammatory disease.

2. Materials and Methods

2.1. Animal experiments

Four-week-old female SJL/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). We infected mice i.p. with 1×10^6 plaque forming units (PFU) of VV_{PLP} or VV_{SC11} (Barnett et al., 1993). Five weeks after VV infection (day 0), mice were injected subcutaneously (s.c.) at the base of the tail with 200 μ l of CFA, which was a mixture of equal volumes of phosphate-buffered saline (PBS) and Imject[®] Freund's Incomplete Adjuvant (Pierce Biotechnology, Rockford, IL) containing *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories, Detroit, MI). The final concentration of *M. tuberculosis* in PBS/CFA solution was 1 mg/ml. Groups of mice received 1) two i.v. injections of 1×10^{10} BP cells (Michigan Department of Public Health, Lansing MI) in 0.1 ml of PBS on days 0 and 2; 2) a single i.p. injection of 1 μ g of recombinant murine interleukin (IL)-12 (BioShop Canada Inc., Burlington, ON) in 200 μ l PBS on day 0; 3) i.p. injection of 1×10^6 PFU of VV_{SC11} on day 0; or 4) i.p. injection of PBS.

In the next set of experiments, 3–5 weeks after PBS, VV_{SC11} or VV_{PLP} injection, mice were infected i.p. with 1×10^6 PFU of wild-type VV (WR strain), 2.9×10^4 PFU of LCMV (Armstrong strain), 2×10^4 PFU of MCMV (Smith strain), or mice were infected i.v. with 1×10^6 PFU of Daniels (DA) strain of TMEV. VV (WR), LCMV (Armstrong strain) and MCMV (Smith strain) were kindly provided by Dr. Liisa Selin (University of Massachusetts, Worcester, MA).

In the last set of experiments, mice were injected with 2×10^5 , 2×10^4 , 2×10^2 , 20, or 2 PFU of VV_{PLP}, or recombinant VVs encoding glial fibrillary acidic protein (VV_{GFAP}), myelin-associated glycoprotein (VV_{MAG}), or myelin basic protein (VV_{MBP}) (Theil et al., 2001), intracerebrally (i.c.). Mice were sacrificed when moribund or after a 4-month observation period. Another group of mice was infected with 1×10^2 to 1×10^6 PFU of MCMV, i.c., and killed 2 weeks after infection.

2.2. Righting reflex

Clinical signs of mice were weight change and an impaired righting reflex (Tsunoda et al., 2001). When the proximal end of the mouse's tail is grasped and twisted to the right and then to the left, a healthy mouse resists being turned over (score of 0). If the mouse is flipped onto its back but immediately rights itself on one side or both sides, it is given a score of 1 or 1.5, respectively. If it rights itself in 1 to 5 seconds, the score is 2. If righting takes more than 5 seconds, the score is 3.

2.3. Histology

Mice were euthanized with halothane and perfused with PBS, followed with a 4% paraformaldehyde solution. We divided brains into 5 coronal slabs and spinal cords into 10 to 12 transverse slabs, and tissues were embedded in paraffin. Four- μ m thick tissue sections were stained with Luxol fast blue for myelin visualization as described previously (Tsunoda et al., 1998,2000). Neuropathology was scored as described previously with slight modification in a double-blinded fashion: 0, no lesions; 1, meningeal thickening, or mononuclear cell (MNC) and/or polymorphonuclear (PMN) cell collection in the meninges, or a small number of T cells detected in the meninges by immunohistochemistry against CD3; 2, perivascular cuffing in the parenchyma and/or meningitis (Tsunoda et al., 1998,2000).

2.4. Immunohistochemistry

B and T cells were visualized by the avidin-biotin peroxidase complex (ABC) technique, using biotin-conjugated anti-mouse CD45R/B220 antibody (1:3000 dilution, PharMingen, San Diego, CA) (Tsunoda et al., 2000) and anti-CD3 ϵ antibody (following trypsinization, 1:30 dilution, DakoCytomation, Carpinteria, CA) (Mason et al., 1989;Tsunoda et al., 2000,2003), respectively. TMEV antigen positive cells were detected with hyperimmune serum against TMEV (Tsunoda et al., 2001). To detect proliferating cells, antibody against Ki-67 (clone TEC-3, 1:400 dilution, DakoCytomation) was used following incubation in Target Retrieval Solution (DakoCytomation) as per instructions. The thymus, intestine and subventricular (subependymal) zone cells in the brain (neural stem cell) were used as positive controls for Ki-67 reactivity (Eisch and Mandyam, 2004;Lois and varez-Buylla, 1993;Morshead et al., 1994). To detect damaged axons, we used SMI 311, a cocktail of monoclonal antibodies to nonphosphorylated neurofilament, with an antigen retrieval method using autoclave treatment (Tsunoda et al., 2003,2007).

2.5. Serum PLP antibody assay

Mice were bled when sacrificed. We used an enzyme-linked immunosorbent assay (ELISA) to measure levels of serum anti-PLP antibody as described previously (Tsunoda et al., 1998;Wang and Fujinami, 1997). Ninety-six well plates were coated with PLP_{139–151} peptide overnight. After blocking, serial dilutions of sera were added to the plates and incubated for 90 min. After washing, a peroxidase-conjugated anti-mouse IgG (H + L) (Invitrogen, Carlsbad, CA), IgG1 (Caltag laboratories, Burlingame, CA), or IgG2c antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) (Martin et al., 1998;Martin and Lew, 1998;Tsunoda et al., 2005a,2005b) was added for 90 min. The plates were colorized with *o*-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO) and were read at 492 nm on a Titertek Multiskan Plus MK II spectrophotometer (Flow Laboratories, McLean, VA).

2.6. Lymphoproliferation assay

Inguinal lymph nodes and spleens were removed and pooled, and MNCs were isolated with Histopaque®-1083 (Sigma-Aldrich). A volume of 200 μ l containing 2×10^5 cells in RPMI supplemented with 1% glutamine, 1% antibiotics, 50 μ M 2-mercaptoethanol and 10% fetal bovine serum was added to each well. PLP_{139–151}, PLP_{178–191}, PLP_{104–117} or myelin oligodendrocyte glycoprotein (MOG)_{92–106} peptide was added at 50 μ g/ml (Tsunoda et al., 1998,2000). The cells were cultured for 4 days, pulsed with 1 μ Ci of tritiated thymidine per well, and cultured for another 18 to 24 h. Cultures were harvested onto filters using a multiwell cell harvester and 3 H uptake was counted using standard liquid scintillation techniques. All cultures were performed in triplicate and stimulation indices (experimental cpm/control cpm) were calculated as described previously (Tsunoda et al., 1998).

3. Results

3.1. CFA/BP challenge induces CNS inflammation with high spontaneous lymphoproliferation in VV_{PLP}-primed mice

Previously, we have demonstrated that VV_{PLP}-primed mice, but not VV_{SC11}-primed mice, developed clinical signs, such as impairment of the righting reflex, during the 3-week observation period after the second challenge with CFA/BP (Theil et al., 2001). Histologically, these mice had meningitis and perivascular cuffs composed of MNCs (Fig. 1a). Immunohistochemistry visualizing CD3 indicated that the majority of MNCs were T cells (Fig. 1b). This time, 5 weeks after VV_{PLP} infection, mice were injected with PBS, CFA alone, or IL-12, or infected with VV_{SC11} to determine which immunomodulatory treatment could result in CNS disease (Table 1). Three weeks after the second challenge, we examined tissues for neuropathology and found that no mice in any of these groups developed substantial CNS pathology. Although IL-12 has been reported to enhance EAE (Gran et al., 2004), we did not observe any pathology among 5 VV_{PLP}-primed mice challenged with IL-12, although CD3 immunohistochemistry showed that some mice had mild T cell accumulations in the meninges after 3 weeks. We observed 5 additional VV_{PLP}-primed mice challenged with IL-12 for 2 months. Although none of the mice developed clinical signs, one mouse had perivascular cuffing composed of T cells in the cerebral cortex and the midbrain (Fig. 1c, d), and another mouse had mild T cell infiltration in the meninges, 2 months after IL-12 administration. Demyelination or axonal damage was not seen in any groups of mice.

To determine whether lymphoproliferative responses were correlated with clinical and histological disease, MNCs were isolated from spleens and lymph nodes and cultured for 5 days with no stimulation (spontaneous proliferation) or with stimulation with PLP_{139–151}, PLP_{178–191} or MOG_{92–106}. VV_{PLP}-primed mice receiving CFA/BP had high levels of spontaneous lymphoproliferation of both spleen and lymph node MNCs with no stimulation (Fig. 1e). The extent of spontaneous proliferation was similar among mice receiving the same treatment in two experiments. However, lymphoproliferative responses against myelin antigens were inconsistent even among mice receiving the same treatment. Some VV_{PLP}-primed mice that received CFA alone or CFA/BP had high levels of lymphoproliferation against all three myelin antigens (stimulation index >3), while the other mice in the same group had MNCs that proliferated against one myelin antigen or had no significant proliferative response (data not shown). These results suggest that CFA/BP challenge induced high levels of polyclonal T cell activation, which could mask antigen specific proliferative responses. CFA/BP challenge also induced similarly high levels of spontaneous lymphoproliferation in mice primed with VV_{SC11}, compared with VV_{PLP}-primed mice. Thus, high levels of polyclonal activation alone was not enough to induce CNS disease.

3.2. MCMV challenge induces CNS inflammation in VV_{PLP}-primed mice

In our above experiments, VV_{SC11}, VV encoding β galactosidase, did not induce CNS disease in mice primed with VV_{PLP}. We tested whether other virus challenges could lead to CNS inflammation in VV_{PLP}-primed mice. After VV_{PLP} infection, mice cleared the virus and were infected with wild-type VV, LCMV or MCMV. Neuropathology was examined 12 and 24 days after the second virus infection (Table 2). At each time point, 8 to 9 mice per group were examined. On day 12, we found no lesions in VV-challenged mice (Fig. 2a). In LCMV challenge, no mice had substantial CNS pathology, although half of the mice had mild meningeal cell infiltrates (Fig. 2c). In contrast, among 9 mice challenged with MCMV, 5 mice (56%) developed lesions in the CNS, 3 mice (33%) had mild cell infiltrates in the meninges and 1 mouse had no lesions (Table 2). The MCMV challenged mice had perivascular cuffing in the CNS parenchyma, including the hippocampus, medial habenular nucleus, internal capsule, periaqueductal gray matter, pontine base, cerebellar white matter and anterolateral

funiculus of the thoracic segment of the spinal cord (Fig. 2e). On day 24, we examined the CNS of mice challenged with VV, LCMV and MCMV. We found mild meningitis and perivascular cuffing only in 1 mouse challenged with MCMV, but none in mice challenged with VV or LCMV. Demyelination or axonal damage was not seen in any groups of mice on day 12 or 24. We also compared neuropathology scores among groups (Table 2). On day 12, the VV_{PLP}-MCMV group had significantly higher scores than the VV_{PLP}-VV and VV_{PLP}-LCMV groups ($P < 0.01$, ANOVA), while no significant difference was seen between the VV_{PLP}-VV and VV_{PLP}-LCMV groups. On day 24, there was no statistical difference among groups.

To further characterize the lesions seen on day 12, we compared T cell infiltration in the CNS of mice challenged with VV, LCMV and MCMV (Fig. 2b, d, f). Using CD3 as a T cell-specific marker, we immunostained T cells in the CNS. Following VV challenge, T cells were not present in most areas of the CNS, although a few T cells were seen occasionally scattered in the meninges and brain parenchyma (Fig. 2b). After LCMV challenge, we were able to detect only mild inflammation in some mice by routine Luxol fast blue stain (Fig. 2c). To our surprise, however, in consecutive brain sections, we detected a mild T cell infiltration in the meninges, periventricular areas, hippocampus, cerebral cortex, cerebellar white matter and brain stem (Fig. 2d). Although a few T cells were present in the spinal cord, some mice had T cells in the anterior horn. The T cell infiltration in the CNS of SJL/J mice infected with LCMV is interesting, since there have been only a few reports infecting SJL/J mice ($H-2^s$) with LCMV (Oldstone et al., 1992) and the neuropathology was unknown. After LCMV infection, CNS inflammation differs among mouse strains and severe T cell infiltration in the CNS has been described in other mouse strains, such as strains with the $H-2^b$ or $H-2^d$ haplotype (Doherty and Zinkernagel, 1975).

Following MCMV challenge, while T cells were present in the perivascular cuffs, we found large numbers of T cells infiltrating into otherwise “normal-appearing white matter” as demonstrated by Luxol fast blue staining of consecutive sections. We found T cells in the meninges, choroid plexus, periventricular areas, cerebral cortex, hippocampus, caudoputamen, internal capsule, midbrain and cerebellar white matter (Fig. 2f). We used immunohistochemistry against Ki-67 (Eisch and Mandym, 2004), a large nuclear protein preferentially expressed during all active phases of the cell cycle (G_1 , S, G_2 , and M phases) but absent in resting cells (G_0), demonstrating that many of the infiltrating T cells were dividing (Fig. 2g). In contrast, B cells were absent from most inflammatory lesions, and only a few B cells were detected in some areas (Fig. 2h). In the spinal cord, a small number of T cells were found in both gray and white matters, nerve roots, and dorsal root ganglions.

3.3. MCMV challenge induces splenomegaly and PLP antibody responses

In general, infiltration of T cells into the CNS requires T cells activation in the periphery (Ohmori et al., 1992) with upregulation of adhesion molecules. Thus, if memory encephalitogenic (myelin specific) cells were induced by VV_{PLP} infection, such encephalitogenic cells may gain access to the CNS upon activation. Some systemic virus infections, including LCMV and MCMV, induce not only virus-specific immune responses but also non-specific bystander activation of immune cells (Christensen et al., 1996). Bystander activation has been shown to cause enlargement of lymphoid organs. After LCMV and MCMV infections, an increase in the size of the spleen is accompanied by activation of immune cells during the early stage of infection, and apoptosis of activated immune cells results in a decrease in the size of the spleen at the later time point (Cheung et al., 1981; Christensen et al., 1996; Razvi et al., 1995; Yoshida et al., 1995). Thus, splenomegaly can be an indicator of bystander activation of immune cells. Since we found splenomegaly in MCMV-challenged mice macroscopically on day 12, we weighed spleens and compared mean weights between

the groups (Fig. 3a). The mean spleen weight of MCMV-challenged mice was significantly heavier than that of LCMV- ($P < 0.05$, ANOVA) and VV-challenged mice ($P < 0.01$). The mean spleen weight of LCMV-challenged mice was significantly heavier than that of VV-challenged mice ($P < 0.01$). Interestingly, on day 24, spleen weights of both LCMV- and MCMV- challenged mice had declined (Razvi et al., 1995), and there were no differences in mean spleen weights among the 3 groups. Mean spleen weight (g) $\pm$ mean standard error (SEM) on day 24 was as follows: VV, 0.19 ± 0.01 ; LCMV, 0.21 ± 0.01 ; MCMV, 0.21 ± 0.01 ($P > 0.05$, ANOVA).

PLP_{139–151} has been shown to act as an epitope for both CD4⁺ T cells and antibody (Tsunoda et al., 1998; Wang and Fujinami, 1997). To determine whether VV_{PLP} infection induces PLP antibody responses, we compared serum PLP antibody titers between the groups. On day 12, MCMV-challenged mice had the highest PLP antibody [IgG (H+L)] titers among the groups (Fig. 3b). LCMV-challenged mice also showed significantly higher antibody titers to PLP_{139–151} than naïve mice ($P < 0.01$, ANOVA). There was no statistical difference in PLP antibody titers between VV-challenged mice and naïve mice. We also measured anti-PLP IgG1 and IgG2c (formerly IgG2a of *Igh-1b* allotype) (Jouvin-Marche et al., 1989; Martin and Lew, 1998; Tsunoda et al., 2005a, 2005b). We found that anti-PLP IgG1 and IgG2c titers were low in all mouse groups. LCMV-challenged mice had higher anti-PLP IgG1 titer than MCMV-challenged mice (Fig. 3c, $P < 0.01$, ANOVA). Interestingly, MCMV-challenged mice had higher IgG2c titer (Fig. 3d) and significantly higher anti-PLP IgG2c/G1 ratio than VV-challenged mice (Fig. 3e, $P < 0.01$, ANOVA). However, by day 24, we did not see any difference in PLP antibody titers between VV-, LCMV- and MCMV-challenged mice (mean OD_{492nm} at 1:256 dilution $\pm$ SEM: VV, 0.1 ± 0.01 ; LCMV, 0.08 ± 0.01 ; MCMV, 0.1 ± 0.01 , each group consisted of 8 or 9 mice).

3.4. MCMV challenge following VV infection, but not MCMV infection alone, induces CNS disease

We examined whether i.v. MCMV infection alone could induce CNS disease, compared to co-infection with VV_{PLP} or VV_{SC11}. Mice were primed with PBS, VV_{SC11} or VV_{PLP}, first, and then challenged 4 weeks later i.p. with MCMV. Clinically, VV_{PLP}-primed mice had the least weight increase and the worst impaired righting reflex score among the 3 groups (Fig. 4a, b, $P < 0.01$, compared with PBS group, ANOVA). There was no significant difference between the VV_{SC11}-primed group versus the PBS group ($P > 0.05$), although the VV_{SC11}-primed group showed less weight increase and worse impaired righting reflex compared with the PBS group.

Luxol fast blue staining showed that, 1 week after MCMV challenge, no group of mice had substantial lesions in the CNS (Table 3). However, 2 weeks after MCMV challenge, all 5 VV_{PLP}-primed mice and 3 of 5 VV_{SC11}-primed mice had meningitis and perivascular cuffing in the corpus callosum, the hippocampus, around the lateral ventricles, the midbrain and the cerebellar white matter (Fig. 4d). In the spinal cord, 3 of 5 mice had meningitis and perivascular cuffing following both VV_{PLP} and VV_{SC11} infections. Demyelination or axonal damage was not seen in VV_{PLP} or VV_{SC11} infections. The PBS-injected group had no lesions in the CNS, 2 weeks after infection (Fig. 4c). This lack of CNS disease in mice receiving MCMV alone is compatible with the age-dependent resistance of mice to CNS MCMV infection that was previously reported by Tsutusi et al (1995). We also compared neuropathology scores among groups (Table 3). At 2 weeks after infection, the VV_{PLP}-MCMV group had significantly higher scores than the PBS-MCMV group ($P < 0.05$, ANOVA, Table 3), although there was no statistical difference between the groups at 1 week. Therefore, MCMV infection alone was not sufficient for induction of CNS pathology; primary infection with VV_{PLP} was required.

Using immunohistochemistry with CD3 antibody, we looked for T cells in the CNS. One week after MCMV challenge, we found parenchymal CD3⁺ T cells in the cerebral cortex and the

cerebellar white matter in some of the VV_{SC11}- or VV_{PLP}-primed mice, while only a few T cells were detected in the meninges and surrounding vessels in the PBS-injected group (Fig. 4e, f). Two weeks after MCMV challenge, VV_{SC11}- and VV_{PLP}-primed mice had perivascular and parenchymal T cell infiltration in the cerebral cortex, hippocampus, cerebellar white matter and spinal cord (Fig. 4h). Interestingly, at 2 weeks post challenge, the PBS-injected group also had mild T cell infiltration in the CNS parenchyma (Fig. 4g), while no perivascular or meningeal inflammation was seen.

We compared serum antibody titers against PLP_{139–151} between naïve mice versus MCMV-infected mice following PBS, VV_{SC11} or VV_{PLP} infection. At 1 week, there was no difference in PLP antibody titers among the groups. However, at 2 weeks we detected significantly higher PLP antibody titers in VV_{SC11}- and VV_{PLP}-primed mice than naïve mice ($P < 0.01$, ANOVA). Although MCMV-infected mice receiving PBS also had high PLP antibody titer, there was no difference between PBS-injected mice versus naïve mice or between PBS-injected mice versus VV_{SC11}- or VV_{PLP}-primed mice. Using spleen MNCs, we also compared lymphoproliferative responses against PLP_{139–151}, PLP_{178–191}, or PLP_{104–117} between MCMV-infected mice following PBS, VV_{SC11} or VV_{PLP} infection. However, none of the PLP-specific responses had stimulation indices higher than 3 (data not shown). All groups of mice had splenomegaly when sacrificed at 1 and 2 weeks after MCMV challenge, and there was no difference between the groups. The mean spleen weight of each group was as follows: PBS groups, 1 week, 0.27 g, 2 weeks, 0.31 g; VV_{SC11} group, 1 week, 0.3 g, 2 weeks, 0.31 g; VV_{PLP} group, 1 week, 0.28 g, 2 weeks, 0.31 g. This suggests that splenomegaly is due to MCMV infection, and co-infection with VV was not required. The findings of antibody responses and splenomegaly suggest MCMV infection induces polyclonal activation of the lymphoid system and that polymicrobial infection with VV and MCMV enhances autoimmune responses against PLP.

3.5. TMEV challenge has a weak adjuvant effect on VV_{PLP}-primed mice

Induction of CNS pathology by a MCMV challenge in VV_{PLP}-primed mice could be due to bystander activation of autoimmune cells. TMEV has been shown to induce proinflammatory cytokine responses in infected mice (Theil et al., 2000). In addition, infection with recombinant TMEV encoding PLP_{139–151} has been shown to lead to rapid onset of paralytic disease (Olson et al., 2002). Thus, TMEV can be another candidate virus that can act as an adjuvant. We injected mice with PBS, VV_{SC11} and VV_{PLP}, and then challenged mice i.v. with TMEV, 5 weeks later. Clinically, no mice showed signs, while the VV_{PLP}-primed group tended to show very mild righting reflex abnormalities (mean righting reflex scores $\pm$ SEM: PBS group, 0.17 ± 0.17 ; VV_{SC11} group, 0; VV_{PLP} group, 0.45 ± 0.15 , $P > 0.05$, ANOVA). Histologically, 3 of 13 (23%) VV_{PLP}-primed mice had CNS MNC inflammatory lesions such as perivascular cuffing in the cerebellar white matter and meningitis (Fig. 5b), comprised of mainly CD3⁺ T cells (Fig. 5d). In the lesions TMEV antigen positive cells were not detected by immunohistochemistry (Fig. 5c). This suggested that CNS inflammation in VV_{PLP}-primed mice following TMEV infection was not induced by direct CNS infection by TMEV. In contrast, only one VV_{SC11}-primed mouse had mild meningitis and the remaining nine VV_{SC11}-primed mice (Fig. 5a) and all eight PBS-injected mice had no CNS lesions. Demyelination was not seen in any groups of mice.

3.6. CNS infection with VV results in acute encephalitis and chronic hydrocephalus

Neuropathology seen in VV_{PLP}-primed mice followed with MCMV challenge could be due to enhancement of the neuropathology of VV or MCMV infection and/or direct virus replication in the CNS. Anti-VV antibody, which can be used for immunohistochemistry to detect VV infection on mouse paraffin sections, was not available. Thus, we were not able to associate possible VV persistence with CNS pathology in our experiments [although this is unlikely: VV persistence in the CNS in SJL/J mice has never been reported. See also Discussion]. Thus,

we decided to use a different approach, testing whether direct VV infection in the CNS could induce distinct neuropathology or not. To determine the difference between direct CNS infection of VV versus peripheral polymicrobial infections associated with VV, we injected mice i.c. with different amounts of VV_{PLP} from 2 PFU to 2×10^5 PFU. During the first 1 week after infection, all infected mice showed encephalitic signs, such as hunched back and ruffled fur, with severe weight loss (Fig. 6a). All mice infected with more than 2000 PFU died between 4 and 7 days after infection. Mice infected with less than 200 PFU survived and recovered completely or had mild impaired righting reflex.

Histologically, during the first week, mice had meningitis consisting of both PMNs and MNCs with amorphous exudates and nuclear fragmentation (karyorrhexis) of infiltrates (Fig. 6b). During the chronic stage, mice had hydrocephalus with enlargement of all ventricles, including the lateral, 3rd and 4th ventricles (Fig. 6c), and no parenchymal or meningeal lesions were detected in the CNS at this time. We also infected mice by the i.c. route with other recombinant VVs: VV_{GFAP}, VV_{MAG}, VV_{MBP} (Theil et al., 2000). We did not see any difference in clinical signs and neuropathology between mice infected with VV_{PLP} and other VVs (data not shown).

We also injected mice with 1×10^2 to 1×10^6 PFU of MCMV i.c. and observed clinical signs for 2 weeks. Mice did not show weight change or clinical signs and no lesions were found in the CNS at 2 weeks after infection (data not shown).

4. Discussion

To determine what cytokine adjuvants could induce CNS inflammatory disease, we first tested whether IL-12 alone could induce CNS inflammatory disease in mice primed with VV_{PLP}. IL-12 is one of several candidate molecules that play an important role in MS and EAE (Gran et al., 2004). For example, administration of IL-12 has been shown to elicit clinical EAE in rats suboptimally sensitized with MBP (Jee and Matsumoto, 2001), induce relapses of EAE in mice (Constantinescu et al., 1998), and unmask the latent potential of MBP-specific T cells to induce EAE upon adoptive transfer into resistant mice (Segal and Shevach, 1996). Although VV_{PLP}-primed mice received a 10 times higher dose of IL-12 than that required for induction of a relapse (Constantinescu et al., 1998), only a small percentage of the mice developed very mild CNS inflammation; it was not comparable to EAE-like pathology seen in VV_{PLP}-primed mice challenged with CFA/BP.

We next tested whether virus infections could induce CNS inflammatory disease in mice primed with VV_{PLP}. We found that peripheral MCMV and TMEV infections induced overt CNS inflammation in VV_{PLP}-primed mice, compared to VV and LCMV infections. Although the precise mechanism of CNS inflammation is currently under investigation, one possible mechanism is that immune responses (against PLP or some other antigens) primed by VV_{PLP} infection could be enhanced by a second virus challenge via bystander activation. In bystander activation, initial/innate immune responses to the second viral infection can play an important role. There appears to be two types of initial immune responses to virus infection, depending on the virus [reviewed in (Biron, 1999)]. The first type of virus infection, where the prototypic infection is LCMV, high levels of interferon (IFN)- α/β are produced, which activate natural killer (NK) cells that have lytic function, leading to the generation of cytotoxic T lymphocytes (CTLs). These CD8⁺ CTLs can make IFN- γ , but the IFN- γ is produced later during the adaptive phase of the immune response. IFN- α/β can also block IL-12 production. The second type of infection is MCMV infection where high levels of IL-12 are produced. IL-12 would then facilitate IFN- γ production from NK cells, early after infection (Biron et al., 1999; Kosugi et al., 2002). IL-12 and IFN- γ would then activate autoimmune T helper (Th) 1 cells. In this study, we found that MCMV challenge induced higher anti-PLP IgG2c (formerly IgG2a of *Igh-1b* allotype) (Jouvin-Marche et al., 1989; Martin and Lew, 1998; Tsunoda et al.,

2005a,2005b) responses and that the anti-PLP IgG2c/G1 ratio in MCMV challenge was higher than in VV and LCMV challenge. Since Th1 and Th2 cells help with the Ig isotype switch to IgG2a (2c) and IgG1, respectively, our results are consistent with the fact that MCMV infection skews immune responses toward Th1. TMEV infection can be categorized into the second type of infection, since i.c. infection with the DA strain of TMEV can induce IL-12 mRNA (Monteyne et al., 1999), but not IFN- β mRNA (Theil et al., 2000) in the brain during the acute stage of infection. In addition, *in vitro* DA virus infection of macrophages increased IL-12 expression, but decreased IFN- α/β expression (Petro, 2005; van Pesch et al., 2001).

Although VV infection can induce production of IFN- α/β , IFN- γ and IL-12 (Xu et al., 2004), we did not see CNS disease in VV-challenged mice primed with VV_{PLP}. This could be due to enhanced clearance of VV by VV-specific immune responses generated by the first VV_{PLP} infection. Indeed, in our system, we observed induction of VV-specific CD8⁺ CTL responses in the spleens of SJL/J mice, 1 week after VV infection, using ⁵¹Cr release assays (Tsunoda et al., 2002). In addition, VV has been shown to express proteins that target different aspects of the innate response by binding cytokines such as both type I and II IFNs, inhibiting cytokine synthesis, and interfering with different signaling pathways, as those triggered by IFNs and toll-like receptors (Haga and Bowie, 2005). Thus, expression of these proteins could also contribute to a lack of bystander activation.

VV_{PLP}-primed mice developed CNS disease around 2 weeks after the second challenge, while the CNS disease subsided by 3 weeks after the second challenge, regardless of whether we use CFA/BP, MCMV, or TMEV as adjuvant. This time course is similar to what has been described in monophasic EAE, where acute CNS disease can be seen 2 weeks after EAE induction with complete remission by day 20. Kawakami et al. (2005) demonstrated a rapid T cell contraction in the periphery after EAE induction. However, the authors showed lifelong persistence of encephalitogenic T cells that mediated EAE after a second EAE challenge. These authors suggested that autoreactive memory T cells can persist throughout life within the lymphoid system, where they can be reactivated at a later time point. Autoimmune T cells generated by molecular mimicry between pathogen and host molecules have two features: 1) anti-microbial immunity and 2) autoimmunity. In this context, if VV_{PLP} infection induces immune responses against PLP, autoimmune responses against PLP can show different kinetics from other memory T cell responses. In the future it would be worth investigating how long such pathogen-induced autoimmune cells remain in the periphery.

Welsh and Selin's group showed that previous immunity to a heterologous virus infection can alter the outcome of a subsequent virus infection, influencing immune responses and pathology (heterologous immunity) (Chen et al., 2003). For example, immunity to influenza virus inhibits VV replication but enhances LCMV and MCMV viral titers and alters cytokine profiles and pathology. One explanation is that the second infection results in bystander activation of memory T cell proliferation via proinflammatory cytokines (Karrer et al., 2001). Currently, we do not know the specificity of T cells that cause CNS inflammation in our model.

Interestingly, MCMV infection induced mild CNS disease not only in VV_{PLP}-primed mice but also in VV_{SC11}-primed mice. Since there were no significant differences in neuropathology and clinical signs between VV_{PLP}-primed versus VV_{SC11}-primed mice, one could argue that immune responses generated by heterologous virus infections (VV and MCMV) solely play a role in CNS inflammation. However, we were also unable to see pathological and clinical differences between VV_{SC11}-primed versus PBS groups in MCMV-infected mice. This suggests that immune responses generated against the PLP encoded in VV_{PLP} could play a role in enhancement of CNS inflammation. However, in VV_{PLP} infection, we did not see a significant enhancement of PLP-specific T cell responses, using lymphoproliferative assays, which can be a measure of major histocompatibility complex (MHC) class II-restrictive

CD4⁺ T cells. We are investigating roles of the other potential immune responses against PLP, such as MHC class I-restricted CD8⁺ T cells or PLP antibody responses to whole PLP protein, not just to PLP peptides.

Autoreactive responses have been shown to be surprisingly frequent, although they are usually low-grade and seem to be harmless (von Herrath et al., 2003). Previously, we (Tsunoda et al., 1998) and others (Takács et al., 1997) have demonstrated that naïve SJL/J mice had low levels of anti-PLP_{139–151} immunoreactivity, and that non-specific immune stimulation, such as CFA or CpG DNA injection (Tsunoda et al., 1999), enhances autoimmune responses against PLP_{139–151}. In our current experiments, we detected low yet statistically significant levels of antibody responses to PLP_{139–151} in MCMV-infected mice primed with not only VV_{PLP}, but also VV_{SC11}, compared with naïve mice (Fig. 4i). In addition, both VV_{PLP}- and VV_{SC11}-primed mice developed CNS inflammation following MCMV challenge. Thus, we cannot as yet exclude the possibility that anti-PLP immune responses play some role in CNS disease not only in VV_{PLP}-primed mice, but also in VV_{SC11}-primed mice followed with MCMV infection.

Another explanation for induction of distinct pathology by heterologous virus infections is that T cell receptors (TCRs) participating in the first response recognize epitopes from both viruses (Karrer et al., 2001). So far, there is no report demonstrating that MCMV or TMEV possesses T cell epitopes that cross-react with VV or PLP in SJL/J mice. In this context, however, it should be worth noting that TMEV infection can induce CD8⁺ T cells that can recognize not only viral protein but also autoantigens, although T cell epitopes of the TMEV-specific CD8⁺ autoreactive cells are as yet characterized (Tsunoda et al., 2002).

Since CNS inflammation can be due to enhancement of the neuropathology of VV or MCMV infection, we inoculated VV or MCMV directly into the CNS and compared neuropathology with VV_{PLP}-primed mice. Since neither VV or MCMV infection alone caused a CNS inflammatory disease similar to that seen in VV_{PLP}-primed mice, CNS inflammatory disease found in VV_{PLP}-primed mice was likely not due to enhancement of viral pathology by VV or MCMV challenge. We did not see neuropathology in SJL/J mice infected i.c. with MCMV. In contrast, mice infected i.c. with VV developed acute meningitis with no parenchymal involvement and mice receiving high doses of VV died within 1 week after infection. This is compatible with previous reports, where mice infected i.c. with VV develop an acute meningitis composed of MNCs and PMNs (Bosse et al., 1982; Ginsberg and Johnson, 1976; Soekawa et al., 1974) with karyorrhexis of inflammatory cells (Blinzinger et al., 1977). Generally, i.c. infection of VV results in high mortality during the early stage of infection, and virus is cleared and does not persist in surviving mice (Ginsberg and Johnson, 1976). At a later time point, we found that VV-infected mice developed hydrocephalus. Development of communicating hydrocephalus following VV i.c. infection has also been reported in hamsters and cats (Davis, 1981). The disease course and pathology seen in mice infected i.c. with VV are similar to what we have reported in communicating hydrocephalus caused by TMEV mutant H101 (Tsunoda et al., 1997). In H101 infection, mice develop acute meningitis composed of TUNEL⁺ apoptotic meningeal infiltrates with no involvement of parenchyma. With time, surviving mice infected with H101 developed severe communicating hydrocephalus.

Neuropathology seen in VV_{PLP}-primed mice followed by TMEV infection is also unlikely the result of enhancement of TMEV infection. In the VV_{PLP}-primed mice, we found inflammation in the cerebellum with no TMEV persistence. This neuropathology is different from mice infected with TMEV alone. Intracerebral TMEV injection causes polioencephalitis in the gray matter with virus infection in neurons during the acute stage, and inflammatory demyelinating disease in the spinal cord white matter with virus persistence in glial cells and macrophages during the chronic stage (Tsunoda and Fujinami, 1999). The cerebellum is spared in TMEV

infection. Intravenous TMEV injection causes no CNS disease in SJL/J mice, while it induces anti-TMEV immune responses in the periphery (Tsunoda et al., 2002).

Regardless of whether the challenge was with CFA/BP, MCMV or TMEV, VV_{PLP}-primed mice developed similar neuropathology, particularly in the cerebellar white matter. It is intriguing that the cerebellum is the favorite target in PLP-induced EAE (Tsunoda et al., 1998), but is spared in MCMV (Lussier, 1975) or TMEV infection (Tsunoda and Fujinami, 1999) even when virus is given i.c. in susceptible mice. Therefore, we are currently investigating a role of PLP-specific cells in CNS inflammatory disease induced in VV_{PLP}-primed mice.

The importance of these experiments is that they provide a working model mirroring what may be occurring in human CNS autoimmune inflammatory disease. In MS, genetically susceptible individuals carry the risk association of where they lived the first 15 years of life [reviewed in (Libbey and Fujinami, 2002)]. This could reflect the type and kinds of infections having molecular mimicry or environmental agents that they are exposed to (priming phase). However, this priming alone does not result in overt clinical disease. Our VV_{PLP} infection may be similar to this early phase where virus infection having molecular mimicry with myelin antigens may be required. The second phase (challenge phase) that results in overt disease can be due to an innocuous infection or environmental challenge. This would be analogous to our CFA/BP or MCMV challenge. There may be a window after the priming phase where the challenge infection results in disease (Inada and Mims, 1986). At an earlier or later time, different outcomes would result where no CNS disease is seen and/or individuals could become resistant.

Here we present a model where initial virus infection could subclinically prime for CNS inflammatory disease and then a later different virus infection could cause an attack, suggesting a role of bystander activation in heterologous immunity. This study could explain the paradox of virus infections triggering MS, but no single virus being the cause.

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Fig 1.

(a–d) Neuropathology of VV_{PLP}-primed mice. Five weeks after VV_{PLP} infection, mice received injection of CFA/BP (a, b) or IL-12 (c, d). VV_{PLP}-primed mice had meningitis (a) comprised of CD3⁺ T cells (b), 3 weeks after CFA/BP challenge. Two months after IL-12 challenge, VV_{PLP}-primed mouse had perivascular cuffing (c) in the midbrain with T cell infiltration (d). (a, c) Luxol fast blue staining. (b, d) Immunohistochemistry against CD3. Magnification, (a, b) $\times 100$; (c, d) $\times 150$. (e) Spontaneous proliferation of MNC from VV_{PLP}-primed mice 3 weeks after immunomodulation. Five weeks after VV_{PLP}-sensitization, mice were injected with PBS, CFA alone, CFA and BP, IL-12 or VV_{SC11}. Three weeks after the second challenge, MNCs were isolated from the spleen and inguinal lymph nodes and cultured

for 5 days without stimulation. CFA/BP challenged group showed high levels of spontaneous proliferation both in the spleen and the lymph node. Results are means of 2 lymphoproliferative assays using MNCs pooled from 2 to 3 mice.

Fig 2.

Neuropathology of VV_{PLP}-primed mice, 12 days after challenge with VV (a, b), LCMV (c, d) or MCMV (e, f). (a, b) In VV challenge, the brain parenchyma around the internal capsule (IC) appeared normal and only a few CD3⁺ T cells were seen in the meninges (b, inset). (c) In LCMV challenge, the brain parenchyma appeared normal and mild cell infiltration was seen in the meninges by routine Luxol fast blue staining. (d) However, a consecutive section immunohistostained with CD3 antibody showed substantial T cell infiltration (arrow) in the meninges and parenchyma. (e) In MCMV challenge, perivascular cuffing was obvious around the IC. (f) Infiltrates were predominantly composed of CD3⁺ T cells. (g) A consecutive section immunostained with Ki-67 antibody showed many infiltrating T cells were proliferating. (h)

In MCMV challenge, only a few B cells were seen in the meninges (arrow). (a, c, e) Luxol fast blue staining. Immunohistochemistry against CD3 (b, d, f), Ki-67 (g), and B220 (h). Magnification, a–g, $\times 88$; h, $\times 177$; insets b–d, f, g, $\times 120$.

Fig 3.

(a) Mean spleen weight was compared between VV_{PLP}-primed mice challenged with VV, LCMV or MCMV on day 12. Both MCMV- and LCMV- challenged mice had larger spleens than those of VV-challenged mice (*, $P < 0.05$; **, $P < 0.01$, by ANOVA). Values are the means $\pm$ SEM. Each group consisted of 8 or 9 mice. (b-e) Serum antibody against PLP₁₃₉₋₁₅₁ was titrated by ELISA in VV_{PLP}-primed mice challenged by VV, LCMV MCMV or naïve mice. (b) MCMV-challenged mice had higher PLP IgG (H+L) titers than VV-challenged mice and naïve mice (*, $P < 0.05$, ANOVA). LCMV-challenged mice also had higher PLP antibody titers than naïve mice (**, $P < 0.01$). (c) LCMV-challenged mice had higher IgG1 titer than MCMV-challenged mice (**, $P < 0.01$). (d) MCMV-challenged mice had higher anti-PLP IgG2c titers than VV and LCMV-challenged mice. (e) MCMV-challenged mice had higher anti-PLP IgG2c/G1 ratio than VV-challenged mice (**, $P < 0.01$). Values are the means $\pm$ SEM of optical density (OD)_{492 nm} at 1:256 (b) or 1: 128 (c-e) dilution of serum, harvested 12 days after VV, LCMV or MCMV challenge. Each group consisted of 5 to 9 mice.

Fig 4.

(a, b) Clinical course of MCMV challenged mice following PBS (○), VV_{sc11} (●) and VV_{PLP} (▲) infection. Mice were first injected i.p. with PBS, VV_{sc11}, or VV_{PLP} on day -25. On day 0, mice were challenged with MCMV i.p. VV_{PLP}-primed mice showed significantly less weight increase and higher righting reflex score than PBS-injected mice (*, $P < 0.05$; **, $P < 0.01$). Shown are means of 5 to 10 mice per group. (c) MCMV challenge of PBS-injected mice did not cause lesions in the white matter (WM) or the granular layer (gr) of the cerebellum. (d) MCMV challenge induced perivascular cuffing (arrow) in the cerebellar white matter of mice primed with VV_{PLP}. (e) One week after challenge, a CD3⁺ T cell was seen around the vessel in a PBS-injected mouse challenged with MCMV, (f) while MCMV challenge induced

T cell infiltration (arrow) in the thalamus of VV_{PLP}-primed mice. (g) Two weeks after infection, PBS-injected mice challenged with MCMV had minor T cell infiltration (arrow) in the cerebellar white matter (WM), where a consecutive section, Fig. 4c, appeared normal. (h) A consecutive section of Fig. 4d showed substantial T cell infiltration in the parenchyma and perivascular area. (c, d) Luxol fast blue staining. (e–h) Immunohistochemistry against CD3. Magnification, (c, d) $\times 200$; (e–h), $\times 100$. (i) Serum PLP antibody responses. One (open column) and 2 weeks (closed column) after MCMV challenge, serum PLP_{139–151} antibody titers were compared between mice injected with PBS, VV_{SC11} or VV_{PLP}, 25 days before MCMV challenge. At 2 weeks after MCMV challenge, VV_{SC11}- and VV_{PLP}-primed mice had higher PLP antibody titers, compared with that of naïve mice (shown by dotted line) (**, $P < 0.01$). Shown are mean OD_{492 nm} at 1:256 dilution of sera $\pm$ SEM. Each group consisted of 5 to 10 mice.

Fig 5.

Adjuvant effect of TMEV challenge in VV_{PLP}-primed mice. (a) TMEV challenge of VV_{SC11}-primed mice caused no parenchymal lesions in the cerebellar white matter (WM). Gr: granular cell layers of the cerebellum. (b–d) TMEV challenge of VV_{PLP}-primed mice resulted in inflammation (b) comprised of CD3⁺ T cells (d) in the cerebellum, but viral antigen (c) was negative. (a, b) Luxol fast blue staining. (c, d) Immunohistochemistry against TMEV antigen (c) and CD3 (d). (a–d) Magnification, ×300.

Fig 6.

Weight change and neuropathology of mice infected i.c. with VV_{PLP}. (a) Mean body weight $\pm$ SEM of mice infected i.c. with VV_{PLP}. After infection, all mice showed significant weight loss during the first week of infection. Mice that survived this acute stage recovered completely with no obvious clinical signs during the 4-month observation period. (b) One week after i.c. VV_{PLP} infection, MNC and PMN infiltrates with amorphous material and fragmented nuclei (arrow) were seen in the subarachnoid space (sub), but no lesions were seen in the CNS parenchyma (pr). (c) Hydrocephalus was seen at 4 months after VV_{PLP} infection. Note severe dilatation of lateral ventricles (*), compared with normal mouse brain (inset). (b) Hematoxylin and eosin staining. (c) Luxol fast blue staining. Magnification: b, $\times 300$; c, $\times 10$; c, inset $\times 4$.

Table 1

Neuropathology scores and incidence^a in VV_{PLP}-primed mice^b

Challenge		Time after 2 nd challenge	Neuropathology score			
[[First]]	[[Second]]	[[]]	[[0]]	[[1]]	[[2]]	[[Mean]] ^c
VV _{PLP}	[[PBS]]	[[3 weeks]]	[[4/5]]	[[1/5]]	0/5	[[0.2 ± 0.2]]
VV _{PLP}	[[CFA alone]]	[[3 weeks]]	[[4/5]]	[[1/5]]	0/5	[[0.2 ± 0.2]]
VV _{PLP}	VV _{SC11}	[[3 weeks]]	[[5/5]]	[[0/5]]	0/5	[[0]]
VV _{PLP}	[[IL-12]]	[[3 weeks]]	[[2/5]]	[[3/5]]	0/5	[[0.6 ± 0.2]]
VV _{PLP}	[[IL-12]]	[[2 months]]	[[3/5]]	[[0/5]]	2/5	[[0.8 ± 0.5]]

^aIncidence; number of mice with each pathology score/total number of mice examined

^b SJL/J mice were infected with vaccinia virus encoding PLP. Five weeks after infection, mice were injected with PBS, CFA, vaccinia virus encoding β-galactosidase (VV_{SC11}), or IL-12. Mice were killed 3 weeks or 2 months after the second challenge.

^cMean ± SEM of neuropathology scores.

Table 2
Neuropathology scores and incidence^a in VV_{PLP}-primed mice followed by second virus challenge^b

Challenge		Day 12			Day 24		
[[First]]	[[Second]]	[[0]]	[[1]]	[[2]]	[[0]]	[[1]]	[[2]]
[[Mean]] ^c							
[[V]]	[[V]]	[[8/8]]	[[0/8]]	0/8	[[8/9]]	[[1/9]]	0/9
[[V]]	[[LCMV]]	[[4/8]]	[[4/8]]	0/8	[[6/9]]	[[3/9]]	0/9
[[V]]	[[MCMV]]	[[1/9]]	[[3/9]]	5/9	[[6/9]]	[[2/9]]	1/9
[[0.1 ± 0.1]]							
[[0.3 ± 0.2]]							
[[0.4 ± 0.2]]							

^aIncidence; number of mice with each pathology score/total number of mice examined

^b SJL/J mice were infected with VV_{PLP}, followed by infection with wild type vaccinia virus (VV), lymphocytic choriomeningitis virus (LCMV), or murine cytomegalovirus (MCMV). Histology was examined 12 or 24 days after the second virus infection.

^cMean ± SEM of neuropathology scores.

^{**}, *P* < 0.01, compared with VV_{PLP}-VV and VV_{PLP}-LCMV groups.

Table 3

Neuropathology scores and incidence^a in MCMV-infected mice^b

Challenge		1 week			2 weeks		
[[First]]	[[Second]]	[[0]]	[[1]]	[[2]]	[[0]]	[[1]]	[[2]]
[[Mean]] ^c							
[[PBS]]	[[MCMV]]	[[2/2]]	[[0/2]]	[[0/2]]	[[1/3]]	[[2/3]]	[[0.7 ± 0.6]]
VV _{SC11}	[[MCMV]]	[[2/5]]	[[3/5]]	[[0/5]]	[[1/5]]	[[1/5]]	[[1.4 ± 0.9]]
VV _{PLP}	[[MCMV]]	[[3/5]]	[[2/5]]	[[0/5]]	[[0/5]]	[[0/5]]	[[2]] [*]

^aIncidence; number of mice with each pathology score/total number of mice examined

^bMice were first injected with PBS, VV_{SC11}, VV_{PLP}, and followed by infection with MCMV. Histology was examined 1 or 2 weeks after MCMV infection.

^cMean ± SEM of neuropathology scores.

^{*}, *P* < 0.05, compared with PBS-MCMV group at 2 weeks. At 2 weeks, no significant difference was seen between the PBS-MCMV and VV_{SC11}-MCMV groups or between the VV_{PLP}-MCMV and VV_{SC11}-MCMV groups (*P* > 0.05).