Published in final edited form as:

J Neuroimmunol. 2007 October ; 190(1-2): 80–89.

TGF- β 1 Suppresses T Cell Infiltration and VP2 Puff B Mutation Enhances Apoptosis in Acute Polioencephalitis Induced by Theiler's Virus

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Abstract

GDVII and DA strains of Theiler's murine encephalomyelitis virus (TMEV) differ in VP2 puff B. One week after GDVII virus infection, SJL/J mice had large numbers of TUNEL⁺ apoptotic cells with a relative lack of T cell infiltration in the brain. DA viruses with mutation in puff B induced higher levels of apoptosis than wild-type DA virus, but levels of inflammation in brains were similar between DA and DA virus mutants. The difference in inflammation among TMEVs could be due to TGF- β 1 expression that was seen only in GDVII virus infection and negatively correlated with CD3⁺ T cell infiltration.

Keywords

CNS autoimmune demyelinating diseases; Cytokines; Multiple sclerosis; *Picornaviridae* infections; Sialic acid; Virus receptors

1. Introduction

Theiler's murine encephalomyelitis virus (TMEV) belongs to the family *Picornaviridae*, and is divided into two subgroups, according to virulence in the central nervous system (CNS) (Roos, 2002; Tsunoda and Fujinami, 1996, 1999). The Theiler's original (TO) subgroup viruses, including the Daniels (DA) and BeAn strains, have relatively low neurovirulence and cause a biphasic disease. During the acute phase, 1 week post infection (p.i.), DA virus infects neurons in the gray matter, mainly in the brain, and causes polioencephalomyelitis. Infected mice survive the acute phase and develop a demyelinating disease in the white matter of the spinal cord, 1 month p.i. (chronic phase). During the chronic phase, virus persists in glial cells and macrophages in the white matter of the spinal cord, but not in neurons. Viral persistence is associated with ongoing inflammatory demyelinating lesions, reminiscent of those observed in multiple sclerosis (MS). In contrast, the GDVII subgroup viruses, including the GDVII and FA strains, are highly neurovirulent (Theiler, 1941; Tsunoda et al., 1996). GDVII virus replicates in neurons in the gray matter of the CNS, and kills its host within ten days due to a polioencephalomyelitis. No virus is detected in the white matter or other areas of the CNS in the rare animals that survive infection (Lipton, 1980).

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During the acute phase, GDVII and DA viruses cause distinct neuropathology. Like other picornaviruses, such as poliovirus (Gosselin et al., 2003; López-Guerrero et al., 2000), both GDVII and DA viruses induce apoptosis (Tsunoda et al., 1997). Infection of mice with GDVII virus causes apoptosis in relatively high numbers of neurons in the CNS. In contrast, DA virus induces low levels of neuronal apoptosis. While significant numbers of activated macrophage/microglia are detected in the brain in both GDVII and DA virus infections, only DA virus infection causes significant perivascular cuffing with T cell recruitment. GDVII virus infection results in only small numbers of T cells infiltrating the CNS (Tsunoda et al., 1996).

Although the precise mechanism for the biological differences between the two subgroups is unknown, the differences in the receptor-binding site between GDVII and DA viruses likely contribute to their distinct tropisms. TMEV encodes four capsid proteins: VP1, VP2, VP3 and VP4. Several sequence differences between the two viruses are located in VP1 loop II (between the CD strands) and VP2 puff B (between the EF strands), which are located in close proximity to each other, close to the proposed receptor binding site, “pit,” or depression surrounding the five-fold axis of TMEV (Grant et al., 1992; Luo et al., 1996; McCright et al., 1999; Tsunoda et al., 2001). VP1 loop II and VP2 puff B are highly exposed at the virion’s surface and constitute an important site for neutralizing antibodies [reviewed in (Tsunoda and Fujinami, 2007)].

The possible role for VP2 puff B of TMEV in cell binding was first reported by Nitayaphan et al. (1985), using a DA monoclonal antibody DAmAb1, whose neutralization site is VP2 puff B (Sato et al., 1996). They demonstrated that DAmAb1 inhibited the hemagglutination activity of the TO subgroup, but not of the GDVII subgroup (Nitayaphan et al., 1985). Kumar et al. (2003) also showed that amino acid substitutions in VP2 puff B of the BeAn virus resulted in the loss of hemagglutinating activity and loss of the ability to infect baby hamster kidney (BHK)-21 cells. These results support the hypothesis that VP2 puff B is important for cell binding. In addition, studies of recombinant viruses and monoclonal antibody escape mutant viruses have provided support for the involvement of this region, formed by VP1 loop II (Jnaoui and Michiels, 1998; Lin et al., 1998; Wada et al., 1994; Zurbriggen et al., 1989) and VP2 puff B (Jnaoui and Michiels, 1998; Sato et al., 1996), in viral persistence and differences in disease phenotype.

Previously, we reported the construction of two DA virus mutants: DApB and DApBL2M viruses (Tsunoda et al., 2001). DApB virus contains the VP2 puff B of GDVII virus with one conservative change from GDVII virus (A to V at position 173) in the background of DA virus. DApBL2M virus has the VP1 loop II of GDVII virus and has a point mutation (S to R at position 171) in VP2 puff B in the background of DA virus. DApB virus persistently infects glial cells in the white matter of the spinal cord and causes demyelination during the chronic phase in SJL/J mice. In contrast, DApBL2M virus induces a prolonged gray matter disease without demyelination and fails to persist in the spinal cord white matter. This suggests that conformational differences via interactions of VP1 loop II and VP2 puff B between GDVII and DA viruses play an important role in making the transition from infection of the gray matter in the brain to infection of the white matter in the spinal cord during TMEV infection.

We investigated the role of VP2 puff B and VP1 loop II in neuropathology during the acute phase of TMEV infection, comparing GDVII, DA, DApB, and DApBL2M virus infections. We found that DApB and DApBL2M viruses induced large numbers of apoptotic cells as determined by the terminal deoxynucleotidyl transferase-mediated dUTP biotin nick-end labeling (TUNEL) staining, similar to GDVII virus infection. Interestingly, DApB and DApBL2M virus infections induced the recruitment of large numbers of CD3⁺ T cells into the CNS, similar to DA virus infection. In contrast, very few T cells were seen in GDVII virus infection. Since transforming growth factor (TGF)- β 1 has been shown to modulate induction

of apoptosis and inflammation, we tested whether TGF- β 1 expression was associated with neuropathology induced by four different TMEV infections. We found that TGF- β 1 was observed only in lesions of GDVII virus-infected mice and that TGF- β 1 expression negatively correlated with CD3⁺ T cell infiltration in the brain.

2. Materials and Methods

2.1. Animal experiments

Four-week-old female SJL/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were infected intracerebrally with 1×10^3 plaque forming units (PFU) of GDVII virus or 2×10^5 PFU of DA, DApB or DApBL2M virus. Each group consisted of 13 to 20 mice. One week after infection, mice were perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO).

2.2. Histology

Brains were divided into five coronal slabs and embedded in paraffin. Four-micrometer-thick sections were stained with Luxol fast blue. Apoptosis was detected using the TUNEL method (Tsunoda et al., 1997). TUNEL⁺ cells were visualized by the avidin-biotin peroxidase complex (ABC) technique (Vector, Burlingame, CA) with 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) as chromogen. Following antigen retrieval by trypsin, T cells were detected by immunohistochemistry with anti-CD3 ϵ antibody (1:30 dilution, Dako Corporation, Carpinteria, CA) (Mason et al., 1989; Tsunoda et al., 2000). TGF- β 1 was detected by immunohistochemistry using polyclonal antibody TGF β 1(V) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which is specific for TGF- β 1 and does not cross-react with other TGF- β family members. The adrenal cortex and the kidney were used as positive controls (Thompson et al., 1989). Laminin was detected by immunohistochemistry, using anti-laminin antibody (Sigma), following a 30-minute pretreatment with 0.0125% of pronase[®] protease (*Streptomyces griseus*, Calbiochem, San Diego, CA) in PBS at pH 7.5 (Kirkpatrick and d'Ardenne, 1984).

We enumerated all perivascular cuffs and TUNEL⁺ cells in each representative area, such as the cerebral cortex and thalamus, as well as the total area of five coronal brain sections from five mice per infection, with a light microscope at a magnification of $\times 100$, as described previously (Tsunoda et al., 2001). Correlation of TGF- β 1⁺ cells versus CD3⁺ T cells or versus vessels that were strongly laminin⁺ was carried out by enumeration of all positive cells in the microscopic field of brain lesions at a magnification of $\times 400$ or $\times 200$, respectively.

2.3. Enzyme-linked immunosorbent assay (ELISA)

An astrocyte cell line derived from SJL/J mice (McCright and Fujinami, 1997) was cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), antibiotics (Mediatech) and glutamine (Mediatech). A murine neuroblastoma cell line, Neuro-2a (American Type Culture Collection, Manassas, VA), was cultured in minimum essential media (MEM, Mediatech). Spleen cells were isolated from SJL/J mice with Histopaque[®]-1083 (Sigma), and cultured in RPMI 1640 medium (Mediatech). Cells were infected with GDVII, DA, DApB or DApBL2M virus at a multiplicity of infection (MOI) of 1. After a 1-hour adsorption, cells were re-fed with media supplemented with 1% FBS. TMEV- or sham-infected tissue culture supernatants were harvested, 24 hours p.i. We measured TGF- β 1 in the supernatants, using an ELISA kit (TGF- β 1 Quantikine[®] ELISA, R&D System, Inc., Minneapolis, MN) (Wang et al., 2003; Yamamoto et al., 1999).

3. Results

3.1. VP2 puff B mutant viruses induce severe neuronal apoptosis, similar to that of GDVII virus infection

One week after infection of SJL/J mice with GDVII, DA, DApB or DApBL2M virus, we compared the extent of apoptotic cells in the brains of infected-mice, using TUNEL staining (Tsunoda et al., 1997). Apoptotic neurons were prominent in the brains of mice infected with GDVII virus (Fig. 1a), while the number of apoptotic neurons in mice infected with DA virus was relatively low [Fig. 1c, (Tsunoda et al., 1997)] in all regions of the brain examined (Table 1). Interestingly, in mice infected with DApB or DApBL2M viruses, we found extensive neuronal apoptosis, particularly in the thalamus, the hippocampus and the pons (Table 1). In most mice, the majority of neurons in the pyramidal cell layer of the hippocampus were TUNEL⁺ (Fig. 1e, g). The extent of apoptotic neurons in DApB and DApBL2M infections was greater than in DA virus infection in most regions examined, while mice infected with GDVII virus tended to have more apoptotic cells than mice infected with the DA virus mutants (Table 1).

3.2. VP2 puff B mutant viruses induce substantial perivascular inflammation with T cell infiltration, similar to that of DA virus infection

We compared the extent of CD3⁺ T cell infiltration in the brains of mice infected with GDVII, DA, DApB or DApBL2M virus by immunohistochemistry. In GDVII virus infection, CD3⁺ T cell infiltrates were only sporadically present in the brain, and perivascular cuffing with CD3⁺ T cells was inconspicuous (Fig. 1b). T cell infiltration was more intense in regions of the brain containing no apoptotic cells rather than areas in which TUNEL⁺ neurons were present [data not shown, (Theil et al., 2000; Tsunoda et al., 1997)]. In contrast, T cell infiltrates were numerous in DA virus infection [Fig. 1d, (Theil et al., 2000; Tsunoda et al., 1996)]. Interestingly, in DApB and DApBL2M virus infections, large numbers of T cells were present in the perivascular cuffs and within the parenchyma of the brain. These areas contained large numbers of TUNEL⁺ cells, when consecutive sections were examined (Fig. 1f, h). The overall distribution and extent of the T cell infiltration in DA mutant virus infections were comparable to that seen in DA virus infection. Thus, alterations in VP1 loop II and VP2 puff B in DA mutant viruses did not affect the extent of T cell infiltration in the CNS.

3.3. Neuronal apoptosis is negatively correlated with inflammation only in GDVII virus infection

In GDVII virus infection, TUNEL⁺ cells were seen in areas where inflammatory cells were scarce (Fig. 2a). In contrast, overt inflammatory lesions contained only a few TUNEL⁺ cells (Fig. 2b). The numbers of TUNEL⁺ cells negatively correlated with perivascular inflammation ($r = -0.54$, $P < 0.05$, Fig. 3a). This is consistent with the general observation that apoptosis *per se* does not induce an inflammatory response (Kerr et al., 1972; Majno and Joris, 1995). While the numbers of TUNEL⁺ cells were greater in GDVII virus infection than in DA virus infection, we found more extensive perivascular inflammation in DA virus infection than in GDVII virus infection in most regions examined (Table 2).

Mice infected with DApB or DApBL2M virus also had more perivascular cuffing than mice infected with GDVII virus, particularly in the hippocampus ($P < 0.01$, compared with GDVII virus infection, ANOVA) (Table 2). Interestingly, in DApBL2M virus infection, the numbers of TUNEL⁺ cells positively correlated with perivascular inflammation ($r = 0.69$, $P < 0.01$, Fig. 3d). In DA and DApB virus infection, there was no significant correlation between TUNEL⁺ cells and perivascular cuffing (DA virus infection, $r = 0.07$, $P = 0.8$; DApB virus infection, $r = 0.23$, $P = 0.43$, Fig. 3b, c).

3.4. TGF- β 1 upregulation is associated with a lack of inflammation during GDVII virus infection

TGF- β 1 has been shown to modulate induction of apoptosis and suppress immune responses (Letterio and Roberts, 1998; Miyajima et al., 2000; Prud'homme and Piccirillo, 2000; Sasaki et al., 1992). Using immunohistochemistry, we investigated whether TGF- β 1 in the CNS was associated with mononuclear cell (MNC) infiltration and apoptosis in the CNS, 1 week after TMEV infection. During GDVII virus infection, we detected TGF- β 1 in areas where viral infection and apoptosis were seen (Fig. 2c), including the cerebral cortex, hippocampus, hypothalamus, mammillary nucleus and the injection site (Fig. 4a–d). TGF- β 1 was present predominantly in cells that were morphologically identified as neurons and their neurites (Fig. 2c).

TGF- β 1⁺ neurons were seen in areas where perivascular cuffing was absent or minimal (Fig. 2c). Although perivascular MNC infiltration was minimal in most areas of the brain infected with GDVII virus, MNC infiltration was seen in a few perivascular areas. Interestingly, around these perivascular cuffs, we found few if any TGF- β 1⁺ cells (Fig. 2d). This suggested that TGF- β 1 expression could contribute to suppression of MNC infiltration in the brain. Thus, we enumerated TGF- β 1⁺ neurons and CD3⁺ T cells in brain lesions of mice infected with GDVII virus. We found that the number of TGF- β 1⁺ cells negatively correlated with CD3⁺ T cell infiltration during GDVII virus infection ($r = -0.8$, $P < 0.001$, Fig. 5a). In contrast, in DA, DApB and DApBL2M virus infections, no TGF- β 1 was detected in the brain, including inflammatory lesions or areas containing large numbers of apoptotic cells (Fig. 4e–h). No TGF- β 1 was observed in the CNS of uninfected SJL/J mice, as previously reported [data not shown, (Thompson et al., 1989)].

TGF- β 1 is secreted in an inactive (latent) form that requires activation before it can exert a biologic effect (Border and Noble, 1994). The active form of TGF- β 1 is known to stimulate the deposition of extracellular matrix proteins, such as laminin and fibronectin (Border and Noble, 1997; Noble and Border, 1997). Upregulation of the matrix proteins indicates that TGF- β 1 in the tissue is biologically active (Logan et al., 1994; Wyss-Coray et al., 1995). Laminin is a consistent component of cerebral basement membrane, which is normally restricted to blood vessels, pia mater, ependyma and choroid plexus (Jucker et al., 1996). Using immunohistochemistry, we detected laminin in vessels, meninges, ependyma and choroid plexus (Fig. 4i) in uninfected SJL/J mice. In GDVII virus infection, we found upregulation of laminin in the endothelia of vessels, including endothelial cells in capillaries (Fig. 4j). The numbers of TGF- β 1⁺ cells positively correlated with the numbers of vessels strongly immunostained with laminin in lesions of GDVII virus infection ($r = 0.7$, $P < 0.001$, Fig. 5b). These results indicated the presence of the active form of TGF- β 1. In contrast, no upregulation was seen in the brains of mice infected with DA virus (Fig. 4k).

3.5. GDVII virus induces TGF- β 1 in vitro

Next, we tested whether direct TMEV infection could induce TGF- β 1 in murine cells *in vitro*. An astrocyte cell line, Neuro-2a, and spleen MNCs were infected with GDVII, DA, DApB or DApBL2M virus. We measured TGF- β 1 in TMEV- or sham-infected tissue culture supernatants, 24 hours p.i., using an ELISA. GDVII virus infection, but not the other TMEV- or sham-infection, induced TGF- β 1 in MNCs above the background levels present in media containing 1% FBS (TGF- β 1 concentration: 140 pg/ml in GDVII virus infection, 64 pg/ml in media). Although TGF- β 1 was detected in the supernatant from both uninfected Neuro-2a and astrocyte cultures (150 and 350 pg/ml, respectively), none of the different TMEV infections upregulated TGF- β 1 production in Neuro-2a cells or astrocytes above the background levels present in sham-infected cultures (data not shown).

4. Discussion

Here we report that two DA virus VP2 puff B mutants induced higher levels of neuronal apoptosis than wild-type DA virus. This suggests that VP2 puff B of TMEV is important for induction of apoptosis: alteration of VP2 puff B from DA virus (or GDVII-like VP2 puff B) contributes to higher levels of apoptosis. This is of interest since we previously reported that DApB and DApBL2M viruses replicate poorly in the CNS, compared to DA virus (Tsunoda et al., 2001). The negative association between induction of apoptosis and virus growth has been reported in picornavirus infections *in vitro* (Belov et al., 2003; Jelachich and Lipton, 1996, 2005). In *in vitro* poliovirus infection, productive infection resulted in necrotic cytopathic effect (CPE), whereas abortive infection caused by some mutant viruses or the presence of some inhibitors triggered apoptosis (Belov et al., 2003; Jelachich and Lipton, 1996, 2005). Similarly, induction of apoptosis by TMEV *in vitro* appears to depend on the cell's permissiveness for virus infection. Cells undergoing abortive TMEV infection apoptose, while permissive cells with a productive TMEV infection show CPE (Jelachich and Lipton, 1996, 2005). In our *in vivo* results, vigorous induction of apoptosis in DA mutant virus infection can be considered a defensive reaction, which prevents generation and spread of the viral progeny by premature cell death, resulting in poor virus replication in the CNS (Tsunoda et al., 1997). On the other hand, productive virus replication in *in vivo* GDVII virus infection could be attributed to one or more of the other sequence differences, outside the VP2 puff B, between DA and GDVII viruses.

We observed that both DA mutants induced high levels of inflammation comparable to that of DA virus infection, while GDVII virus infection resulted in low levels of inflammation. The recruitment of large numbers of T cells in apoptotic lesions in DA mutant virus infections was intriguing, since the presence of apoptotic cells generally does not lead to the recruitment of inflammatory cells (Kerr et al., 1972; Majno and Joris, 1995). Since T cell infiltration occurred regardless of mutations in DA mutants, the genomic differences between GDVII versus DA virus outside the VP1 loop II and VP2 puff B seem to be important in regulation of inflammation in the CNS.

One potential mechanism contributing to the distinct differences in pathology between TMEV infections is the differential production of cytokines between virus infections. Among cytokines, the multifunctional nature of TGF- β 1 could explain several neuropathological features in GDVII virus infection, including significant neuronal apoptosis, relative lack of T cell infiltration, as well as activation of macrophages and microglia (Tsunoda et al., 1996). TGF- β 1 has been reported to enhance apoptosis of neurons, suppress lymphocyte function, and activate and recruit macrophages (Letterio and Roberts, 1998; Miyajima et al., 2000; Prud'homme and Piccirillo, 2000; Sasaki et al., 1992). Previously, using RNase protection assays, we demonstrated similar levels of mRNA expression of TGF- β 1, TGF- β 2 and TGF- β 3 among CNS tissues from uninfected mice and mice infected with GDVII or DA virus, 1 week after infection (Theil et al., 2000). However, in our current study, using TGF- β 1 specific antibody, we found TGF- β 1 protein expression in neurons only in the brains of mice infected with GDVII virus, but not in the brains of mice infected with DA virus or uninfected mice. The discrepancy between mRNA versus protein expression of TGF- β 1 between our two studies could be explained by the different translation efficiencies of TGF- β 1 mRNA between mice infected with GDVII virus versus mice infected with DA virus or uninfected mice. On the other hand, Chang et al. (2000) did not detect TGF- β 1 mRNA expression in the CNS of uninfected mice, but did detect TGF- β 1 mRNA in DA virus infected mice (GDVII virus infected mice were not examined). They also detected TGF- β protein in meningeal MNC infiltrates in DA virus infection, using TGF- β antibody that recognize all three TGF- β isoforms (TGF- β 1, 2 and 3). The reasons for differences between their results versus our results are not known, but could be due to different sensitivities of the detection methods for TGF- β 1 mRNA and due to different

TGF- β 1 specificities of the antibodies between the two groups. Administration of another TGF- β isoforms, TGF- β 2, was shown to reduce demyelination and virus persistence in DA virus infection (Drescher et al., 2000).

Overall in TMEV infection of SJL/J mice, TGF- β 1 was associated with a lack of T cell infiltration in the CNS, but not with neuronal apoptosis. Upregulation of TGF- β 1 in neurons was seen only during GDVII virus infection, but not in infections with DA, DApB or DApBL2M viruses. TGF- β in neurons has also been reported in mice with experimental allergic encephalomyelitis (Fujinami, 2006; Issazadeh et al., 1998) and in rats with spinal cord injury (O'Brien et al., 1994). Although *in vivo* induction of TGF- β 1 specifically in CNS neurons by virus infections has not been reported, TGF- β 1 is produced or activated in other cell types by several virus infections [reviewed in (Fitzpatrick and Bielefeldt-Ohmann, 1999)], including Epstein-Barr virus (Xu et al., 2000), cytomegalovirus (Haagmans et al., 1997; Michelson et al., 1994), herpes simplex virus (Méndez-Samperio et al., 2000), human T-lymphotropic virus (HTLV)-I, human immunodeficiency virus (HIV) (Lotz and Seth, 1993; Wahl et al., 1991; Yamamoto et al., 1999), influenza virus (Schultz-Cherry and Hinshaw, 1996) and yellow fever virus (Quaresma et al., 2006).

Although the precise role of TGF- β 1 in GDVII virus infection remains to be clarified, endogenous immunosuppressive factors, including TGF- β 1, can play a protective role during host immune responses to prevent damage by the inflammatory response (protection versus immunopathology). However, in the case of GDVII virus infection, the immunosuppressive effect of TGF- β 1 might result in a fatal outcome due to the lack of anti-virus immune responses (Tsunoda et al., 1996). The unique pathology seen in GDVII virus infection, which is characterized by TGF- β 1 expression with poor inflammatory cell infiltration in apoptotic lesions, was similar to what was reported in the liver in human yellow fever virus infection (Quaresma et al., 2006).

TGF- β has also been shown to stimulate apoptosis of cells, including neurons (Miyajima et al., 2000), [reviewed in (Flanders et al., 1998)]. In influenza virus infection, TGF- β has been suggested to be activated by virus to induce apoptosis in the surrounding cells, thus limiting virus spread (Schultz-Cherry and Hinshaw, 1996). However, despite large numbers of apoptotic cells in DApB and DApBL2M virus infections, we could not detect TGF- β 1 in DA mutant virus infections. Therefore, in TMEV infection, TGF- β 1 is associated with suppression of T cell infiltrates, but not with neuronal apoptosis.

In vitro TGF- β 1 production has been shown in several cell types, including splenocytes (Haagmans et al., 1997; Méndez-Samperio et al., 2000) and astrocytes (Michelson et al., 1994; Morganti-Kossmann et al., 1992; Yong, 1996). Using ELISAs, we found induction of TGF- β 1 protein in spleen MNCs in GDVII virus infection, but not in the other TMEV infections. None of the TMEV infections upregulated the level of TGF- β 1 protein in Neuro-2a or astrocyte cell lines. We do not know why TGF- β 1 induction by TMEV differed among cell lines. However, induction of other cytokines by TMEV has been reported to be altered by several conditions such as cell type, maturation of cells, MOI, and incubation time after infection (Jelachich and Lipton, 2005; Kim et al., 2005; Palma et al., 2003). Although we did not detect upregulation of TGF- β 1 protein in an astrocyte cell line infected with DA virus at an MOI of 1, Palma et al. (2003) reported that infection of a primary astrocyte culture with the BeAn strain of TMEV at an MOI of 10 upregulated the mRNA of TGF- β 1 *in vitro* (Palma et al., 2003). In this case, the discrepancy in astrocyte infection between the two studies could be due to differences in detection methods (TGF- β 1 protein versus mRNA), MOIs, virus strains, and cell types (primary versus cell line). Although these results demonstrate that direct GDVII virus infection could induce TGF- β 1 production in some murine cells *in vitro*, we do not know whether *in vivo* TGF- β 1 production is induced by direct GDVII virus infection of neurons or

by some other factors associated with GDVII virus infection. Indeed, HIV infection has been shown to induce not only TGF- β production by infected cells, but also a factor(s) capable of triggering uninfected cells to secrete TGF- β (Wahl et al., 1991). Therefore, one virus most likely has several different ways of inducing TGF- β , depending on the site of infection and tissues/cells infected.

VP1 loop II and VP2 puff B have been suggested to influence the shape of the sialic acid binding site, reported as the “gap” (Zhou et al., 1997). In separate experiments, we found that DApBL2M virus showed decreased sialic acid binding similar to that of GDVII virus (Table 3, Tsunoda I et al., manuscript was submitted for publication). This supports the hypothesis that VP1 loop II and VP2 puff B form the sialic binding site “gap.” In contrast, sialic acid binding of DApB virus was similar to that of wild-type DA virus. Since DApB virus persistently infects and induces demyelination of the white matter in the spinal cord similar to wild-type DA virus (Table 3), the use of sialic acid could play a role in virus persistence *in vivo*. In reovirus infection, virus binding to sialic acid was shown to potentiate virus-induced apoptosis (Barton et al., 2001; Connolly et al., 2001). In our current experiments, however, we found similar levels of neuronal apoptosis in infections with two DA virus mutants that differ in sialic acid binding. Thus, the sialic acid-independent attachment step of virus might be a critical determinant for apoptosis induction in TMEV infection.

In summary, the alterations in VP2 puff B of DA virus mutants did not alter the numbers of T cells infiltrating the brain, compared with wild-type DA virus infection. TGF- β 1 upregulation was seen only in GDVII virus infection and TGF- β 1 expression negatively correlated with T cell infiltration. Therefore, TGF- β 1 could be responsible for the relative lack of T cell recruitment into the brain during GDVII virus infection in SJL/J mice. To further clarify the role of VP2 puff B and its interaction with VP1 loop II, we are currently investigating a GDVII virus mutant that has sequences of DA virus in VP1 loop II and VP2 puff B.

Acknowledgements

We thank Melina V. Jones, PhD, Lisa K. Peterson, BS and Yoshiaki Wada, MD, PhD for many helpful discussions, and Nancy K. Burgess, BS, Sarah E. Doyle, BS, Nikki J. Kirkman, BS, Li-Qing Kuang, MD, Faris Hasanovic, Daniel Scott Kennett, Russell J. Palmer, and J. Wes Peterson for excellent technical assistance. We are grateful to Ms. Kathleen Borick for preparation of the manuscript. This work was supported by NIH grant NS34497.

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Fig. 1. Neuronal apoptosis and T cell infiltration of the hippocampus of SJL/J mice one week post GDVII (a, b), DA (c, d), DAPB (e, f) or DAPBL2M (g, h) virus infection. Terminal deoxynucleotidyl transferase-mediated dUTP biotin nick-end labeling (TUNEL) was used to detect apoptosis by DNA fragmentation (a, c, e, g). In DA virus infection, a few neurons were TUNEL⁺ in the pyramidal cell layer of the hippocampus (c, arrowhead). In contrast, the majority of neurons in comparable areas were TUNEL⁺ in GDVII (a), DAPB (e), and DAPBL2M (g) virus infection (arrowhead). T cell infiltration was detected by immunohistochemistry, using anti-CD3 antibody (b, d, f, h). In GDVII virus infection, there were only a few T cells around vessels (b, arrow, inset), and parenchymal T cell infiltration

was sporadically seen. In contrast, we detected significant parenchymal T cell infiltration as well as perivascular cuffing, which contained more than two layers of T cells around vessels (inset), in DA (d), DApB (f), or DApBL2M (h) virus infections. Magnification: $\times 60$; inset $\times 180$. Results are representative of each group consisted of 13 to 20 mice.

Fig. 2.

TUNEL⁺ and transforming growth factor (TGF)- β 1⁺ cells were negatively associated with perivascular cell infiltration in the brains of mice infected with GDVII virus. (a, b) Apoptosis of neurons was detected with TUNEL. (a) Numerous TUNEL⁺ nuclei were detected in areas where cell infiltration was not seen in the parenchyma and perivascular space. TUNEL⁺ cells had fragmented nuclei (inset). Note: no cell infiltration around vessels (v). (b) In contrast, inflammatory lesions contained only a few TUNEL⁺ nuclei (arrowhead). (c, d) TGF- β 1 was detected by immunohistochemistry. (c) Substantial numbers of TGF β 1⁺ cells (arrow) were detected in areas where inflammatory cell infiltration was scarce both in the parenchyma and around vessels (v). TGF- β 1 was detected in the cell bodies and processes of neurons (inset). (d) In areas of severe inflammation, only a few TGF- β 1⁺ cells (arrow) were detected. Magnification: (a–d), $\times 100$; (a, inset), $\times 760$; (c, inset), $\times 370$.

Fig. 3.

Correlation of TUNEL⁺ cells with perivascular inflammation in GDVII (a), DA (b), DApB (c) or DApBL2M (d) virus infection. We enumerated numbers of TUNEL⁺ apoptotic neurons and perivascular cuffs in regions of the brain, including the cerebral cortex and thalamus, 1 week after TMEV infection. (a) TUNEL⁺ cells negatively correlated with perivascular cuffing in GDVII virus infection. (b, c) No correlation was found in DA or DApB virus infection. (d) In DApBL2M virus infection, there was a positive correlation between TUNEL⁺ cells and perivascular inflammation. Each dot represented the number of TUNEL⁺ cells and perivascular cuffs in one brain region, such as the cerebral cortex and thalamus. In each TMEV infection, 14 to 16 brain regions were examined.

Fig. 4. GDVII virus, but not DA or DA virus mutants, upregulated TGF- β 1 and laminin expression in the brain. (a–h) Immunohistochemistry against TGF- β 1. GDVII virus infection induced TGF- β 1 expression in neurons (a–d, arrow). (c, d) Higher magnification showed TGF- β 1 in processes and the cytoplasm, but not in the nucleus of neurons, whose cell bodies appeared triangular, with the typical morphology of neurons. No TGF- β 1 was detected in DA, DApB, or DApBL2M virus infections in the brain (e–h). (a, e), cerebral cortex; (b, f), hypothalamus; (c, g), mammillary nucleus; (d, h), injection site. (i–k) Immunohistochemistry against laminin in the cerebral cortex. In normal brain parenchyma, laminin was detected in blood vessels (i, arrow). In GDVII virus infection, upregulation of laminin was seen in endothelia (j). In DA virus infection, however, levels of laminin were not altered (k). Magnification: (a, b, e, f, i–k), $\times 90$; (c, g), $\times 180$; (d, h) $\times 360$. Results are representative of each group consisted of 13 to 20 mice.

Fig. 5.

Correlation of TGF- $\beta 1^+$ cells with CD3⁺ T cells (a) or vessels that strongly expressed laminin (b) in GDVII virus infection. (a) CD3⁺ T cell infiltrates negatively correlated with TGF- $\beta 1^+$ cells. (b) In contrast, numbers of laminin⁺ vessels positively correlated with numbers of TGF- $\beta 1^+$ cells. Brains were harvested from mice 1 week after GDVII virus infection, and immunostained against TGF- $\beta 1$, CD3, or laminin. Enumeration was carried out at a magnification of $\times 400$ (a) or $\times 200$ (b) in 15 (a) or 16 (b) microscopic fields.

Table 1
Numbers of TUNEL⁺ cells in the brain in GDVII, DA or DA mutant virus infection^a

Infection	Total ^b	Cerebral cortex	Thalamus	Hippocampus	Brainstem
GDVII	2995 ± 603 ^{**}	739 ± 184 ^{**}	879 ± 214 ^{**}	331 ± 187	229 ± 71
DA	827 ± 290	170 ± 75	60 ± 26	308 ± 151	5 ± 2
DΔpB	1424 ± 266	198 ± 64	377 ± 90 [*]	581 ± 83	179 ± 74
DΔpBL2M	1326 ± 184	142 ± 30	333 ± 82 [*]	572 ± 187	76 ± 54

^a Five brains per group were examined 1 week after infection with GDVII, DA, DΔpB, or DΔpBL2M virus. Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method.

^b Mean total numbers ± standard error of the mean (SEM) of TUNEL⁺ cells in five coronal brain sections per mouse.

^{*} , $P < 0.05$;

^{**} , $P < 0.01$: statistical difference, compared with DA virus infection by ANOVA

Table 2
Numbers of perivascular cuffing in the brain in GDVII, DA or DA mutant virus infection^a

Infection	Total ^b	Cerebral cortex	Thalamus	Hippocampus	Brainstem
GDVII	66 ± 6	11 ± 2	11 ± 3	2 ± 1	11 ± 1
DA	98 ± 8	24 ± 2 [*]	9 ± 1	44 ± 5 ^{***}	3 ± 1
DΔpB	73 ± 14	20 ± 4	11 ± 3	23 ± 7 ^{***}	3 ± 1
DΔpBL2M	93 ± 5	13 ± 3	11 ± 2	41 ± 4 ^{***}	10 ± 4

^a Five brains per group were examined 1 week after infection with GDVII, DA, DΔpB, or DΔpBL2M virus.

^b Mean total numbers ± SEM of perivascular cuffs in five coronal brain sections per mouse.

^{*} , $P < 0.05$;

^{***} , $P < 0.01$: statistical difference, compared with GDVII virus infection by ANOVA

Table 3

Comparison of GDVII, DA and DA mutant viruses

Virus	VP1 loop II	VP2 puff B	Sialic acid binding ^a	Acute phase		Chronic phase	
				Apoptosis	T cell infiltration	TGF-β1 expression	White matter infection and demyelination ^b
GDVII	GDVII ^c	GDVII ^c	–	+++	+	+	–
DA	DA ^d	DA ^d	+	+	+++	–	++
DAPB	DA ^d	GDVII ^c	+	++	+++	–	+
DAPBL2M	GDVII ^c	GD-DA ^e	–	++	+++	–	–

^a Pretreatment of baby hamster kidney (BHK)-21 cells with neuraminidase (sialidase) inhibited DA and DAPB virus replication but not replication of GDVII or DAPBL2M virus (Tsunoda et al., manuscript was submitted for publication).

^b DA and DAPB viruses caused demyelination in the white matter of the spinal cord with virus persistence during the chronic phase, while GDVII and DAPBL2M viruses infected the gray matter and did not induce demyelination (Tsunoda et al., 2001).

^c GDVII virus amino acids in either VP1 loop II or VP2 puff B.

^d DA virus amino acids in either VP1 loop II or VP2 puff B.

^e The VP2 puff B of DAPBL2M virus (RRT) has a two-amino-acid reversion from GDVII VP2 puff B (RQA) to DA VP2 puff B (SRT).