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Apoptosis of infiltrating T cells in the central nervous system of mice infected with Theiler's murine encephalomyelitis virus

Emilia L. Oleszak,[a,b,*] Brad E. Hoffman,[c] J. Robert Chang,[c] Ewa Zaczynska,[b] John Gaughan,[d] Christos D. Katsetos,[c,e] Chris D. Platsoucas,[c] and Nile Harvey[b]

[a] *Department of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA 19140, USA*
[b] *Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA 19140, USA*
[c] *Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, PA 19140, USA*
[d] *Department of Physiology (Biostatistics), Temple University School of Medicine, Philadelphia, PA 19140, USA*
[e] *Department of Pediatrics (Neurology) and Pathology and Laboratory Medicine, St. Christopher's Hospital for Children, and Department of Pediatrics, Drexel University College of Medicine, Philadelphia, PA 19134, USA*

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Abstract

Theiler murine encephalomyelitis virus (TMEV), DA strain, induces in susceptible strain of mice a biphasic disease consisting of early acute disease followed by late chronic demyelinating disease. Both phases of the disease are associated with inflammatory infiltrates of the central nervous system (CNS). Late chronic demyelinating disease induced by TMEV serves as an excellent model to study human demyelinating disease, multiple sclerosis. During early acute disease, the virus is partially cleared from the CNS by $CD3^+$ T cells. These T cells express Fas, FasL, negligible levels of Bcl-2 proteins and undergo activation-induced cell death as determined by TUNEL assay leading to resolution of the inflammatory response. In contrast, during late chronic demyelinating disease, and despite dense perivascular and leptomeningeal infiltrates, only very few cells undergo apoptosis. Mononuclear cells infiltrating the CNS express Bcl-2. It appears that the lack of apoptosis of T cells during late chronic demyelinating disease leads to the accumulation of these cells in the CNS. These cells may play a role in the pathogenesis of the demyelinating disease.

Keywords: Apoptosis; Theiler's virus; T cells

Introduction

Theiler murine encephalomyelitis virus (TMEV), a picoma virus, induces in susceptible strains of mice, such as SJL, biphasic disease, consisting of early acute disease and late chronic demyelinating disease (Oleszak et al., in press; Oleszak et al., 1995; Rodriguez et al., 1987; Theiler, 1937; Tsunoda and Fujinami, 1996). During early acute disease, the virus replicates in the gray matter of the brain predominantly in the neurons, but also in astrocytes, microglia/macrophages, and oligodendrocytes (Brahic et al., 1981; Levy et al., 1992; Lipton, 1975; Rodriguez et al., 1987). After early acute disease-resistant strains of mice, such as C57BL/6 (B6), clear the infection in contrast susceptible strains, such as SJL, develop chronic, inflammatory demyelinating disease associated with a low level of viral persistence mainly in macrophages, astrocytes, and oligodendrocytes (Brahic et al., 1981; Rodriguez et al., 1987; Theiler, 1937). This disease closely resembles multiple sclerosis (MS). Both phases of the disease are associated with perivascular and parenchymal inflammatory infiltrates comprising T cells, monocytes/macrophages, and some B cells (Dal Canto et al., 1996; Oleszak et al., in press; Oleszak et al., 1995; Tsunoda and Fujinami, 1996). Although both $CD4^+$ and $CD8^+$ T cells contribute to the clearance of the virus, lack of complete clearance has been attributed to low

* Corresponding author. Department of Anatomy and Cell Biology and Fels Institute for Cancer Research and Molecular Biology; Temple University School of Medicine, 3307 N. Broad Street, Philadelphia, PA 19140. Fax: +1-215-829-1320.
E-mail address: eoleszak@astro.temple.edu (E.L. Oleszak).

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level and late appearance of virus-specific cytotoxic T lymphocytes (CTL) (Dethlefs et al., 1997; Larsson-Sciard et al., 1997). Myelin damage during late chronic demyelinating disease is thought to be immunologically mediated and both $CD4^+$ and $CD8^+$ T cells may contribute to the process (Azoulay-Cayla et al., 2000; Inoue et al., 1999; Kang et al., 2000; Katz-Levy et al., 2000; Kim et al., 1999; Murray et al., 1998; Rivera-Quinones et al., 1998). Thus, T cells play a very important role in TMEV-induced disease, participating both in the clearance of the virus and in the damage of myelin.

Apoptosis regulates virus-induced T cell responses, immune suppression, appearance of memory T cells, and survival of target cells and it is essential for maintaining homeostasis in the immune system (Alcami and Koszinowski, 2000; Antia et al., 1998; Butz and Bevan, 1998; Grayson et al., 2000; Lenardo, 1996; Marten et al., 2000; Murali-Krishna et al., 1998; Van Parijs and Abbas, 1998). It is well established that acute infections with a number of viruses including lymphocytic choriomeningitis (LCMV), human immunodeficiency virus (HIV), measles virus, Epstein–Barr virus (EBV), and a number of others lead to 5- to 50-fold increase in virus-specific T cells, which eliminate the virus-infected cells, clearing the virus (Blattman et al., 2000; Butz and Bevan, 1998; Doherty and Christensen, 2000; Kottilil et al., 2000; Maini et al., 2000; Mongkolsapaya et al., 1999; Murali-Krishna et al., 1998). After elimination of the virus, there is a decline in T cell numbers through the apoptotic mechanism of activation-induced cell death (AICD), which involves T cell receptor (TCR) activation (Bossu et al., 1993; Green and Scott, 1994; Russell, 1995; Singer and Abbas, 1994). Activated T cells produce potentially hazardous molecules, such as TNF-α, IL-1, perforin, and granzymes. AICD is necessary to silence this massive immune/inflammatory response after clearance of the virus (Ahmed and Gray, 1996; Akbar and Salmon, 1997; Akbar et al., 1994; Matloubian et al., 1999; Swain et al., 1996). Thus, the expansion of T lymphocytes after the immune response to the virus during early acute disease is followed by elimination of these cells by apoptosis. Only a small proportion of virus-specific T cells survive, to become memory cells (reviewed by Ahmed and Gray, 1996; Swain et al., 1996). AICD is mediated by the Fas (CD95)/FasL (CD95L) and TNF-receptor type I (TNF-RI) pathways, both of which share a common cell death domain (Brunner et al., 1995; Dhein et al., 1995; Strasser et al., 1995; Swain et al., 1996; Zheng et al., 1995). Fas belongs to the TNF/nerve growth factor receptor family; it is ubiquitously expressed on lymphoid cells and is significantly induced following activation of T cells (for review see Wallach et al., 1998). FasL belongs to the TNF family and is expressed constitutively only in immune-privileged sites, such as eyes or testes levels on naive T cells, but its expression is significantly upregulated following T cell activation (Beutler and van Huffel, 1994; Brunner et al., 1995; Cohen and Eisenberg, 1991; Dhein et al., 1995). Activation of the Fas/FasL pathway results in induction of a specific death-inducing signaling complex (DISC), which triggers the sequential activation of caspases (reviewed in Budihardjo et al., 1999). On the cellular level, activation of Fas/FasL on T cells through TCR leads to apoptotic cell death characterized by DNA fragmentation, chromatin condensation, and cytoplasmic shrinkage with preservation of membrane integrity (Odaka et al., 1990; Wyllie et al., 1984). Fas-mediated death is regulated by a number of molecules, including Bcl-2, a protooncogene, which is expressed on the mitochondrial outer membrane and protects cells against many mechanisms of cell death (reviewed by Chao and Korsmeyer, 1998).

Cytokines also play an important role in regulating apoptotic cell death of T cells (reviewed by Lenardo et al., 1999). IL-2 is a particularly important cytokine, because, while it predisposes T cells to apoptosis, it also prevents apoptosis of activated T cells through upregulation of Bcl-2 (Refaeli et al., 1998; Van Parijs et al., 1999). Thus, virus-induced T cell responses are tightly regulated by the Fas/FasL pathway, members of Bcl-2 family, and cytokines. The importance of Fas-mediated apoptosis in controlling homeostasis of the immune response has been demonstrated in both mice and humans. Mice or humans with genetically defective Fas or FasL develop systemic autoimmunity, leading to such diseases as Hashimoto thyroiditis in humans and autoimmune diabetes in nonobese diabetic mice, as well as other diseases (Chervonsky et al., 1997; Cohen and Eisenberg, 1991; Giordano et al., 1997). Tsunoda et al. (1997) have shown that during early acute disease neurons from TMEV-infected mice are the predominant cell types that undergo apoptosis. However, during late chronic demyelinating disease, cells stained with antibodies to carbonic anhydrase (presumably, oligodendrocytes) and macrophages showed apoptotic features (Tsunoda et al., 1997). In vitro studies using the GDVII strain of TMEV, which induces only gray matter disease, revealed that this virus induces apoptosis in restrictive but not in permissive cells (Jelachich and Lipton, 1996). However, the fate of the central nervous system (CNS)-infiltrating T cells in TMEV-infected mice has not been examined.

The purpose of this study has been to determine the fate of inflammatory infiltrating cells in the CNS of TMEV-infected mice during early acute and late chronic demyelinating disease. We have also examined the expression of molecules participating in Fas/FasL-induced cell death, antiapoptotic genes such as Bcl-2, and certain cytokines.

Results

Apoptosis in the CNS of TMEV-infected mice

Histopathological examination of the CNS of TMEV-infected SJL mice, which are susceptible to TMEV-induced demyelination, at various time intervals postinfection (p.i.)

(3, 6, 10, 42, 62, 107, and 170 days p.i.) revealed a typical biphasic disease comprising early acute disease that resembles polioencephalomyelitis followed by late chronic demyelinating disease. In contrast, B6 mice, which are resistant to TMEV-induced demyelinating disease, developed only early acute disease and cleared the virus without developing subsequently late chronic demyelinating disease. These histopathological results are typical of those previously reported by ourselves (Chang et al., 2000; Oleszak et al., 1988, 1995, 1997), and others (reviewed by Dal Canto et al., 1996; Monteyne et al., 1997; Tsunoda and Fujinami, 1996). In this study, we investigated whether apoptotic cell death occurs in the CNS of TMEV-infected mice during early acute disease and late chronic demyelinating disease.

Apoptosis during early acute disease

At 3 days p.i. very few infiltrating mononuclear cells were detected in the CNS of TMEV-infected SJL and B6 mice. None of them were apoptotic (data not shown). However, the titer of the virus in the CNS of TMEV-infected SJL mice reached maximum levels at 3 days p.i. (1.3×10^6 PFU/g) and it was reduced by approximately 3 logs (5×10^2 PFU/g) at 10 days p.i. At 6, 7, and 10 days p.i. a brisk inflammatory response with microscopic foci of brain tissue necrosis was present in the CNS of TMEV-infected SJL mice (focally necrotizing encephalitis). It consisted of inflammatory infiltrates of mononuclear cells (MNC) involving predominantly the regions of the basal ganglia and thalamus and exhibiting a distinctive perivascular topographic predilection. A noteworthy number of MNC particularly in perivascular cuffs (and also in the intervening neuropil) were TUNEL+ (Figs. 1A–C). At 6 days p.i. there were no differences in the extent and distribution of TUNEL + MNC between TMEV-infected SJL and B6 mice (data not shown). By light microscopy, TUNEL+ cells were morphologically suggestive of apoptotic cells (characterized by chromatin condensation and cellular shrinkage). TUNEL staining of CNS tissue from TMEV-infected SJL mice showed that cells undergoing apoptosis were located predominantly in perivascular cuffs. Fig. 1B shows a higher magnification of a blood vessel in the basal ganglia (7 days p.i.), demonstrating dense perivascular cuffing of lymphocytes and to a lesser degree monocytes. Several TUNEL+ apoptotic cells were predominately lymphocytes, some of which were engulfed within the cytoplasm of monocytes/macrophages.

To identify the cell types undergoing apoptosis, brain tissue sections immediately adjacent to tissue shown in Fig. 1B have been double-labeled for $CD3^+$ T cells and for TUNEL+ cells. TUNEL+ cells stained brown (DAB), whereas CD3-positive cells show a purple membrane/cytoplasmic staining (VIP) (Fig. 1C). Apoptotic cells identified within the perivascular cuffs were predominantly $CD3^+$ T cells (Fig. 1C). Fig. 1 shows the topographical relationships of these mononuclear inflammatory cells during early acute disease. It should be noted that the bulk of inflammatory cells (predominantly $CD3^+$ T lymphocytes) undergoing apoptosis is concentrated in the leptomeninges and in the perivascular (Virchow-Robbin spaces) of the CNS parenchyma. Figs. 1B, E, and F depict unequivocal containment of TUNEL+ lymphocytes in the cytoplasm of larger cells situated at the rim of the perivascular inflammatory cuffs, which exhibit overt, unambiguous morphological features of monocytes/macrophages.

Immunohistochemical staining using a TMEV-specific monoclonal antibody (mAb) revealed robust cytoplasmic staining of presumptive macrophages within an area of acute encephalitis at 7 days p.i. (Fig. 1D, left). The same anti-TMEV mAb stained a number of cells presumably infected with TMEV (data not shown), as described (Tsunoda et al., 1997). No evidence of staining with the anti-TMEV antibody was observed (Fig. 1D, right) in a deeper section of the same blood vessel as in Fig. 1B (×1000). This suggests that the primary role of macrophages in the perivascular cuffs is to remove apoptotic T cells and not to serve as a viral reservoir. Light background staining of endothelial cells is not specific and has been observed in tissue stained with control antibodies (data not shown).

TUNEL staining of CNS tissue from TMEV-infected SJL mice showed that cells undergoing apoptosis were located predominantly in perivascular cuffs. In addition, a number of TUNEL+ neurons (Fig. 1E) or macrophages (Fig. 1F) were detected in the CNS in areas of active encephalitis at days 3, 6, and 10 p.i. in TMEV-infected SJL or B6 mice. TUNEL+ neurons in areas of necrotizing encephalitis do not show an apoptotic morphology. It is possible that the TUNEL+ neurons are undergoing incipient necrosis (Fig. 1E). Apoptosis involving predominantly lymphocytes (l), and to lesser extent macrophage/microglia (m), were identified in the neuropil of the basal ganglia away from perivascular cuffs (Fig. 1F). This process ultimately leads to resolution of the inflammatory cuffs. Few TMEV-infected macrophages infiltrating parenchyma were also TUNEL+, in agreement with the findings of Tsunoda et al. (1997).

Apoptosis during late chronic demyelinating disease: immunohistochemical analysis

This phase of the disease is associated with spinal cord parenchymal (intramedullary) demyelinating lesions and heavy leptomeningeal and perivascular infiltration of MNC. Fig. 2 (as well as Fig. 1, see above) shows the topographical relationships of the infiltrating mononuclear inflammatory cells during TMEV-induced disease. Quantitative comparison of TUNEL+ $CD3^+$ cells between early acute disease (brain) and late chronic demyelinating disease (spinal cord) is shown in Fig. 3 and has been carried out by double staining determinations for $CD3^+$ cells and ex vivo TUNEL+ cells using flow cytometry.

In contrast to early acute disease induced by TMEV (Fig. 1), very few TUNEL+ MNC (presumptive apoptotic lymphocytes) were detected in the spinal cord of TMEV-infected SJL mice at 42 days p.i. (Fig. 2A), 62 days p.i. (Fig.

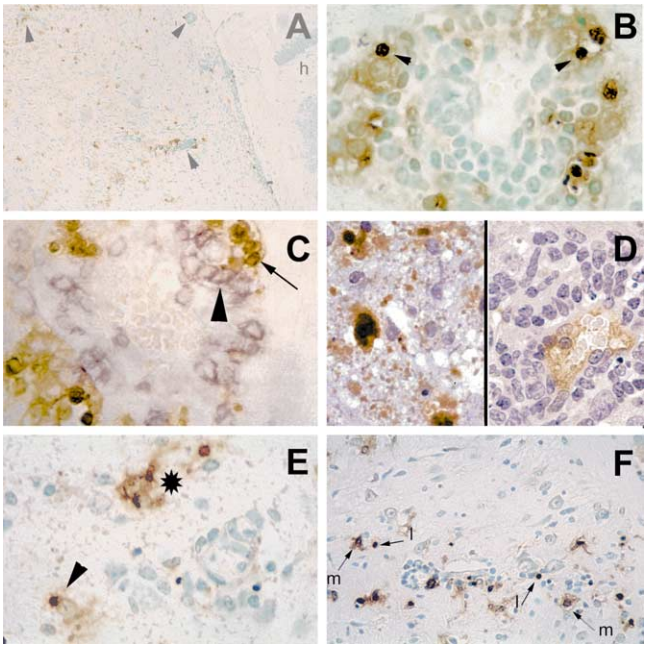

Fig. 1. Apoptosis in the brain of TMEV-infected SJL mice during the encephalitic phase of the disease. (A) Staining was performed on paraffin-embedded brain tissue, 6 days p.i., using the TUNEL method and DAB as the immunoperoxidase chromogen. Florid deep gray matter inflammation involving the basal ganglia is observed. Perivascular mononuclear cell infiltrates (arrowheads) egress into the surrounding brain substance. Increased TUNEL localization is restricted to areas of inflammation. No evidence of apoptosis in neurons of the nearby unaffected hippocampus (h), ×100. (B) High magnification of a blood vessel in the basal ganglia (7 days p.i.) showing dense perivascular cuffing of lymphocytes and to a lesser degree of monocyte/macrophages. Several TUNEL+ apoptotic cells are predominantly lymphocytes, some of which are engulfed within the cytoplasm of macrophages (arrowheads), ×1000. (C) Colocalization of TUNEL+ and $CD3^+$ cells in inflammatory mononuclear cells in the brain of TMEV-infected SJL mice at 7 days p.i. Double immunoperoxidase staining of perivascular infiltrates using TUNEL and anti-CD3 monoclonal antibody. TUNEL+ cells appear brown (DAB substrate; arrows), whereas $CD3^+$ T cells show a purple membranous/cytoplasmic staining using VIP substrate (arrowheads). Numerous TUNEL+ apoptotic mononuclear cells are morphologically similar to the adjoining $CD3^+$ T lymphocytes, ×1000. (D) Robust cytoplasmic staining with anti-TMEV antibody of presumptive macrophages within an area of acute encephalitis at 7 days p.i. (left), and no evidence of staining with anti-TMEV antibody (right) in a deeper section of the same blood vessel as in B, ×1000. Light brown staining of endothelial cells is not specific and has been observed in tissue slides stained with control antibodies (not shown). (E) 7 days p.i. showing TUNEL localization in a rare neuron in a field of inflammation (asterisk) and in a lymphocyte within the cytoplasm of a macrophage (arrowhead), ×400. (F) A nearby field in the basal ganglia showing apoptosis involving predominantly lymphocytes (l), and to lesser extent macrophage/microglia (m), ×400.

2B), 107 days p.i. (Fig. 2C), and 170 days p.i. (Fig. 2D). Few cells (dark stain) present outside of the perivascular cuff in Fig. 2B represent cells of the monocyte/macrophage lineage or oligodendrocytes. In contrast, inflammatory MNC within perivascular cuffs are not TUNEL positive. Numerous apoptotic cells, present in the upper corner of Fig. 2C, are cells of bone marrow of vertebral spine adjacent to spinal cord. Cells of bone marrow are continuously undergoing apoptosis and this is a normal physiological process. In contrast, very few cells in perivascular cuffs present in Fig. 2A to D are undergoing apoptosis.

Quantitative analysis by flow cytometry of mononuclear cells undergoing apoptosis in the CNS of TMEV-infected mice during early acute and late chronic demyelinating disease employing ex vivo TUNEL assay combined with anti-CD3 staining

Quantitative analysis of apoptosis of $CD3^+$ T cells infiltrating the CNS of TMEV-infected mice was carried out by

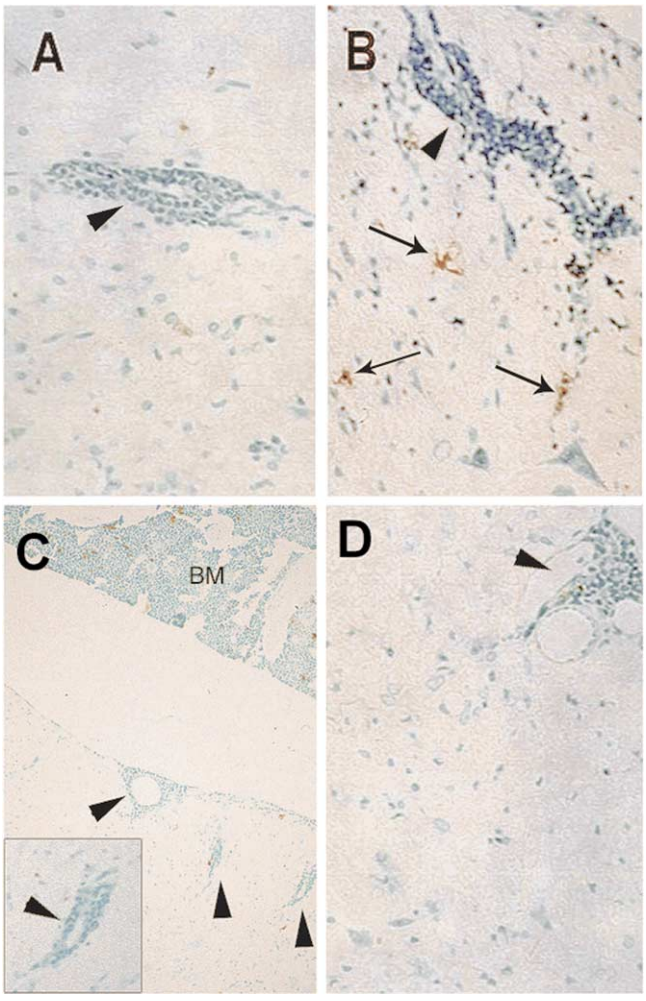

Fig. 2. Paucity of apoptosis during late chronic demyelinating disease in TMEV-infected SJL mice (spinal cords) at 42 days p.i. (A), 62 days p.i. (B), 107 days p.i. (C), and 170 days p.i. (D). Despite the presence of heavy perivascular and leptomeningeal infiltrates, only rare apoptotic mononuclear inflammatory cells are identified. Arrowheads depict aggregates (cuffs) of perivascular inflammatory cells (A, B, C, D). There are only rare TUNEL+ cells outside the perivascular cuffs, which may represent cells of monocyte/macrophage lineage or oligodendrocytes (arrows) (B). Inset in C is a higher magnification of one of the cellular cuffs showing paucity of TUNEL staining in inflammatory cuffs. Note the presence of numerous TUNEL+ cells within the bone marrow (BM) of the vertebral spine at the corresponding level of the spinal cord (Fig. 2C). Original magnifications (A, B, and C) ×100 [inset in C, ×400], and D, ×400.

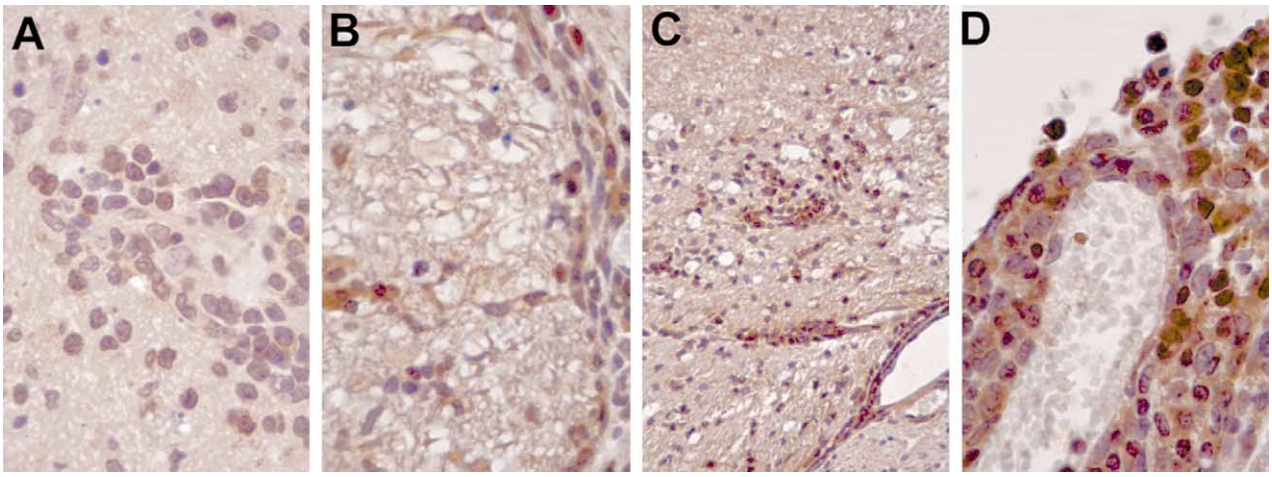

Fig. 7. Immunohistochemical detection of Bcl-2 in the CNS of TMEV-infected SJL mice at (A) 7 days p.i. (brain), (B) 40 days p.i. (spinal cord), (C) 107 days p.i. (spinal cord), and (D) 170 days p.i. (spinal cord). Staining was performed on 5 μM paraffin-embedded sections with ABC immunoperoxidase (DAB) method with hematoxylin counterstaining. (A) Early acute disease (encephalitic phase): lack of Bcl-2 expression in mononuclear inflammatory cell infiltrates in perivascular cuff in the thalamic region, ×1000. (B) Late chronic demyelinating disease (demyelinating phase): Bcl-2 expression in mononuclear inflammatory cells in the subarachnoid space overlying an area of demyelination, ×400. (C and D) Late chronic demyelinating disease (demyelinating phase): widespread localization of Bcl-2 in densely aggregated perivascular mononuclear inflammatory cells in the leptomeninges of the spinal cord observed at 107 and 170 days p.i., respectively. C: ×400; D: ×1000. Although these immunohistological preparations (A to D) were examined at different magnifications, identical results were obtained in each panel.

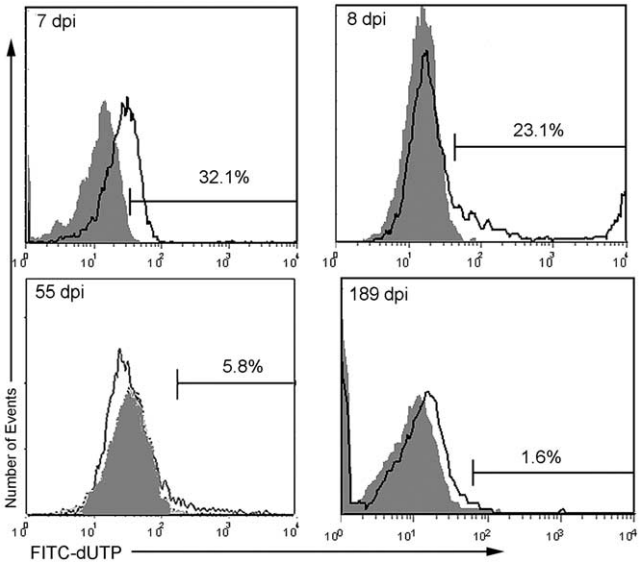

Fig. 3. Apoptosis of $CD3^+$ T cells infiltrating the CNS of TMEV-infected mice determined during early acute disease (brain; at 7 and 8 days p.i.) and at late chronic demyelinating disease (spinal cord at 55 and 189 days p.i.). Apoptosis ($CD3^+$ TUNEL+ cells) was measured by ex vivo TUNEL assay. Mononuclear cells were isolated from either the brain or the spinal cord, as described under Materials and methods. Cells were first labeled with PerCP-conjugated anti-CD3ε mAb, followed by staining with terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP (ex vivo TUNEL assay), as described under Materials and methods. Samples were analyzed by flow cytometry. Unshaded histograms represent fluorescence intensity of TUNEL+ cells electronically gated on the $CD3^+$ T cell population. Shaded histograms show fluorescence intensity of control samples, where TdT was omitted, electronically gated on the $CD3^+$ T cell population.

flow cytometry in double staining experiments employing ex vivo TUNEL assay combined with $CD3^+$ T cell surface staining. To assure that all T cells (present in perivascular cuffs, parenchyma, and meninges of TMEV-infected mice) are included in the determination, we isolated mononuclear cells from the CNS of TMEV-infected mice using Percoll gradients, as described under Materials and methods. Staining for $CD3^+$ TUNEL+ cells was carried out as described under Materials and methods. For the ex vivo TUNEL staining FITC-conjugated dUTP was used. For staining of $CD3^+$ T cells PerCP-anti-CD3 mAb was employed. Results from four experiments of double immunofluorescence staining of $CD3^+$ TUNEL+ cells is shown in Fig. 3. Two of the experiments shown are from early acute disease; the remaining two are from late chronic demyelinating disease. Each data point was obtained using pooled cells from the CNS of five to seven TMEV-infected mice. Cells were first gated for $CD3^+$ T cells. During early acute disease, at 7 and at 8 days p.i. 32.1 and 23.1% of the $CD3^+$ cells, respectively, were undergoing apoptosis ($CD3^+$ TUNEL+). In contrast, during late chronic demyelinating disease, at 55 and at 189 days p.i. 5.8 and 1.6% of the $CD3^+$ cells, respectively, were undergoing apoptosis ($CD3^+$ TUNEL+). Statistical analysis (five independent experiments) was carried out as described under Materials and methods. The differences obtained in the $CD3^+$ TUNEL+ fractions between early acute disease and late chronic demyelinating disease were significant ($P = 0.0311$).

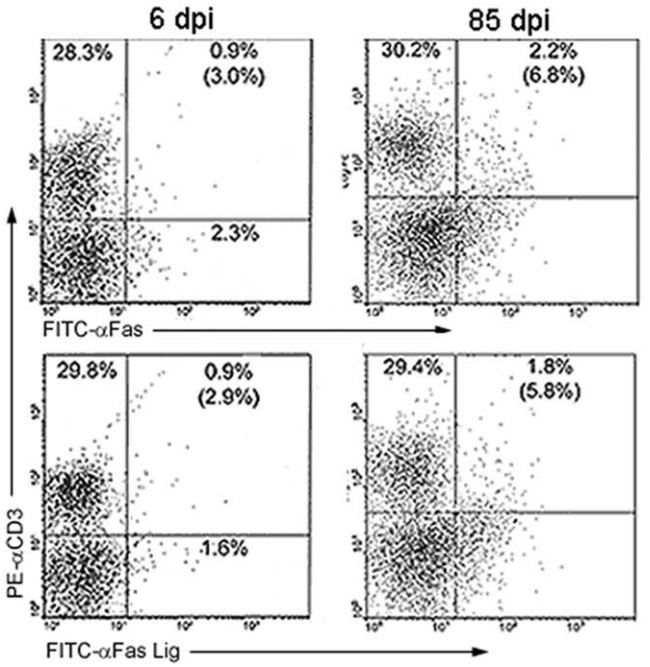

Fig. 4. Expression of Fas and FasL on $CD3^+$ T cells in spleen of TMEV-infected mice at 6 and 85 days p.i. using flow cytometry. Cells were first labeled individually with PE-conjugated anti-CD3 antibodies, washed, and then labeled with purified monoclonal antibodies to either Fas or FasL, followed by labeling with biotinylated secondary antibodies FITC-conjugated as described under Materials and methods. The fluorescence intensity was recorded in the log mode. Numbers in brackets indicate percentages of double-positive cells as a proportion of $CD3^+$ T cells only.

Expression of Fas and FasL in the CNS of TMEV-infected mice during early acute and late chronic demyelinating disease

The Fas/FasL (CD95/CD95L) pathway has been implicated in AICD of clonally expanded T cells. Using flow cytometric analysis low levels of Fas+ $CD3^+$ (5.4%) and FasL+ $CD3^+$ (5.7%) cells were found in the spleen of mock-infected SJL mice at 6 days p.i. Similar levels of Fas+ $CD3^+$ and FasL+ $CD3^+$ cells have been reported by others in the spleen of SJL mice using the same mAbs (Gao et al, 2001).

Expression of Fas+ $CD3^+$ and FasL+ $CD3^+$ cells in the spleen of TMEV-infected SJL mice at 6 days p.i. was 3.0 and 2.9%, respectively (Fig. 4). At 85 days p.i., the proportions of Fas+ $CD3^+$ and FasL+ $CD3^+$ cells in the spleen of TMEV-infected SJL mice were 6.8 and 5.8%, respectively (Fig. 4). Statistical analysis of five independent determinations revealed that there were no significant differences in the expression of Fas+ $CD3^+$ cells in spleens of TMEV-infected SJL mice during early acute vs late chronic demyelinating disease ($P = 0.2116$). Statistical analysis of four independent determinations revealed that there were no significant differences in the expression of FasL+ $CD3^+$ cells ($P = 0.5203$) in the spleens of TMEV-infected SJL mice during early acute vs late chronic demyelinating disease.

In contrast, expression of Fas+ $CD3^+$ and FasL+ $CD3^+$ cells was much higher in the brain than the spleen of TMEV-infected SJL mice. At 6 days p.i. 40.6 to 48% of the inflammatory infiltrates were $CD3^+$ T cells. Fas+ $CD3^+$ cells accounted for 24% of the brain infiltrating $CD3^+$ T cells at 6 days p.i. (Fig. 5) and for 21% at 7 days p.i. FasL+ $CD3^+$ cells accounted for 9% of the brain infiltrating $CD3^+$ T cells at 6 days p.i. (Fig. 5) and for 19% at 7 days p.i. Representative results are shown in Fig. 5. Expression of Fas and FasL on $CD3^+$ T cells suggests T cell receptor engagement resulting likely in triggering of AICD of infiltrating T cells during early acute disease.

We have also examined the expression of Fas and FasL antigens on T cells infiltrating the spinal cord during late chronic demyelinating disease. At 85 days p.i. 13 to 15% of the mononuclear inflammatory infiltrates in the spinal cord were $CD3^+$. The proportions of Fas+ $CD3^+$ T cells infil-

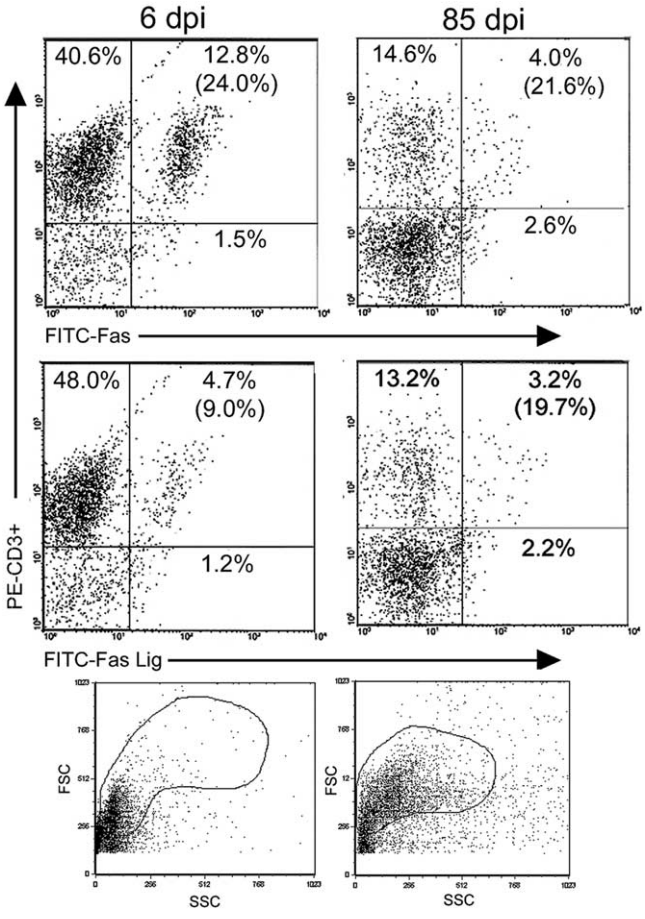

Fig. 5. Expression of Fas and FasL on $CD3^+$ T cells infiltrating the CNS of TMEV-infected SJL mice at 6 (brain) and 85 (spinal cord) days p.i. using flow cytometry. The fluorescence intensity was recorded in the log mode. The percentages of $CD3^+$, Fas+, and FasL+, and double-positive cells $CD3^+$/Fas+ and $CD3^+$/FasL+ in total inflammatory infiltrate are given in each panel. Numbers in parentheses indicate percentages of double-positive cells as a proportion of $CD3^+$ T cells only. For details, see legend to Fig. 4. Representative FSC and SSC patterns, including the lymphocyte gate used for the analysis, is shown for both time points at the bottom of Fig. 5.

Table 1
Expression of Bcl-2 on $CD3^+$ T cells in the CNS and spleen of TMEV-infected and mock-infected SJL mice

dpi	Percentage of $CD3^+$ Bcl-2+ cells (%)		
	TMEV CNS	TMEV spleen	Mock spleen
	Early acute disease[a]		
8[b]	1.3	11.04	18.5
8[b]	0.7		
	Before onset of demyelinating disease		
33[c]	9.8	ND	ND
	Late chronic demyelinating disease[a]		
100[c]	21.54	12.23	20.18
101[c]	24.4	ND	ND
140[c]	52.9	ND	ND
142[c]	53.0	39.7	22.0
145[c]	32.5	38.1	ND

Note. SJL mice were TMEV-infected or mock-infected as described under Materials and methods. At the indicated times p.i. mice were perfused with PBS and mononuclear cells were isolated from the CNS (either brain or spinal cord) and spleen as described under Materials and methods. Mononuclear cells were double labeled with PerCP-conjugated anti-CD3 mAb and FITC-conjugated anti-Bcl-2 mAb and analyzed by flow cytometry as described. ND, not done.

[a] Early vs late disease $P = 0.0099$.

[b] Brain of TMEV-infected mice.

[c] Spinal cord of TMEV-infected mice.

trating the spinal cord were examined at 85 (Fig. 5), 102, and 142 days p.i. and were 21.6 (Fig. 5), 12.8 and 9%, respectively. Statistical analysis of five independent determinations revealed significant differences ($P = 0.0088$) in the expression of Fas+ $CD3^+$ cells in the brain of TMEV-infected SJL mice during early acute disease vs the spinal cord of these mice during late chronic demyelinating disease. The proportions of FasL+ $CD3^+$ T cells infiltrating the spinal cord during late chronic demyelinating disease were determined at 85 (Fig. 5) and 100 days p.i. and were 19.7 (Fig. 5) and 20%, respectively. Statistical analysis of four independent determinations revealed no significant differences ($P = 0.1039$) in the expression of FasL+ $CD3^+$ cells in the brain of TMEV-infected SJL mice during early acute disease vs the spinal cord during late chronic demyelinating disease.

Expression of Bcl-2 in the CNS of TMEV-infected SJL mice during early acute and late chronic demyelinating disease

Flow cytometric analysis of Bcl-2 expression on $CD3^+$ T cells infiltrating the brain of TMEV-infected SJL mice during early acute disease at 8 days p.i. revealed negligible levels of expression of this antiapoptotic gene (range 0.7–1.3%) (Table 1 and Fig. 6). In contrast to negligible levels of Bcl-2 expression on T cells infiltrating the brain of TMEV-infected mice during early acute disease, $CD3^+$ Bcl-2+ cells accounted for 9.8% of the $CD3^+$ T cells in the spinal cord of TMEV-infected SJL mice before onset of demyelinating disease at 33 days p.i., 21.5% of $CD3^+$ T cells in the spinal cord of TMEV-infected SJL mice during late chronic demyelinating disease at 100 days p.i., 24.4% of $CD3^+$ T cells at 101 days p.i., 52.9% of $CD3^+$ T cells at 140 days p.i., 53% of $CD3^+$ T cells at 142 days p.i., and 32.5% of $CD3^+$ T cells at 145 days p.i. (Table 1 and Fig. 6). Statistical analysis was carried out as described under Materials and methods and revealed significant differences in the expression of $CD3^+$ Bcl-2+ cells in the brain of TMEV-infected SJL mice during early acute disease vs the spinal cord of these mice during late chronic demyelinating disease ($P = 0.0099$) (Table 1).

Analysis of T cells in the spleen of TMEV-infected mice at 8 days p.i. revealed that $CD3^+$ Bcl-2+ cells accounted for 11% of $CD3^+$ T cells (Table 1), which is 10 times higher than that of the proportions (1%) of $CD3^+$ Bcl-2+ cells in the brain of TMEV-infected mice at 8 days p.i. These results suggest that the $CD3^+$ T cells, which infiltrate the brain of TMEV-infected SJL mice at 8 days p.i., either downregulate the expression of Bcl-2 or that the only T cells that are able to migrate to the brain are those which express minimal levels of Bcl-2. In either case, expression of Fas+ $CD3^+$ and FasL+ $CD3^+$ T cells and virtual absence of Bcl-2 on these cells may account for efficient elimination of infiltrating T cells by AICD from the brain of TMEV-infected SJL mice after viral clearance. These results support the hypothesis that viral clearance in TMEV-infected SJL mice is followed by downsizing of the inflammatory response through AICD. The proportions of $CD3^+$ Bcl-2+ cells in the spleen of TMEV-infected mice during late chronic demyelinating disease were 12.2% at 100 days p.i., 39.7% at 142 days p.i., and 38.1% at 145 days p.i. (Table 1).

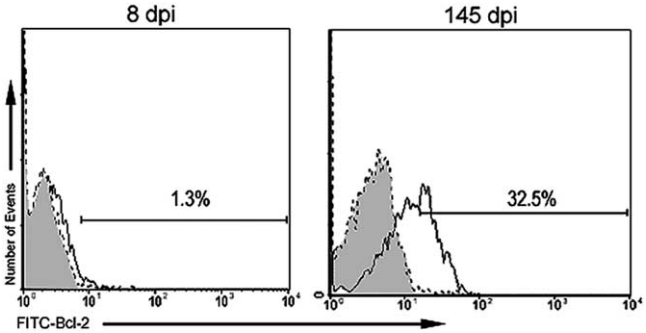

Fig. 6. Expression of Bcl-2 on $CD3^+$ T cells infiltrating the CNS of TMEV-infected SJL mice at 8 (brain) and 145 (spinal cord) days p.i. Infiltrating mononuclear cells were isolated from the CNS, labeled with PE-conjugated anti-CD3 mAb, permeabilized, stained with FITC-conjugated anti-Bcl-2 mAb, and analyzed by flow cytometry, as described under Materials and methods. Unshaded histograms show fluorescence intensity of Bcl-2 expressing cells electronically gated on the CD3-positive population. Shaded histograms show fluorescence intensity of cells stained with the isotype control of the anti-Bcl-2 mAb, electronically gated on the CD3-positive population. The percentage of double-stained ($CD3^+$ Bcl-2+) cells in inflammatory infiltrates are indicated.

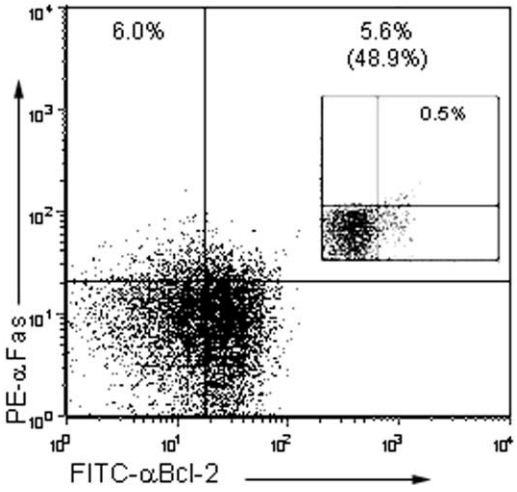

Fig. 8. Expression of Bcl-2 on double-positive $CD3^+$/Fas+ T cells infiltrating the CNS of TMEV-infected mice (three-color analysis). Mononuclear cells were first labeled with PerCP-conjugated anti-CD3 mAb, followed by anti-Fas mAb. $CD3^+$/Fas+ double-positive cells were fixed, permeabilized, and labeled with FITC-conjugated anti-Bcl-2 mAb and were analyzed by flow cytometry as described under Materials and methods. Isotype control antibody staining is shown in the upper right quadrant of the figure.

Expression of Bcl-2 on $CD3^+$ T cells isolated from the spleen of mock-infected SJL mice is shown in Table 1. There were no statistically significant differences ($P = 0.1439$) in the expression of $CD3^+$ Bcl-2+ cells in the spleen of mock-infected SJL mice at 8 days p.i. (18.5%) vs 100 days p.i. (20.2%) and 142 days p.i. (22%).

Immunohistochemical staining confirmed lack of expression of Bcl-2 in inflammatory brain cuffs during early acute disease (Fig. 7A). There are no positive cells in Fig. 7A. In contrast, high expression of Bcl-2 in perivascular mononuclear inflammatory cells was evident in the spinal cord during late chronic demyelinating disease, at 40 (Fig. 7B), 107 (Fig. 7C), and 170 (Fig. 7D) days p.i. Three-color flow cytometric analysis of the inflammatory infiltrate isolated from the spinal cord of TMEV-infected mice (CD3, Fas, and Bcl-2) demonstrated that indeed at 142 days p.i. approximately 50% of double-positive $CD3^+$ Bcl-2+ cells also expressed Fas. (Fig. 8).

We have also examined the expression of Bcl-2 in macrophages infiltrating the spinal cord of TMEV-infected SJL mice. Although Mac-1-positive cells constitute only 8.5% of the total infiltrate of the spinal cord during late chronic demyelinating disease, approximately 40% of Mac-1-positive macrophages in the spinal cord expressed Bcl-2 (data not shown). This finding may account for the low number of macrophages undergoing apoptosis during late chronic demyelinating disease in our studies.

Discussion

The objective of this study has been to determine the fate of CNS-infiltrating mononuclear cells in TMEV-infected mice. We have demonstrated that during TMEV-induced early acute disease infiltrating T cells are eliminated from the CNS through AICD. This process follows the clearance of the virus from the CNS of infected mice. In SJL mice the virus titer dropped from approximately 1.3×10^6 PFU/g of tissue on day 3 p.i., to 5×10^2 PFU/g of tissue on day 10 p.i. In the CNS of B6 mice the virus titer reduced to almost undetectable levels 10 days p.i. (data not shown). Double labeling experiments, using TUNEL and anti-CD3 mAb staining, demonstrated that the majority of the cells undergoing apoptosis were $CD3^+$ T cells, and that they were located mainly in the perivascular cuffs in areas of encephalitis. By 20 days p.i. the mononuclear cells disappear completely from the CNS of both strains of mice. Previous reports suggested that neurons (ex vivo) and macrophages (in vitro) infected with two different stains of TMEV undergo apoptotic cell death (Jelachich and Lipton, 1996; Kang et al., 2000; Kim and Palma, 1999; Tsunoda et al, 1997). However, apoptosis of mononuclear cells infiltrating the CNS of TMEV-infected mice was not examined by these investigators. In agreement with the reports of Jelachich and Lipton (1996) and Tsunoda et al. (1997), we also observed that the few cells other than infiltrating mononuclear cells which stained positively in the TUNEL assay were macrophages or neurons or possibly oligodendrocytes (Fig. 1).

While this article was under revision, Schlitt et al. (2003) reported that apoptotic T cells and macrophages were detected in the spinal cord of SJL mice infected with the BeAn strain of TMEV at 31 to 99 days p.i. Demyelinating disease is observed during this period in the spinal cord of mice infected with the BeAn strain of TMEV. The DA strain of TMEV induces clearly two phases of the disease, early acute disease (gray matter disease) followed by late chronic demyelinating disease, while the encephalitic stage of the disease is heavily attenuated in mice infected with the BeAn strain of TMEV (reviewed by Dal Canto et al., 1996; Monteyne et al., 1997; Oleszak et al., in press; Tsunoda and Fujinami, 1996). Although both BeAn and DA induce chronic demyelinating disease, BeAn-infected mice develop early clear clinical signs of demyelinating disease, such as waddling gait and hind leg paralysis, at 50 days p.i. (Iwahashi et al., 1999), while DA-infected mice develop such signs much later, at 140–180 days p.i. Mononuclear infiltrates in mice infected with the BeAn strain begin to resolve at about 150 days p.i. (Iwahashi et al., 1999), whereas we have observed dense mononuclear infiltrates in the CNS of mice infected with the DA strain as late as 270 days p.i. These differences in the level of apoptosis of mononuclear cells infiltrating the CNS of mice infected with the BeAn or the DA strain may be due to (i) different kinetics of the disease induced by these strains; or (ii) the dose of the viral inoculum, which with the BeAn strain used by Schlitt et al. (2003), was 10-fold-higher than that of the DA strain we routinely used (Oleszak et al., 1997). Thus apoptotic death of mononuclear cells is observed predominantly during

early acute disease in the CNS of mice infected with the DA strain, but mostly during chronic demyelinating disease in mice infected with the BeAn strain. Also iNOS expression in the CNS of mice infected with the DA strain is also different than that of mice infected with the BeAn strain (Oleszak et al., 1997, 2003; Iwahashi et al., 1999).

Elimination of infiltrating T cells from the CNS by apoptosis was associated with upregulation of the expression of Fas and FasL on T cells and downregulation of the expression of the major antiapoptotic gene Bcl-2. Less than 1.3% of $CD3^+$ T cells expressed Bcl-2 during early acute disease. Upregulation of Fas and FasL expression was limited to CNS infiltrating cells, since T cells in the spleen of TMEV-infected mice expressed only low levels of Fas and FasL. Recruitment of $CD3^+$ T cells into the CNS of TMEV-infected SJL mice during both early acute disease and late chronic demyelinating disease appears to be associated with upregulation of the Fas/FasL molecules and T cell activation, which may be antigen-induced. These T cells may be activated in the periphery, in response to antigen, before crossing into the CNS; they may upregulate the expression of Fas/FasL because of the activation. Alternatively, these cells may become activated in the CNS. It has been reported that mouse brain cerebrovascular endothelial cells could be infected by TMEV (Welsh et al., 1995). These endothelial cells may act as antigen-presenting cells and may be responsible for the activation of infiltrating T cells. Expression of Fas and FasL on $CD3^+$T cells and virtual absence of Bcl-2 on these cells during early acute disease may account for the efficient elimination by AICD of infiltrating T cells from the CNS of TMEV-infected SJL mice after viral clearance. These results support the hypothesis that viral clearance in TMEV-infected SJL mice is followed by downsizing of the inflammatory response through AICD.

A major difference in the mononuclear infiltrates found during early acute disease and late chronic demyelinating disease is on the expression of the major antiapoptotic gene Bcl-2. T cells and monocytes infiltrating the CNS of TMEV-infected SJL mice with early acute disease are devoid of Bcl-2-positive cells. It appears that these infiltrating T cells either downregulate their expression of Bcl-2, or that the only T cells capable of infiltrating the CNS are those which express minimal levels of Bcl-2. In contrast, during late chronic demyelinating disease, infiltrating T cells are strongly Bcl-2-positive and they are essentially devoid of apoptotic figures.

The expression of Bcl-2 on $CD3^+$ T cells in spleens from mock-infected SJL mice were substantially higher (11 vs 1%; Table 1) than that on T cells infiltrating the CNS of TMEV-infected SJL mice at 8 days p.i.. These results suggest that T cells recruited and/or expanded in situ in the CNS of TMEV-infected mice are under different regulatory mechanisms than spleen T cells. Upregulation of Fas/FasL system has also been demonstrated on T cells in the CNS but not in the spleens of animals with EAE (Bonetti et al., 1997; McCombe et al., 1996; White et al., 1998). Thus, during early acute disease, TMEV is cleared (although not completely) from the CNS of TMEV-infected SJL mice by T cells that in turn are eliminated from the CNS by AICD.

Although expression of Fas and FasL on T cells in the CNS suggests that these T cells are undergoing apoptotic cell death transduced by Fas, we cannot exclude the fact that cell death is transduced by TNFR. We have previously reported high levels of TNF-α transcripts in the CNS of TMEV-infected SJL mice (Chang et al., 2000). The downsizing of inflammatory, virus-specific T cells by AICD is obligatory for the host to return to homeostasis. Presumably, some T cells survive and enter the memory pool (reviewed by Ahmed and Gray, 1996; Swain et al., 1996). Elimination of autoreactive T cells through the Fas/FasL pathway (AICD) from the CNS has also been described in animals with EAE (McCombe et al. 1996; Waldner et al. 1997; White et al. 1998). Although a similar level of apoptosis within inflammatory infiltrates has been observed in the CNS of TMEV-infected SJL and B6 mice, only the latter cleared the virus completely. In contrast to B6 mice, TMEV-infected SJL mice develop late chronic demyelinating disease associated with fulminate inflammation in perivascular spaces, meninges, and parenchyma. It has been described previously that the virus persists at a low level in the CNS of SJL mice (Kim and Palma, 1999; Monteyne et al. 1997; Tsunoda and Fujinami, 1996). TMEV antigens and RNA genome have been detected in macrophages, astrocytes, and oligodendrocytes (Brahic et al., 1991; Kim and Palma, 1999; Lipton, 1975; Oleszak et al., 1995; Rodriguez et al, 1987). In contrast to early acute disease, we have detected only rare apoptotic T cells infiltrating the spinal cord of TMEV-infected SJL mice during late chronic demyelinating disease. However, Fas and FasL were expressed on T cells in the CNS during both early acute and late chronic demyelinating disease. Lack of apoptosis of Fas+ T cells in the CNS during late chronic demyelinating disease may be explained by the expression of the antiapoptotic Bcl-2. Indeed, high levels of expression of Bcl-2 have been found on T cells in the CNS at 40, 107, and 170 days p.i. (Fig. 7). This is in contrast with early acute disease, where Bcl-2 was virtually absent on T cells infiltrating the CNS of TMEV-infected mice (Figs. 6 and 7A). Bcl-2 proteins are found on both naive and memory cells and they provide survival stimuli (Akbar and Salmon, 1997; Akbar et al., 1994; Chao and Korsmeyer, 1998; Grayson et al., 2000).

During late chronic demyelinating disease certain T cells that have survived AICD formed a pool of memory T cells (unpublished observations B.E. Hoffman, J.R. Chang, C.D. Platsoucas, E.L. Oleszak). However, the specificity of most T cells that accumulate in the CNS is not known. T cell proliferative responses to viral epitopes of BeAn and DA strains of TMEV have been shown during late chronic demyelinating disease (Katz-Levy et al., 2000; Kim et al., 1999; Kang et al., 2002). These were followed by T cell responses to a number of autoantigens, such as proteolipid (PLP) and myelin oligodendrocyte protein (MOG) (Katz-

Levy et al., 2000). The de novo generated responses to sequestered autoantigens secondary to TMEV-induced T cell responses have been thought to be responsible for the propagation of demyelinating disease. The phenomenon has been designated as "epitope spreading" (Sercarz, 2000; Voskuhl et al., 1996; Yu et al., 1996). Whether Bcl-2 is expressed on long-term memory T cells continuously stimulated with viral antigen(s) during persistent infection or on T cells specific for neoantigens generated in the process of epitope spreading remains to be established. Despite Fas and FasL expression on T cells infiltrating the CNS during late chronic demyelinating disease, they do not undergo apoptotic cell death. Bcl-2 may prevent transduction of apoptosis by Fas. The mechanism of interference by Bcl-2 in the CD95 signaling pathway is subject to intensive studies. It has been suggested that Bcl-2 effectively blocks activation of caspases triggered by CD95 signaling and that it blocks apoptosis only in so-called Type II cells (Scaffidi et al., 1999; Strasser et al., 1995). In these cells apoptogenic activity depends on mitochondria, while in Type I cells, CD95 ligation triggers large amounts of active caspase 8 formed at DISC, followed by direct cleavage of caspase 3. Type I cells are less dependent on apoptogenic activity of mitochondria and overexpression of Bcl-2 in them does not block apoptosis. Thus T cells present in the CNS of TMEV-infected mice during late chronic demyelinating disease resemble typical Type II cells, such as in the case of hepatocytes. A Bcl-2 transgene expressed in hepatocytes protects mice from anti-CD95 antibody-induced apoptosis (Lacronique et al., 1996).

An important question is why the expression of Bcl-2 on T cells infiltrating the CNS of TMEV-infected mice is substantially increased during late chronic demyelinating disease versus early acute disease. Cytokine(s) may play a role in upregulating Bcl-2. One of these cytokines is IL-2 (Refaeli et al., 1998; Van Parijs et al., 1999). It has been demonstrated that the removal of IL-2 from in vitro activated T cells reduces Bcl-2 expression and triggers apoptosis (Refaeli et al., 1998; Van Parijs et al., 1999). On the other hand, addition of exogenous IL-2 upregulates Bcl-2 expression and prevents apoptosis (Van Parijs et al., 1999). However, Fas-mediated AICD is potentiated by IL-2, which suggests that IL-2 has a dual function: it may promote apoptosis, or it could be a survival factor for the cells in question (Blattman et al., 2003). Recently, it has been revealed that the distinct effect of IL-2 on T cells is induced through a particular IL-2Rβ-chain signaling motif. IL-2 promotes T cell proliferation, FasL expression on T cells, and AICD by activation of Stat5 pathway (Van Parijs et al., 1999; Lin and Leonard, 2000). In contrast, IL-2 can increase the expression of Bcl-2, a survival molecule, by activating Akt (Ahmed et al., 1997; Van Parijs et al., 1999). Other cytokines, such as IL-4, IL-7, and IL-15, which signal via IL-2Rγ-chain, may also prevent apoptosis of activated T cells by induction of Bcl-2 gene (Ahmed et al., 1997; Akbar et al, 1994; Dai et al., 1999; Li et al., 2000; Vella et al., 1998). We have previously reported the induction of high levels of IL-2 (both transcripts and protein) before the onset of the demyelinating disease at 25–39 days p.i. coinciting with a new wave of T cells of unknown specificity that infiltrate the spinal cord of TMEV-infected SJL mice (Chang et al., 2000). These relatively high levels of IL-2 could trigger the induction of Bcl-2, which begins to be expressed on infiltrating T cells approximately at the same time as IL-2. Furthermore, IL-4, another cytokine that can induce Bcl-2 in T cells, is expressed in the CNS during late chronic demyelinating disease in TMEV-infected SJL mice (Chang et al., 2000). Much lower levels of IL-2 (Chang et al., 2000) may be sufficient to support antigen-driven clonal expansion of T cells in the CNS in response to the virus, as well as Fas/FasL expression, during early acute disease. However, after viral clearance, these T cells are induced to undergo apoptosis by a mechanism(s) that very likely involves AICD.

Our results are in agreement with recent studies in patients with MS. These studies revealed remarkably few apoptotic T cells in the brains of these patients with massive perivascular infiltrates in acute lesions (Dowling et al., 1997). Recently, impaired apoptotic deletion of myelin basic protein-reactive T cells has also been described in MS patients (Ichikawa et al., 1999; Zang et al., 1999). In general, T lymphocytes from the peripheral blood of patients with MS are less susceptible to AICD through numerous impairments of the apoptotic pathways (Sharief and Semra, 2001; Sharief et al., 2002; Waiczies et al., 2002). In a separate study, the expression of Bcl-2 and T lymphocytes has been examined in MS plaques. High levels of Bcl-2 have been found in T cells infiltrating the CNS of patients with chronic progressive MS (Zettl et al., 1998). The mechanism(s) leading to T cell persistence in the CNS is not known. However, similarities between low levels of apoptosis of T cells in the CNS of TMEV-infected animals during late chronic demyelinating disease and T cells in the CNS of patients with MS are striking. These similarities suggest the existence of a common pathway of T cell regulation during inflammatory demyelinating disease in both mouse and human.

Materials and methods

Virus

The wild-type Daniel strain of TMEV was used in all experiments. The origin and propagation of this virus has been previously reported (Oleszak et al., 1988, 1997).

Animals

Five- to-six-week-old female SJL and B6 mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Mice were housed in microisolators in a biohazard level II facility. All manipulations and changes of cages were performed in a biohazard hood. Mice were maintained in accordance with the standards of the American Association for Accreditation of Laboratory Animal Care. Mice were slightly anesthetized with isoflurane and inoculated with approximately 10^5 PFU of TMEV in a 20-μl volume in the right cerebral hemisphere. Control, mock-infected animals were injected with Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum (FCS) and standard amounts of penicillin, streptomycin, and glutamine. Mice were euthanized by the administration of an overdose of isoflurane, and their spinal cords and brains were immediately removed and placed in 10% buffered formalin. Formalin-fixed spinal cords and brains were embedded in paraffin and cut into 5-μm-thick sections. Additionally, brain and spinal cords of TMEV-infected mice were aseptically removed and homogenized, and viral titer was determined using plaque assay, as described (Oleszak et al., 1988, 1997).

Antibodies

Phycoerythrin (PE)- or PerCP-conjugated hamster anti-mouse CD3ε monoclonal antibody (mAb), PE-, or PerCP-conjugated hamster isotypic control mAb, FITC-conjugated hamster anti-mouse Bcl-2 mAb, and FITC-conjugated hamster isotypic control mAb, biotinylated hamster anti-mouse CD95 mAb, and biotinylated hamster isotypic control mAb were used in the immunofluorescence determinations by flow cytometry described here and were purchased from BD Pharmingen (San Diego, CA). CD95L+ cells were determined in the CNS and spleen of TMEV-infected mice at 227 days p.i., using a PE-conjugated mouse anti-mouse CD95L mAb (BD Pharmingen). The following polyclonal antibodies used in the experiments described here were purchased from Serotec Ltd. (Oxford, UK): purified rabbit anti-mouse CD95, purified rabbit anti-mouse CD95L, purified rat anti-human CD3ε peptide (which also recognizes mouse CD3), purified rabbit anti-mouse Bcl-2, and purified rat anti-F4/80 antigen (macrophage marker). Biotinylated goat anti-rabbit and streptavidin-conjugated FITC were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The DamAb2 monoclonal anti-TMEV antibody was generously provided by Dr. R. Roos (University of Chicago, IL) (Nitayaphan et al., 1985).

Immunohistochemistry

Apoptotic cells were detected by immunohistochemistry using a TUNEL kit (Intergen, Purchase, NY). According to this method terminal deoxynucleotidyl transferase (TdT) extends the 3′ end of DNA with digoxigenin-11-dUTP. The procedure was carried out in accordance with the manufacturer's recommendations. Tissue sections were developed with 3,3-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA) and were counterstained with methyl green (Sigma Chemical Co., St. Louis, MO).

For single labeling, the slides were rehydrated in xylene and alcohols followed by washing with phosphate-buffered saline (PBS) or Tris-buffered saline (TBS). Endogenous peroxidases were blocked by incubation of the slides for 10 min in 3% hydrogen peroxide in methanol. Antigen retrieval was performed in 0.1 M citrate buffer (pH 6.0) using a pressure cooker in a microwave oven for 1.25 min. Tissue sections used for macrophage marker detection were pretreated for 30 min at 37°C with a trypsin solution (1 mg/ml, pH 7.8) (Sigma). Specific antigens were detected by the immunoperoxidase technique (Vectastain Elite ABC kit; Vector Laboratories). DAB was used as the substrate. Tissue sections were counterstained with Mayer's hematoxylin (Sigma).

Double labeling for TUNEL+ $CD3^+$ cells was carried out by sequential staining for TUNEL+ cells (DAB substrate) followed by staining with the anti-CD3ε peptide antibody by an immunohistochemistry (ABC kit; Vector Laboratories) with the final peroxidase complex being developed with Vector VIP substrate (Vector Laboratories) according to the manufacturer's instruction and without counterstaining.

Virus-infected cells were stained using the DamAb2 mouse anti-TMEV MAb as described (Hierck et al., 1994). Anti-TMEV antibody was incubated with 1:200 dilution of biotinylated anti-mouse secondary antibody (Vectastain Elite Anti-Mouse ABC kit) overnight at 4°C. To remove any excess of secondary antibody, 0.1% (v/v) normal mouse serum was added to the cocktail and incubated for 1 h. Tissue sections were incubated with blocking solution (Vectastain Elite Anti-Mouse ABC kit) for 30 min followed by 1 h incubation with precomplexed primary/secondary antibody mixture. Slides were washed twice in PBS. The sections were then incubated with ABC solution for 30 min. Slides were developed using DAB and counterstained with Mayer's hematoxylin.

Isolation of mononuclear cells from CNS or spleen

Mononuclear cells were isolated from the CNS as described (Clatch et al., 1990). Briefly, virus-infected or mock-infected mice were anesthetized with isoflurane and perfused with approximately 30 ml of sterile PBS through the left ventricle. To obtain a single-cell suspension the CNS tissue or spleens were dissociated through a no. 100 stainless steel mesh. After letting the connective tissue debris settle for 1 min, the supernatant was centrifuged at 300 *g* for 7 min at room temperature. The supernatant was discarded and the cells (isolated from four spinal cords or two brains which were pooled together) were resuspended in 3 ml of 68% Percoll (Sigma) and overlaid with 30% Percoll (Sigma), as described (Clatch et al., 1990). The cells were centrifuged at 400 *g* for 25 min at room temperature.

Cells at the interphase were collected and washed twice with PBS.

Flow cytometry

Double labeling for determination of Fas+/$CD3^+$ and FasL+/$CD3^+$ cells was carried out using single-cell suspensions of mononuclear cells isolated from the CNS or spleens of mice resuspended in DMEM supplemented with 2% FCS. Cells (1×10^5 to 1×10^6 per tube) were incubated with PE-labeled hamster anti-CD3ε mAb (or control PE-labeled hamster isotypic control mAb) for 1 h on ice and were washed twice with cold PBS. In most experiments Fc Block (0.5 μg/10^6 cells) (BD Pharmingen) was added to the cell suspension to prevent nonspecific binding of antibodies and the cells were incubated for 5 min at room temperature before the addition of primary antibodies. The cells were then incubated with either rabbit anti-mouse CD95 antibody or with rabbit anti-CD95L antibody for 1 h on ice and washed with PBS. Purified rabbit IgG was used for controls. Subsequently cells were incubated with the secondary biotinylated goat anti-rabbit antibody for 1 h on ice and washed again twice in PBS. Streptavidin-FITC was added to each tube and after 30 min of incubation cells were washed twice. After 1 h incubation on ice, cells were washed twice and fixed in 1% buffered paraformaldehyde (BPFA).

Three-color analysis was used for the simultaneous detection of surface (Fas and CD3ε) and intracellular antigens (Bcl-2). Mononuclear cells isolated from the CNS or from spleens of TMEV-infected mice were first labeled with PerCP-conjugated anti-CD3ε mAb (or PerCP-conjugated control isotypic mAb) for 1 h on ice. Cells were washed with PBS and subsequently labeled with biotinylated anti-Fas mAb for 30 min on ice. After removal of unbound antibodies by two washes with PBS, cells were incubated with streptavidin–PE complex for an additional 30 min. CD3/Fas double-labeled cells were fixed with 1% of BPFA for 30 min on ice and permeabilized with 0.2% saponin solution in PBS for 30 min at room temperature. FITC-conjugated anti-Bcl-2 or FITC-conjugated control isotypic mAb was added to double-labeled cells and incubated for 1 h at RT. Cells were washed in 0.2% saponin solution in PBS followed by several washings with PBS. Final pellets were resuspended in 1% PFA. All samples were analyzed using an EPICS-XL flow cytometer (Coulter Corp., Hialea, FL).

In certain experiments cells were double labeled with PerCP-conjugated anti-CD3ε and FITC-conjugated anti-Bcl-2 as described above.

Apoptosis TUNEL analysis ex vivo

Apoptosis of $CD3^+$ T cells was evaluated by the FITC labeling of the 3′ end of DNA by terminal deoxynucleotidyl transferase assay using the Apo-Direct kit from Pharmingen. Briefly, mononuclear cells were isolated from the CNS as described above and were first labeled with PerCP-conjugated anti-CD3ε mAb (or with PerCP-conjugated control isotypic mAb). After 1 h incubation on ice the cells were washed twice with PBS and samples were each resuspended in 0.5 ml of PBS. Cells were fixed by adding 5 ml of 1% of ice-cold 70% ethanol per tube and the tubes were kept for 30 min at 20°C. Cells were washed twice, and TdT and FITC-dUTP were added in reaction buffer and incubated at 37°C for 60 min. TdT was omitted from control samples. Samples were analyzed by flow cytometry as described above.

Statistical analysis

Flow cytometry data were analyzed as the proportion, q2/q1+q2, under each set of experimental conditions, using a logit transformation in a generalized linear model with the binomial distribution (PROC GENMOD, SAS Institute, Cary, NC). Treatment comparisons were adjusted using the Bonferroni–Holm step-down procedure for multiple comparisons. Differences were considered significant if the adjusted P value was 0.05.

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