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B cell Apoptosis in the Central Nervous System in Experimental Autoimmune Encephalomyelitis: Roles of B cell CD95, CD95L and Bcl-2 Expression

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Abstract

The role and fate of B cells in the central nervous system (CNS) in experimental autoimmune encephalomyelitis (EAE) are unknown. Using enzyme-linked immunospot assays we now show that B cells reactive to myelin basic protein (MBP) accumulate in the CNS of Lewis rats with acute EAE induced by immunization with MBP and adjuvants. We also report that B cells are eliminated from the CNS by apoptosis during spontaneous recovery from this disease. Apoptotic B cells were identified by flow cytometry of inflammatory cells extracted from the spinal cord and by histological sections of the spinal cord using light and electron microscopic immunocytochemistry. B cell apoptosis occurred preferentially in the CNS rather than in the peripheral lymphoid organs and was maximal just prior to the onset of spontaneous clinical recovery. Three colour flow cytometry indicated that B cells expressing CD95 (Fas) or CD95 ligand (CD95L) were highly vulnerable to apoptosis, whereas B cells expressing Bcl-2 were relatively protected from apoptosis. We propose that B cells are eliminated from the CNS by the interaction of CD95L and CD95 on the same B cell and that this contributes to the spontaneous resolution of CNS inflammation and clinical recovery in acute EAE.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is a T-cell-mediated inflammatory demyelinating disease of the central nervous system (CNS) and is widely studied as an animal model of multiple sclerosis. It can be induced in susceptible animals by active immunization with myelin antigens, such as myelin basic protein (MBP) and adjuvants or by the passive transfer of T lymphocytes activated by these antigens. The inflammatory infiltrate in the CNS in EAE is composed predominantly of $CD4^+$ T lymphocytes and macrophages with a smaller proportion of $CD8^+$ T lymphocytes and B lymphocytes[1–3].

The overall role of B cells in EAE is unclear. Some studies have suggested that intact B cell function is required for the induction of EAE by active immunization[4–6] but recent studies in B-celldeficient mice have suggested that B cells are not necessary for the development of EAE [7–9]. The role of B cells in the CNS in the pathogenesis of EAE is also unclear. They may act as antigen-presenting cells (APC) in the CNS and, if myelin-reactive B cells accumulate in the CNS, may locally produce antimyelin antibodies that augment demyelination [3]. Another important unanswered question concerns the fate of B cells in the CNS during spontaneous recovery from EAE. One possibility is that B cells are eliminated from the CNS by apoptosis, as are T cells [10, 11] and macrophages/microglia [12–14]. The present study was undertaken to determine whether myelin-reactive B cells accumulate in the CNS in EAE and whether B cells undergo apoptosis in the CNS during spontaneous recovery from this disease.

Materials and Methods

Animals

Female Lewis rats (JC strain), 7–10 weeks old, were obtained from the Central Animal Breeding House of the University of Queensland.

Preparation of inoculum and induction of EAE

MBP was prepared from guinea-pig brains by the method of Deibler *et al*. [15]. MBP in 0.9% saline was emulsified in an equal volume of incomplete Freund's adjuvant containing 4 mg/ml *Mycobacterium butyricum*. Under anaesthesia, rats were inoculated in a footpad of one hindfoot with 0.1 ml emulsion. The total dose of MBP was 50 μg per rat.

Clinical assessment

Tail, hindlimb and forelimb weakness were each graded on a scale of 0 (no weakness) to 4 (total paralysis) as previously described [16]. The total clinical score was obtained by adding these three scores (maximum =12).

Extraction of cells from the spinal cord and the lymphoid organs

Cells were isolated from the spinal cords of rats perfused with ice-cold saline using previously described methods [17]. The entire spinal cord was removed by insufflation, weighed, and a single-cell suspension in ice-cold RPMI containing 1% foetal calf serum (FCS) was prepared by passage of the spinal cord through a 200-mesh stainless steel sieve. The cell suspension was mixed with isotonic Percoll (Percoll: Hank's Balanced Salt Solution 9:1) in a 3:2 ratio (density 1.052) in 50 ml centrifuge tubes and spun for 25 min at 640×g at 4°C. The cell pellet and the last 9 ml supernatant were resuspended, transferred to a conical centrifuge tube, underlaid with 1 ml Ficoll and spun for 20 min at 600×g at 4°C. The cells from the interface above the Ficoll were collected, washed and counted. Single-cell suspensions were prepared from the draining and non-draining popliteal lymph nodes and the spleen by teasing and passage through a fine nylon mesh. Erythrocytes were removed from the spleen cell suspension using Ficoll. Because the present study was focused on B lymphocytes, we did not pass the spinal cord inflammatory cells through a nylon wool column to enrich for T lymphocytes as we have done in some of our previous studies [17–19].

Antibodies

Mouse monoclonal antibodies specific for the αβ T cell receptor (TCRαβ) (R73), CD45RA or A/B (CD45RA/B) (OX33; specific for rat B cells [20)] and CD11b/c (OX42; macrophages/microglia) were obtained from Dr J Sedgwick (DNAX Research Institute, Palo Alto, CA, USA). Rabbit polyclonal antibodies specific for rat CD95 (M-20), CD95 ligand (CD95L, N-20) and Bcl-2 (N-19) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). The secondary antibodies employed were FITC-conjugated swine $F(ab2)_2$ anti-rabbit immunoglobulin (Ig) (Dako, Carpinteria, CA, USA) and PE-conjugated goat anti-mouse Ig (Dako). Mouse IgG_1 (Dako) and normal rabbit serum (Dako) were used as control antibodies. The primary and secondary antibodies were diluted in phosphate buffered saline (PBS)/azide (1% FCS/1% sodium azide in PBS) plus 10% autologous rat serum.

Labelling of cells and flow cytometric analysis

Three-colour analysis was used for the simultaneous detection of surface and intracellular antigens and for the analysis of DNA content for assessment of apoptosis. Briefly, 10^5–10^6 cells were stained for expression of cell-surface antigens as previously described [18]. After washing, cells were fixed with 1 ml ice-cold 0.25% paraformaldehyde in PBS (pH 7.2) overnight at 4°C. They were then washed in PBS and permeabilized by gentle resuspension in 1 ml 0.2% Tween 20 in PBS and incubated at 37°C for 15 min. Samples were washed, and the intracellular antigen Bcl-2 was labelled in the same manner as for the surface antigens. To stain for DNA, the washed samples were then resuspended in an appropriate volume (100– 300 μl) of propidium iodide (PI)-staining solution which was freshly prepared by diluting stock solution (RNase [5 mg/ml] and PI [250 μg/ml] in 0.1 M PBS containing 0.1 mM EDTA, pH 7.4) with PBS/azide. Samples were kept on ice in the dark and analysed within 1 h. Immunofluorescence and DNA analysis were performed on a Becton Dickinson (BD) FACScan using CellQuest software, which was routinely calibrated using BD CaliBRITE Flow Cytometry Beads and BD FACSComp software. Electronic compensation for three-colour analysis ensured unchanged FITC and PE distributions following PI labelling of DNA. For each sample, 40,000 events were scored. To avoid detecting nuclear debris, events with a low level of PI fluorescence were not collected. For surface and intracellular antigen labelling, the Ig-control sample values were subtracted from all other sample values to remove FITC and PE background fluorescence. Apoptotic events were defined as those having a lower PI fluorescence than the sharply defined G_0/G_1 peak.

Light and electron microscopic immunocytochemistry

For immunocytochemistry the rats were perfused through the aorta with 4% paraformaldehyde/0.05% glutaraldehyde in 0.1 M PBS (pH 7.4). Segments of the spinal cord were removed and further fixed in the above fixative for a total of 3–4 h before being transferred to 0.01 M PBS. Sections (50 μm and 100 μm) were cut on a TPI vibratome. For both light and electron microscopic immunocytochemistry, a modified protocol of Hsu's avidin/biotin peroxidase [21] preembedding immunolabelling technique [22] was employed as described previously [23] and briefly as follows. After endogenous peroxidase and nonspecific binding sites had been blocked with 1% H_2O_2/0.1% sodium azide solution and with normal goat serum (Dako) (1:50 in PBS) respectively, vibratome sections were incubated with the OX33 mouse monoclonal antibody against CD45RA/B which was diluted 1:25 in 0.01 M PBS. Sections were washed with PBS and then incubated with biotinylated anti-mouse IgG (Vector, Burlingame, CA, USA) diluted to 1:300 in PBS containing 1% normal rat serum.

After being washed again with PBS, the sections were incubated with an avidin-biotinylated peroxidase complex (Vector). The sections were washed with PBS and then reacted with 3,32- diaminobenzidine-tetrahydrochloride (Sigma, Castle Hill, Australia). Reactions were stopped with PBS, and the sections were then washed with saline, re-fixed with modified Karnovsky's fixative (2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3–7.4) and washed in saline again. The sections were then flat-osmicated and flat-embedded [24]. Semithin sections (1 μm) were examined unstained or counterstained with toluidine blue. Ultrathin sections were examined with a Jeol JEM-1200 EXII electron microscope.

Enzyme-linked immunospot assays

We used the enzyme-linked immunospot (ELISPOT) assay [25, 26] to determine the frequencies of B cells producing antibodies specific for MBP or $MBP_{72–89}$ (sequence: PQKSQRSQDENPVVHF), the major encephalitogenic region of guinea-pig MBP in the Lewis rat (synthesized by the Queensland Institute of Medical Research), in the spinal cord, the spleen and the popliteal lymph node draining the inoculation site of rats with acute MBP-EAE 13 days after inoculation (day 13). Cells were prepared as described above. The culture medium used for this assay was RPMI 1640 supplemented with 216 mg/l l-glutamine (Gibco, Rockville, MD, USA), 100 IU penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol and 10% heat-inactivated FCS. Millipore 96-well filtration plates with mixed cellulose and ester membrane bottoms (type HATF, pore size 0.45 μm) were coated with antigen by the addition of 100 μl/well of 500 μg/ml MBP or 1 mg/ml $MBP_{72–89}$ at 4°C overnight. Nonbound antigen was then flicked out and the residual binding capacity of the plates was removed by adding 100 μl/well of 5% bovine serum albumin in PBS (blocking buffer) for 30 min at 37°C. The blocking solution was then flicked out and the plates washed twice with 0.0005% Tween 20/PBS (washing solution), then twice with PBS. One hundred microlitres of culture medium was then added to each well and incubated at room temperature, and flicked out immediately prior to addition of the cells.

Triplicates of increasing numbers of responder cells prepared from the spinal cord, the spleen and the lymph node were incubated in the wells for 120 h in 5% CO_2/95% air at 37°C. The cells were then discarded by flicking the plates and washing the wells nine times with washing solution and once with distilled water. As a positive control, 100 μl of a 1/50 dilution of serum from a rat with EAE (previously shown in an enzyme- immunosorbent assay to respond to MBP) was to control wells precoated with antigen and no responder cells had been added, and incubated room temperature for 30 min; the serum flicked out, and the plates washed thoroughly times with washing solution, then once with water. One hundred microlitres of peroxidaseconjugated goat anti-rat IgG (Cappel) diluted in blocking buffer was then added to all control wells for 2 h at room temperature. After flicking out the non-bound peroxidase-conjugated antibody, the wells were again washed four times washing solution, then once with distilled Sigma AED Chromogen Kit was used to detect binding. After incubation with the substrate reagent 15 min, the plates were given a final six washes distilled water, then allowed to air dry. Spots quantified using a dissecting microscope.

Table 1. *Clinical details of rats with EAE and phenotypes of spinal cord inflammatory cells 11–15 days after inoculation*

Day of study[a]	Total number of rats	Number of groups studied	Mean total clinical score on day of study	Mean day of onset of EAE	% CD45RA/B$^+$ cells (cells/g)[b]	% TCRαβ$^+$ cells (cells/g)	% CD11b/c$^+$ cells (cells/g)
11	3	1	1.7	10	9.8 (2.2×10^5)	23.5 (5.3×10^5)	41.7 (9.4×10^5)
12	11	3	2	10.8	10.8 (2.1×10^5)	30.3 (7.9×10^5)	32.0 (8.0×10^5)
13	67	13[c]	4.9	9.9	15.4 (9.0×10^5)	28.8 (17.5×10^5)	28.7 (18.5×10^5)
14	7	2	3.5	9.9	14.3 (4.8×10^5)	17.4 (5.6×10^5)	38.6 (15.9×10^5)
15	7	2	3.3	9.4	9.6 (5.2×10^5)	14.2 (6.5×10^5)	60.0 (32.8×10^5)

a) Number of days after inoculation.
b) Total number of cells per gram of spinal cord.
c) For the co-staining of CD95 and CD95L with CD45RA/B, TCRαβ and CD11b/c, four groups were studied on day 13.

Table 2. *Frequencies of MBP-reactive B cells and $MBP_{72–89}$-reactive B cells in rats with EAE on day 13*

	MBP	$MBP_{72–89}$
CNS	1/713[a] (1/2,936–1/406)[b]	1/645 (1/2,139–1/380)
Spleen	1/5,808 (1/11,723–1/3,860)	1/9,479 (1/18,070–1/6,425)
Draining LN[c]	1/2,424 (1/14,397–1/1,323)	1/6,315 (1/68,094–1/3,311)

a) Frequency.
b) 95% confidence limits.
c) Lymph node.

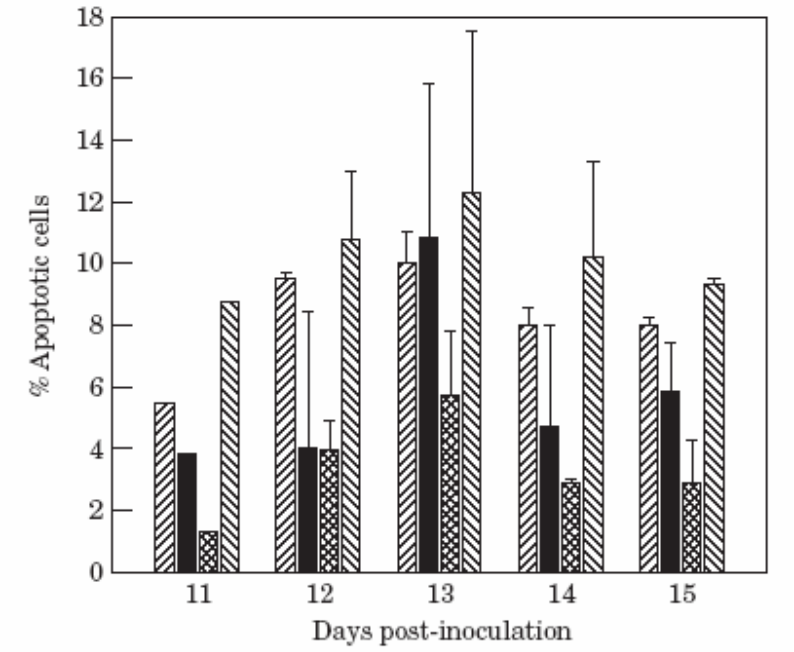

Figure 1. Percentages of the CNS-infiltrating total inflammatory cell population (total), B cell (CD45RA/B^+) population, TCR$\alpha\beta^+$ population and macrophage/microglial (CD11b/c^+) population that were apoptotic, in rats with EAE from 11–15 days after inoculation. Total (▨), CD45 RA/B^+ (■), TCR$\alpha\beta^+$ (▩), CD11b/C^+ (▧).

Statistical analysis

Percentages of the inflammatory cell populations which were apoptotic were analysed using analysis of variance (ANOVA) to compare the cell populations from all organs simultaneously, followed by Student's *t*-test to compare cell populations in pairs of organs.

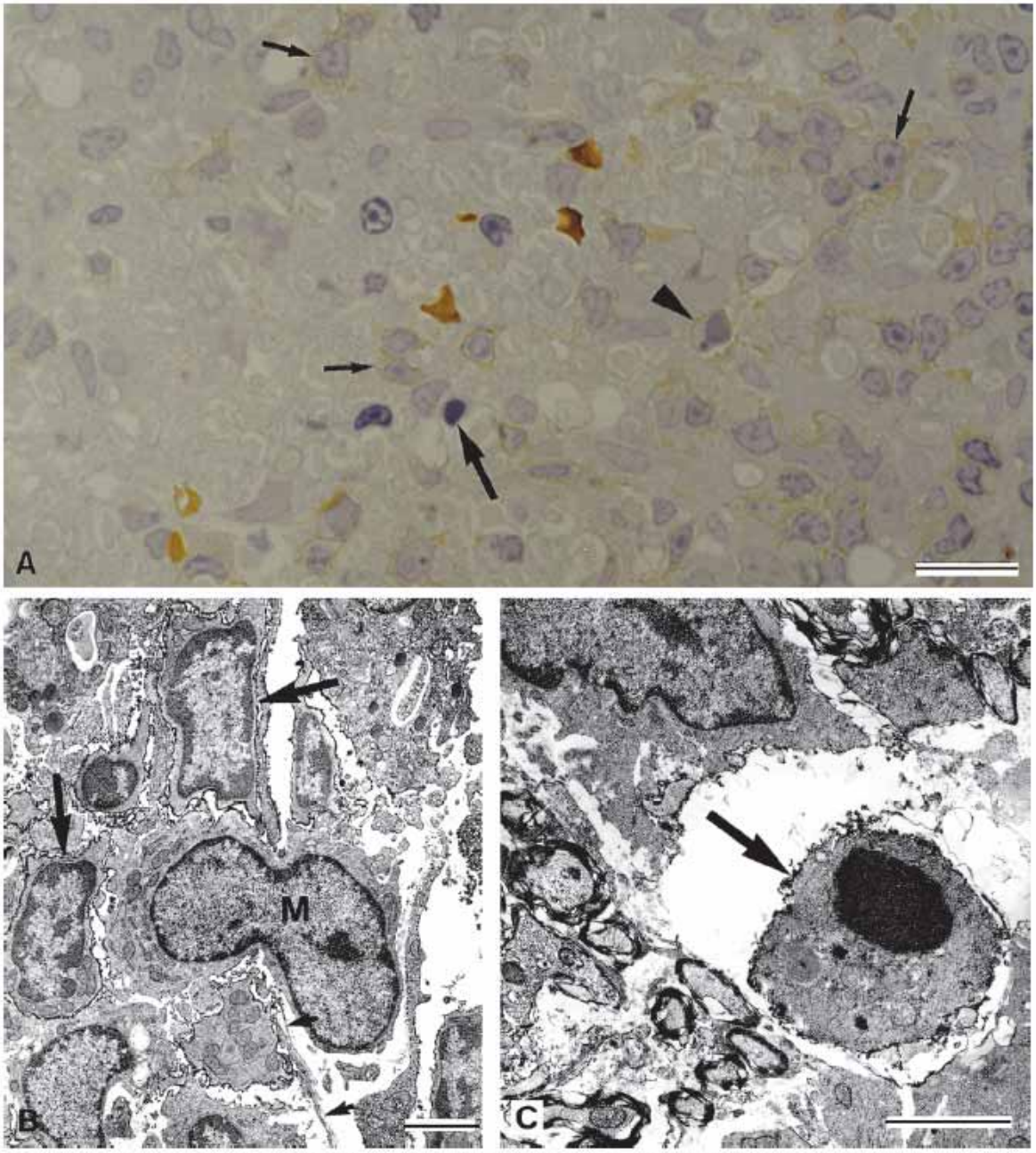

Figure 2. Light micrograph (A) and electron micrographs (B & C) of the sacral spinal cord obtained from rats with EAE 13 days after inoculation and labelled with anti-CD45RA/B antibody. (A) Numerous non-apoptotic CD45RA/B$^+$ cells (small arrows) can be seen. An apoptotic CD45RA/B$^+$ cell (arrowhead) as well as an apoptotic CD45RA/B$^-$ cell (large arrow) are also present. (B) Non-apoptotic CD45RA/B$^+$ cells (large arrows) have the morphology of lymphocytes with clumped nuclear chromatin and a thin layer of cytoplasm. An unlabelled macrophage [M] appears to be entering the parenchyma from the meninges through a broken glia limitans (small arrows). (C) An apoptotic CD45RA/B$^+$ cell (arrow) in an inflammatory lesion. (A) Semithin section (1 µm) counterstained with toluidine blue. Bars: A=10 µm; B & C=2 µm.

Results

Course of disease and spinal cord inflammatory cell phenotype

Rats developed neurological signs of EAE nine to 11 days after inoculation (Table 1). The peak of neurological signs was on day 13, after which the rats gradually recovered. Pooled spinal cord inflammatory cells extracted from groups of three to seven rats were analysed on days 11–15 after inoculation. The proportions of the three major inflammatory cell populations in the spinal cord (CD45RA/B+ B lymphocytes, TCRαβ + T lymphocytes, and CD11b/c+ macrophages and microglia) are shown in Table 1. The highest proportion of B cells (15.4%) was found at the peak of disease on day 13.

Detection of specific antibody-secreting cells by the immunospot assay

To determine the frequency of MBP-specific B cells and $MBP_{72–89}$-specific B cells in the CNS, the spleen and the draining popliteal lymph node, we performed ELISPOT assays on cells isolated from rats with EAE on day 13. As shown in Table 2, the frequencies of B cells reactive to MBP or to the major encephalitogenic epitope $MBP_{72–89}$ were higher in the CNS than in the peripheral lymphoid organs.

Apoptosis of B cells

To assess B cell apoptosis, we employed simultaneous flow cytometric analysis of surface antigens and DNA content. Figure 1 shows the means and population standard deviations of the percentages of spinal cord CD45RA/B+ B cells, TCRαβ + T lymphocytes, and CD11b/c+ macrophages/microglia undergoing apoptosis on days 11–15 after inoculation. As shown previously in the CNS for the percentage of all inflammatory cells that are apoptotic [17] and the percentage of TCRαβ+ cells that are apoptotic [18], the percentage of apoptotic cells within each of the cell populations studied increased until the peak of neurological signs on day 13, and then decreased during clinical recovery. On day 13, 10.8% of the B cells in the CNS were apoptotic. The presence of apoptotic B cells in the CNS was confirmed histologically by light and electron microscopic immunocytochemistry which revealed apoptotic B cells in the parenchyma of the spinal cord (Figure 2).

B cell apoptosis occurs preferentially in the CNS rather than in the peripheral lymphoid organs

To determine whether B cell apoptosis occurs in the peripheral lymphoid organs as well as in the CNS, we measured by flow cytometry the levels of apoptosis in the total inflammatory, CD45RA/B+, TCRαβ+ and CD11b/c+ populations obtained from the CNS, the spleen, and the draining and non-draining popliteal lymph nodes. Figure 3 and Table 3 show that B cell apoptosis in EAE mainly occurs in the CNS, as has been previously reported for T cells [10, 11, 27].

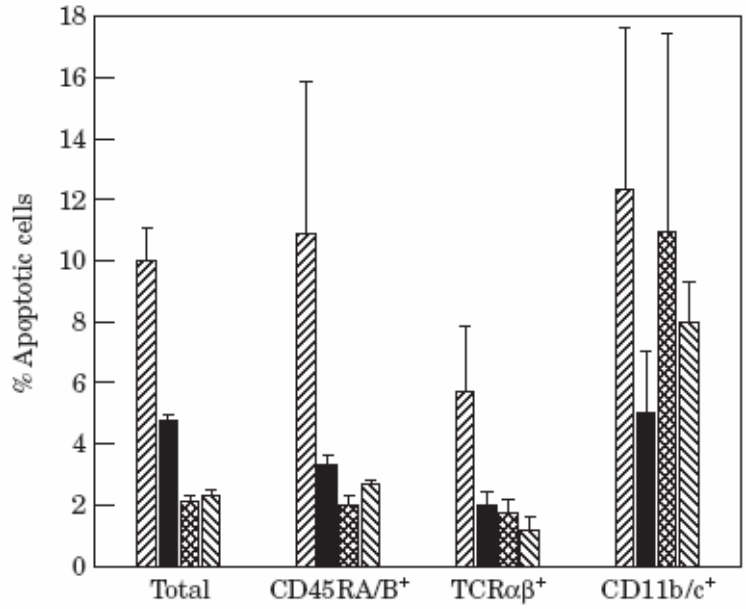

Figure 3. Percentages of the total inflammatory cell populations (total), B cell (CD45RA/B$^+$) populations, TCR$\alpha\beta^+$ populations and macrophage/microglial (CD11b/c$^+$) populations from the CNS, spleen, draining lymph node (LN) and the non-draining LN that were apoptotic in rats with EAE 13 days after inoculation. The ANOVA *P*-values for the simultaneous comparison of the levels of apoptosis in the four organs, together with the *P*-values (Student's *t*-test) for the comparisons of pairs of organs, are shown directly below the cell population to which they refer. CNS (▨), Spleen (■), Draining LN (▩), Non-draining LN (▧).

Table 3.

ANOVA *P*-value	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.225$
t-test *P*-values				
CNS *vs.* Spleen	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.007$
CNS *vs.* Draining LN	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.378$
CNS *vs.* Non-draining LN	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.031$

Expression of apoptosis-regulating proteins by B cells

We have previously shown that CD95$^+$ T cells and CD95L$^+$ T cells are particularly vulnerable to apoptosis in the CNS in EAE whereas Bcl-2$^+$ T cells are relatively protected from apoptosis [19]. To determine whether apoptosis-regulating proteins play a similar role in B cell apoptosis, we analysed the expression of these proteins by CNS-infiltrating CD45RA/B$^+$ cells 13 days after inoculation. The percentage of CD45RA/B$^+$Bcl-2$^+$ cells undergoing apoptosis (10.3±4.7) was much lower than the percentage of CD45RA/B$^+$Bcl-2$^-$ that B cells expressing Bcl-2 are relatively protected from apoptosis in the CNS in EAE. The percentage of

CD45RA/B+CD95+ cells that were apoptotic (16.8±16.9) and the percentage of CD45RA/ B+CD95L+ cells that were apoptotic (24.3±22.6) were more than twice the percentages of CD45RA/ B+CD95– cells and CD45RA/B+CD95L– cells that were apoptotic (7.1±2.6 and 7.6±2.6, respectively). These results indicate that the expression of CD95 or CD95L by B cells renders them susceptible to apoptosis. The four groups of rats used to obtain these results could be subdivided according to the clinical course of EAE (early onset or late onset). Rats with late onset EAE developed neurological signs two days later than the rats with early onset EAE, and the mean total clinical score was less in the late onset group (Table 4). Subdividing the rats in this way revealed marked differences in the percentages of CD45RA/B+ cells that expressed CD95 or CD95L and in the percentages of CD45RA/B+CD95+ cells and CD45RA/B+CD95L+ cells that were apoptotic. These results indicate that CNS-infiltrating B cells from rats that are more advanced in the course of EAE are more likely to undergo apoptosis through the CD95-mediated pathway. In the spleen and draining and non-draining popliteal lymph nodes the B cell expression of CD95 and CD95L was also associated with an increased susceptibility of B cells to apoptosis (data not shown).

Discussion

In the present study we have shown by immunospot assays that myelin-reactive B cells accumulate in the CNS in acute EAE. We have also shown that B cell apoptosis occurs in the CNS and is maximal just prior to the onset of spontaneous clinical recovery from this disease. To our knowledge, B cell apoptosis in the target organ of an autoimmune disease has not previously been reported. The B cell apoptosis occurs preferentially in the CNS rather than in the peripheral lymphoid organs. Three colour flow cytometry indicated that B cells expressing CD95 (Fas) or CD95L were highly vulnerable to apoptosis, whereas B cells expressing Bcl-2 were relatively protected from apoptosis. Although CNS-infiltrating B cells have not been shown to play a pathogenic role in MBP-induced EAE, their presence in the CNS raises the possibility that they may contribute to the pathogenesis, for example by acting as professional APC. Myers *et al.* [6] have shown that anti-MBP antibodies enhance the induction of EAE by passively transferred MBP-specific T cells and have proposed that the antibodies increase the presentation of myelin antigens in the CNS to the encephalitogenic T cells. If B cells in the CNS do have a pathogenic role, B cell apoptosis may contribute to the spontaneous resolution of inflammation in the CNS and clinical recovery.

There are two main mechanisms for the induction of apoptosis in mature B cells. One occurs after hyper-crosslinking of the B cell antigen receptor (BCR) [28, 29] and does not involve the interaction of CD95 and CD95L [30, 31]. The degree of cross-linking of surface Ig (sIg) receptors on mature B cells determines whether the B cells proliferate or are deleted; weak sIg cross-linking induces mature B cells to proliferate, whereas strong sIg cross-linking by multivalent antigens such as cell membrane antigens eliminates mature B cells by apoptosis [32, 33]. BCR-induced apoptosis is dependent on the absence of T cell signals, because it can be reversed by interleukin-4 [32], tumour necrosis factor-_ [30] and ligation of CD40, which is constitutively expressed on B lymphocytes [34, 35]. B cell and T cell interactions therefore play an important role in regulating B cell apoptosis. The second main mechanism of mature B cell apoptosis is mediated through CD95, the expression of which is upregulated by the ligation of B cell CD40 by activated T cells expressing CD40L [36, 37]. This CD40L-triggered CD95-mediated apoptosis can be overcome by BCR antigen recognition [37], but antigen-specific B cells that have been activated for a prolonged period can become sensitive to CD95- mediated cell death [38]. The CD95-mediated pathway is postulated to result from the ligation of B cell CD95 by T cell CD95L [39].

However, activated B cells express functional CD95L [40] as well as CD95[38], which raises the possibility that the interaction of CD95L and CD95 on the same B cell may mediate apoptosis. In the present study we have shown that B cells expressing CD95 or CD95L in the CNS are much more vulnerable to apoptosis than B cells not expressing these proteins. This strongly suggests that B cell apoptosis in the CNS in EAE is mediated by the interaction of CD95L and CD95 on the same B cell, just as the interaction of CD95L and CD95 on the same T cell appears to lead to T cell apoptosis in the CNS [19]. We also found that Bcl-2 expression appears to protect against B cell apoptosis in the CNS. As Bcl-2 can inhibit CD95-mediated apoptosis in some cell types [41], we hypothesize that CD95-mediated apoptosis is occurring in CNS B cells that are expressing low levels of Bcl-2. Low Bcl-2 expression may result from cytokine withdrawal following activation-induced apoptosis of autoreactive T cells. This CD95-mediated mechanism could be responsible for the deletion of both autoreactive and non-autoreactive B cells. It is also

possible that some B cells are being deleted in the CNS by non-CD95-dependent mechanisms, for example BCR hyper-crosslinking [28, 29], or by the action of endogenous glucocorticosteroids [42] which are produced during recovery from acute EAE [43]. In conclusion, we have reported the new finding of B cell apoptosis in the CNS in EAE and suggested that it might contribute to the spontaneous resolution of CNS inflammation and clinical recovery in acute EAE. A detailed understanding of the process of inflammatory cell elimination in the target organ and how it might fail in chronic autoimmune disorders may lead to a better understanding of diseases such as multiple sclerosis.

Table 4. *Expression of CD95 and CD95L by, and susceptibility to apoptosis of, spinal cord CD45RA/B$^+$ cells 13 days after inoculation*

Apoptosis-regulating protein	Clinical course	Mean day of onset of EAE	Mean total clinical score	% of CD45RA/B$^+$ cells that were apoptosis-regulating protein$^+$	% of CD45RA/B$^+$ protein$^+$ cells that were apoptotic[a]	% of CD45RA/B$^+$-protein$^-$ cells that were apoptotic[a]	Mean ratio[b]
CD95	Late onset	10.8±0.5	4.4±0.9	3.2±0.0	1.9±1.9	7.3±3.6	0.2±0.2
	Early onset	9.1±0.4	6.6±0.6	11.5±1.0	31.7±11.0	6.9±0.6	4.5±1.2
CD95L	Late onset	10.8±0.5	4.4±0.9	1.0±0.9	2.6±2.6	6.0±2.2	0.7±0.7
	Early onset	9.1±0.4	6.6±0.6	7.1±0.2	46.0±8.8	9.1±1.9	5.1±.01

a) Mean and population standard deviation.
b) For each experiment the percentage of apoptotic cells in the CD45RA/B$^+$ population expressing the apoptosis-regulating protein was divided by the percentage of apoptotic cells in the CD45RA/B$^+$ population not expressing the protein. The mean and population standard deviation of these values were then calculated.

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