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Microglia are More Susceptible Than Macrophages To Apoptosis in the Central Nervous System in Experimental Autoimmune Encephalomyelitis Through a Mechanism not Involving Fas (CD95)

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Abstract

Morphological studies have shown that macrophages and microglia undergo apoptosis in the central nervous system (CNS) in acute experimental autoimmune encephalomyelitis (EAE) in the Lewis rat. To assess the relative levels of macrophage and microglial apoptosis, and the molecular mechanisms involved in this process, we used three-colour flow cytometry to identify $CD45^{low}CD11b/c^{+}$ microglial cells and $CD45^{high}CD11b/c^{+}$ macrophages in the inflammatory cells isolated from the spinal cords of Lewis rats 13 days after immunization with myelin basic protein (MBP) and complete Freund's adjuvant. Simultaneously, we analyzed the DNA content of these cell populations to assess the proportions of cells undergoing apoptosis and in different stages of the cell cycle or examined their expression of three apoptosis-regulating proteins, i.e. Fas (CD95), Fas ligand (FasL) and Bcl-2. Microglia were highly vulnerable to apoptosis and were over-represented in the apoptotic population. Macrophages were less susceptible to apoptosis than microglia and underwent mitosis more frequently than microglia. The different susceptibilities of microglia and macrophages to apoptosis did not appear to be due to variations in Fas, FasL or Bcl-2 expression, as the proportions of microglia and macrophages expressing these proteins were similar, and were relatively high. Furthermore, in contrast to T cell apoptosis, apoptosis of microglia/macrophages did not occur more frequently in cells expressing Fas or FasL, or less frequently in cells expressing Bcl-2. These results indicate that the apoptosis of microglia and CNS macrophages in EAE is not mediated through the Fas pathway, and that Bcl-2 expression does not protect them from apoptosis. Expression of FasL by macrophages and microglia may contribute to the pathogenesis and immunoregulation of EAE through interactions with Fas^{+} oligodendrocytes and Fas^{+} T cells. The high level of microglial apoptosis in EAE indicates that microglial apoptosis may be an important homeostatic mechanism for controlling the number of microglia in the CNS following microglial activation and proliferation.

Introduction

Apoptosis (programmed cell death) of inflammatory cells occurs in the central nervous system (CNS) in experimental autoimmune encephalomyelitis (EAE) (1–3). EAE is a T cell-mediated inflammatory demyelinating disease of the CNS and is an animal model of multiple sclerosis. It can be induced in susceptible animals by immunization with myelin antigens, such as myelin basic protein (MBP), and adjuvants or by the passive transfer of T lymphocytes activated by these antigens (4). The encephalitogenic T cells release cytokines (5) that attract and activate other cells, including macrophages which are essential for the pathogenesis of EAE (6). Activated macrophages phagocytose myelin and act as antigen-presenting cells (APC) in the CNS in EAE (7–9). Microglia, the resident tissue macrophages of the CNS, also become activated and express increased levels of class II MHC molecules during the course of EAE (10–12) but are not effective APC *in vitro* (13).

We have previously reported that in acute EAE in the Lewis rat, encephalitogenic $V_\beta 8.2^+$ MBP-reactive T cells are selectively eliminated from the CNS by apoptosis and that this contributes to the spontaneous clinical recovery from EAE (14–16). We have also provided evidence that the apoptosis of autoreactive T cells in the target organ of this autoimmune disease represents activation-induced cell death through the Fas (CD95) pathway (17). Morphological studies using immunocytochemical techniques have shown that, in addition to T cells, $CD11b/c^+$ cells (macrophages/microglia) undergo apoptosis in the CNS in EAE (18); however, these studies were not able to distinguish definitively between apoptotic macrophages and apoptotic microglia. In an earlier study, using standard light and electron microscopy we demonstrated apoptotic macrophages in the CNS in EAE, particularly in chronic persistent EAE, on the basis of the presence of myelin debris within apoptotic cells (19). One possible mechanism for the apoptosis of macrophages and microglia in EAE is involvement of the Fas pathway, as in the case of T cells (17). Ashany and colleagues (20) have demonstrated that Fas expression is up-regulated on activated macrophages and that they are thus prone to Fas-mediated cytotoxicity by $CD4^+$ T cells expressing Fas ligand (FasL). The present study was undertaken to quantify CNS macrophage and microglial apoptosis in EAE, to determine whether these cells express Fas and FasL, and to determine whether Fas plays a role in macrophage/microglial apoptosis as it does in the apoptotic deletion of autoreactive T cells in the CNS during spontaneous recovery from EAE.

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.* (21). 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 (22). The total clinical score was obtained by adding these three scores (maximum = 12).

Extraction of cells from the spinal cord

On day 13 after inoculation, cells were isolated from the spinal cords of rats perfused with ice-cold saline using our previously described methods (14). The entire spinal cord was removed by insufflation, weighed and a single-cell suspension in ice-cold RPMI containing 1% 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:HBSS 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. Because the present study was focussed on macrophages and microglia, we did not pass the

spinal cord inflammatory cells through a nylon wool column to enrich for T lymphocytes as we have done in previous studies (14,16,17).

Antibodies

Mouse mAb specific for CD45 (OX1; leukocyte common antigen), CD5 (OX19; T lymphocytes), αβ TCR (R73), CD8α (OX8; cytotoxic/suppressor T cells, NK cells and a subset of dendritic cells), NKR-P1A (10/78; NK cells), CD45RA or A/B (OX33; B lymphocytes) were obtained from Dr J. Sedgwick. Antibody to V_β8.2 (R78) was kindly provided by Dr T. Hünig (23). Rabbit polyclonal antibodies specific for rat Fas (M-20), FasL (N-20) and Bcl-2 (N-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The directly labelled antibodies employed were mouse phycoerythrin (PE)-conjugated anti-rat CD11b/c (PharMingen, San Diego, CA) and mouse FITC-conjugated anti-rat CD8α (Serotec, St Louis, MO). The secondary antibodies employed were FITC-conjugated sheep anti-mouse IgG (Sigma, St Louis, MO), FITC-conjugated goat anti-rabbit IgG F(ab')2 (Rockland, Gilbertsville, PA), PE-conjugated goat anti-mouse Ig (Dako, Glostrup, Denmark), PE-conjugated goat anti-rabbit IgG (Sigma) and TriColor-conjugated goat anti-mouse IgG1 (Caltag, San Francisco, CA). Mouse IgG1 (Dako) and rabbit IgG (Rockland) were used as control antibodies. The primary and secondary antibodies were diluted in PBS/azide (1% FCS/1% sodium azide in PBS) plus 10% autologous rat serum.

Labeling of cells and flow cytometric analysis

Three-colour analysis was used for the simultaneous detection of surface and intracellular antigens, and for 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 (16). The cells were then incubated with mouse Ig for 10 min at 4°C to prevent any free fluorochrome-conjugated anti-mouse Ig secondary antibodies from binding to the PE-conjugated mouse anti-rat CD11b/c and FITC-conjugated mouse anti-rat CD8α antibodies which were added to certain cell samples in the next step (30 min at 4°C in the dark). 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 being gently resuspended 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. If DNA staining was to be performed, 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 analyzed within 1 h. Immunofluorescence and DNA analysis were performed on a FACSCalibur using CellQuest software (Becton Dickinson, Mountain View, CA), which was routinely calibrated using Becton Dickinson CaliBRITE flow cytometry beads and FACSComp software. Electronic compensation for three-colour analysis ensured unchanged FITC and PE distributions following TriColor labelling of antigens or 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, PE and TriColor background fluorescence. Apoptotic events were defined as those having a lower PI fluorescence than the sharply defined G_0/G_1 peak.

Table 1. Phenotype of spinal cord inflammatory cells 13 days after inoculation

Cell marker	Percent of cells expressing the cell marker	No. of groups studied[a]
CD45	81.6 ± 5.3	13
CD5	38.9 ± 3.0	11
CD8	11.4 ± 4.0	11
αβ TCR	27.7 ± 5.0	8
V_β8.2	3.5 ± 2.5	3
CD45RA or A/B	16.1 ± 2.9	6
NKR-P1A	2.6 ± 1.2	5
CD11b/c	24.8 ± 3.2	13
$CD45^{low}CD11b/c$	8.1 ± 2.0	11
$CD45^{high}CD11b/c$	13.6 ± 3.3	11
Apoptotic	8.9 ± 1.2	10

[a]Each group comprised five to seven rats.

Statistical analysis

Percentages of the different inflammatory cell populations which were apoptotic or which expressed Fas, FasL or Bcl-2 were compared using ANOVA to compare all cell populations simultaneously, followed by Student's *t*-test to compare pairs of cell populations.

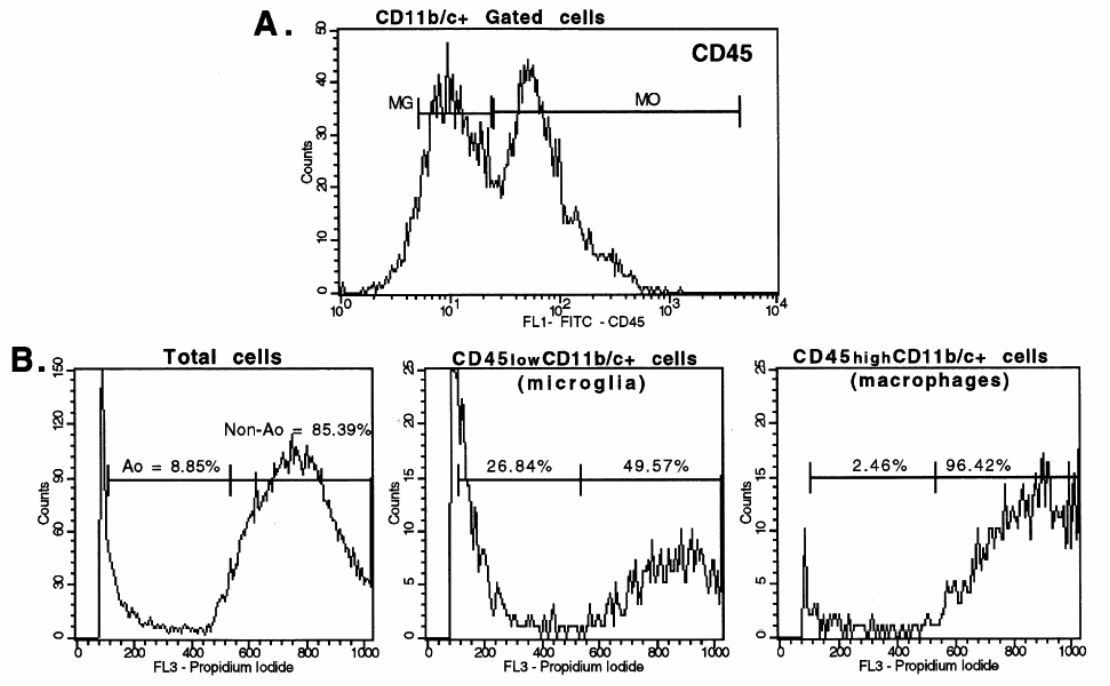


Fig. 1. (A) Representative flow cytometric histogram of CNS-infiltrating cells extracted from the spinal cords of rats 13 days after inoculation and double stained for CD45 and CD11b/c, showing CD45 expression by $CD11b/c^+$ cells, with typical analysis gates (MG = microglia; MO = macrophages). (B) DNA staining profiles of the total inflammatory cell population (total cells) and the $CD45^{low}CD11b/c^+$ (microglia) and $CD45^{high}CD11b/c^+$ (macrophage) populations shown in (A). The numbers in the profiles represent the percentages of apoptotic (Ao) and non-apoptotic (Non-Ao) cells in each population.

Results

Spinal cord inflammatory cell phenotype

Pooled spinal cord inflammatory cells were analyzed on day 13 after inoculation (previous studies have shown that the peak of clinical disease occurs on days 12–14 after inoculation) from 13 groups of rats with five to seven rats per group. The mean total clinical score was 4.8 ± 0.9, with the mean day of onset being 9.6 ± 0.7. The phenotypes of the spinal cord inflammatory cells isolated from these rats are shown in Table 1. Haemopoietic ($CD45^+$) cells constituted 81.6% of the total extracted cell population. Of the $CD45^+$ population, 48% were $CD5^+$ (T lymphocytes), 20% were CD45RA or A/B^+ (B lymphocytes), 3% were $NKR\text{-}P1A^+$ (NK cells), 10% were $CD45^{low}CD11b/c^+$ (microglia) and 17% were $CD45^{high}CD11b/c^+$ (macrophages), with the last two cell types, which are both $CD11b/c^+$, being identified according to their level of expression of CD45 (Fig. 1A) (24,25).

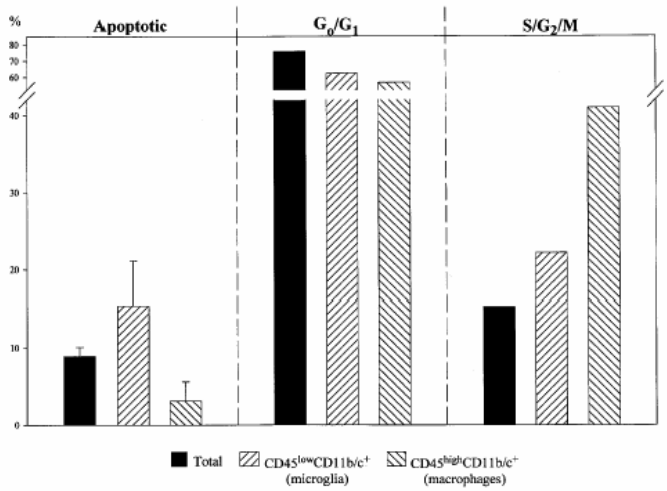


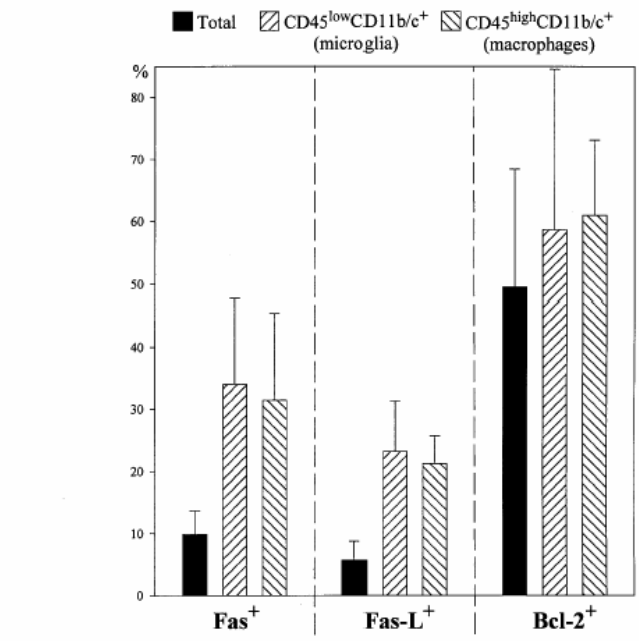
Fig. 2. Percentages of the total CNS-infiltrating cell population (total), the microglial ($CD45^{low}CD11b/c^+$) population and the macrophage ($CD45^{high}CD11b/c^+$) population in the different phases of the cell cycle, including apoptosis, in rats with EAE 13 days after inoculation. The values for the G_0/G_1 and $S/G_2/M$ phases of the cell cycle were calculated from one experiment. The ANOVA *P* value for the comparison of apoptosis in the three cell populations was <0.001. Student's *t*-test indicated that there were significant differences in the levels of apoptosis when comparisons were made within each pair of cell populations (microglia versus total, $P = 0.01$; microglia versus macrophages, $P < 0.001$; macrophages versus total, $P < 0.001$). (G_0 = resting; G_1 = pre-DNA synthesis; S = DNA synthesis; G_2 = period between DNA synthesis and mitosis; M = mitosis).

Apoptosis of microglia and macrophages

To determine the susceptibility of microglia and macrophages to apoptosis, we employed simultaneous flow cytometric analysis of surface antigens and DNA content. Figure 1(B) shows representative DNA staining of the total inflammatory cell population, $CD45^{low}CD11b/c^+$ cells and $CD45^{high}CD11b/c^+$ cells, and Fig. 2 shows the means and population SD of the percentages of these three populations undergoing apoptosis. The proportion of the $CD45^{low}CD11b/c^+$ cells (microglia) undergoing apoptosis was higher than the proportion of the total inflammatory cell population undergoing apoptosis and much higher than the proportion of $CD45^{high}CD11b/c^+$ cells (macrophages) undergoing apoptosis. The average ratio of the percentage of microglia undergoing apoptosis to the percentage of macrophages undergoing apoptosis in seven separate experiments was 7:1. These results indicate that microglia are highly vulnerable to apoptosis and that macrophages are relatively resistant to apoptosis, when compared with the total inflammatory cell population. The vulnerability of microglia to apoptosis was also indicated by a 2.4 ± 1.4-fold enrichment of

$CD45^{low}CD11b/c^{+}$ cells in the total apoptotic population compared to the total non-apoptotic population. In contrast, $CD45^{high}CD11b/c^{+}$ cells were under-represented in the total apoptotic population compared to the total non-apoptotic population (0.3 ± 0.2).

Figure 2 also shows the proportion of cells in the G_0/G_1 and $S/G_2/M$ phases of the cell cycle, and reveals that microglia and especially macrophages are proliferating more frequently than the total inflammatory cell population.



ANOVA *P*-value	$P < 0.001$	$P < 0.001$	$P = 0.295$
t-test *P*-values			
MG vs MO	$P = 0.386$	$P = 0.325$	$P = 0.350$
MG vs Total	$P = 0.006$	$P = 0.002$	$P = 0.215$
MO vs Total	$P = 0.010$	$P < 0.001$	$P = 0.029$

Fig. 3. Percentages of the total CNS-infiltrating cell population (total), the microglial (MG) ($CD45^{low}CD11b/c^{+}$) population and the macrophage (MO) ($CD45^{high}CD11b/c^{+}$) population expressing apoptosis-regulating proteins in rats with EAE 13 days after inoculation. The ANOVA *P* values for the comparison of the expression of each apoptosis-regulating protein by the three cell populations, together with the *P* values (Student's *t*-test) for the comparisons of the pairs of cell populations, are shown directly below the apoptosis-regulating protein to which they refer.

Expression of apoptosis-regulating proteins by microglia and macrophages

We have recently shown that Fas^{+} T cells and $FasL^{+}$ T cells are particularly prone to undergo apoptosis in the CNS in experimental autoimmune encephalomyelitis (EAE), whereas $Bcl\text{-}2^{+}$ T cells are relatively resistant (17). To determine whether these apoptosis-regulating proteins play a similar role in microglial and macrophage apoptosis, we determined their expression by $CD45^{low}CD11b/c^{+}$ cells (microglia) and $CD45^{high}CD11b/c^{+}$ cells (macrophages) (Fig. 3). The proportions of microglia expressing Fas, FasL or Bcl-2 were similar to the proportions of macrophages, and higher than the proportions of the total inflammatory cell population, expressing these proteins. Because our flow cytometer was limited to a maximum of three colours, we were unable to determine the proportions of apoptotic cells in the microglial and macrophage populations expressing apoptosis-regulating proteins. However, 85.2 6 17.7% of $CD11b/c^{+}$ cells were $CD45^{+}$ (the remainder were probably endothelial cells) and expression of the apoptosis-regulating proteins by $CD11b/c^{+}$ cells had no

relationship to the level of CD11b/c$^+$ cell apoptosis observed (Table 2). These results indicate that the Fas pathway is not involved in the apoptosis of microglia and macrophages, and that Bcl-2 expression does not protect these cells from apoptosis.

Table 2. Susceptibility of CD11b/c$^+$ cells to apoptosis

Apoptosis-regulating protein	Apoptotic versus Non-apoptotic	CD11b/c$^+$protein$^+$ cells that were apoptotic/ non-apoptotic (%)[a]	CD11b/c$^+$protein$^-$ cells that were apoptotic/ non-apoptotic(%)[a]	Mean ratio[b]
Fas	apoptotic	8.7 ± 4.4	11.8 ± 4.6	0.8 ± 0.4
	non-apoptotic	86.7 ± 7.6	80.2 ± 10.4	1.1 ± 0.1
FasL	apoptotic	10.7 ± 5.9	9.7 ± 3.8	1.0 ± 0.6
	non-apoptotic	84.8 ± 9.3	82.9 ± 7.0	1.0 ± 0.1
Bcl-2	apoptotic	8.0 ± 3.8	9.1 ± 8.7	0.8 ± 0.3
	non-apoptotic	85.6 ± 10.2	86.0 ± 17.7	1.1 ± 0.4

[a]Mean and population SD.

[b]For each experiment the percentage of apoptotic cells in the CD11b/c$^+$ population expressing the apoptosis-regulating protein was divided by the percentage of apoptotic cells in the CD11b/c$^+$ population not expressing the protein. The mean and population SD of these values were then calculated.

Discussion

In the present study we have used flow cytometry to identify microglia and macrophages in the CNS in acute EAE on the basis of their expression of CD45 and CD11b/c, and we simultaneously have used DNA staining to identify apoptotic cells and cells at different stages of the cell cycle. At the clinical peak of acute EAE, we found both apoptotic microglia and apoptotic macrophages, which confirms our previous *in situ* studies (18,19). Furthermore, the present study has extended these observations to show that CD45^{low}CD11b/c$^+$ microglia are highly vulnerable to apoptosis, whereas CD45^{high}CD11b/c$^+$ macrophages are less susceptible. As the density of some cell surface antigens is lower on apoptotic cells (defined by cell size) than on viable cells (26), it is possible that the level of CD45 expression on some apoptotic macrophages was reduced and that these cells were thus incorrectly classified as microglia. However, in CNS-infiltrating cells obtained from rats with EAE and enriched for T cells, the majority of apoptotic cells were CD45^{high}, indicating that apoptotic cells are still capable of expressing high levels of CD45 (data not shown). We would therefore expect that only a small proportion of the apoptotic macrophages might have been incorrectly classified as CD45^{low}CD11b/c^1 microglia, and that this would not account for the observed differences in the susceptibility of microglia and macrophages to apoptosis.

We have also shown that microglia and CNS macrophages express Fas and FasL in EAE. However, the different susceptibilities of microglia and macrophages to apoptosis do not appear to be due to variations in Fas or FasL expression, or Bcl-2 expression, as the proportions of microglia and macrophages expressing these apoptosis-regulating proteins were the same. Furthermore, CD11b/c$^+$ cells (of which 85% were also CD45$^+$) expressing Fas, FasL or Bcl-2 underwent apoptosis to the same extent as CD11b/c$^+$ cells not expressing these proteins. These results suggest that the Fas pathway, which mediates T cell apoptosis in the CNS in acute EAE (17), is not involved in the apoptosis of microglia and macrophages. Our study also suggests that microglial and macrophage apoptosis is not controlled by Bcl-2. This is similar to the findings of Gehrmann and Banati (27) who found that expression of *bcl*-2 and c-*myc* was unchanged throughout a period of microglial activation, proliferation and apoptosis in the CNS following peripheral nerve injury.

Non-Fas-dependent mechanisms which may be responsible for the microglial and

macrophage apoptosis include: the effect of NO which induces macrophage apoptosis *in vitro* (28, 29), although microglia have been reported to be resistant to NO-induced damage (30, 31); the release of down-regulatory cytokines such as IL-4, IL-6 and transforming growth factor-β (32–34); and the withdrawal of pro-inflammatory cytokines such as IL-1, tumour necrosis factor-α and IFN-γ , which are produced in the CNS in EAE (5,35), or colony stimulating factors, which are produced by stimulated astrocytes (36) and which prevents monocyte and microglial apoptosis *in vitro* (37,38). Withdrawal of T cell-derived cytokines could occur when T cells die by apoptosis or are inhibited by other means. One possible explanation for the different susceptibilities of microglia and macrophages to apoptosis could be the differential expression of CD40 which, when ligated, may provide protection from apoptosis (39). A lack of CD40 expression by microglia could possibly account for their greater vulnerability to apoptosis.

We also found that macrophages and microglia undergo mitosis more frequently than the total inflammatory cell population. Furthermore, in contrast to our findings on apoptosis, the proportion of macrophages undergoing mitosis was twice the proportion of microglia undergoing mitosis. Microglial/macrophage proliferation during EAE has previously been reported (12,40–42). Reid and colleagues (43) demonstrated that microglia in the normal CNS are activated and induced to proliferate or undergo apoptosis after exposure to an anti-CD11b/CD18 antibody. They suggested that the apoptosis occurred because of a lack of the growth factors necessary to support a sustained microglial proliferative response. As suggested above, macrophages may possess some mechanism which protects them from the apoptotic fate of microglia and allows continuation of proliferation. The ultimate fate of these CNS macrophages is unclear, as the low incidence of macrophage apoptosis observed in the present study suggests that this mechanism may play only a minor role in their removal from the CNS. It is likely that they emigrate from the site of inflammation, as observed in a murine model of resolving peritonitis (44). The high level of microglial apoptosis in EAE indicates that microglial apoptosis may be an important homeostatic mechanism for controlling the number of microglia in the CNS following microglial activation and proliferation.

Our finding that relatively high proportions of microglia and macrophages express FasL compared to the total CNS inflammatory cell population in EAE may have implications for the pathogenesis of this disease. Recent studies have shown that the interaction between Fas and FasL is necessary for the development of EAE (45,46). $FasL^+$ macrophages and microglia, in addition to $FasL^+$ T cells, could contribute to the development of demyelination by damaging Fas^+ oligodendrocytes.

The expression of FasL by microglia could also have immunoregulatory effects by contributing to the activation-induced apoptosis of T cells, as the expression of FasL in some organs, such as the testis (47) and the eye (48), has been suggested to mediate immune privilege by inducing apoptosis of infiltrating Fas^+ lymphocytes. This could add to the T cell apoptosis resulting from the interaction between Fas and FasL on the same T cell (17). A detailed understanding of the process of inflammatory cell elimination and how it might fail in chronic autoimmune disorders may lead to a better understanding of diseases such as multiple sclerosis.

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Abbreviations

APC	antigen-presenting cells
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
FasL	Fas ligand
MBP	myelin basic protein
PE	phycoerythrin
PI	propidium iodide

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