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Apoptosis of αβ T Lymphocytes in the Nervous System in Experimental Autoimmune Encephalomyelitis: Its Possible Implications for Recovery and Acquired Tolerance

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We have recently shown that apoptosis, an active process of cellular self-destruction, occurs in the central nervous system in Lewis rats with acute experimental autoimmune encephalomyelitis (EAE) induced by inoculation with myelin basic protein (MBP) and adjuvants. Conventional light and electron microscopic studies suggested that some of the apoptotic cells were oligodendrocytes and that others were hematogenous mono-nuclear cells. To determine whether any of the apoptotic cells were T lymphocytes, we used the technique of pre-embedding immunolabelling which allows sufficient preservation of the ultrastructure to permit recognition of apoptotic changes while at the same time preserving surface antigens so that the identity of the apoptotic cells can be determined by immunocytochemistry. Light microscopic immunocytochemistry using the mono-clonal antibodies OX-34 (CD2) and R73 (αβ T-cell receptor) revealed that 10% of the $CD2^+$ cells and 5% of the αβ T lymphocytes in the parenchyma of the spinal cord were dying by apoptosis. The presence of apoptotic αβ T cells was confirmed by electron microscopy. About half of all the apoptotic cells within the spinal cord were labelled by these antibodies. It is possible that some of the unlabelled apoptotic cells were also T lymphocytes but that others were glial cells such as oligodendrocytes. One possible interpretation of this T-cell apoptosis is that it represents activation-induced cell death, which has recently been shown to provide a mechanism of clonal elimination of mature as well as immature autoreactive T cells. Another possible interpretation is that it is a result of corticosterone released during the course of EAE. The apoptotic elimination of target-antigen-specific lymphocytes within the target organ in this autoimmune disease may contribute to the subsidence of inflammation and, if ongoing, to the development of tolerance.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is a T-cell-mediated demyelinating disease of the central nervous system (CNS) and a putative model of the human demyelinating disease, multiple sclerosis [1]. It has been shown that EAE is mediated by $CD4^+$ T lymphocytes with a cytotoxic capacity [2-4]. In Lewis rats, acute EAE induced by inoculation with myelin basic protein (MBP) [MBP-EAE] causes a self-limited disease in which paralysis is followed by rapid clinical recovery [5, 6]. Convalescent rats are tolerant to MBP, as evidenced by resistance to re-induction of EAE by active immunization [7]. The question of the fate of T lymphocytes that have entered the nervous system in EAE has received little attention. Yet knowledge of this fate may shed new light on the mechanisms responsible for the subsidence of inflammation in acute MBP-EAE and the acquisition of tolerance.

We have recently reported that apoptosis, an active process of cellular self-destruction [8], occurs in the spinal cord in rats with acute MBP-EAE [9]. Some apoptotic cells were located within myelin sheaths, the meninges and perivascular spaces and were most likely blood-derived mononuclear cells; the sparseness of their cytoplasm and the absence of phagocytosed material indicated that they were mainly lymphocytes rather than macrophages. Other apoptotic cells located in the parenchyma of the white and grey matter of the spinal cord could not be identified by their morphology, but the size and location of some of them suggested that they were oligodendrocytes.

Because of the potential significance of the death of T lymphocytes in the target organ in this autoimmune disease, we performed the present study to determine whether any of the apoptotic cells are T lymphocytes. Our results show that there is apoptosis of αβ T lymphocytes in the spinal cord in acute MBP-EAE.

Materials and methods

Induction of EAE

MBP was prepared from guinea pig spinal cord by the method of Deibler *et al. [10]*. MBP in 0.9% saline was emulsified in an equal volume of incomplete Freund's adjuvant containing 4 mg/ml of *Mycobacterium butyricum.* Male Lewis rats (JC strain), aged 8-10 weeks, were inoculated with 0.1 ml of emulsion in one footpad of each hindfoot. The total dose of MBP was 50-75 μg/rat.

Pre-embedding immunolabelling

To determine whether any of the apoptotic cells in the spinal cord are T lymphocytes, we have used the technique of pre-embedding immunolabelling which allows sufficient preservation of ultrastructure to permit recognition of apoptotic changes while at the same time preserving surface antigens so that the identity of the apoptotic cells can be determined by immunocytochemistry. A modification of the technique of Lassmann *et al.* [11] was used. After the development of neurological signs the rats were anaesthetized and perfused through the left ventricle with 4% formaldehyde in 0.1 M phosphate buffer. The spinal cord was cut into slices which were immersed overnight in foetal calf serum at 4°C. The slices were incubated with OX-34 (rat CD2) [12] or R73 (rat αβ T-cell receptor) [13] mouse monoclonal antibodies (Serotec, Oxford, UK), then thoroughly washed with phosphate-buffered saline (PBS) and incubated with biotinylated anti-mouse immunoglobulin (Amersham, UK). After further thorough washing with PBS, the slices were incubated with avidin horseradish peroxidase complex (Sigma, St Louis, MO, USA), washed with PBS and reacted with 3,3'-diaminobenzidine-tetrachloride (Sigma) dissolved in 0.1 M Tris buffer (pH 7.6), and with 0.01% hydrogen peroxide. The slices were then washed in PBS, immersed in 2.5% glutaraldehyde/2% formaldehyde in 0.1 M sodium cacodylate buffer, washed in PBS, post-fixed in osmium tetroxide, washed and embedded in Epox 812 (Ernest F. Fullam, Schenectady, NY, USA). One-μm-thick sections were counterstained with toluidine blue. For electron microscopy,

ultrathin sections were cut from epoxy-embedded immunostained tissue blocks, stained with lead citrate and examined with a Jeol JEM-1200 EXII electron microscope.

Lymph node assessment

To assess the extent of T cell apoptosis in the normal lymph node and in the antigen-primed lymph node, we examined the popliteal lymph nodes of two normal rats and of nine rats studied 12-14 days after footpad inoculation with incomplete Freund's adjuvant containing *Mycobacterium butyricum.* This inoculum was prepared and given in the same manner as was the inoculum to produce MBP-EAE, except that the MBP was omitted. The rats were perfused through the left ventricle with 0.9% saline followed by 2.5% glutaraldehyde/2% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3-7.4). After removal, the popliteal lymph nodes were post-fixed with 2% osmium tetroxide in phosphate buffer and embedded in HistoResin (Reichert-Jung), sectioned (1μm thick) and stained with cresyl fast violet as previously described [14].

Results

Clinical findings

Eleven days after inoculation with MBP and adjuvants, the rats developed an ascending paralysis of the tail followed by hindlimb weakness or paralysis. Most animals had recovered clinically by 18 days.

Histological findings

Histological examination of rats with neurological signs showed meningeal and perivascular inflammation associated with limited primary demyelination in the spinal cord, and inflammation and demyelination of the dorsal and ventral spinal nerve roots (see reference 5). As previously reported [9], cells undergoing apoptosis were recognized at light and electron microscopy by the presence of either crescentic masses of condensed chromatin lying against the nuclear envelope or rounded masses of uniformly dense chromatin. Apoptotic cells were present in the CNS from the day of onset of neurological signs to the time of clinical recovery.

Table 1. *Apoptosis of T lymphocytes labelled with monoclonal antibodies to CD2 (OX-34) and to the a(3 T-cell receptor (R73) in transverse sections of the lumbosacral spinal cord of rats with MBP-EAE, 2-S days after the onset of neurological signs*

	OX-34		R73	
Rat number	Total number of labelled cells per section	Number (%) of apoptotic labelled cells per section	Total number of labelled cells per section	Number (%) of apoptotic labelled cells per section
804-JMO	125	20 (16)	287	12 (4)
805-JMO	177	21 (12)	186	6 (3)
807-JMO	101	9 (9)	50	5 (10)
810-JMO	291	11 (4)	257	9 (4)
862-JMO	NT	NT	304	9 (3)
863-JMO	NT	NT	106	5 (5)
Mean percentage of labelled cells that were apoptotic		10		5

Sections were prepared from separate blocks of tissue stained with either OX-34 or R73. NT = Not tested.

Table 2. *Labelling of apoptotic cells with monoclonal antibodies to CD2 (OX-34) and to the αβ T-cell receptor (R73) in transverse sections of the lumbosacral spinal cord in rats with MBP-EAE, 2–5 days after the onset of neurological signs*

Rat number	OX-34 Percentage of apoptotic cells that were labelled per section	R73 Percentage of apoptotic cells that were labelled per section
804-JMO	69	41
805-JMO	65	38
807-JMO	19	29
810-JMO	50	39
862-JMO	NT	55
863-JMO	NT	26
Mean percentage of apoptotic cells that were labelled	51	38

Sections were prepared from separate blocks of tissue stained with either OX-34 or R73.
NT = Not tested.

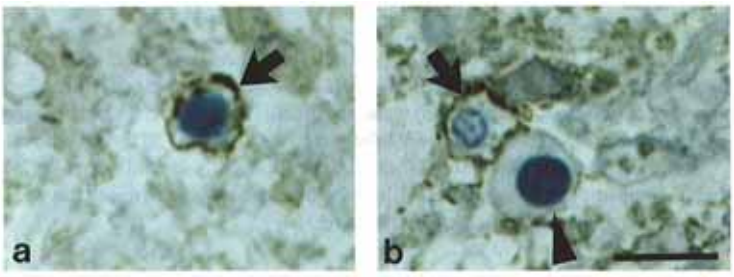

Figure 1. Transverse Epox 812 sections through the lumbosacral spinal cord of rats with neurological signs of acute MBP-EAE. Each section was immunostained with the specified monoclonal antibody and counterstained with toluidine blue. (a) OX-34. A labelled apoptotic cell can be seen (arrow). (b) R73. A labelled normal T lymphocyte (arrow) lies adjacent to a non-labelled apoptotic cell (arrowhead). Bar=5 μm.

Immunocytochemical findings

Light microscopic immunocytochemistry using the monoclonal antibodies OX-34 (CD2) [12] and R73 *(αβ* T-cell receptor) [13] revealed that 10% of the $CD2^+$ cells and 5% of the αβ T lymphocytes in the parenchyma of the lumbosacral spinal cord of rats with neurological signs of MBP-EAE (12-14 days after inoculation; 2-5 days after the onset of neurological signs) were apoptotic (Figure IA; Table 1). Fifty-one per cent of all the apoptotic cells were labelled by OX-34 and 38% were labelled by R73 (Table 2). Occasionally non-labelled apoptotic cells lay adjacent to labelled normal T lymphocytes (Figure 1B). Electron microscopy confirmed that there was labelling of the apoptotic cells by R73 (Figure 2).

Lymph node findings

In the normal lymph nodes no apoptosis was observed in the cortex or paracortex, although occasional apoptotic cells were observed in the germinal centres. In the

popliteal lymph nodes draining the sites of inoculation with *Mycobacterium butyricum,* there was also some apoptosis in the germinal centres but very rare apoptosis in the cortex or paracortex. Thus apoptosis in the normal and antigen-primed lymph nodes was essentially restricted to the germinal centres which are B-cell areas.

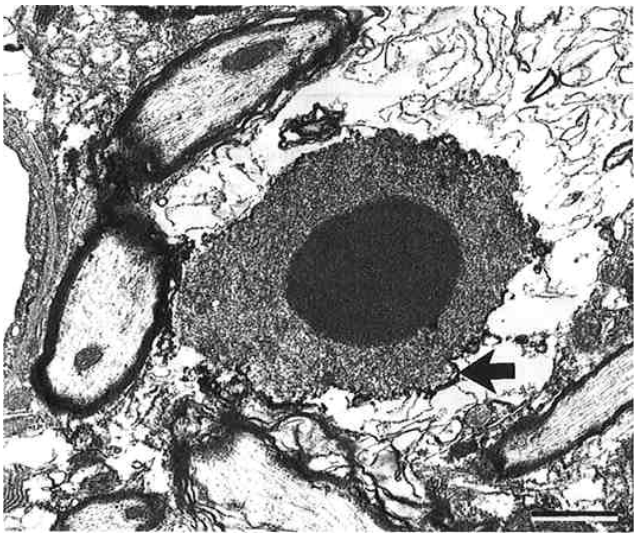

Figure 2. Electron micrograph of the lumbosacral spinal cord of a rat with neurological signs of acute MBP-EAE showing an apoptotic cell labelled with horseradish peroxidase reaction product (arrow) following immunostaining with R73 monoclonal antibody. Bar = 1 μm.

Discussion

In the present study we have shown by immunocytochemistry that there is apoptosis of T cells in the spinal cord in rats with acute MBP-EAE. Using the monoclonal antibodies OX-34 (CD2) and R73 (αβ T-cell receptor) we have found that 10% of the $CD2^+$ cells and 5% of the αβ T lymphocytes in the spinal cord are dying by apoptosis. Our studies demonstrate that at least half of all the apoptotic cells are T cells. Because of the variable penetration of the monoclonal antibody into the tissue blocks, it is possible that some of the apoptotic cells were not exposed to the antibody and that the true figure is higher. However, as shown in Figure 1B, unlabelled apoptotic cells were sometimes found near labelled normal T cells, thus indicating that such a technical problem cannot account for all the unlabelled apoptotic cells. Another factor that might lead to non-labelling of apoptotic T cells is down-regulation of cell surface molecule expression, which has been described early in apoptosis [15]. Selective downregulation of the αβ T-cell receptor may explain why there appear to be more $CD2^+$ cells than αβ T cells, although it is possible that some of the $CD2^+$ apoptotic cells are not αβ T lymphocytes but γδ T lymphocytes or natural killer cells.

It is likely that some of the apoptotic cells are not T cells but glial cells such as oligodendrocytes, the apoptosis having been induced by cytotoxic MBP-specific T cells, as we have previously suggested [9]. Such oligodendrocyte apoptosis may be an important mechanism leading to demyelination in EAE [9]. Since oligodendrocytes myelinate multiple axons and since there is limited demyelination in the spinal cord in rats with acute MBP-EAE [16], only a small number of apoptotic oligodendrocytes would be expected in any spinal cord section. We are performing immunocytochemical studies using oligodendrocyte markers to determine what proportion of the apoptotic cells are oligodendrocytes.

At present we do not know what proportion of the apoptotic T cells in the spinal cord in MBP-EAE are MBP-specific. Since only a minority of the infiltrating cells in the CNS in EAE are MBP-specific [17, 18], it is possible that all the apoptotic T cells that we observe are autoreactive. Selective elimination by apoptosis within the spinal cord may contribute to the low yield of MBP-specific cells in lymphocytes extracted from the spinal cord of rats with acute MBP-EAE [19].

Several mechanisms could be responsible for apoptosis of αβ T lymphocytes in the target organ of this autoimmune disease. Firstly, T cells may undergo apoptosis in the CNS as a result of an intrinsic programme to self-destruct after fulfilling their effector function. This seems unlikely, since cytotoxic T cells can recycle after killing their targets [20]. Secondly, apoptosis of encephalitogenic T cells may occur as a result of cytotoxicity by T cells specifically targeted against the encephalitogenic T cells (cytolytic T-T-cell interactions) [21]. Thirdly, the endogenous corticosterone released during the course of EAE [22] may cause the T-cell apoptosis: glucocorticoids induce apoptosis of lymphoid cells [23, 24]. Lastly, T-cell apoptosis in the CNS may represent activation-induced cell death. This process results in clonal deletion of autoreactive immature T cells in the thymus during normal development [25, 26]; it also eliminates mature T cells *in vitro* [27-30] and *in vivo,* in the latter case providing a mechanism of extra thymic (peripheral) tolerance to foreign or self antigen [31-34].

T-cell activation by occupancy of the antigen-specific receptor can result in proliferation, anergy or apoptosis, the outcome being determined by the presence and timing of other signals such as the costimulatory signal [35, 36] and the cytokines, interleukin-2 [30, 37] and interferon-γ [28]. In the early induction phase of acute MBP-EAE, activation of MBP-specific T cells in lymphoid organs leads to proliferation; however, we hypothesize that in the later effector phase of the disease, activation of MBP-specific T cells within the CNS may lead to apoptosis, because of the failure of non-professional antigen-presenting cells such as astrocytes to produce the costimulatory signal or because of the different availability of cytokines. This hypothesis is supported by our finding that the proportion of T cells dying by apoptosis in the CNS in EAE is much higher than that observed in normal or antigen-primed lymph nodes, which suggests that the environment in the CNS may be an important factor contributing to the induction of T-cell apoptosis in this autoimmune disease.

Whatever the mechanism of its induction, apoptotic elimination of MBP-specific T lymphocytes in the CNS may play an important role in the subsidence of inflammation in acute MBP-EAE. Our findings are consistent with the conclusion of others that effector T cells in EAE 'turn themselves off' [38]. An acute elimination of T cells within the CNS would not be expected to produce longstanding tolerance, since precursors in the lymphoid organs (see reference 7) could expand to produce new effector T cells; however, an ongoing low level of T-cell apoptosis in the CNS could contribute to the tolerant state that develops after an attack of acute MBP-EAE [7]. We hypothesize that

T-cell apoptosis in the target organ may be a protective mechanism that also occurs in other self-limited, T cell-mediated autoimmune diseases.

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