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Apoptosis in the Nervous System in Experimental Allergic Encephalomyelitis

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Abstract

We report here for the first time the occurrence of apoptosis of cells in the spinal cord in experimental allergic encephalomyelitis (EAE), an autoimmune, T-cell-mediated demyelinating disease. Four different forms of EAE were studied in the Lewis rat: (i) acute EAE induced by inoculation with whole spinal cord and adjuvants; (ii) acute EAE induced by inoculation with myelin basic protein (MBP) and adjuvants; (iii) acute EAE induced by the passive transfer of MBP-sensitized spleen cells; (iv) chronic relapsing EAE induced by inoculation with whole spinal cord and adjuvants followed by treatment with low-dose cyclosporin A. 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. They were found in both the white and grey matter of the spinal cord in all 4 forms of this disease. Although it was not possible to identify definitively the types of cells undergoing apoptosis, the size and location of some of the affected cells suggested that they were oligodendrocytes. As there is now a large body of evidence that T-cell-induced target cell death takes the form of apoptosis, it is attractive to hypothesize that oligodendrocyte apoptosis is occurring in EAE as a result of oligodendrocyte-directed T-cell cytotoxicity. However, other apoptotic cells were located within the myelin sheath, meninges and perivascular spaces and were clearly not oligodendrocytes but were most likely blood-derived mononuclear cells. The sparsity of their cytoplasm and the absence of phagocytosed material suggested that they were mainly lymphocytes rather than macrophages. Apoptosis has been shown to be involved in deleting autoreactive T-cells during the normal development of tolerance. Thus apoptotic deletion of myelin/oligodendrocyte-specific lymphocytes in the central nervous system in EAE might explain both the subsidence of inflammation and the acquisition of tolerance in this autoimmune disease.

Keywords: apoptosis; autoimmunity; cell death; demyelination; experimental allergic encephalomyelitis; lymphocyte; oligodendrocyte; tolerance

Introduction

Cell death has been reported to occur in the central nervous system (CNS) in experimental allergic encephalomyelitis (EAE) (Lampert 1965; Field and Raine 1966; Lampert and Kies *1967;* Lassmann 1983; Lassmann et al. 1988; Blakemore et al. 1989). The dead cells have variously been considered to be oligodendrocytes (Lampert 1965; Field and Raine 1966), invading mononuclear cells (Lampert and Kies 1967) or lymphocytes (Blakemore et al. 1989). However, identification of the origin of the dead cells has been conceded to be difficult and prone to error (Lampert and Kies 1967; Lassmann 1983). Moreover, the mode of cell death occurring in EAE has not been determined.

There are two basically different mechanisms of cell death, necrosis and apoptosis. Apoptosis is a new and rapidly evolving concept of cell death. Necrosis is a degenerative phenomenon that follows irreversible injury; apoptosis, in contrast, is an active process of cellular self-destruction that often serves a biologically meaningful, homeostatic function (Walker et al. *1988).* Biochemically, there is distinctive internucleosome cleavage of DNA in apoptosis, which is different from the random DNA degradation observed in necrosis (Wyllie 1980). In the early stages of its evolution, apoptosis can be reliably distinguished from necrosis by its characteristic pattern of chromatin condensation and by the absence of cytoplasmic degeneration; however, at a late stage of the process, degeneration supervenes, and separation from necrosis on morphological grounds becomes difficult (Walker et al. 1988). Here we report the occurrence of apoptosis in the CNS in EAE.

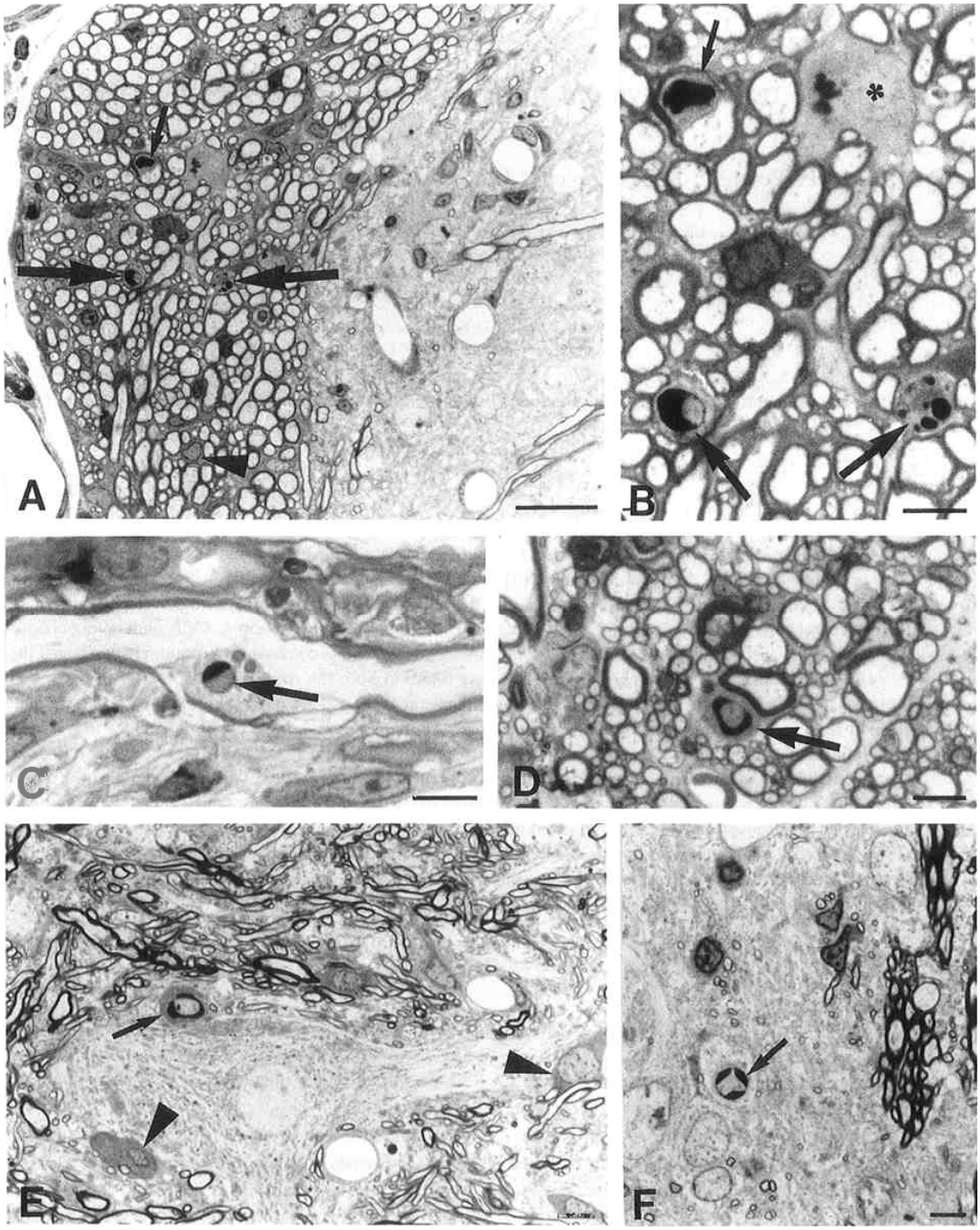

Fig. 1. A: S3 spinal cord of a rat with actively induced MBP-EAE 16 days post-inoculation (DPI) showing apoptotic cells (large arrows) adjacent to myelinated fibres. An intramyelinic pyknotic cell (small arrow) can also be seen. A normal oligodendrocyte is indicated by an arrowhead. B: higher magnification of (A) showing an apoptotic cell with a crescent of condensed chromatin (left large arrow), an apoptotic cell with multiple rounded masses of uniformly dense chromatin (right large arrow), an intramyelinic pyknotic cell (small arrow) and a mitotic figure (asterisk). C: S3 spinal cord, actively induced MBP-EAE 16 DPI. An intramyelinic apoptotic cell with the characteristic distribution of compacted chromatin (arrow) is present. D: T6 spinal cord, chronic relapsing EAE 29 DPI, showing an apoptotic cell (arrow) adjacent to normal myelinated fibres. E: L5 spinal cord grey matter, actively induced MBP-EAE 13 DPI. An apoptotic cell (arrow) lies adjacent to a motor neurone cell body. Normal oligodendrocytes can be seen (arrowheads). F: L5 spinal cord dorsal horn, actively induced MBP-EAE 13 DPI, showing an apoptotic cell (arrow). Epox 812 sections stained with toluidine blue. Bars: A = 25 μm; B-F = 10 μm.

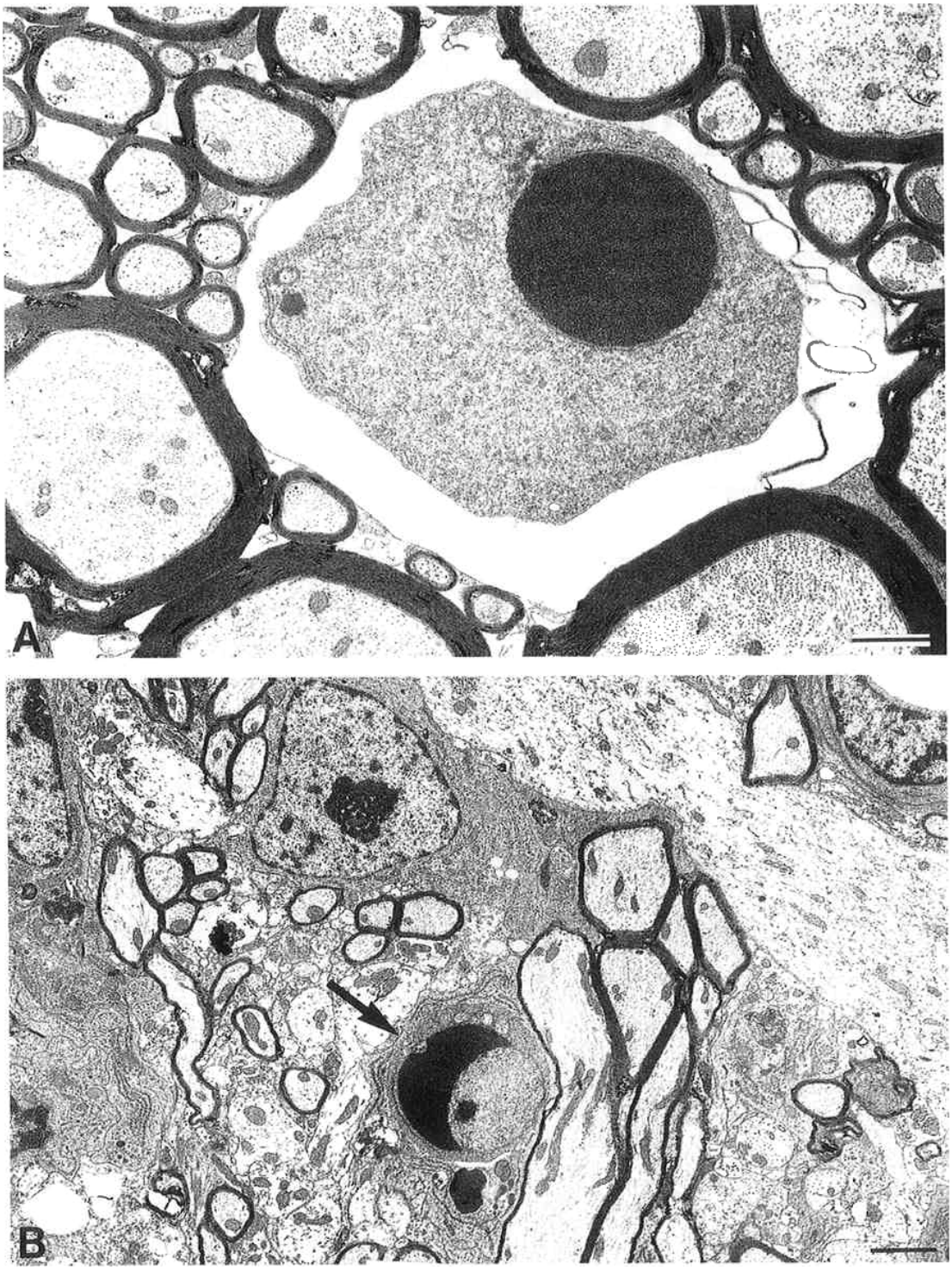

Fig. 2. Electron micrographs of the S3 spinal cord of a rat with actively induced MBP-EAE 16 DPI. A: an apoptotic cell containing a uniformly dense nuclear fragment lies adjacent to myelinated fibres in the white matter. B: apoptotic cell with a characteristic crescent of marginated compacted chromatin in its nucleus (arrow) lies adjacent to a myelinated fibre in the grey matter. Bars: A = 1 μm; B = 2 μm.

Materials and methods

Animals

Lewis rats (JC strain) were kept in cages of five and were fed rat and mouse cubes and water ad libitum.

Induction of EAE

Four different forms of EAE were induced.

Whole-spinal-cord-induced acute EAE. The inoculum was an homogenate of equal volumes of a 30% suspension of guinea pig spinal cord (the spinal roots having been removed) in 0.9% saline and a suspension of 4 mg of killed and dried *Mycobacterium butyricum* (Difco) per ml of incomplete Freund's adjuvant (Commonwealth Serum Laboratories, Melbourne, Australia). Under anaesthesia, male rats 8-10 weeks old were given 0.05 ml of inoculum in the footpad of each of the 4 feet or 0.1 ml in one footpad of each hindfoot.

Myelin basic protein (MBP)-induced acute EAE (actively induced acute MBP-EAE). MBP was prepared from guinea pig spinal cord (after removal of the spinal roots) by the method of Deibler et al. (1972). MBP in 0.9% saline was emulsified in an equal volume of incomplete Freund'sadjuvant containing 4 mg/ml of added *Mycobacterium butyricum.* Male rats, 8-10 weeks old, were inoculated with 0.1 ml of emulsion in one footpad of each hindfoot. The total dose of MBP was 50 μg/rat.

Passively transferred acute MBP-EAE. Passive EAE was induced as previously described (Pender et al. 1989). Single-cell suspensions were prepared from the spleens of donor rats sensitized 10-12 days previously with MBP as described above. Cells were cultured at a concentration of 2×10^6 cells/ml in RPMI 1640 with added 5% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 200 mM L-glutamine, penicillin and streptomycin. Concanavalin A was added at 2 μg/ml, and 50 ml cultures were incubated at 37°C in an atmosphere of CO_2 (10%), O_2 (7%) and N_2. Cells were harvested after 72 h and washed with Hank's balanced salt solution. 5 x 10^7 viable cells were injected into a lateral tail vein of each recipient male rat.

Chronic relapsing EAE. Chronic relapsing EAE was induced as previously described (Pender et al. 1990). Each batch of inoculum was prepared by homogenizing a mixture of 1 g guinea pig spinal cord, 1 ml 0.9% saline, 1 ml complete Freund's adjuvant (Difco) and 10 mg *Mycobacterium tuberculosis* H37RA (Difco). Female rats, 7-10 weeks old, were inoculated by the intradermal injection of 0.05 ml inoculum into the medial footpad of the right hindfoot. Commencing on the day of inoculation, the rats were given subcutaneous injections of cyclosporin A (Sandoz; 4 mg/kg) on alternate days until 22 days post-inoculation inclusive.

Histological studies

Under anaesthesia 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 spinal cord was immersed in fixative and postfixed with 1% osmium tetroxide (Dalton's solution) and embedded in Epox 812 (Ernest F. Fullam, Schenectady, NY). 1 pm sections were stained with toluidine blue for light microscopy. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined with an Hitachi H-300 electron microscope.

Results

Inflammation and demyelination were present in the spinal cord, spinal roots and dorsal root ganglia of rats with all forms of the disease, the appearances being similar to those described previously (Pender and Sears 1986; Pender 1988; Pender et al. 1989, 1990). By light microscopy pyknotic, dead cells were found in the spinal cord in animals with neurological signs in each case. Many of these cells were in the advanced stages of degeneration, and it was not possible to determine whether they had undergone necrosis or apoptosis (Fig. 1A,B). However, in some cases, the cell death could be unequivocally identified as apoptosis by the presence of either crescentic masses of condensed chromatin lying against the nuclear envelope or rounded masses of uniformly dense chromatin (Fig. 1A-F). The occurrence of apoptosis was confirmed by electron microscopy (Fig. 2A,B).

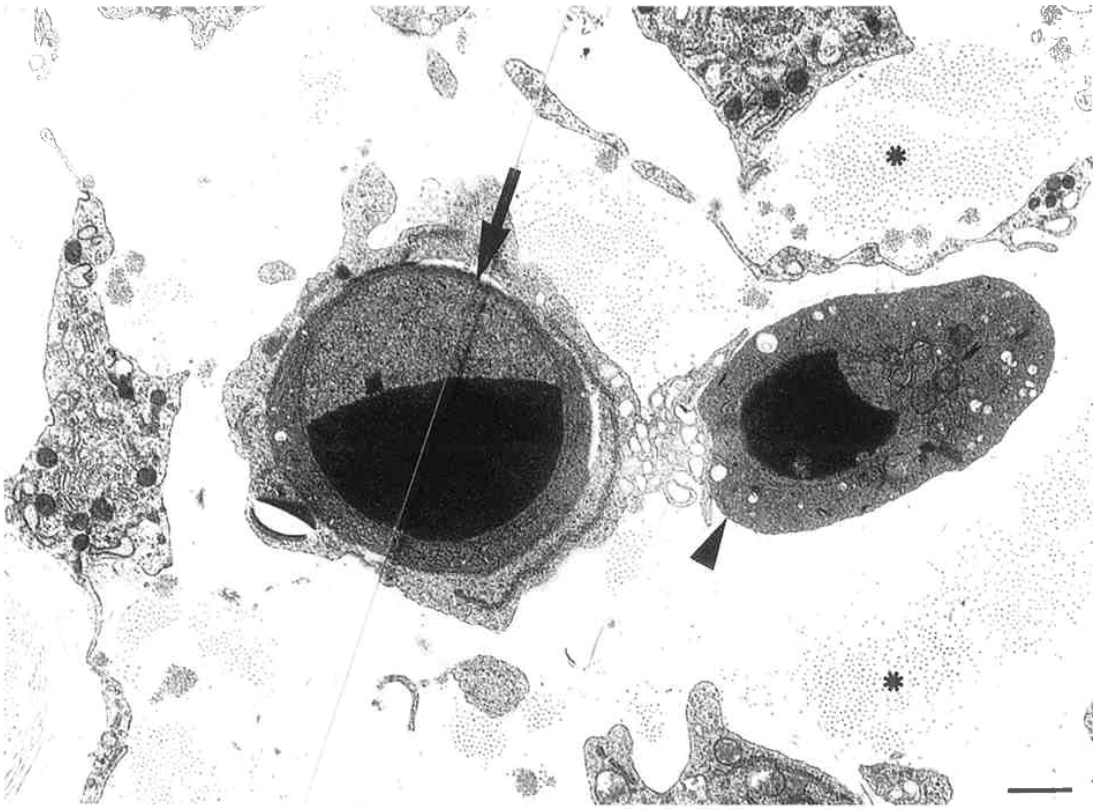

Fig. 3. Electron micrograph of the meninges of the T6 spinal cord of a rat with chronic relapsing EAE 29 DPI showing a macrophage that has ingested an apoptotic cell (arrow) and that is contacting another apoptotic cell (arrowhead). Extracellular collagen can also be seen (asterisks). Bar = 1 μm.

The apoptotic cells were present in both the white and grey matter of the spinal cord (Figs. 1 and 2) and were occasionally present in the meninges (Fig. 3) and perivascular spaces. In the white matter, they usually lay adjacent to myelinated fibres that were normal in appearance (Figs. 1A,B,D, 2A), but in chronic relapsing EAE they were also found adjacent to demyelinated fibres. Occasionally apoptotic cells were present within otherwise normal myelin sheaths (Fig. 1C) or dilated myelin sheaths. In the grey matter, apoptotic cells lay adjacent to neuronal cell bodies (Fig. 1E) and myelin sheaths (Fig. 2B) as well as in other positions. Phagocytosis of apoptotic cells was observed (Figs. 3 and 4) and apoptotic bodies were sometimes found within macrophages that contained myelin debris and that were adjacent to demyelinated axons (Fig. 4).

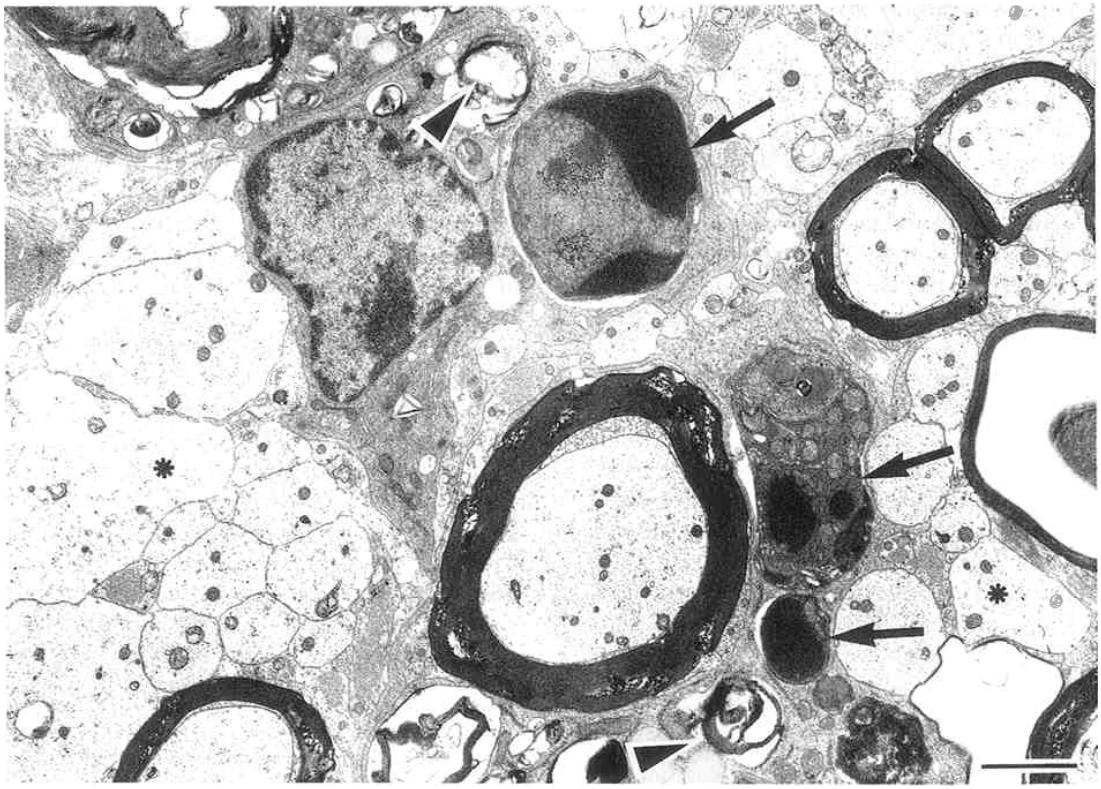

Fig. 4. Electron micrograph of the T6 spinal cord white matter of a rat with chronic relapsing EAE 29 DPI showing macrophages containing apoptotic bodies (arrows) and myelin debris (arrowheads) lying adjacent to demyelinated axons (asterisks). Bar = 2 μm.

Discussion

The present study is the first to recognize the occurrence of apoptosis in the CNS in EAE. In previous reports of cell death in this condition the cells have been referred to as degenerating, necrotic or pyknotic. However, inspection of the published micrographs reveals appearances diagnostic of apoptosis in some cases (see Fig. 5B and C in Lampert and Kies (1967) and Fig. 1B in Blakemore et al. (1989).

The identity of the apoptotic cells in the CNS in EAE is unclear. The location of some of them in the white matter and their size suggest that they are oligodendrocytes. How-ever, definitive identification of these cells as oligodendrocytes has not yet been possible. Oligodendrocyte apoptosis has been described in the postnatal period in the jimpy mouse and has been suggested as the explanation for the failure of CNS myelination in this mutant (Knapp et al. 1986). After normal myelination, oligodendrocyte death results in changes in the myelin sheath, phagocytosis of the sheath by macrophages and resultant primary demyelination (Blakemore 1982). Although oligodendrocyte apoptosis after myelination has not previously been recognized as such, Blakemore (1972) noted that, in cuprizone intoxication in mice, the nuclei of some of the dying oligodendrocytes showed more marked peripheral clumping of chromatin than normal or showed separation of nuclear components with the formation of several large masses of electron-dense material. Inspection of Fig. 7 of his paper reveals changes typical of apoptosis. In this model, oligo-dendrocyte death is followed by marked vacuolation of the myelin sheath resulting either from splitting of the intraperiod line or, less commonly, from expansion of the periaxonal space, and the myelin is phagocytosed (Blakemore 1972). Pyknotic oligodendrocytes are seen 2 weeks before demyelination occurs (Blakemore 1982), indicating the delay that may occur between oligodendro-cyte death and demyelination.

MBP-specific or oligodendrocyte-specific T-cells have been shown, using cytotoxicity assays, to kill oligodendrocytes in vitro although the mode of oligodendrocyte death has not been determined (Kawai and Zweiman 1988, 1990; Jewtoukoff et al. 1989). In other systems, apoptosis has been shown to be the mechanism of T-cell-induced target cell death (see Walker et al. 1988). We

therefore propose that oligodendrocyte apoptosis may be occurring in EAE asa result of oligodendrocyte-directed T-cell cytotoxicity, and that this may contribute to the primary demyelination in this disease. It is possible that the dilatation of the myelin sheath (intramyelinic oedema) observed in EAE (Lassmann et al. 1980; Brosnan et al. 1988; Pender et al. 1990) is the result of oligodendrocyte apoptosis.

While it is possible that some of the apoptotic cells in the CNS in EAE are oligodendrocytic in nature, it is clear that the non-phagocytosed apoptotic cells within the myelin sheath, the meninges and the perivascular spaces could not be oligodendrocytes. These cells are most likely blood-derived mononuclear cells, either lymphocytes or monocyte-macrophages. The sparsity of their cytoplasm and the absence of phagocytosed material suggest that they are mainly lymphocytes. The definitive identification of the origin of these apoptotic haematogenous cells will require immunocytochemical studies. Blakemore et al. (1989) have reported the occurrence of intramyelinic pyknotic cells in acute EAE induced by the passive transfer of MBP-specific lymphocytes. They considered that these cells were lymphocytes specific for MBP, but immunocytochemical studies were not performed.

If apoptosis of myelin/oligodendrocyte-specific lymphocytes occurs in the CNS in EAE, this could be important in the immunoregulation of this autoimmune disease. Rats rapidly recover from acute EAE and acquire a long lasting tolerance to MBP (Willenborg 1979). Activation-induced apoptosis of immature T-cells in the thymus has been proposed as the mechanism responsible for clonal deletion of autoreactive cells leading to tolerance during normal development (Smith et al. 1989; Murphy et al. 1990). Activation-induced apoptosis of mature T-cells has been observed in vitro (Newell et al. 1990) and also occurs in vivo as a mechanism of extrathymic (peripheral) tolerance to foreign antigen (Kawabe and Ochi 1991). The deletion of target antigen-specific lymphocytes in the target organ in EAE could explain both the clinical recovery and the acquisition of tolerance; it would be consistent with the conclusion of others that effector T-cells turn themselves off in EAE (Willenborg et al. 1986).

In conclusion, we have reported the new finding that apoptosis occurs in the CNS in EAE, suggested that the apoptotic cells are oligodendrocytes or lymphocytes and discussed the possible significance of this observation. As apoptosis is an active process that can {be controlled, agents that modulate this process may prove useful in the therapy of inflammatory demyelinating disease.

Acknowledgements

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