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Macrophage Apoptosis in the Central Nervous System in Experimental Autoimmune Encephalomyelitis

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Abstract
Using light and electron microscopy, we have demonstrated that macrophage apoptosis (programmed cell death) occurs in the central nervous system (CNS) in Lewis rats with acute experimental autoimmune encephalomyelitis (EAE) and chronic relapsing EAE. Apoptotic macrophages were identified by the presence of an apoptotic nucleus in a cell with cytoplasm containing myelin debris but no intermediate filaments. They were found in the meninges, perivascular spaces and in the parenchyma of the white and grey matter of the spinal cord. In acute EAE the apoptotic macrophages were most frequently seen at the time of maximal neurological signs and during the early stages of clinical recovery. Several possible mechanisms may be responsible for the macrophage apoptosis: the release or withdrawal of cytokines; T-cell cytotoxicity; the effect of activated macrophage products, such as nitric oxide; and a direct effect of endogenous glucocorticoids. Macrophage apoptosis, together with the T-cell apoptosis we have previously described in the CNS in EAE, may contribute to the down-regulation of this autoimmune disease.

Introduction
Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease, which serves as a putative animal model of the human demyelinating disease, multiple sclerosis [1]. We have recently reported the occurrence of apoptosis (programmed cell death) in the central nervous system (CNS) in acute EAE and chronic relapsing EAE in the Lewis rat [2]. On the basis of their morphology, size and location, we suggested that some of the apoptotic cells were oligodendrocytes and that others were blood-derived mononuclear cells (lymphocytes or possibly macrophages) [2]. Using a pre-embedding immunolabelling technique we subsequently demonstrated that about half of the apoptotic cells in the spinal cord in acute EAE induced by inoculation with myelin basic protein (MBP-EAE) are a[3 T lymphocytes [3]. We now report that some of the apoptotic cells in the spinal cord in acute EAE and chronic relapsing EAE are macrophages. To our knowledge, this is the first report of macrophage apoptosis in the target organ of any autoimmune disease.

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
Whole-spinal-cord-induced acute EAE
The inoculum was a homogenate of an equal volume of a 30% suspension of guinea pig spinal cord 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.

Acute MBP-EAE
MBP was prepared from guinea pig CNS tissue by the method of Deibler et al. [4]. MBP in 0.9% saline was emulsified in an equal volume of incomplete Freund's adjuvant 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.

Chronic relapsing EAE

Chronic relapsing EAE was induced as previously described [5]. 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. As previously described [5], some of the inoculated rats had a chronic persistent or chronic progressive instead of a chronic relapsing clinical course.

Histological studies

Under anaesthesia, rats were perfused via the aorta with 0.9% saline followed by 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3-7.4). Specimens of the spinal cord were post-fixed in 1% osmium tetroxide in dichromate buffer (Dalton's solution). Some specimens were embedded in HistoResin, sectioned (1 μm) and stained with cresyl fast violet for light microscopy as previously described [6]. Others were stained en bloc with uranyl acetate, dehydrated in a series of graded ethanol solutions followed by absolute acetone, embedded in epoxy resin, sectioned (0.5 μm) and stained with toluidine blue for light microscopy. Ultrathin epoxy sections were stained with lead citrate and examined with a Jeol JEM-1200 EXII electron microscope.

Results

Apoptosis was recognized by the presence of either crescentic masses of condensed chromatin lying against the nuclear envelope or discrete rounded masses of uniformly dense chromatin within the nucleus [7]. In all three forms of EAE, light microscopy revealed apoptotic cells in the spinal cord. Many of the apoptotic cells had the appearance of lymphocytes, in accordance with our previous immunocytochemical study [3]. However, some of the apoptotic cells had a large amount of cytoplasm containing lipid droplets, vacuoles and myelin debris, indicating that they were macrophages. In some instances, serial sections were cut through these cells to ensure that the only nucleus present was an apoptotic one; this excluded the possibility that the apoptotic nucleus belonged to another cell that had been ingested by a normal macrophage (see Figure IA).

At electron microscopy, we confirmed that some of the apoptotic cells in the spinal cord in all three forms of EAE were apoptotic macrophages, as determined by the presence of an apoptotic nucleus in a cell with cytoplasm containing myelin debris but no intermediate filaments (Figure 1B). The absence of intermediate filaments was required to exclude the possibility that the cell was an astrocyte, which also has a phagocytic capacity. When apoptotic macrophages contained a large amount of myelin debris, the nuclei had an irregular shape, being indented or compressed by cytoplasmic vacuoles laden with debris (Figures 1B, 2A, 2B). In many instances, apoptotic macrophages, like other apoptotic cells, were engulfed by other macrophages (Figure 2A). Some of the apoptotic macrophages showed secondary degeneration [8] (Figure 2B). Apoptotic macrophages were located in the meninges and perivascular spaces and in the parenchyma of the white and grey matter of the spinal cord. They were most readily identified in sections containing macrophages heavily laden with myelin debris, as in chronic persistent EAE. In acute EAE, apoptotic macrophages were most frequently seen at the time of maximal neurological signs and during the early stages of clinical recovery.

Discussion

EAE is a demyelinating disease characterized by the infiltration of the nervous system by lymphocytes and macrophages. Encephalitogenic T cells initiate the inflammatory process and release cytokines [9] which attract and activate other cells including macrophages which are essential for the pathogenesis of EAE [10]. In EAE, macrophages invade myelinated fibres and

phagocytose myelin [5] and are easily recognized when they contain myelin debris. Little is known about the fate of macrophages in tissue inflammation [11]. In the present study we have shown that some macrophages die by apoptosis in the CNS in EAE. We used strict morphological criteria, including the presence of myelin debris, to identify apoptotic macrophages. Some apoptotic cells which did not contain myelin debris had features such as stringy rough endoplasmic reticulum, multiple mitochondria, lipid droplets, lysosomes and pinocytotic vesicles suggesting that they might also be macrophages but immunocytochemistry would be needed to determine this.

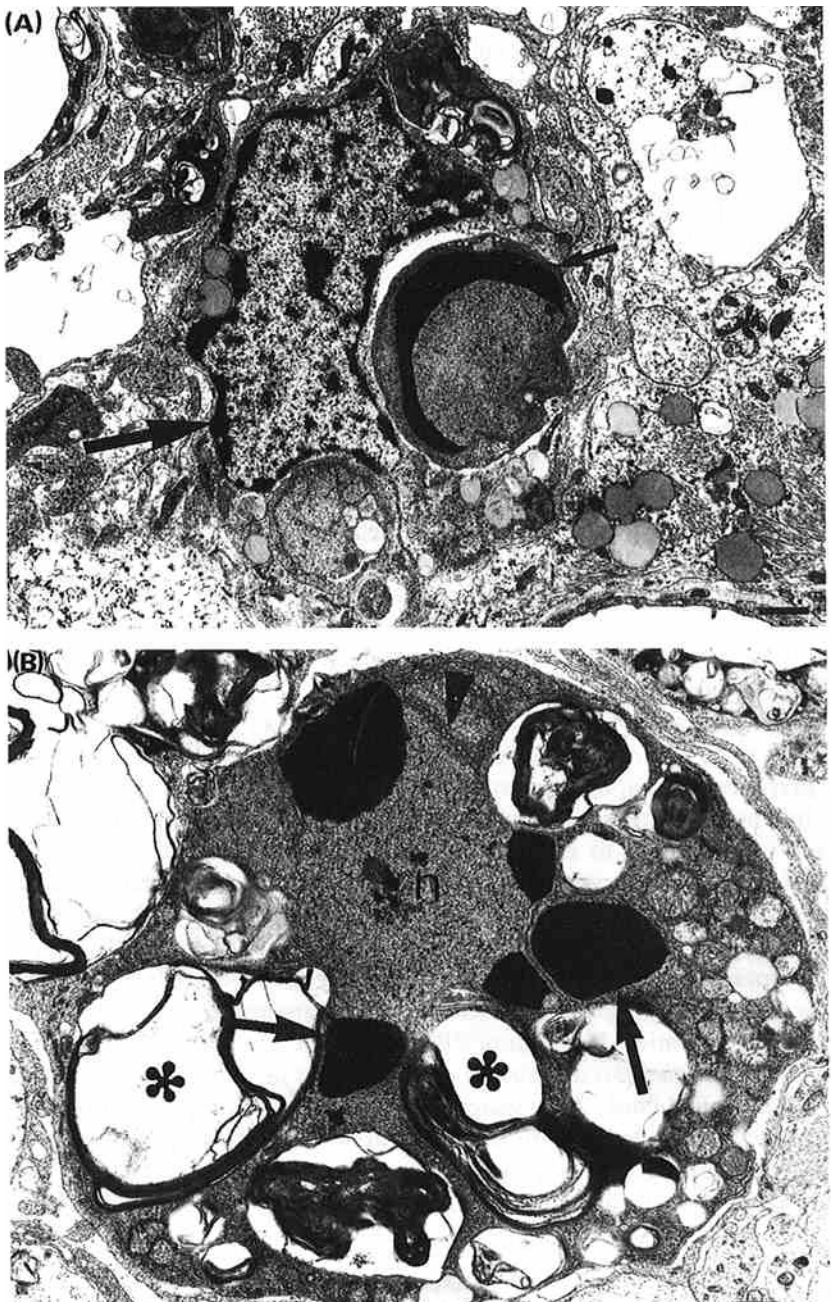

Figure 1. Electron micrographs of the spinal cord of a Lewis rat with chronic persistent EAE (33 days after inoculation) showing: (A) An apoptotic cell phagocytosed by a normal macrophage. The apoptotic cell has a small round nucleus and sparse cytoplasm (small arrow), which does not contain myelin debris or any visible organelles and is likely to be a lymphocyte. The normal nucleus of the macrophage is clearly seen (large arrow). (B) An apoptotic macrophage with vacuoles containing myelin debris (asterisks). The apoptotic nucleus (n) is budding (arrows). Nuclear pores can be seen adjacent to euchromatin (arrowhead), but not next to condensed chromatin. Bar= 1μm.

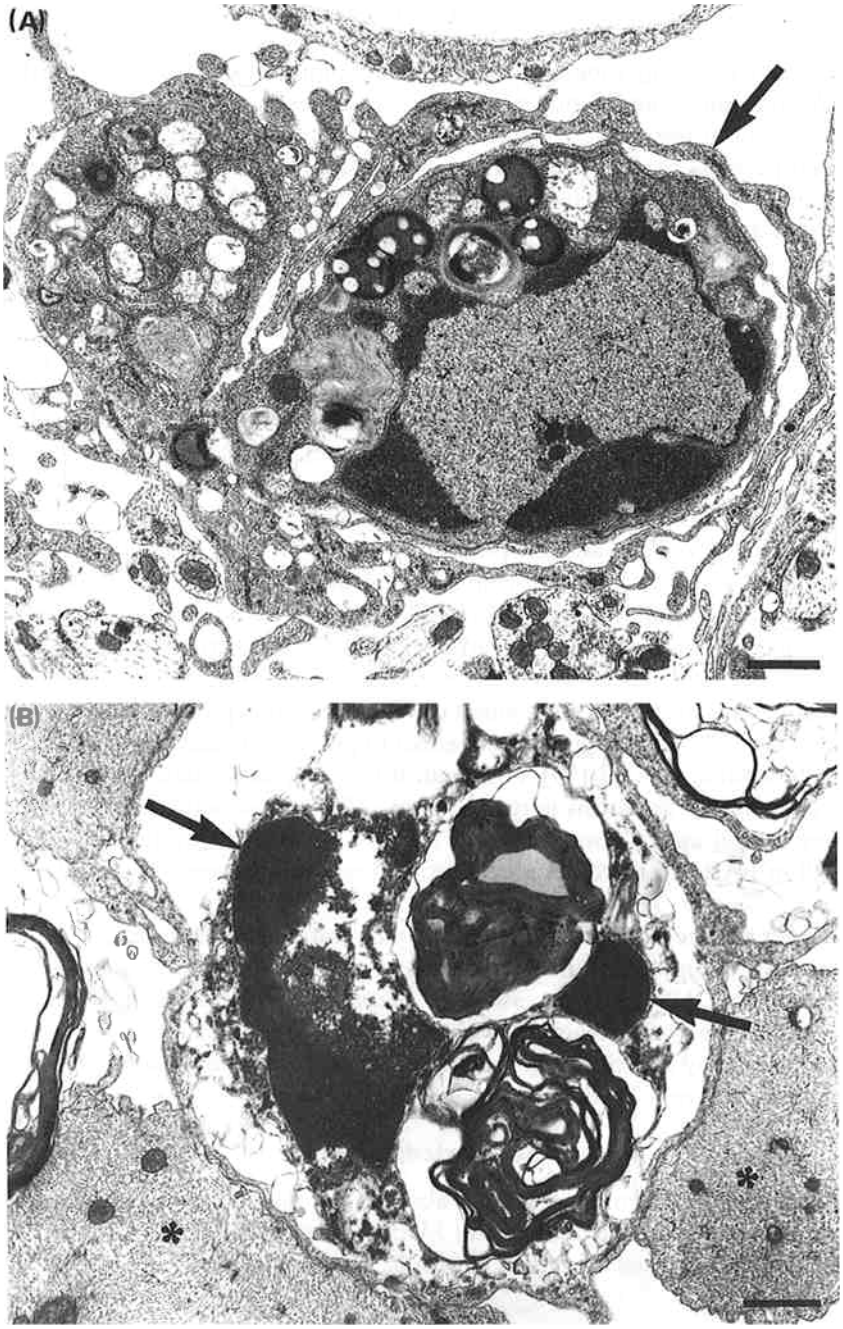

Figure 2. Electron micrographs of the spinal cord of a Lewis rat with chronic persistent EAE (33 days after inoculation) showing: (A) An apoptotic macrophage surrounded by the processes of another macrophage (arrow). (B) An apoptotic macrophage showing secondary degeneration. The nucleus (arrows) and myelin debris can be seen. Demyelinated axons (asterisks) are also present. Bars=l μm.

Several possible mechanisms may be responsible for the macrophage apoptosis. Firstly, macrophage apoptosis may be due to the release or withdrawal of cytokines. Interleukin-4 (IL-4) and IL-6, which are found in the CNS in EAE [9], can induce apoptosis of monocytes or macrophage cell lines [12, 13]. Withdrawal of cytokines such as IL-1, tumor necrosis factor-a and interferon-7 (IFN-y), which are also produced in the CNS in EAE [9, 14] and which prevent monocyte apoptosis in vitro [15], might lead to macrophage apoptosis. Withdrawal of T-cell-derived cytokines could occur when T cells die by apoptosis or are inhibited by other means. Secondly, macrophage apoptosis might be caused by cytotoxic T cells, which kill their targets by inducing apoptosis. Encephalitogenic MBP-specific T cells are cytotoxic to MBP-pulsed macrophages in vitro [16]. In EAE, encephalitogenic T cells

might kill macrophages that have ingested myelin debris. Cytotoxic T-cell activity directed against antigen-presenting cells plays an important role in the down-regulation of the immune response and in the prevention of tissue damage by hyperactivated macrophages [17]. Thirdly, the toxic products of activated macrophages themselves may be responsible for the macrophage apoptosis. For example, nitric oxide, a prominent cytotoxic product of activated macrophages, can induce apoptosis in these cells in vitro [18]. Fourthly, it is possible that macrophage apoptosis could be caused by a direct effect of glucocorticoids which are produced during the course of EAE [19]. Although it is not known whether glucocorticoids can directly induce apoptosis in macrophages, they can directly induce apoptosis in thymocytes [20] and T lymphocytes [21]. IFN-y, which is produced by T cells in the CNS in EAE [9], increases glucocorticoid receptor expression on macrophages [22]

Whatever the mechanism, the apoptotic elimination of macrophages in the CNS in EAE may be important in down-regulating this autoimmune disease. Macrophages in the CNS play an essential role in the development of EAE [10]. Macrophage apoptosis, together with the T-cell apoptosis we have previously described [3], may contribute to the resolution of inflammation in the CNS during clinical recovery from EAE.

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