Apoptotic Cells Are Present in the CNS throughout Acute and Chronic-Progressive EAE in the Absence of Clinical Recovery

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**Abstract.** Experimental allergic encephalomyelitis (EAE) is an autoimmune, demyelinating disorder of the central nervous system induced in susceptible animals as a model for the human disease multiple sclerosis. Antibodies against the leukocyte adhesion molecule α4 integrin have been shown to prevent and reverse acute and chronic EAE of the guinea pig. The results presented in this paper implicate apoptosis as the mechanism of reversal of EAE following treatment with anti-α4 integrin antibody. Apoptotic cells were observed in the central nervous system (CNS) throughout chronic-progressive EAE of the guinea pig in the absence of clinical recovery. Many of the apoptotic cells were identified as T cells using immunohistochemistry. Similarly, apoptotic cells were present in the CNS of animals during anti-α4 integrin-mediated recovery from acute and chronic disease. Therefore, anti-α4 integrin-mediated recovery from EAE is due to the prevention of the influx of new inflammatory cells into the CNS that are required to replace those undergoing apoptosis.

**Key Words:** Apoptosis; Autoimmune disease; CNS; EAE; Multiple sclerosis.

**INTRODUCTION**

Acute experimental allergic encephalomyelitis (EAE) has a monophasic course in which a period of neurological symptoms is followed by a spontaneous recovery. Similarly, in chronic-relapsing EAE, periods of disease activity (relapses) are followed by periods of clinical quiescence (remission). The neurological recovery that takes place in monophasic disease and at the time of each remission of chronic-relapsing disease is accompanied by resolution of the inflammation in the central nervous system (CNS). In actively-induced, acute EAE of the Lewis rat, clinical recovery is evident approximately 10 days following the onset of neurological signs. At this time, much of the inflammation associated with acute disease has resolved and remyelination of damaged myelin sheaths has begun (1). In passive EAE of the Lewis rat, a complete clinical recovery occurs 7 days following the onset of neurological symptoms and is concurrent with the onset of remyelination (2). Similarly, remission during chronic-relapsing EAE of the SJL/J mouse is not due to suppression of the activity of the cells within the CNS, but rather due to the loss of these cells from the CNS within 48 hours (3).

The resolution of the inflammatory response in acute and chronic-relapsing EAE and the ensuing clinical recovery appear to be consequences of apoptotic deletion of the infiltrating cells. T cell and macrophage apoptosis have been demonstrated in passive and active models of acute EAE of the rat (4–6) and in chronic-relapsing EAE of the rat (4, 6). The number of apoptotic cells was maximal during the recovery from disease; in one study, up to 49% of all T lymphocytes were apoptotic at the time of recovery (5).

We have previously demonstrated a reversal of the neurological symptoms of acute and chronic-progressive EAE and a resolution of the inflammatory activity following a short course of treatment with an antibody against α4 integrin (7–9). In the absence of antibody treatment, neither clinical recovery nor resolution of inflammation was observed. The mechanism of clearance of the inflammatory cells from the CNS following administration of the anti-α4 integrin antibody is unclear. However, the time course of the rapid recovery and resolution of the inflammation is comparable to that seen in acute monophasic disease. Although apoptotic cells have been identified at the time of recovery from EAE in many studies, an investigation of apoptosis in chronic-progressive disease (in which animals do not normally recover) has not been done previously. The experiments described herein implicate apoptosis as a mechanism for continual, local elimination of inflammatory cells from the CNS and as the mechanism of recovery following anti-α4 treatment in chronic-progressive EAE of the guinea pig.

**MATERIALS AND METHODS**

**Animals and EAE Induction**

Female Hartley guinea pigs (Charles River Canada, St-Constant, Quebec) were maintained in a controlled light environment and allowed food and water ad libitum. EAE was induced in 78 animals (200–250 g) by intradermal, nuchal injection of 0.2 ml of a 1:1 mixture of homogenized isologous CNS tissue (in saline) and CFA (Difco, Detroit, MI) with the addition of 10 mg of inactivated Mycobacterium tuberculosis (Difco) per ml of CFA (10, 11). The animals were assessed daily using a 4-point clinical scale as follows 0: no abnormality; 0.5: more than one day of weight loss; 1.0: hind limb weakness, poor righting reflex; 2.0: paresis, urinary incontinence, fecal impaction; 3.0: paralysis; 4.0: terminal paralysis. Three guinea pigs were used as control non-EAE animals.

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A. Parenchymal perivascular infiltration
   0. No changes.
   1. One to three parenchymal vessels infiltrated in Virchow-Robin spaces.
   2. Four to six vessels involved.
   3. More than six vessels involved.
   4. Virtually all or nearly all vessels involved.
B. Myelitis
   0. No invasion of the neural parenchyma; microglial or inflammatory cells invading neural parenchyma.
   1. A few scattered cells.
   2. Invasion by cells from several perivascular cuffs.
   3. Large areas of neural parenchyma involved.
   4. Virtually the entire section is infiltrated.

For the purposes of calculating the cellularity index, each slide was assigned a score from 0 to 4 for each category, and the 2 values were combined to give a range of 0 to 8.

### Antibodies and Treatment Regimens

AN100226m (Athena Neurosciences/Elan, South San Francisco, Calif) is an anti-α4 integrin antibody used as an in vivo inhibitor of α4 integrin. AN100226m reacts with guinea pig lymphocytes and monocytes, but not with neutrophils. This pattern of reactivity is the same with human blood cells and is consistent with the expression of α4 integrin (7). TM2a (Athena Neurosciences/Elan) is a control antibody of identical isotype to AN100226m. Injected solutions contained less than 1.2 (0.8-0.07 EU/mg) U of endotoxin as determined by the limulus amoebocyte assay (Associates of Cape Cod, Inc., Woods Hole, Mass).

Animals were treated with AN100226m (4 mg/kg) or TM2a (4 mg/kg) during the acute and chronic-progressive phases of EAE. AN100226m (n = 9) or TM2a (n = 9) were administered by subcutaneous injection on days 12 and 15 during the acute phase of disease. Another 18 animals were treated with AN100226m (n = 9) or TM2a (n = 9) on days 42 and 45 during the chronic-progressive phase of disease.

### Animal Sacrifice and Tissue Collection

Untreated, control EAE animals were sacrificed prior to disease onset (days 7 and 9) and throughout the acute (days 12 and 26) and chronic-progressive (days 42 and 87) disease. Animals were sacrificed on days 14, 16, and 18 from the groups treated with AN100226m or TM2a on days 12 and 15. Animals were also sacrificed on days 44, 46, and 48 from the AN100226m or TM2a groups treated on days 42 and 45. Three animals were sacrificed from each treatment group at each time point.

At the time of sacrifice (0.25 ml sodium pentobarbital), animals were exsanguinated and their brains and spinal cords were dissected as described. One half of each brain and spinal cord was fixed in 10% formalin and embedded in paraffin. The other half of each brain and spinal cord was flash frozen and stored at −70°C. Sections of paraffin-embedded tissue were stained with hematoxylin-eosin or solochrome-R-cyanin and assigned a score from 0–8 (cellularity index) by a blinded observer, which reflected the extent of inflammation in the perivascular spaces and tissue parenchyma (Table).

### Detection of Apoptotic Cells

Cells undergoing apoptosis were identified using the ApopTag® peroxidase kit (Oncor). Briefly, 5-μm sections of formalin-fixed, paraffin-embedded spinal cord were mounted on positively charged slides, deparaffinized in xylene, and rehydrated in ethanol. Following permeabilization with protease K (Sigma-Aldrich Canada Ltd, Oakville ON), the tissue was incubated with terminal deoxynucleotidyl transferase and digoxigenin-labeled nucleotides. Incorporated nucleotides were detected with anti-digoxigenin antibodies conjugated to horse-radish peroxidase and the color was developed in diaminobenzidine. Sections were counterstained with Mayer’s-hematoxylin and celestine blue and sealed in glycerol. The number of apoptotic cells in a standard sagittal-section of spinal cord were counted by a blinded observer. An average of 8 spinal sections per animal were counted in each of 3 animals per day to calculate the average number of apoptotic cells per spinal cord cross-section.

### Immunohistochemical Identification of Apoptotic Cells

For determination of the cell type undergoing apoptosis in the CNS, tissue sections were sequentially processed for immunohistochemical detection of T cells and the presence of cells undergoing apoptosis using the ApopTag® technique. Eight-μm frozen spinal cord sections were mounted on positively charged slides, fixed, and post-fixed. Subsequently, the tissue was permeabilized with protease K, and nonspecific antigenic sites were blocked with 2% milk proteins. Sections were incubated in primary antibody (1:500 mouse anti-guinea pig T cells, Serotec) followed by alkaline-phosphatase-conjugated goat anti-mouse antibodies. The sections were developed with Fast-red TR/naphthol containing 0.15 mg/ml levamisole (Sigma) to block endogenous alkaline phosphatases. Following thorough washing in PBS, the tissue was processed for detection of apoptotic cells as described above.

### Statistical Analysis

Statistical analysis was performed using SigmaStat v2 software. One way ANOVA with Tukey test or Kruskal-Wallis one
Fig. 1. The number of apoptotic cells observed in spinal cord sections of EAE animals throughout chronic-progressive EAE of the guinea pig. EAE was induced in all animals on day 0 and animals were sacrificed during the disease course for analysis of the number of cells undergoing apoptosis. The number of apoptotic cells was counted by a blinded observer in an average of 8 sections of spinal cord per animal. The data is expressed as mean (± SEM) number of apoptotic cells per section of spinal cord for all sections of spinal cord examined in all animals sacrificed on a particular day. The numbers at the end of each bar are the clinical scores of the animals at the time of sacrifice. Despite significant numbers of apoptotic cells throughout acute and chronic EAE, clinical recovery was not apparent. *Indicates a significant difference from days 0, 7, and 9 (p < 0.05; Kruskal-Wallis one way ANOVA on Ranks with Dunn’s multiple comparison procedure). Temporal divisions of the disease course are labeled.

way ANOVA were used where appropriate, with p < 0.05 considered significant. A linear regression was performed on the apoptotic cell counts and the cellularity score.

RESULTS

Apoptosis Throughout Acute and Chronic-Progressive Disease

Formalin-fixed, paraffin-embedded sections of spinal cord were processed with the ApopTag® kit to identify cells undergoing apoptosis. Apoptotic cells were present throughout acute and chronic-progressive EAE of the guinea pig in the absence of clinical recovery (Fig. 1). Untreated EAE animals were sacrificed prior to disease onset (days 7 and 9) and throughout the acute (days 12 to 26) and chronic-progressive (days 39 to 87) disease. The number of cells undergoing apoptosis was counted in an average of 8 sections per animal (range 4 to 11 sections per animal) in each of 3 animals at each time point. The spinal sections used for each animal were selected from lumbar, thoracic, and cervical regions of the cord. Very few apoptotic cells were found in non-EAE animals (day 0), in EAE animals sacrificed prior to (day 7), or at the onset of neurological symptoms (day 9). Beginning on day 12, apoptotic cells were present in spinal sections of all animals. Each of these animals displayed neurological signs of disease at the time of sacrifice. The clinical scores of the individual animals sacrificed each day are indicated above each bar. On day 12, elevated numbers of apoptotic cells were found in all spinal cord sections examined, with an average of 57 ± 7 apoptotic cells per sagittal section of spinal cord (p < 0.05; Kruskal-Wallis one way ANOVA on ranks with Dunn’s multiple comparison procedure). This elevation in the number of apoptotic cells in spinal cord sections was maintained throughout the course of the chronic-progressive disease. Apoptotic cells were found in all animals studied at all stages of EAE, although no signs of clinical recovery were apparent.

Most of the apoptotic cells were found in regions of inflammation; within the perivascular cuffs of inflammatory cells, within regions of meningeal infiltration, and throughout regions of parenchymal infiltration. Greater numbers of apoptotic cells were found in regions of more severe infiltration. Few apoptotic cells were found within
the white and gray matter of the spinal cord, usually in close proximity to myelin tracts. Figure 2 depicts apoptotic cells detected in control EAE animals. Figures 2A, B, and C are photomicrographs of spinal cord from an EAE animal sacrificed on day 87. Figure 2A is a solochrome-R-cyanin stained section showing a small patch of inflammation (and demyelination) in the meninges and surrounding a radial blood vessel. Figures 2B and C illustrate apoptotic cells around this small lesion. Cells can be seen within the perivascular cuff and in the surrounding parenchyma. In Figure 2B, several apoptotic cells can be seen in the white matter at a distance from any inflammation. Figures 2D, E, and F are photomicrographs of spinal cord from an animal with chronic progressive disease (d46). Figure 2D is a solochrome-R-cyanin stained section illustrating a large demyelinated plaque with extensive myelitis and perivascular inflammation. Figures 2E and F are low and high power photomicrographs of the same section of spinal cord showing many apoptotic cells within this large lesion.

Clinical Course of EAE

The animals were treated with either TM2a or AN100226m on days 12 and 15 during the acute phase of EAE, or days 42 and 45 during the chronic-progressive phase of EAE. Others received no treatment.

Acute Treatment

The onset of the clinical signs of disease was apparent on day 8 in all groups of animals (Fig. 3). The mean clinical scores of the control and acute treatment TM2a or AN100226m group of animals were not significantly different on day 12 (Fig. 3A). The mean clinical score for the control and TM2a-treated animals was unchanged during the treatment period. The mean clinical score for the AN100226m-treated animals decreased rapidly following treatment on day 12 to a score of 0 ± 0 on day 18. The pooled clinical scores from all days following the onset of treatment (days 13 to 18) for the AN100226m-treated group was significantly different from the control and TM2a-treated groups (p < 0.05; Kruskal-Wallis one way ANOVA on ranks with Dunn’s multiple comparison procedure).

Chronic Treatment

The clinical course of disease in the control and chronic treatment TM2a or AN100226m groups were not different from day 0 to 42 (Fig. 3B). During the treatment period, the mean clinical score of the AN100226m-treated animals decreased slightly. One AN100226m-treated animal improved from a clinical score of 3 to a score of 2, and one improved from a score of 2 to a score of 1. The mean clinical scores of the control and TM2a-treated groups were unchanged during the treatment period.

Apopotosis Throughout Anti-α4 Antibody-mediated Recovery from Acute and Chronic-Progressive EAE

The number of cells undergoing apoptosis was determined in paraffin-embedded sections of spinal cord from animals treated with AN100226m or TM2a during the acute and chronic-progressive phases of disease. Figure 4 illustrates the mean number of apoptotic cells in spinal cord sections of animals treated with AN100226m or TM2a on days 12 and 15 and sacrificed on days 14, 16, or 18 or treated on days 42 and 45 and sacrificed on days 44, 46, or 48. Apoptotic cells were observed in spinal cord sections of all animals regardless of treatment. The number of apoptotic cells in spinal cord sections of acute EAE animals was decreased following treatment with AN100226m on days 12 and 15 (p < 0.05; one way ANOVA with Tukey test). Fewer apoptotic cells were also observed in the spinal sections from the animals treated with AN100226m on days 42 and 45, although this was significant only on day 46. In contrast, there was no change in the number of apoptotic cells observed in animals treated with TM2a (the control antibody) during either the acute or chronic-progressive phase of EAE.

Figure 5 illustrates cells undergoing apoptosis during anti-α4 integrin-mediated recovery from chronic-progressive EAE. Figure 5A is a solochrome-R-cyanin stained section of spinal cord from a guinea pig treated with AN100226m during chronic-progressive disease. There was a notable reduction in the number of inflammatory cells present in this lesion when compared with an untreated animal (Fig. 2D). Figures 5B and C illustrate apoptotic cells within the lesion in panel A.

Relationship between the Degree of Inflammation and Extent of Apoptotic Cell Death

The inset of Figure 6 illustrates the relationship between cellularity index and time course following immunization. The cellularity index, derived from sequential hematoxylin-eosin and solochrome-R cyanin-stained sections, scores the infiltration of immune cells within the perivascular spaces and parenchyma of the spinal cord. No infiltrates were observed until day 9 postimmunization. Subsequently, the index increased rapidly as cellular infiltrates accumulated, rising to a score between 6 and 8, which persisted from day 20 and later. Therefore, immune cells rapidly accumulate during the acute portion of the experimental disease (d10 to 20) and the levels of infiltrates are maintained throughout the disease course despite substantial apoptosis (Fig. 1). A highly significant and direct relationship was observed between the cellularity index and the number of apoptotic cells counted on the same tissue section (R = 0.604, p < 0.001) (Fig. 6, main panel). The higher the cellularity index, the higher the number of apoptotic
Fig. 2. Apoptotic cells in spinal cord sections of animals with chronic-progressive EAE of the guinea pig. A: Solochrome-R-cyanin–stained section of a day 87 EAE animal demonstrating inflammation around a penetrating blood vessel (magnification, 120×). B and C: Apoptotic cells surrounding the same blood vessel shown in A (magnification 120×, 310×). Apoptotic cells are identified by the presence of a brown reaction product in the nucleus. Few apoptotic cells can be seen at a distance from the inflammatory lesion (arrowheads). D: Solochrome-R-cyanin–stained section of a day 46 EAE animal demonstrating extensive inflammation, myelitis, and demyelination of a large section of spinal cord (magnification, 120×). E and F: Apoptotic cells in the same lesion shown in D (magnification 120×, 310×).

cells counted per slide, with some sections reaching over 1000 cells. Although there are likely many factors contributing to the number of cells undergoing apoptosis, the extent of cellular infiltration present in the spinal cord is an important factor.

Identification of the Cells Undergoing Apoptosis

Immunohistochemistry was combined with the ApopTag® staining to identify the cell types undergoing apoptosis. Since other studies have demonstrated that the
The red reaction product identifies T cells and the brown reaction product indicates apoptotic nuclei. In panel A, double-stained apoptotic T cells are visible along with many nonapoptotic T cells. In panel B, double-stained cells and single-stained apoptotic or T cells can be identified. T cells were stained in the various stages of apoptosis; staining was apparent in cells in the early stages of apoptosis with chromatin condensation and in cells that were undergoing fragmentation into apoptotic bodies. Technical difficulties with the double-labeling procedure did not permit quantification of the percentage of T cells undergoing apoptosis. Fewer apoptotic cells were observed in sections that had previously been processed for immunohistochemistry than were observed in adjacent sections of spinal cord from the same animal processed for ApopTag staining alone. Therefore, it was not possible to provide convincing evidence for the exact number of apoptotic T cells. However, the majority of apoptotic cells in these double-stained sections were T cells. Most of the apoptotic cells were found in regions of inflammation and are therefore assumed to be T cells and macrophages.

**DISCUSSION**

Apoptosis is believed to be responsible for the recovery of animals from passive-transfer, active whole-spinal-cord-induced and active MBP-induced, acute monophasic EAE (4, 5, 12). Up to 49% of all T cells have been observed undergoing apoptosis at the time of recovery from the acute lesions of EAE (5). Apoptosis may also contribute to the onset of clinical remissions in chronic-relapsing EAE, since apoptotic T cells and macrophages have been identified at this time (4, 6). Each of these studies demonstrated the presence of apoptotic cells at the time of clinical recovery from EAE. The highest levels of apoptosis coincided with a decreasing number of infiltrating cells, and clinical recovery was attributed to the apoptotic clearance of inflammatory cells. There are no reports that examine the presence of apoptotic cells in chronic-progressive EAE, in which animals show worsening neurological symptoms throughout the disease course without recovery.

Herein we have demonstrated the presence of apoptotic cells throughout chronic-progressive EAE of the guinea pig. Cells undergoing apoptosis were observed as early as day 12, three days following the onset of neurological symptoms, and were continually present in the CNS as far as day 87, eleven weeks after the onset of clinical symptoms. None of the untreated EAE animals in this study showed signs of clinical recovery; in fact, several of the CNS tissue samples were obtained from animals that were sacrificed during the disease course after entering a moribund state. Therefore, apoptotic elimination from the CNS (Fig. 1) occurs throughout chronic disease in animals that show no signs of clinical recovery (Fig.
Fig. 4. The number of apoptotic cells observed in spinal cord sections of animals treated with AN100226m or TM2a during acute or chronic-progressive EAE. EAE was induced on day 0 and animals were treated on days 12 and 15 during the acute phase of disease, or days 42 and 45 during the chronic-progressive phase of disease. Apoptotic cells were detected in 5-μm paraffin sections of spinal cord using the ApopTag® kit. A decrease in the number of apoptotic cells was observed following treatment with AN100226m on days 12 and 15. The number of apoptotic cells was unchanged by TM2a-treatment. *Indicates a significant difference from the number of apoptotic cells on day 14 following AN100226m-treatment and † indicates a significant difference from the same day following TM2a-treatment (p < 0.05 one way ANOVA with Tukey test).

2) where inflammatory cellular infiltrates are maintained (Fig. 6, inset). We have also determined that the extent of this apoptosis relates to the degree of inflammation (Fig. 6).

In the present study, double-labeling was used to determine whether any of the cells undergoing apoptosis were T cells. Although quantification of the number of apoptotic T cells was not performed, the majority of apoptotic cells in double-stained sections of spinal cord were T cells (Fig. 7). In other studies, 50% and 64% of all apoptotic cells were identified as αβ T cells (5, 12). However, in both of these studies, apoptotic cells were observed in an advanced stage of degeneration that did not stain with antibodies against T cell, macrophage or oligodendrocyte markers. Many of these unidentified cells may have been degenerating T cells. With the anti-T cell antibody used in the present study, apoptotic T cells were identified in all stages of apoptosis: after chromatin condensation, after formation of apoptotic bodies, and in one case after engulfment by another cell, which may account for the higher proportion of apoptotic cells identified as T cells.

Other cell types have been shown to undergo apoptosis in the CNS during EAE, and this may explain the other apoptotic cells seen in this study. Oligodendrocytes undergo apoptosis in MS and EAE; they may be killed specifically by cytotoxic γδ T cells or antibody-mediated killing, or they may die nonspecifically due to cytokotoxic cytokines (TNF), proteolytic enzymes, or reactive oxygen species (reviewed in 13). Schmied et al reported that 9% of the apoptotic cells in active spinal cord–induced acute EAE of the Lewis rat were oligodendrocytes (5). In contrast, in MBP-induced EAE of the Lewis rat, no apoptotic oligodendrocytes were observed (14). Similarly, in adoptive transfer chronic-relapsing EAE of the mouse, apoptotic oligodendrocytes were not observed at the height of disease activity when one third of all lymphocytes were apoptotic (15). In the present study, although immunolabeling for oligodendrocytes was not performed, it is unlikely that the high numbers of apoptotic cells counted in many of the sections could have all been oligodendrocytes. Several large apoptotic cells were observed in close proximity to myelin tracts; their size and location suggested that they may be degenerating oligodendrocytes. In all of the sections examined in the present study (n = 350), few large cells of this type were observed, indicating that the majority of the apoptotic cells were not oligodendrocytes.

Apoptotic macrophages have also been demonstrated in the CNS of rats with acute EAE. Macrophages undergoing apoptosis have been observed in the perivascular spaces, the meninges, and the parenchyma of the gray and white matter during the time of maximal clinical signs and early clinical recovery from acute EAE (6). However, macrophages make up a small proportion of apoptotic cells in the CNS (16). Schmied et al (5) could
identify no ED1+ apoptotic macrophages in acute EAE. In another study, less than 1% of all macrophages were observed undergoing apoptosis (16). Immunolabeling of macrophages was not performed in the present study, but it is certainly possible that some of the apoptotic cells within the inflammatory foci in chronic-progressive EAE were macrophages or microglia. Immunostaining of macrophage markers was not performed due to technical difficulties inherent in using a guinea pig model of EAE. Immunological reagents for use in the guinea pig are rare, and none of the available antibodies against guinea pig macrophages were able to label macrophages in the ApopTag-positive sections. Nguyen et al noted a similar problem with preservation of apoptotic cells in sections previously processed for immunohistochemistry (14).

Recently, it has been suggested that autoreactive, encephalitogenic T cells undergo selective apoptosis in the CNS of animals with EAE. During clinical recovery, there was a selective loss of VB 8.2+ cells, and VB 8.2+ cells were highly enriched in the apoptotic population (17). In MBP-induced active acute EAE of the Lewis rat, the encephalitogenic T cells are also VB 8.2+ (18, 19). VB 8.2+ T cells were also selectively eliminated from the CNS by apoptosis in this model of EAE (20). Furthermore, it has been hypothesized that apoptotic deletion of autoreactive T cells during acute EAE may contribute to the tolerance to reinduction of a new episode of EAE following the recovery from acute disease (12). The antigen-specificity of the apoptotic T cells in the present study was not determined. It has been reported that between 1% and 45% of all infiltrating T cells are myelin-antigen specific in EAE (21, 22). The high numbers of apoptotic T cells observed in this study suggest that other nonautoreactive T cells were also being eliminated.

The presence of apoptotic cells in the CNS of patients with MS has been reported (13). Degenerating inflammatory cells were found primarily within acute lesions. In acute MS, focal areas of apoptotic cells (mostly macrophages) were found and in chronic MS, most of the degenerating cells were found at the lesion border. The majority of the apoptotic cells in all lesions were identified by immunohistochemistry as macrophages/microglia or T cells, although apoptotic oligodendrocytes and astrocytes were also seen.

Apoptosis is a physiological process in which a cell participates in a cascade of biochemical events that lead to its own death. The stimulus directing the entry into the apoptotic cascade varies among different cell types and in different tissues. Several mechanisms have been demonstrated to induce apoptosis in T cells in vitro including corticosteroids, high levels of antigenic stimulation, IL2, and immunosuppressive cytokines (reviewed in 23). A strong glucocorticoid response is generated during EAE, high levels of specific antigen are present in the CNS of animals with EAE, and cytokines are present within EAE lesions. There is evidence to suggest that several of these factors may combine to induce apoptotic deletion of the inflammatory cells in EAE. Adrenalec- tomy aggravates EAE and inhibits the apoptotic elimination of T cells from the CNS (16), while corticosteroid treatment increases the number of T cells and macrophages undergoing apoptosis in the CNS, but not in other tissues (14, 24). Since apoptosis was selectively stimulated in the CNS but not in other organs following dexamethasone treatment, other factors must play a role in the regulation of apoptosis (14). The preferential apoptosis of

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**Fig. 6.** The relationship between the number of apoptotic cells in a tissue and the corresponding cellularity index (see Table 1) from control (●), TM2a (○) and AN100226m (▼) treated animals is shown in the main panel. A linear relationship between cellularity and apoptosis was observed (R = 0.604; p < 0.001) with no significant differences between the treatment groups. The cellularity index for control animals (inset) increased with disease course. The cellularity index was zero before day 9, rose to 8 by day 20 (acute phase), and was maintained at a high level throughout the chronic disease course.

**Fig. 5.** Apoptosis during anti-α4 integrin-mediated recovery from chronic-progressive EAE. A: Solochrome-R-cyanin-stained section of the spinal cord from a day 48 EAE animal treated with AN100226m on days 42 and 45 (magnification, 120×). Fewer inflammatory cells are present in this lesion. B: Apoptotic cells surrounding the lesion illustrated in panel A (magnification, 120×). C: A higher magnification photomicrograph of apoptotic cells surrounding the same lesion in panels A and B (magnification, 310×). Many of the apoptotic cells in this photomicrograph are on the edge of regions of the lesion that have been cleared of inflammatory cells. Apoptotic cells are identified by the presence of a brown reaction product in the nucleus.
V8 8.2*, MBP-specific T cells suggests that the apoptosis may be antigen-induced (20). Furthermore, T cell deletion has been identified as the mechanism of action of high dose antigen therapy in EAE (25, 26). CNS-resident cells may be active participants in the antigen-induced apoptosis. Astrocytes do not express the necessary co-stimulatory molecules for antigen presentation, although they express MHC. Presentation of specific antigen by an astrocyte to a T cell led to incomplete activation and increased susceptibility to apoptosis (27). A third mechanism that may be involved in deletion of T cells during EAE is cytokine-mediated apoptosis. TGFβ, an immunosuppressive cytokine, improves the clinical course of EAE (28) and induces apoptosis of T cells in vitro (29). The mechanisms that induce apoptosis of macrophages in EAE are less well studied, but may include cytokines, T cell cytotoxicity, activated macrophage products, and endogenous glucocorticoids (6). Administration of exogenous glucocorticoids increased the number of macrophages undergoing apoptosis in acute EAE, suggesting that glucocorticoid-induced apoptosis may play an important role in the resolution of inflammation of the CNS (14).

The presence of apoptotic cells throughout chronic-progressive EAE implies that a continual influx of new inflammatory cells from the periphery is required to maintain an inflammatory lesion and disease activity in EAE. In accord with this hypothesis is data suggesting that each new relapse in chronic-relapsing EAE is accompanied by a fresh influx of inflammatory cells rather than the reactivation of cells that had entered the CNS at an earlier time in the disease course (3). The continual recruitment of inflammatory cells to the CNS would require that several special conditions be met in the microvasculature of the CNS. First, a signal for recruitment of inflammatory cells (T cells and macrophages) to the CNS is required such as chemokines (32–34). Secondly, the appropriate adhesion molecules must be expressed on the surface of the brain microvessel endothelium to permit extravasation of the inflammatory cells into the tissue. An increased expression of VCAM-1 has been demonstrated in MS and EAE; however, it has not been determined whether the level of VCAM-1 expression remains elevated throughout chronic disease (35–37).

Treatment of acute or chronic-progressive EAE with antibodies against α4-integrin leads to a clinical reversal and resolution of inflammation. The data presented here suggests that the clearance of the infiltrated cells in anti-α4-integrin-treated animals arises due to apoptotic deletion. Spinal cord samples from AN100226m-treated guinea pigs contained apoptotic T cells in both acute and chronic disease. With increasing time after treatment, the number of apoptotic cells in AN100226m-treated animals decreased, coincident with the resolution of inflammation (Fig. 5). After treatment, new cells were prevented from entering the CNS and those that had already entered continued to undergo apoptosis. Without an influx of new cells, there was a decrease in the total number of cells present to undergo apoptosis. We propose that the mechanism of action of anti-α4-integrin antibodies in the prevention and treatment of EAE is identical: the prevention of inflammatory cell entry into the CNS. Infiltrating inflammatory cells are spontaneously eliminated from the CNS through apoptosis at all times after immunization. Prevention of the entry of new cells into an inflammatory lesion in the CNS allows resolution of the inflammation through apoptosis.

Tabi et al suggested that it may be the failure of apoptosis as a normal protective mechanism that contributes to the perpetuation of chronic autoimmune disease (17). On the contrary, we have demonstrated that apoptosis occurs throughout chronic EAE. Therefore, recovery is not solely due to apoptosis since apoptosis is present in the absence of recovery. Recovery from EAE is due to cessation of the influx of new inflammatory cells into the CNS that are required to replace those undergoing apoptosis.

Regardless of the mechanism that is ultimately responsible for inducing apoptosis in the T cells that invade the CNS of EAE, the elimination of the autoimmune cells has important consequences to the progression of disease. Further understanding of the role of the apoptotic mechanism in EAE will provide insights into the pathogenesis of MS, other immune disorders of the CNS, and autoimmune diseases of other organs, and may provide a possible therapeutic avenue for MS.

ACKNOWLEDGMENTS

We are grateful to Drs Heidi Horner and Ted Yednock of Athena Neurosciences/Elan for their kind donation of the antibodies AN100226m and TM2a for these studies.

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Fig. 7. Immunohistochemical identification of apoptotic T cells. Frozen sections of EAE spinal cord were processed for alkaline-phosphatase immunohistochemistry using an anti-guinea pig T cell antibody. Following immunohistochemistry, the ApopTag® technique was used to label cells undergoing apoptosis and the sections were counterstained with celestine blue and Mayer's hematoxylin. T cells are identified by the presence of a red reaction product and the brown reaction product identifies apoptotic nuclei. A. This section of frozen spinal cord of a day 14 EAE animal illustrates several apoptotic T cells (arrowheads) and nonapoptotic T cells (magnification 580×). B. This frozen spinal cord section of a d44 EAE animal has apoptotic (arrowheads) and nonapoptotic T cells and several apoptotic cells (arrows) that cannot be identified (magnification 360×).
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Received December 12, 1997
Revision received March 16, 1998
Accepted March 20, 1998