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Apoptotic Elimination Of Vβ8.2+ Cells From The Central Nervous System During Recovery From Experimental Autoimmune Encephalomyelitis Induced By The Passive Transfer Of Vβ8.2+ Encephalitogenic T Cells

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Abstract

A $CD4^+V\beta8.2^+$ T cell clone specific for the peptide 72-89 of guinea pig myelin basic protein (GMBP) was used to induce acute experimental autoimmune encephalomyelitis (EAE) in Lewis rats. To assess apoptosis in inflammatory cells infiltrating the central nervous system (CNS), we extracted cells from the spinal cord, enriched them for T cells and performed flow-cytometric analysis of their DNA stained with propidium iodide. The presence of apoptosis was confirmed by the demonstration of DNA fragmentation on gel electrophoresis. A gradual increase in the proportion of apoptotic cells was observed between 4 and 7 days after the transfer of the encephalitogenic T cells. The highest frequency of apoptotic cells (9.2 ± 1.2%) was observed 7 days after cell transfer, when clinical recovery commenced. Passive transfer of ovalbumin-specific cells resulted in only a background level (0.8%) of apoptosis in the CNS. We conclude that the apoptotic process selectively eliminates autoreactive T cells from the CNS as: (a) there was a selective disappearance of disease-relevant $CD5^+V\beta8.2^+$ cells from the CNS during the course of EAE; (b) there was a decrease in the frequency of CNS-infiltrating T cells reactive to the GMBP 72-89 peptide during the course of EAE, and in a standard proliferation assay there was a loss of *in vitro* reactivity of CNS-infiltrating cells to this peptide, but not to a non-CNS antigen (ovalbumin); (c) simultaneous surface labeling and DNA analysis of CNS-infiltrating cells revealed that the frequency of $V\beta8.2^+$ cells was about sevenfold higher in the apoptotic T cell population than in the normal (non-apoptotic) T cell population; and (d) we were unable to detect recirculation of the $V\beta8.2^+$ cells to lymphoid organs after their frequency decreased in the CNS. The selective apoptotic elimination of autoreactive T cells from the target organ of this spontaneously resolving autoimmune disease may have implications for the understanding of the mechanism by which an autoimmune attack is terminated and for the design of therapeutic strategies to facilitate this process.

Abbreviations:

GMBP - Guinea pig myelin basic protein
MBP - Myelin basic protein
CNS - Central nervous system
SC - Spinal cord
EC2 - Encephalitogenic T cell clone
PI - Propidium iodide
MLN - Mesenteric lymph nodes

Keywords: apoptosis; autoreactive T lymphocytes; experimental autoimmune encephalomyelitis; EAE; autoimmunity

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated demyelinating disease of the central nervous system (CNS). It can be induced actively by a single injection of myelin basic protein (MBP) and adjuvants (MBP-EAE) or by the passive transfer of activated MBP-specific $CD4^+$ T cells [1, 2]. Actively induced EAE in the Lewis rat is followed by spontaneous clinical recovery [3] and by the development of tolerance, as indicated by resistance to the active reinduction of the disease by inoculation with the same antigen [4], but not to the passive reinduction by the transfer of MBP-specific T cells [4]. Numerous immunological studies have focused on the entry of T cells into the CNS, antigen presentation and recognition in the CNS, and the phenotype, function and lymphokine production of the inflammatory cells [5], but little is known about the fate of the encephalitogenic T cells after the autoimmune attack, the down-regulation of their activity and the development of tolerance.

In the Lewis rat, encephalitogenic T cells specific for the 72-89 residue of the guinea pig MBP (GMBP) molecule predominantly use the Vβ8.2 chain of the T cell

receptor (TcR) [6, 7]. It has been established by Offner et al. [8] that there is an early overexpression of Vβ8.2 genes in the target organ (spinal cord) and in the cerebrospinal fluid, but not in the lymphoid organs, before the onset of EAE. They also showed that the biased TcR gene expression gradually diminishes after clinical onset and during recovery. Tsuchida et al. [9] have also shown that the Vβ8.2 chain is the predominant TcR Vβ chain used by CNS-infiltrating T cells in EAE induced by inoculation with GMBP or the 72-89 peptide. It is known from the studies of McCombe et al. [10] that T cells disappear from the CNS during recovery from actively induced EAE in the Lewis rat; however, these studies did not reveal the fate of the disease-relevant, encephalitogenic T cells. We have previously reported the presence of apoptotic T cells in the CNS during EAE [11] and suggested that the apoptotic elimination of T cells may contribute to the termination of the immune attack on the CNS [11, 12]. Our finding, based on immunocytochemical and morphological observations, has been confirmed by Schmied et al. [13] who used *in situ* nick translation to quantitate T cell apoptosis in the CNS in EAE.

In this study we asked whether autoreactive T cells are eliminated from the CNS in EAE by apoptosis. Using an anti-Vβ8.2 antibody we monitored the entry, accumulation and disappearance of $V\beta8.2^+$ cells in the CNS after injecting $V\beta8.2^+$ MBP-specific cloned T cells into normal Lewis rats. Our results show that there is a selective loss of $V\beta8.2^+$ T cells from the CNS during clinical recovery and that $V\beta8.2^+$ cells are highly enriched in the apoptotic T cell population. We conclude that autoreactive $V\beta8.2^+$ T cells are selectively eliminated by apoptosis from the target organ during spontaneous recovery from this autoimmune disease.

2. Materials and methods

2.1 Animals

Male and female Lewis rats (JC strain), 7-10 weeks old, were obtained from the breeding facilities of the University of Queensland.

2.2 Development of T cell clones

T cell clones were derived from the lymph nodes of rats injected 10 days earlier in both footpads with 50-75 μg GMBP or with 50 μg ovalbumin (OVA) in incomplete Freund's adjuvant containing 4 mg/ml Mycobacterium butyricum. GMBP used in these experiments was prepared from guinea pig brain [14]. Cells from the popliteal lymph nodes were stimulated with GMBP or OVA in the presence of irradiated syngeneic thymocytes under limiting dilution conditions. Clones specific for MBP or OVA, respectively, were selected and maintained as described by Ben-Nun et al. [15]. The MBP-specific clone EC2 was used in all experiments in this paper.

2.3 Induction of EAE

EC2 cells were stimulated with irradiated thymocytes (2500 rad) in the presence of 10-15 mg/ml MBP for 3 days. T cell blasts were separated on a Ficoll gradient, washed in PBS and transferred to normal Lewis rats, 8-10 weeks old, by intravenous (i.v.) injection of 10^7 cells per rat in 0.5 ml PBS. The severity of EAE was assessed by grading tail, hind limb and fore limb weakness each on a scale of 0 to 4, and by adding these scores to obtain a total clinical score (maximum = 12) as described by Pender [16].

2.4 Isolation of mononuclear cells from the CNS and lymphoid organs

CNS-infiltrating cells were collected from animals perfused with ice-cold saline. The entire spinal cord was removed by insufflation and weighed. A single-cell suspension was prepared through a 200-mesh stainless steel sieve with ice-cold RPMI containing 1% normal rat serum. Mononuclear cells were separated from the myelin by two-step density gradient centrifugation. The cell suspension prepared from the spinal cord (about 10 ml/spinal cord) was mixed with Percoll stock at 3 : 2 ratio (density: 1.052 mg/ml) in 50 ml centrifuge tubes and spun for 15 min at 3000 rpm at 4°C.The supernatant, except about 8 ml, was discarded. The cell pellet was resuspended in the 8 ml RPMI, transferred to a 10 ml conical centrifuge tube, underlaid with 1.5 ml Ficoll and spun for 15 min at 2500 rpm at

4°C. The cells from the interface above the Ficoll were collected, washed and counted. Mesenteric lymph node and spleen cell suspensions were prepared by gently squeezing the lymphoid tissues through a 100-mesh stainless steel sieve. The debris was removed by filtering the suspension through fine nylon gauze.

2.5 T cell enrichment

For all experiments in the present study, except for the quantitation of total CNS-infiltrating cells, T cells were enriched by passaging the cell suspension through a nylon wool column. The method described by Julius et al. [17] was modified for a lower cell number: 0.15 g nylon wool was inserted into a 2.5 ml syringe (up to the 1.5 ml mark) and a maximum of 10^7 cells were loaded in 500 µl final volume, washed into the column with another 200 µl of media and after 45 min of incubation the nylon wool nonadherent cells were removed by washing the column with 20 ml warm RPMI. The recovery of cells from the nylon wool was between 30 and 50%.

2.6 Phenotypic and DNA analysis by flow cytometry; cell sorting

Mouse anti-rat monoclonal antibodies specific for CD4 (W3/25), PE-conjugated or non-conjugated CD5 (OX-19), and FITC-conjugated rabbit anti-mouse $F(ab')_2$ IgG (STAR 41) were purchased from Serotec. FITC-conjugated anti-CD3 antibody (1F4) was purchased from Caltag. The antibody to Vβ8.2 (R78) was kindly provided by Dr. Thomas Hünig (Würzburg, FRG) [18]. Analysis of DNA content for assessment of apoptosis was carried out according to the method of Telford et al. [19]. Briefly, 2×10^5–10×10^5 cells were either resuspended in 1 ml ice-cold 80% ethanol and incubated at 4°C for a minimum of 30 min or resuspended in 50% ethanol, incubated overnight, then washed in PBS and resuspended in 0.4 ml propidium iodide (PI) staining reagent, containing 0.1% Triton-X, 0.1 mm EDTA, 0.05 mg/ml RNase and 50 µg/ml PI in PBS. As the live mononuclear cells were separated from the spinal cord tissue by density gradient centrifugation, the viability of cells, assessed by trypan blue exclusion, was always high (>98%) and the cell suspension was relatively free of debris. However, to avoid detecting nuclear debris during FACS analysis, events with a low level of red (PI) fluorescence were not collected [20]. For simultaneous detection of cell surface antigens and DNA, the cells were first labeled with OX19 or R78 antibodies or with the isotype control antibody (mouse IgG_1, Serotec), followed by labeling with STAR 41, then fixed in ethanol, as described above. The cells were washed in PBS and resuspended in a 4: 1 mixture of PBS containing 0.1% azide and 1% FCS, and PI-reagent containing 5 mg/ml RNase and 250 µg/ml PI just before analysis. Electronic compensation for two-color analysis and "low" sample flow rate ensured unchanged FITC distribution following PI labeling of DNA. Immunofluorescence and DNA analysis were performed on a FACScan using Lysis II software (Becton Dickinson, Mountain View, CA) to collect and analyze the data. Lymphocytes were defined by scatter gates for analysis of cell surface markers. Cell sorting according to DNA content of PI-stained cells was carried out on a FACS Vantage (Becton Dickinson).

2.7 Proliferation assay and *in vitro* frequency of CNS-infiltrating cells

Single-cell suspensions of CNS-infiltrating cells, mesenteric lymph node cells (MLN) and spleen cells were prepared in RPMI 1640 containing 216 mg/l-glutamine (Gibco),100 IU penicillin, 100 µg/ml streptomycin (Sigma), 0.1 mM Napyruvate (Gibco), nonessential amino acids (Gibco), 36 mg/l L-asparagine (Sigma), 5×10^{-5} M 2β-mercaptoethanol (Sigma), 5 µg/ml Fungizone (Squibb) and 1% normal rat serum. In the proliferation assay, 5×10^4 CNS-infiltrating cells were cultured in triplicates in 96-well U-bottom trays in the presence of 5×10^5 irradiated thymocytes, 20 µg/ml antigen or 20 U/ml IL-2. The cultures were incubated for 3 days, and for the last 8 h of the incubation 0.5 µCi [^{3}H] thymidine was added to each well. In the frequency analysis, 12-24 replicates of gradually increasing numbers of responder cells (1×10^3 –20×10^3 cells per well) were plated in 96-well U-bottom tissue culture trays with 5×10^5 γ-irradiated normal rat thymocytes in the presence or absence of the GMBP 72-89 peptide. The peptide (PQKSQRSQDENPVVHF) was syn-

thesized in the Queensland Institute of Medical Research using the "tea-bag" method [21]. Medium (100 µl) was carefully removed from each well 24 and 120 h after initiating the cultures and replaced with medium containing 10% horse serum and 10% IL-2-containing Con A supernatant. The cultures were incubated for 7 days, and 0.5 µCi [^{3}H] thymidine was added to each well for the last 12 h of the incubation. The wells were harvested using a Skatron cell harvester and assayed for incorporated radioactivity. Estimates of minimal frequencies of the cells specifically proliferating in the presence of the peptide were calculated according to the methods of Taswell [22]. Cultures were scored as positive when thymidine uptake (cpm) was higher than the mean plus three times the standard deviation of background cultures without responder cells.

2.8 Analysis of DNA fragmentation in agarose gels

Total DNA from 0.8×10^6–1.0×10^6 cells was isolated according to the method of Smith et al. [23]. Briefly, cell pellets (kept at −20°C before DNA extraction) were resuspended in 20 microlitre lysis buffer containing 10 mM EDTA, 50 mM Tris-HC1, 0.5% sodium lauryl sarkosinate and 0.5 mg/ml proteinase K and incubated for an hour at 50°C and for a further hour after adding 0.5 mg/ml RNase A to each sample. Samples were heated to 70°C and 10 µl 10 mM EDTA containing 1% agarose, 0.25% bromophenol blue and 40% sucrose was mixed with each sample before loading each sample into two dry wells of a 2% agarose gel containing 0.1 mg/ml ethidium bromide. Electrophoresis was carried out in 2 mM EDTA 800 mM Tris-phosphate.

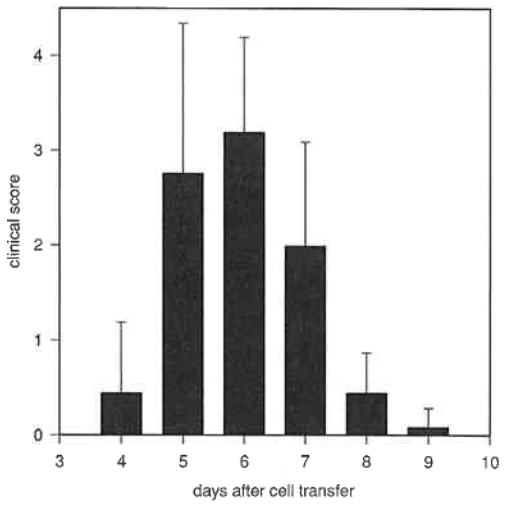

Figure 1. Clinical severity of passively transferred EAE. Lewis rats were injected i.v. with 10^7 EC2 cells (MBP-specific T cell clone). The rats were examined daily and assessed on a scale from 0 to 12 (see Section 2.3). The figure shows the mean and SD of clinical scores of 47 rats on days 3, 4 and 5, of 26 rats on days 6 and 7 and of 6 rats after day 7.

3. Results

3.1 Characterization of the encephalitogenic T cell clone EC2 and induction of acute EAE

An MBP-specific T cell clone (EC2) derived from the popliteal lymph nodes of a Lewis rat with actively induced MBP-EAE was used in the experiments described in this paper. We have characterized this clone as $CD4^+$, $TcR\alpha\beta^+$, $CD44^+$, $OX22^-$ ($CD45RC^-$), specific for the 72-89 amino acid sequence of the GMBP molecule, producing IL-2 upon stimulation with antigen, and cytotoxic for MBP-pulsed syngeneic Con A blasts. The clinical severity of EAE induced by the i.v. injection of EC2 cells into normal Lewis rats was dependent on the number of cells transferred (data not shown). Distal tail weakness was the first clinical sign and commenced 4-5 days after the transfer of 10^7 EC2 cells. The clinical signs were maximal 5 or 6 days post-transfer, and recovery commenced on day 7 with most of the animals having completely recovered by day 9 (Fig. 1).

3.2 Quantitative analysis of CNS-infiltrating cells during EAE

The number of CNS-infiltrating cells per gram of spinal cord is shown in Fig. 2. A substantial increase in cell number was observed 4 days after cell transfer, with a further increase peaking at day 5. At this time the total number of CNS-infiltrating cells was nearly 40-fold higher than in the CNS of normal animals. After the fifth day following cell transfer, a steady decline in the number of CNS-infiltrating cells was observed. This decline might be due to (a) the cells leaving the CNS or (b) death of the inflammatory cells in the CNS. The cell number remained slightly elevated even after complete recovery (see day 10). Passive transfer of ovalbumin-specific cloned T cells resulted in a twofold increase in the number of CNS-infiltrating cells by day 6, compared to that in the normal CNS (not shown).

3.3 Phenotypic analysis of CNS-infiltrating cells

To determine whether there was a preferential loss of certain cells from the CNS during clinical recovery, we analyzed the phenotype of the CNS-infiltrating cells. Fig. 3 A shows that the absolute numbers of CD4+ T cells and Vβ8.2+ T cells in the CNS are maximal on day 5 after cell transfer and gradually decrease thereafter. However, while the decline in the number of CD4+ T cells is only 6.8-fold between day 5 and day 8, that of the Vβ8.2+ T cells is 31.3-fold. When expressed as the proportion of CD4+ or Vβ8.2+ cells within the total CD5+ T cell population (Fig. 3 B), the results reveal that the proportion of CD5+ cells that are CD4+ started to decrease gradually on day 5, reaching the lowest level on day 7 (48% of the CD5+ population), and then increased on day 8. In contrast, the proportion of the CD5+ cells which are Vβ8.2+ was highest on the day of onset (25.6% of CD5+ cells on day 4) and decreased thereafter. Moreover, unlike the proportion of CD4+ cells within the CD5+ population, the proportion of Vβ8.2+ cells in the CD5+ population remained at a very low level after the onset of recovery (<3% on day 8). This cannot be satisfactorily explained by a preferential influx of non-Vβ8.2+ T cells [8] as there is also a major reduction in the total number of non-Vβ8.2+ T cells in the CNS of rats recovering from EAE (Fig. 3 A). Thus, while there is a major decline in the total number of T cells in the CNS during recovery from EAE, there is a preferential loss of the Vβ8.2+ subset.

3.4 Detection of apoptosis

To determine whether apoptosis is responsible for the disappearance of T cells from the CNS during recovery from EAE, the DNA content of the cells isolated from the CNS and enriched for T cells on a nylon wool column was assessed using PI staining (Fig. 4 A). Cells in G_0/G_1, S and G_2/M phases of the cell cycle can be distinguished by their PI uptake, and cells which are undergoing apoptosis (A_0) can be characterized by decreased staining of DNA with PI [19]. To confirm that cells with decreased PI binding are apoptotic we carried out DNA gel electrophoresis to demonstrate DNA fragmentation, which is characteristic of apoptosis [24] (Fig. 5). As we were unable to collect a sufficient number of cells in A_0 by sorting from the CNS to perform gel electrophoresis, we used CTLL cells, which undergo apoptosis in the absence of IL-2 [25], as a positive control. The CTLL cells were deprived of IL-2 overnight, then fixed and stained with PI using the same method as that employed for extracted CNS-infiltrating cells. Cells sorted into the A_0 region on the FACS Vantage were then assessed for DNA fragmentation by gel electrophoresis. These cells showed no band of full-length DNA but showed DNA fragmentation (Fig. 5, lanes 3-4), while normal lymph node cells (not shown) or CTLL cells growing in IL-2 (lanes 5, 6) (0.8 or 2.8% apoptosis, determined by FACS) showed no detectable DNA fragmentation. In unsorted CNS-infiltrating cells, where 9.9% of the cells were in the A_0 region, DNA fragmentation was also detectable (lanes 1, 2).

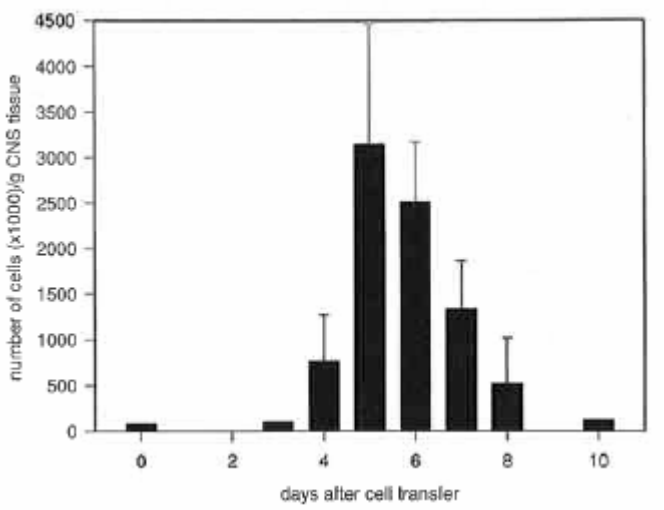

Figure 2. Quantitative analysis of CNS-infiltrating cells. Inflammatory cells were isolated from the spinal cords of rats after the i.v. injection of 107 ec2 cells as described in the section 2.4. The spinal cords were weighed and the number of infiltrating cells is expressed as cells/gram tissue. Day 0 represents normal animals without cell transfer. The mean and standard deviation of cell numbers based on repeated experiments (total of 52 animals) are shown, except for days 0, 3 and 10 where one experiment was done, using 3, 3 and 6 animals, respectively.

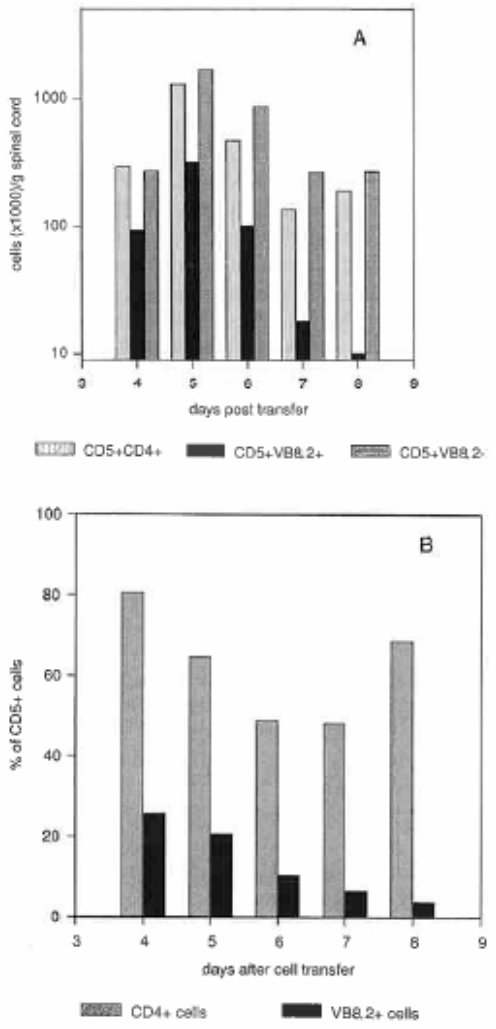

Figure 3. Phenotype of CNS-infiltrating cells in passively induced EAE. Lewis rats were injected with EC2 cells and the inflammatory cells obtained from the spinal cord (two to five rats for each day) were immunostained for two-color FACS analysis. (A) shows the number of cells per gram of spinal cord that are $CD5^{+}CD4^{+}$,

$CD5^+V\beta8.2^+$ or $CD5^+V\beta8.2^-$.The latter results were obtained by subtracting the number of $CD5^+V\beta8.2^+$ cells from the number of $CD5^+$ cells per gram of tissue. (B) shows the percentages of $CD5^+$ lymphocytes that express CD4 or Vβ8.2.

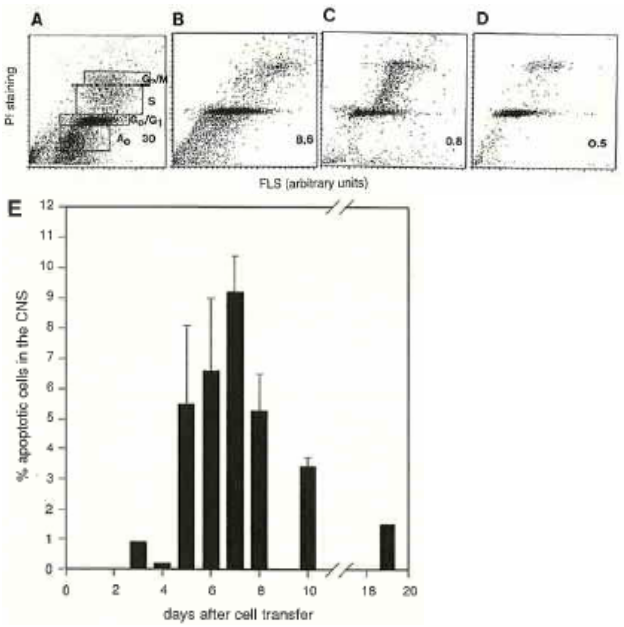

Figure 4. DNA content analysis of CNS-infiltrating cells during passively induced EAE. Lewis rats were injected i .v. with the encephalitogenic EC2 cells or with cloned OVA-specific T cells, and the CNS-infiltrating cells were isolated from three rats in each group 6 days after passive transfer, and enriched for T cells on a nylon wool column. The cells were fixed with ethanol, stained with propidium iodide and analyzed on the FACScan; 10 000 events were collected, and statistical analysis of the DNA profile was carried out using Lysis II software. In (A) which represents unsorted, IL-2-deprived CTLL cells, the regions that were used to determine the different stages of the cell cycle are shown. (B) shows the result of DNA staining of cells from the CNS of rats 6 days after the transfer of 10^7 EC2 cells, (C) shows the results for CNS-infiltrating cells 6 days after the transfer of 10^7 OVA-specific T cells and (D) shows the results for mesenteric lymph node cells isolated from the same animals as in (B). The numbers on (B-D) represent the proportions of apoptotic cells. (E) summarizes the proportions of apoptotic cells among the CNS-infiltrating cells on different days after the injection of the EC2 clone. The bars represent the mean and the SD of the proportion of apoptotic cells in A_0 based on at least three experiments on days 5, 6 and 7, with each experiment using one to six animals. The results at each of the other time points were derived from one experiment on each day using two to six animals.

Figure 4 B shows the PI staining of CNS-infiltrating cells 6 days after passive transfer. The percentage of cells extracted from the CNS that were apoptotic at different stages of EAE is shown in Fig. 4 E. The proportion of apoptotic (A_0) cells increases after the commencement of neurological signs of EAE, is maximal (9.2 ± 1.2%) on the day of commencement of clinical recovery (7 days after passive transfer) and thereafter decreases. These changes were not reflected in the peripheral lymphoid organs: the proportion of cells in A_0 in the mesenteric lymph nodes (MLN) or in the spleens of the same animals never exceeded 0.8% on days 4, 5, 7 and 10 after cell transfer (day 6 MLN is shown in Fig. 4D).

To determine whether similar levels of apoptosis occur when T cells with non-CNS antigen specificity enter the CNS, the proportion of apoptotic cells was measured in the CNS infiltrates of animals injected with the same number of OVA-specific cloned T cells (10^7) as that used for EAE induction. At day 6 after the transfer of OVA-specific cloned T cells, only 0.8% of the CNS cells were apoptotic (Fig. 4 C). The experiment was repeated with the same result. $CD5^+$ T cells constituted about 30% of the CNS-infiltrating cells after nylon wool depletion in animals injected with the OVA-specific clone, and 60% in animals injected with the MBP-specific T cell clone (not shown). Thus, the lower proportion of apoptotic cells in the CNS of rats receiving OVA-specific T cells cannot be adequately explained by the lower proportion of T cells within the CNS infiltrate. From this experiment it can be concluded

that the environment in the CNS itself is not responsible for the apoptotic elimination of activated T cells entering the CNS.

3.5 Phenotypic analysis of apoptotic cells

To determine whether there is preferential apoptosis of the Vβ8.2$^+$ subset, we employed simultaneous flow-cytometric analysis of surface antigens and DNA content. This approach makes it possible to gate on the cells in the A_0 region and to carry out their phenotypic analysis (Table 1). CNS-infiltrating cells were isolated from animals with EAE 6 days after cell transfer. Apoptotic cells had negligible autofluorescence (not shown). The level of nonspecific antibody binding was determined by using an isotype control (mouse IgGl). The level of nonspecific binding was 10.7 ± 4% for apoptotic and 2.0 ± 0.7% for non-apoptotic cells. Table 1 shows the results after subtracting the appropriate background values. The data obtained in this way show that 35.4 ± 8.0% of the apoptotic cells were positive for the CD5 pan T cell marker on day 6, and that Vβ8.2$^+$ lymphocytes constituted 12.4 ± 3.4% of the apoptotic cells. This means that 36% of the apoptotic T cells were Vβ8.2$^+$ whereas similar calculations indicated that only 5% of the normal (non-apoptotic) T cell population were Vβ8.2$^+$ on day 6. Thus there is a sevenfold enrichment of the Vβ8.2$^+$ cells in the apoptotic compared to the non-apoptotic T cell population, indicating that Vβ8.2$^+$ T cells are selectively eliminated from the CNS by apoptosis. Further studies will be required to determine whether the decline in the non-Vβ8.2$^+$ T cell population in the CNS during the course of EAE is solely due to exit from the CNS or whether apoptosis of these cells also contributes to the decline.

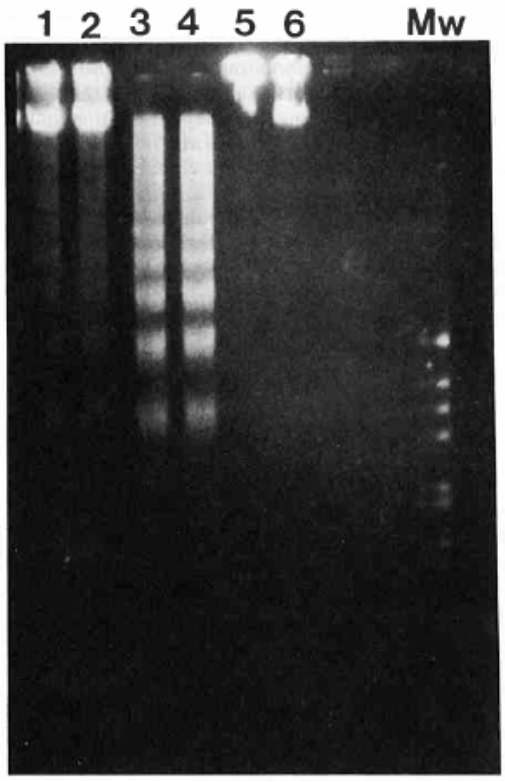

Figure 5. Analysis of DNA fragmentation by agarose gel electrophoresis. DNA was isolated from 10^6 cells and loaded on agarose gel as described in the Section 2.8. Lanes 1 and 2 represent unsorted CNS-infiltrating cells from animals injected with 10^7 EC2 cells 6 days previously. The proportion of cells in A_0 determined by FACS was 9.9%. Lanes 3-4 represent sorted CTLL cells in A_0 obtained by depriving exponentially growing CTLL cells of IL-2 overnight and by sorting them on a FACS Vantage according to their decreased PI uptake. Lanes 5-6 represent the CTLL cells growing exponentially in IL-2 where the proportion of cells in A_0 was 2.8% . The molecular weight standard is shown in lane Mw.

Table 1. Phenotype of apoptotic cells (cells in A_0) in the CNS 6 days after cell transfer[a)]

	CD5+	Vβ8.2+	Percentage of Vβ8.2+ cells within the CD5+ subset
Phenotype of apoptotic cells[b)] (%)	35.4 ± 8.0	12.4 ± 3.4	35.5 ± 8.8
Phenotype of normal cells[c)] (%)	65.0 ± 6.4	3.3 ± 2.0	5.0 ± 2.9

a) Cells were isolated from the CNS of a total of 11 animals in two separate experiments, 6 days after transfer of EC2 cells. The frequency of apoptotic cells was determined as described in Section 2.6. The results show the mean and SD of data obtained from the two experiments.
b) Percentage of apoptotic cells (cells in A_0) that were CDS+ or Vβ8.2+ after subtracting the background values obtained by the isotype-control.
c) Percentage of normal cells (cells in G_0/G_1, S and G_2/M phases, based on gating on non-A_0 cells) that were CD5+ or Vβ8.2+

3.6 Functional analysis of CNS-infiltrating cells

To supplement the information on CNS-infiltrating cells obtained by phenotyping, *in vitro* proliferation assays of CNS-infiltrating cells from animals injected with 10^7 cloned MBP-specific T cells, or with 10^7 cloned MBP-specific T cells and the same number of cloned OVA-specific T cells, were carried out 6 days after the transfer of cells. The results in Fig. 6 show that cells isolated from the CNS 6 days after T cell transfer do not respond to the GMBP 72-89 peptide, regardless of whether T cells specific to this region of the MBP molecule were injected on their own (EC2 cells) or together with OVA-specific cells. However, when OVA-specific T cells were injected with MBP-specific cells, the specific proliferative response to OVA was 71-fold higher than the background. Proliferation of CNS-infiltrating cells in the presence of IL-2 served as a positive control. When OVA-specific cells were injected alone, the cells did not home to the CNS, and thus the number of cells isolated from the CNS was too low to set up a similar assay. These results indicate that the selective elimination of Vβ8.2+ T cells from the CNS during EAE is also reflected in the diminished secondary immune response of the CNS-infiltrating cells against the relevant antigen *in vitro*.

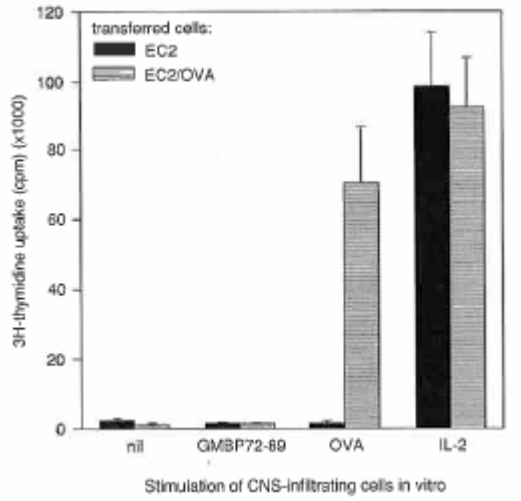

Figure 6. Functional analysis of cells isolated from the CNS of animals 6 days after the passive transfer of 10^7 cloned EC2 cells alone or 10^7 EC2 cells together with 10^7 cloned OVA-specific T cells. T cells 5×10^4, enriched on nylon wool, were cultured in the presence of 5×10^5 irradiated thymocytes (2500 rad) for 3 days in triplicate cultures. GMBP 72-89 peptide or OVA was added to the culture wells at 20 µg/ml final concentration. IL-2 (20 U/ml) was added in the form of MLA 144 supernatant. The cultures were pulsed with [^{3}H] thymidine for the last 8 h of the incubation. The mean proliferative values (cpm) and SD are shown.

3.7 Analysis of recirculation of Vβ8.2$^+$ cells during the course of EAE

To determine whether encephalitogenic T cells leave the CNS after the autoimmune attack, we studied (a) the phenotype of MLN cells and spleen cells from animals with EAE on different days after cell transfer (Table 2) and (b) the frequencies of T cells specific for the GMBP 72-89 peptide in these lymphoid organs (Table 3). Phenotypic analysis revealed a slight decrease in the proportion of Vβ8.2$^+$ cells in the peripheral lymphoid organs after day 6 (Table 2), when the number of inflammatory cells was decreasing in the CNS (Fig. 2), but this change does not seem sufficient to conclude that elimination of some Vβ8.2$^+$ T cells of the recipient also occurred. Frequency analysis of specifically proliferating cells showed no evidence of elevated levels of GMBP 72-89-specific cells in the spleen or MLN during recovery (Table 3). In the CNS the frequency of peptide-specific cells was maximal on day 4 (1/1780) and decreased thereafter (Table 3). These cells were highly enriched in the CNS compared to the spleen and MLN. The frequency of nonspecifically proliferating cells isolated from the CNS on day 6 (1/6080) was higher than on day 4 (1/18 900), but it decreased thereafter. The frequency of nonspecifically proliferating cells became elevated in the spleen on day 8 (1/9520).

Table 2. Phenotype of MLN and spleen cells of animals with passively induced EAE[a])

		day 4[b)]	day 5	day 6	day 7	day 8
MLN	CD5$^+$	60.9%[c)]	54.3%	56.3%	66.5%	65.4%
	CD5$^+$CD4$^+$	45.2%	43.1%	43.5%	47.6%	49.6%
	CD5$^+$Vβ8.2$^+$	2.8%	3.2%	3.4%	2.9%	2.3%
Spleen	CD5$^+$	51.2%	42.2%	46.3%	45.0%	49.2%
	CD5$^+$CD4$^+$	40.7%	31.7%	34.3%	31.7%	32.8%
	CD5$^+$Vβ8.2$^+$	4.1%	3.3%	3.0%	2.3%	2.5%

a) MLN and spleen cells were prepared and immunostained as in Sections 2.4 and 2.6. The background immunofluorescence was determined by staining the cells with Star 41 and CD5-PE. It was generally less than 3.0%. The appropriate background values have been subtracted from the data shown above.
b) Days after injection of EC2 cells.
c) Percentage of total lymphocytes, characterized by their scatter properties.

4. Discussion

In this study we have demonstrated that Vβ8.2$^+$ T cells selectively undergo apoptotic elimination in the CNS of Lewis rats during clinical recovery from EAE induced by the i.v. injection of Vβ8.2$^+$ CD4$^+$ T cells specific for peptide 72-89 of guinea pig MBE As Vβ8.2$^+$ cells comprise only a small proportion (4.6%) of the T cells in peripheral lymphoid organs [18], it is unlikely that host cells contributed significantly to the Vβ8.2$^+$ population in the CNS. Even if host Vβ8.2$^+$ cells can be primed by exposure to MBP in the CNS, this is unlikely to occur within the time frame of our experiments. We have also shown that the selective apoptotic elimination of Vβ8.2$^+$ T cells from the CNS is associated with a loss of the *in vitro* proliferative response of CNS-infiltrating cells to the MBP peptide but not to a non-CNS antigen (ovalbumin).

We found that the maximum level of apoptosis in the T cell-enriched population of cells extracted from the CNS was 8-11% and occurred 7 days after the transfer of encephalitogenic T cells. This is considerably less than the proportion (49%) found by

Schmied et al. [13] at the same stage of recovery in a similar model. One possible explanation for this difference is that their results were obtained by histological analysis of cells within the CNS parenchyma, which they found was the main site of T cell apoptosis, whereas our results are based on the analysis of cells extracted from the meninges and perivascular spaces as well as from the CNS parenchyma. Furthermore, there might be some loss of apoptotic cells during the preparation of single-cell suspensions and gradient centrifugation when isolating CNS-infiltrating cells. The different methods used to identify apoptotic cells might also contribute to the different results. Our method for detecting apoptosis based on decreased staining with PI may underestimate the proportion of apoptotic cells, as it probably does not detect early apoptosis characterized by morphological and biochemical changes preceding DNA fragmentation [26] or the late stages of apoptosis when secondary degeneration supervenes or when apoptotic cells have been phagocytosed by macrophages. On day 6 following cell transfer, 35.4 ± 8.0% of the apoptotic cells were identified as T cells, which is similar to the proportion found in our previous immunocytochemical and morphological study [11] and in the study of Schmied et al. [13]. The other apoptotic cells may be T cells with down-regulated CD5 molecules, macrophages [27] or other non-T cells.

T cell apoptosis in the CNS in EAE could be due to the effect of increased endogenous corticosteroids, anti-clonotypic T cell killing or activation-induced cell death, as we have previously suggested [11, 28]. In view of our present finding that the apoptotic process selectively affects Vβ8.2$^+$ T cells in the CNS and the finding of Schmied et al. [13] that T cell apoptosis is mainly localized to the CNS, it is unlikely that corticosteroids per se are responsible, because elevated levels of circulating corticosteroids would also be expected to induce apoptosis of other T cells within the CNS and of T cells in the peripheral lymphoid organs. Anti-clonotypic T cell killing as found in vitro by Sun et al. [29] is also unlikely to be the mechanism for T cell apoptosis, as we found only very low levels of apoptosis in the CNS when animals were injected with ovalbumin-specific T cells (Fig. 4 C). We suggest that the most likely mechanism responsible for the apoptosis of Vβ8.2$^+$ T cells in the CNS in EAE is activation-induced cell death. This process results in the apoptotic deletion of autoreactive immature T cells in the thymus during normal development [30]; it also eliminates mature T cells in vivo [31-33]. In some in vitro studies, stimulation of the TcR of non-resting T cells, which had recently been activated either by high levels of IL-2 [34, 35] or through the TcR [36], leads to T cell apoptosis. Activation of resting memory T cells through the TcR without providing the co-stimulatory signal can also lead to apoptotic death of T cells [37, 38]. We hypothesize that activation-induced death of encephalitogenicT cells in the CNS in EAE is due to the relative lack of the co-stimulatory signal during the reactivation of these T cells, as there is a paucity of specialized antigen-presenting cells in the CNS. Endogenous glucocorticoids, which are released during the course of EAE [39], may enhance activation-induced apoptosis by inhibiting IL-2 production.

T cell activation in the absence of the co-stimulatory signal has also been reported to lead to T cell anergy [40]. Anergic cells are unable to proliferate in the presence of antigen even if it is presented by specialized antigen-presenting cells, but they can regain their ability to react with their antigen after a resting period in vivo [41] or in the presence of high concentrations of IL-2 in vitro [42]. Anergic cells might be cells that have escaped apoptotic elimination; however, because of the abbreviated life span of anergic cells, the distinction between apoptosis and anergy may be only semantic, as suggested by Sprent [43]. Whether any CNS-antigen-specific T cells that have entered the CNS escape apoptotic elimination in our model is not known. Although encephalitogenic T cells can be isolated from the lymph nodes or spleens of rats that have recovered from actively induced EAE [44], it is possible that such T cells have never entered the CNS, and thus have not had the chance to be eliminated by apoptosis. Using frequency analysis Mor and Cohen [45] found MBP-specific T cells in the CNS of rats that had recovered from actively induced EAE at about a 30-fold lower frequency that at the time of commencement of clinical signs. We found a similar decrease in our model between days 4 and 8 after cell transfer. If MBP-specific T cells can be isolated from the CNS after clinical recovery,

further studies will be required to determine whether they have escaped apoptotic elimination during the course of EAE or have only recently entered the CNS and will ultimately be eliminated. In the actively induced form of EAE, the elimination of autoreactive T cells in the CNS may also contribute to the refractoriness to reinduction of disease by inoculation with the same antigen, although other mechanisms such as intrathymic or extrathymic deletion of newly produced autoreactive T cells may also be involved.

In conclusion, our findings indicate that apoptotic cell death plays an important role in eliminating mature autoreactive T cells from the target organ during spontaneous recovery from an autoimmune disease. It may be feasible to increase the sensitivity of autoreactive T cells to apoptosis and thus reduce the severity of an autoimmune attack.

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