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# Antigen-Specific Down-Regulation of Myelin Basic Protein-Reactive T Cells During Spontaneous Recovery From Experimental Autoimmune Encephalomyelitis: Further Evidence of Apoptotic Deletion of Autoreactive T Cells in the Central Nervous System

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## Abstract

Experimental autoimmune encephalomyelitis (EAE) was induced in Lewis rats by the i.v. injection of $10^7$ cloned $V_\beta 8.2^+$ T cells specific for the 72-89 peptide of guinea pig myelin basic protein (MBP). Some animals were injected simultaneously with $10^7$ cloned T cells specific for ovalbumin (OVA). Lymphocytes were isolated from the spinal cord and from the peripheral lymphoid organs of these rats and the frequencies of MBP-peptide-specific or OVA-specific proliferating cells were estimated by limiting dilution analysis at different times after cell transfer. The frequencies of cells specific for $MBP_{72-89}$ or OVA in the spinal cord were highest 5 days after cell transfer ($MBP_{72-89}$, 1 in 1149; OVA, 1 in 1116). On day 7, when the rats were recovering, the frequency of cells specific for $MBP_{72-89}$ in the spinal cord fell dramatically to <1 in $10^5$, while that of OVA-specific cells decreased to a much lesser extent (1 in 7001). The frequencies of $MBP_{72-89}$-specific cells in the peripheral lymphoid organs during and after recovery were also much lower than those of OVA-specific cells. A similar pattern of down-regulation of the MBP-peptide-specific, but not the OVA-specific, T cell response was observed in the spleen and mesenteric lymph nodes (MLN) of rats 38 days after the active induction of EAE by immunization with equal amounts of MBP and OVA in adjuvants. In the passively transferred model, cells isolated from the spinal cord and MLN on day 7 did not regain responsiveness to $MBP_{72-89}$ after incubation with high levels of IL-2, indicating that the unresponsiveness was not due to T cell anergy. Thus this study demonstrates that there is a specific down-regulation of the $MBP_{72-89}$-specific T cell response during spontaneous recovery from EAE. This conclusion is consistent with our previous observation that $V_\beta 8.2^+$ T cells are selectively eliminated from the CNS by apoptosis during recovery from EAE induced by the passive transfer of $V_\beta 8.2^+$ T cells reactive to this MBP peptide. In contrast to autoreactive T cells, the non-autoreactive T cells that accumulate in the CNS during EAE appear to recirculate to the peripheral lymphoid organs.

**Keywords:** apoptosis, experimental autoimmune encephalomyelitis, T cell deletion, tolerance

## Introduction

Experimental autoimmune encephalomyelitis (EAE) is a T-cell- mediated demyelinating disease of the CNS. In Lewis rats it can be actively induced by a single injection of myelin basic protein (MBP) and adjuvants (1) or passively induced by the transfer of activated MBP-specific T cells (2). The autoimmune attack is followed by spontaneous clinical recovery. After recovery from actively induced EAE there is specific tolerance to MBP as shown by resistance to reinduction of EAE by further immunization with MBP (1). It has been proposed that the down-regulation of EAE is due to various regulatory cells, either macrophages (3), B cells (4), suppressor T cells (5,6) or anti-clonotypic cytotoxic $CD8^+$ cells targeting encephalitogenic T lymphocytes (7,8). Others have proposed that corticosteroids (9) or inhibitory cytokines (10) are responsible for the down-regulation of autoreactive T cells during spontaneous recovery.

We have observed T cell apoptosis in the CNS of Lewis rats with EAE, and have proposed that this contributes to the spontaneous recovery and to the development of tolerance (11,12). Schmied *et al.* (13) have confirmed this observation and have shown that the T cell apoptosis is maximal at the time of clinical recovery. Furthermore, using flow cytometric analysis we have shown that, during recovery from EAE induced by the passive transfer of MBP-specific

$V_\beta 8.2^+$ cloned T cells, the apoptotic process selectively affects $V_\beta 8.2^+$ cells (14). We also found that the co-transfer of MBP-specific T cells and cloned T cells specific for ovalbumin (OVA) resulted in down-regulation of the MBP-specific but not of the OVA-specific proliferative response of T cells isolated from the CNS 6 days after passive transfer (14).

In the present study we further investigated the down-regulation of the T cell response during recovery from EAE in order to answer the following questions: (i) do non-CNS-specific activated T cells that have entered the CNS subsequently exit from the CNS, while CNS-antigen-specific cells are deleted in the CNS?, (ii) does anergy (functional tolerance) contribute to the down-regulation of the MBP-specific T cell response? and (iii) is the pattern of down-regulation similar in passively induced EAE and actively induced EAE? We compared the frequencies of MBP-peptide-specific and OVA-specific cells in the CNS and in the peripheral lymphoid organs of animals during the course of, and after recovery from, EAE following the passive transfer of a MBP-peptide-specific T cell clone and an OVA-specific T cell clone. We also examined the specific T cell frequencies in animals that had recovered from EAE induced by the co-injection of MBP and OVA in adjuvants. In all experiments we used limiting dilution analysis to detect small subsets of T cells that are functionally relevant in mediating EAE. To determine whether T cell anergy could be responsible for the decreased MBP-specific T cell responses following recovery from EAE, we examined the MBP-specific proliferative responses of the isolated cells after *in vitro* culture with a high concentration of IL-2 which can reverse T cell anergy (15). Our results indicate antigen-specific down-regulation, but not T cell anergy, of MBP-reactive T cells, and provide further evidence that autoreactive T cells are deleted in the CNS during spontaneous recovery from EAE.

## Methods

### *Animals*

Male and female Lewis rats (JC strain) 8-10 weeks old, were obtained from the breeding facilities of the University of Queensland.

### *T cell clones*

T cell clones specific for the 72-89 peptide of guinea pig MBP (sequence: PQKSQRSQDENPVVHF) ($MBP_{72-89}$) (clone EC2) or OVA (Sigma) (clone OVA-T) were developed, maintained and characterized as previously described (14). Both T cell clones were $CD4^+$, had similar passage numbers and *in vitro* proliferation characteristics, and were stimulated with their specific antigens in the same way before being transferred into normal Lewis rats (14).

### *Induction of EAE*

EAE was passively induced by the i.v injection of $10^7$ cloned T cells specific for $MBP_{72-89,}$ as previously described (14). Some rats were also given $10^7$ cloned OVA-specific T cells simultaneously. EAE was actively induced in normal Lewis rats by the intradermal inoculation of MBP and OVA in adjuvants. MBP was prepared from guinea pig brain as described by Deibler *et al. (16).* MBP or OVA in 0.9% saline was emulsified in an equal volume of incomplete Freund's adjuvant (Commonwealth Serum Laboratories, Melbourne, Australia) containing 4 mg/ml of killed *Mycobacterium butyricum* (Difco). Each rat received 50 μg MBP in 0.1 ml emulsion in a footpad of one hindfoot, and 50 μg of OVA in 0.1 ml emulsion in a footpad of the other hindfoot.

### *Limiting dilution analysis of antigen-specific cells*

Single cell suspensions from the mesenteric lymph nodes (MLN), spleen and the spinal cords of groups of two or three rats were prepared according to the method described previously (14). RPMI 1640 supplemented with 216 mg/l L-glutamine (Gibco), 100 IU penicillin, 100 μg/ml streptomycin (Sigma), 0.1 mM Na-pyruvate (Gibco), non-essential amino acids

(Gibco), 36 mg/l L-asparagine (Sigma), 5 × $10^{-5}$ M β-mercaptoethanol (Sigma), 5 μg/ml Fungizone (Squibb) and 5% heat-inactivated horse serum (Trace Biosciences) was used as culture medium. Red blood cells were removed from the spleen cell suspension by a 5 min treatment with 0.147 M $NH_4Cl$ at 4°C. The precursor frequency of specifically proliferating cells was determined by incubating 24 replicates (unless otherwise stated) of gradually increasing numbers of responder cells (four to six dilution steps) with 5 × $10^5$ γ-irradiated normal rat thymocytes in the presence or absence of antigen (10 μg/ml $MBP_{72-89}$ or OVA) in 96-well U-bottom tissue culture trays (Costar). The approximate precursor frequencies were determined in preliminary experiments and then dilutions of responder cells were set up around the expected frequencies in order to obtain more accurate results. No cultures contained $>10^5$ responder cells per well, as we found elevated levels of background proliferation (proliferation in the absence of antigen) at that cell concentration in the case of lymphocytes from rats with EAE. When the percentages of negative wells at high cell concentrations were well above the 37% cutoff level, the frequency was taken to be <1 in 150,000 cells (the limit of sensitivity of the assay) (17). Each well was supplemented with 10 U/ml IL-2 in the form of 20 μl MLA 144 supernatant 24 h after initiating the assay. For the last 8-12 h of the 7 day incubation, 0.5 μCi [$^3$H]thymidine was added to each well. The cells were harvested using a Skatron cell harvester and assayed for incorporated radio-activity on a LKB 1025 Betaplate counter. Estimates of minimal frequencies of the cells specifically proliferating in the presence of $MBP_{72-89}$ or OVA were calculated according to the method of Taswell (18). Wells were scored as positive following antigen stimulation when thymidine uptake (c.p.m.) was greater than twice the mean background level (dilutions of responder cells incubated with antigen-presenting cells in the absence of antigen) (i.e. when the SI was >2).

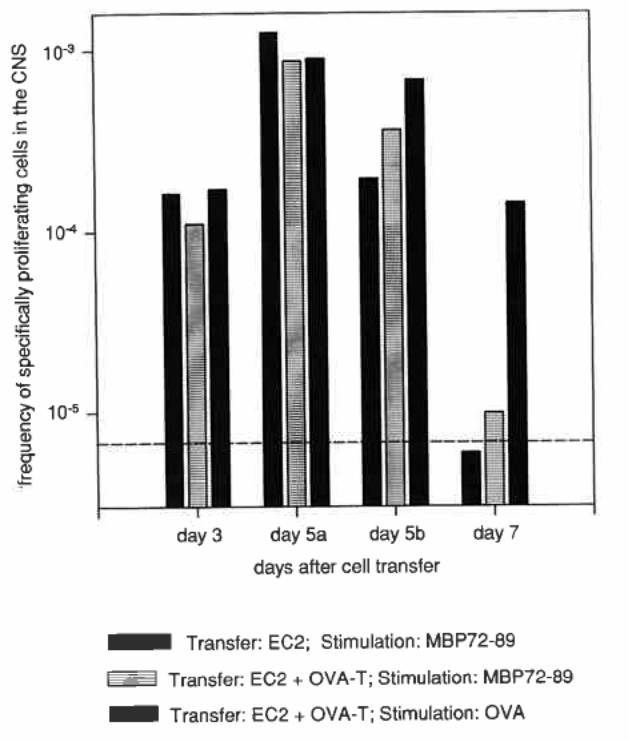


***Fig. 1.*** Frequencies of T cells specific for $MBP_{72-89}$ or OVA in the spinal cord of rats 3, 5 and 7 days after cell transfer. CNS-infiltrating cells were isolated from the spinal cords of rats after the i.v. injection of $10^7$ cloned T cells specific for $MBP_{72-89}$ (EC2) or after the i.v. co-transfer of $10^7$ EC2 cells and $10^7$ cloned OVA-specific T cells (OVA-T), as described in Methods. Day 5a refers to animals with neurological signs commencing on day 5; day 5b refers to animals with neurological signs commencing on day 4. Two animals were used for each combination of time point and type of cell transfer. Between 16 and 24 replicates of gradually decreasing numbers of cells were

cultured in the absence of antigen, or in the presence of 10 μg/ml $MBP_{72-89}$ peptide or 10 μg/ml OVA, and in the presence of $5 \times 10^5$ irradiated thymocytes as antigen-presenting cells for 7 days. The wells were supplemented with 10 U/mI IL-2 24 h after the assays had been initiated. The frequencies of proliferating cells were estimated as described in Methods. The coefficient of linear regression was usually >0.9 for all results above the background level which is represented by the dotted line (1 in 150,000 cells).

### *Effect of IL-2 on lymphocytes isolated from the CNS and MLN*

Cells isolated from the spinal cord and MLN of animals 7 days after cell transfer were used in a standard proliferation assay against $MBP_{72-89}$ or were preincubated with 100 U/ml mouse recombinant IL-2 (Boehringer Mannheim) for 5 days and then tested in a similar proliferation assay. Normal syngeneic irradiated thymocytes ($10^5$/well) were incubated, in the presence or absence of 20 μg/ml $MBP_{72-89,}$ with $2.5 \times 10^4$ CNS-infiltrating cells or $5 \times 10^4$ MLN cells in the primary assay and $1.5 \times 10^4$ CNS-infiltrating cells or $3 \times 10^4$ MLN cells in the secondary assay for 3 days, and pulsed with 0.5 μCi [$^3$H]thymidine per well for the last 6 h of the incubation. The results were expressed as the means of [$^3$H]thymidine uptake (c.p.m.) from duplicate cultures.

## Results

### *Frequencies of T cells specific for $MBP_{72-89}$ or OVA in the spinal cords of rats with passively induced EAE*

Tail weakness developed 4-5 days after cell transfer and was sometimes followed by hindlimb weakness. Clinical recovery usually began on day 7. The frequencies of specifically proliferating cells isolated from the spinal cord 3, 5 and 7 days after cell transfer are shown in Fig. 1. Co-transfer of MBP-peptide-specific and OVA-specific cells did not influence the clinical manifestations of EAE (not shown). On day 3 after co-transfer, and also on day 5 when it was the first day of weakness (day 5a), the frequency of MBP-peptide-specific T cells in the CNS (day 3: 1 in 9001; day 5a: 1 in 1149) was similar to that of OVA-specific T cells (day 3: 1 in 5843; day 5a, 1 in 1116). When the clinical manifestations began on day 4, the frequency of OVA-specific cells on day 5 (day 5b) was higher (1 in 1480) than that of MBP-peptide-specific cells (1 in 2783). By day 7 after co-transfer, when most animals begin to recover from EAE, the frequency of MBP-peptide-specific cells had fallen to <1 in $10^5$, while the frequency of OVA-specific cells remained relatively high (1 in 7001). The results in rats injected with EC2 cells alone were similar to those in rats receiving the combination of EC2 cells and OVA-T cells, with the frequency of MBP-peptide-specific cells in the CNS falling dramatically by day 7 (<1 in 150,000). When OVA-specific T cells alone were injected, there was some increase in the number of CNS-infiltrating cells 5 days after cell transfer compared with that in normal animals, and when cells were plated out $2 \times 10^3$ cells per well (no other dilutions were done), the frequency of OVA-specific cells was estimated to be ~ 1 in $10^4$.

### *Frequencies of MBP-peptide-specific and OVA-specific cells in the MLN and spleens of rats with EAE*

The frequencies of specifically proliferating cells in the peripheral lymphoid organs of animals with EAE are shown in Figs 2 and 3. Both MBP-peptide-specific and OVA-specific cells were present at relatively high frequencies in the MLN on day 3 (Fig. 2), although when OVA-specific cells were transferred alone, the frequency was lower than when they were co-transferred with MBP-peptide-specific cells. The frequency of MBP-peptide-specific cells subsequently decreased to a much lower level than did the frequency of OVA-specific cells, reaching background levels by day 5 (MBP-peptide-specific cells transferred alone) or by day 7 (when co-transferred). OVA-specific cells were still present at relatively high frequencies 5 and 7 days after passive transfer (1 in 41,000 to 1 in 52,000). On day 10 OVA-specific cells were detectable at a low frequency (1 in 110,000), while MBP-peptide-specific cells could not

be detected. In the spleen, OVA-specific cells were present at a very low frequency 3 days after sole transfer or co-transfer (Fig. 3). On days 5 and 7, OVA-specific cells were present at relatively high frequencies in the spleen (1 in 18,058 to 1 in 30,847) regardless of whether the OVA-specific T cells had been given alone or in combination with EC2 cells. The frequency of OVA-specific cells declined to lower levels by day 10 (1 in 50,554 and 1 in 105,786). In contrast to OVA-specific cells, MBP-peptide-specific cells were not detected in the spleens of animals with EAE at any of the studied time points.

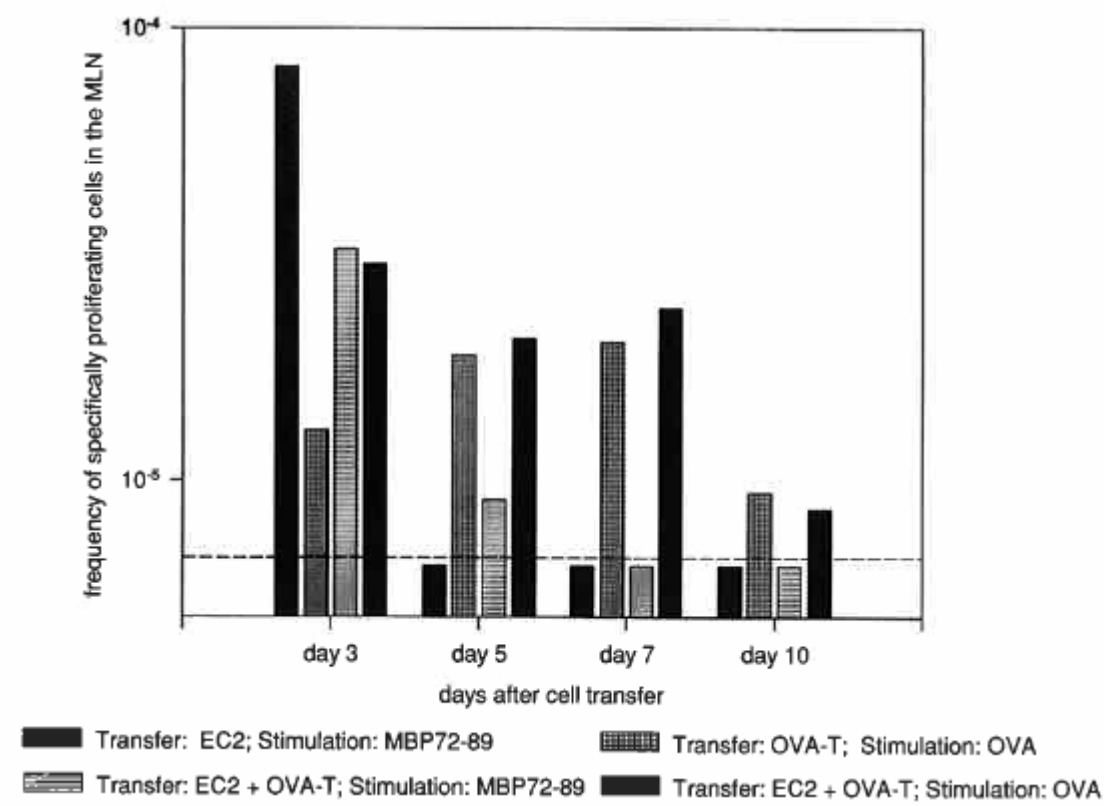


***Fig. 2.*** Frequencies of T cells specific for $MBP_{72-89}$ or OVA in the MLN of rats 3, 5, 7 and 10 days after cell transfer. EC2 cells alone, EC2 cells in combination with OVA-T cells, or OVA-T cells alone were i.v. transferred into normal Lewis rats and MLN cells were subsequently isolated from the animals as described in Methods. Limiting dilution analysis was carried out as described in Methods and in Fig. 1.

## *Persistence of OVA-specific cells in the peripheral lymphoid organs*

The frequencies of specifically proliferating cells isolated from the peripheral lymphoid organs were also estimated 26 and 48 days after the passive induction of EAE by the transfer of MBP-peptide-specific EC2 cells and the co-injection of OVA-specific T cells (Fig. 4). OVA-specific cells were still present at low frequencies in the MLN and at slightly higher frequencies in the spleen 48 days after cell transfer MBP-peptide-specific cells were undetectable in the MLN and spleen at both time points.

## *EAE actively induced by immunization with MBP and OVA*

To determine whether MBP-peptide-specific and OVA-specific cells are present in animals that have recovered from actively induced EAE, we performed frequency analysis of specifically proliferating cells in the spleen 38 days after immunization with MBP and OVA. MBP-peptide-specific cells were undetectable, while there was a relatively high frequency of OVA-specific proliferating cells (1 in 25,020).

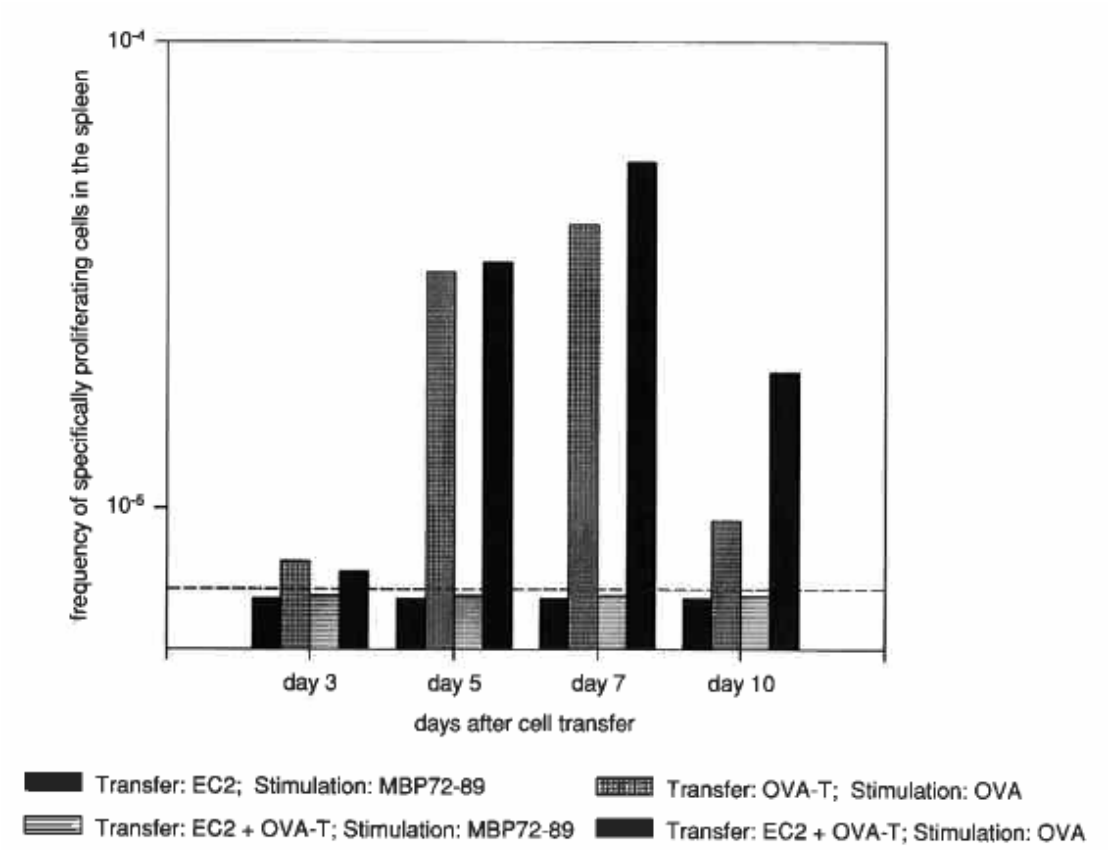


***Fig. 3.*** Frequencies of T cells specific for $MBP_{72-89}$ or OVA in the spleen of rats 3, 5, 7 and 10 days after cell transfer. EC2 cells alone, EC2 cells in combination with OVA-T cells or OVA-T cells alone were i.v. transferred into normal Lewis rats and the spleen cells were subsequently isolated from the animals as described in Methods. Limiting dilution analysis was carried out as described in Methods and in Fig. 1.

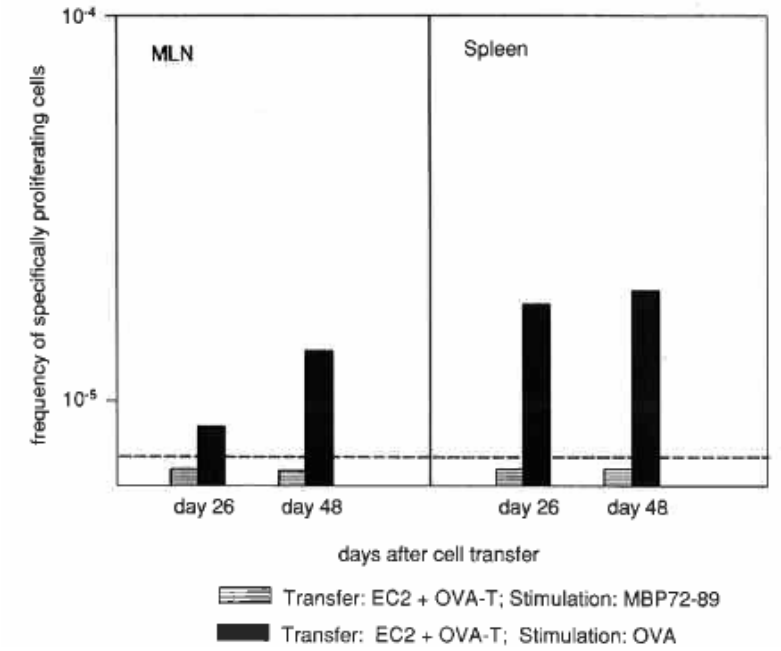


***Fig. 4.*** Frequencies of T cells specific for $MBP_{72-89}$ or OVA in the MLN and spleens of rats that had recovered from EAE. EC2 and OVA-T cells were i.v. co-transferred, and 26 or 48 days later the MLN and spleen were removed as described in Methods. Limiting dilution was carried out as described in Methods and in Fig. 1, with the exception of day 48 when only six wells were set up in the absence of antigen.

*Effect of IL-2 on lymphocytes isolated from the CNS and MLN of rats recovering from EAE*

To determine whether MBP-peptide-specific cells were present, but not responding to antigen, in the CNS infiltrate or MLN of rats recovering from EAE, spinal cord or MLN cells were isolated from rats 7 days after transfer of EC2 cells and incubated for 5 days in culture media containing 100 U/ml mouse recombinant IL-2. Figure 5 shows the MBP-peptide-specific proliferative responses of freshly isolated cells and of those preincubated with IL-2. The preincubation did not increase the level of the MBP-peptide-specific proliferative response above that obtained in the absence of antigen.

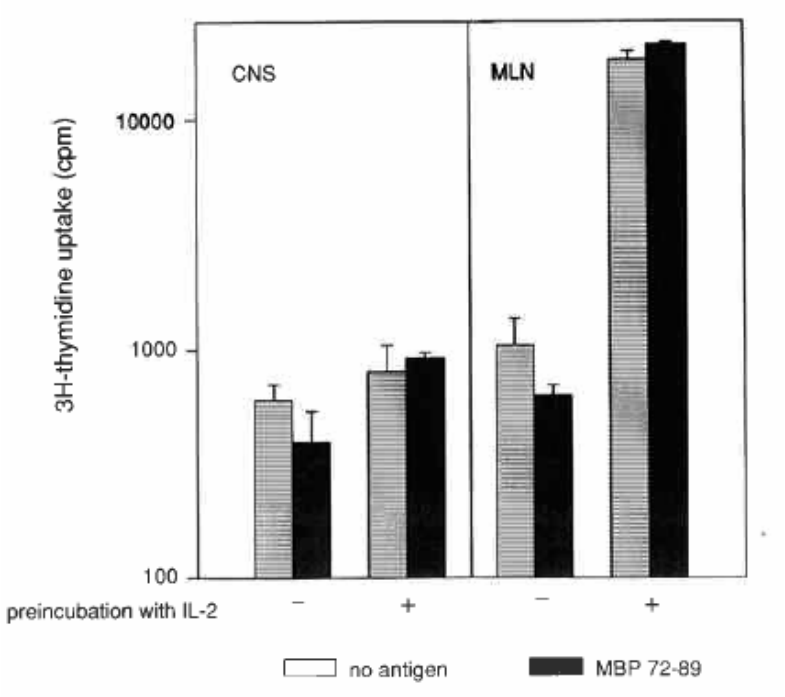


***Fig. 5.*** Effect, on $MBP_{72-89}$-specific proliferation, of *in vitro* IL-2 pretreatment of lymphocytes isolated from the spinal cord and MLN of rats 7 days after the i.v. administration of $10^7$ EC2 cells. A standard 3 day proliferation assay in the presence of $10^5$ irradiated thymocytes per well was carried out either on freshly isolated cells (no preincubation with IL-2) or on cells that had been preincubated with 100 U/ml mouse recombinant IL-2 for 5 days after isolation from the spinal cord or MLN. The mean + SD of [$^3$H]thymidine uptake (c.p.m.) obtained from duplicate cultures in the absence of antigen or in the presence of 20μg/ml $MBP_{72-89}$ are shown.

## Discussion

This study indicates that there is an antigen-specific down-regulation of the T cell response to $MBP_{72-89}$ in the CNS during spontaneous clinical recovery from EAE induced by the passive transfer of activated T cells specific for $MBP_{72-89}$. Furthermore, after recovery there is no detectable T cell response to this peptide in the peripheral lymphoid organs. The responsiveness to $MBP_{72-89}$ was not restored by incubating CNS-infiltrating cells and MLN cells with IL-2. This indicates that deletion and not anergy is responsible for the loss of responsiveness. We have confirmed the findings of Ludowyk *et al.* (19) that activated T cells specific for a non-CNS-antigen (OVA) accumulate in the CNS in EAE to a greater degree than in the normal CNS. Our results also indicate that, in contrast to autoreactive T cells, the non-autoreactive T cells that accumulate in the CNS during EAE recirculate to the peripheral lymphoid organs. T cell responsiveness to OVA persisted in the peripheral lymphoid organs for at least 48 days after the co-transfer of $MBP_{72-89}$-specific T cells and OVA-specific T cells. Similarly, in actively induced EAE, T cell responsiveness to OVA but not to

$MBP_{72-89}$ could be detected in the spleen 38 days after immunization with OVA and $MBP_{72-89}$.

The present finding of antigen-specific down-regulation of T cells reactive to $MBP_{72-89}$ is consistent with our previous observation that $V_\beta 8.2^+$ T cells are selectively eliminated from the CNS by apoptosis during recovery from EAE induced by the passive transfer of $V_\beta 8.2^+$ T cells reactive to $MBP_{72-89}$ (14). Although this mechanism appears to be highly effective in eliminating the transferred encephalitogenic T cells from the CNS and peripheral lymphoid organs, it would not be expected to result in the elimination of all of the recipient's $MBP_{72-89}$-reactive T cells from the peripheral lymphoid organs as these T cells would not have been activated and therefore not have entered the CNS. A frequency of $MBP_{72-89}$-reactive T cells similar to that of the background response in the normal rat would not have been detected in our assays. The present study suggests that the decline in the MBP-peptidespecific T cell response is not mediated by an anti-clonotypic mechanism, which has been demonstrated in the spleens of animals that have recovered from EAE (7,8), as such a mechanism would be expected similarly to affect OVA-specific cells. Furthermore, the selective decline in the frequency of $MBP_{72-89}$-specific cells is not likely to be solely due to the elevated levels of glucocorticoids which are found in EAE (9), as glucocorticoids would be expected to have the same inhibitory effect on all T cells. However, it is possible that glucocorticoids may enhance activation-induced apoptosis of T cells specific for $MBP_{72-89}$ (see below) by inhibiting IL-2 production.

Re-engagement of the T cell receptor of MBP-peptide-specific cells and not that of OVA-specific cells *in vivo* could account for the selective loss of MBP-reactive cells in EAE. After entering the CNS, activated MBP-specific T cells may again encounter antigen on the surface of bone-marrow-derived cells, such as macrophages or microglia (20-22) or possibly on parenchymal cells such as astrocytes or endothelial cells (22-24). If MBP-specific cells encounter antigen on the surface of non-professional antigen-presenting cells such as astrocytes or possibly microglia which are unable to deliver the second signal necessary for proliferation, this suboptimal stimulation might lead to activation-induced apoptotic T cell deletion as has been observed in other models of T cell activation (reviewed in 25). During the course of EAE there is increased class II MHC antigen expression on microglia (26-28) which may increase antigen presentation by these cells and lead to activation-induced T cell apoptosis.

In conclusion, our findings from this study together with those from our previous study (14) indicate that antigen-specific apoptotic deletion of autoreactive T cells in the target organ has an important role in the down-regulation of autoimmune disease. We hypothesize that failure of this normal protective mechanism may predispose to the development and perpetuation of chronic autoimmune disease. As apoptosis is an active process that can be modulated, 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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