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# The Effects of Prophylactic Cyclosporin A on Experimental Allergic Neuritis (EAN) in the Lewis Rat. Induction of Relapsing EAN Using Low Dose Cyclosporin A

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## Abstract

Experimental allergic neuritis (EAN) was induced in Lewis rats by inoculation with bovine intradural root myelin plus adjuvants. Animals treated with high dose (30 mg/kg) cyclosporin A (CsA) 3 times per week did not develop clinical EAN during the period of CsA treatment, but had an episode of EAN after cessation of CsA treatment. Animals treated with low dose (4 mg/kg) CsA 3 times per week developed EAN during the period of treatment, and after cessation of CsA treatment all of these animals developed relapsing EAN with disease continuing for up to four episodes. In contrast, 30-40% of untreated animals had a mild second episode of EAN but no further attacks. Histological studies performed in treated and untreated animals at the time of clinical episodes revealed inflammation and demyelination in the spinal roots and dorsal root ganglia. When animals were challenged with a second inoculation at age 7 months, one of 15 untreated control animals but none of the CsA treated animals developed an episode of EAN.

**Keywords**: experimental allergic neuritis; EAE; Cyclosporin A; demyelination; tolerance; Lewis rat

## Introduction

Cyclosporin A (CsA) has powerful effects on cell-mediated immunity, especially on helper T cells (Thomson and Webster, 1988). CsA reduces interleukin 2 (IL-2) production and reduces the expression of IL-2 receptors by lymphocytes (Prince and John, 1986). CsA prevents graft rejection after transplantation (Uhteg et al., 1985). There has been increasing interest in the use of CsA in the treatment of autoimmune disease. CsA in high doses has been shown to be effective in the prophylaxis against, and treatment of, experimental allergic neuritis (EAN) (King et al., 1983) and to suppress EAN mediated by T cell lines (Hartung et al., 1987). CsA also suppresses experimental allergic encephalomyelitis (EAE) (Bolton et al., 1982a, b; Reiber and Suckling, 1986). However, low dose CsA converts acute EAE into chronic relapsing EAE (Polman et al., 1988). Because EAN has many similarities to EAE, we decided to determine whether low dose CsA treatment of EAN has similar effects on immunoregulation particularly with respect to the development of relapses.

## Materials and methods

*Induction of EAN*

Two-month (8-10-week) or 4-month (17-18-week) Lewis rats of both sexes were inoculated with a total of 0.1 ml of emulsion (containing 2 mg bovine intradural root **(BIR)** myelin, 0.05 ml of complete Freund's adjuvant (Difco), 0.5 mg *Mycobacterium tuberculosis* H37RA (Difco), and 0.05 ml saline) per animal. The inoculum was given in divided doses in the medial footpads of both hindlimbs. The myelin was prepared by sucrose density gradient centrifugation from bovine intradural roots obtained within 1 h of death and dissected immediately. *Re-challenge experiments*

After the initial part of the experiment was concluded, some animals were given a second inoculation (prepared in the same way as the first inoculum) into both medial footpads of the fore-limbs at age 7 months. For these re-challenge experiments, animals of the same age, but with no previous inoculation or CsA treatment, were used as controls.

*CsA treatment*

Animals were divided into groups which were given one of the following treatments: no treatment, sham treatment with saline injections, or treatment with high or low dose CsA injections. CsA was administered by subcutaneous injection into the nuchal region at a dose of 30 mg/kg on 3 days per week ('high dose') or 4 mg/kg on 3 days per week ('low dose'). The high dose CsA was continued

until day 17 or 31 and the low dose CsA was continued until day 29 post-inoculation.

*Neurological assessment*
Animals were weighed and examined for neurological impairment. Weakness was scored using the scale devised by Pender (1986). Tail weakness, hindlimb weakness and forelimb weakness were each assessed on a scale of 0-4. For each animal these three scores were added together to give an overall measure of the severity of disease. Note was also taken of ataxia and posture. Animals were examined daily until day 85 post-inoculation. Some animals were re-inoculated at age 7 months and these were examined daily for a further 25 days. A small group of animals was followed until day 130 after the first inoculation to assess the incidence of late relapses.

*Histological methods*
At appropriate times post-inoculation animals were anaesthetized and perfused through the heart with modified Karnovsky's fixative. The spinal cord, spinal roots, dorsal root ganglia and sciatic nerves were removed and prepared as follows. For light microscopy, the tissues were embedded in HistoResin (LKB, Bromma, Sweden) and processed according to the methods of Nguyen and Pender (1989). Some specimens were embedded in epoxy resin and semi-thin sections were prepared and stained with toluidine blue.

## Results

Table 1 summarizes the clinical features of control animals and animals given high or low dose CsA. Table 2 shows the histological findings. Table 3 summarizes the results of the re-challenge experiments. Fig. 1 shows the clinical course of an individual 4-month-old male animal from each treatment group and Fig. 2 shows the mean clinical course of all male animals observed up to day 85 in the different treatment groups. Figs. 3 and 4 show examples of the histological findings. The details of each group are given below.

*Control group (untreated and saline-treated animals)*
Both 2-month-old and 4-month-old untreated animals developed neurological signs of EAN (tail weakness, limb ataxia and limb weakness) 11-15 days after inoculation and had recovered completely by day 21-24 (Figs. 1 and 2). In the 2-month-old untreated control group, one of three male animals and three of four female animals had a second episode of EAN manifested by mild tail weakness commencing about day 28. In the 4-month-old untreated control group two of eight male and one of four female animals had a second episode of EAN manifested by mild tail weakness commencing about day 33. None of the untreated animals had a third episode of weakness during the period of daily observation up to day 85. A group of five untreated animals was observed 3 times per week until day 130 but none of these animals had a further episode of disease between days 85 and 130. Histological examination revealed typical features of EAN (inflammation and demyelination) in the spinal nerve roots and dorsal root ganglia of animals studied during the first episode (Fig. 3). There was no inflammation or demyelination of the sciatic nerves, spinal nerves or the spinal cord except for occasional involvement of the dorsal root entry and ventral root exit zones of the spinal cord. Animals given sham treatment with saline developed EAN at the same time as untreated animals. One of three saline-treated animals had an early relapse.

When control animals were re-challenged with a further dose of inoculum at age 7 months, one of ten animals developed a further episode of EAN (Table 3). The histological findings in this animal during this episode were typical of EAN. All of the animals which were inoculated for the first time at age 7 months for use as controls in the re-challenge experiments developed clinical EAN. One of these animals was studied histologically, and had inflammation and demyelination in nerve roots and dorsal root ganglia.

*High dose CsA (until day 17)*
In this experiment, 4-month-old animals were inoculated with BIR myelin plus adjuvants. The animals were given prophylactic subcutaneous CsA (30 mg/kg 3 times per week) from the day of inoculation until day 17 post-inoculation, at which time all control animals had developed signs of EAN but no control animals had recovered from the episode. No animals in the high dose CsA treatment group developed EAN during the time of CsA treatment. However, in the nerve roots of an animal treated with high dose CsA and sampled on day 15 post-inoculation, there were occasional inflammatory cells and demyelinated fibres. All animals developed neurological signs of EAN 21-30 days after ceasing CsA (Fig. 1). The disease was mild and was manifested only by tail weakness. However, histological examination demonstrated inflammation and demyelination in the spinal nerve roots and dorsal root ganglia. The animals recovered from this episode and were

TABLE 1

THE EFFECT OF CYCLOSPORIN A ON THE INDUCTION OF EAN AND THE DEVELOPMENT OF EARLY RELAPSE

| Treatment | 1st episode | | | 2nd episode | | | 3rd episode | | |
|---|---|---|---|---|---|---|---|---|---|
| | Number (%) affected | Mean day of onset | Mean max. severity | Number (%) affected [a] | Mean day of onset [b] | Mean max. severity [b] | Number (%) affected [a] | Mean day of onset [b] | Mean max. severity [b] |
| **None** | | | | | | | | | |
| *2 months old* | | | | | | | | | |
| Males | 4/4 (100%) | 12.0 | 7.7 | 1/3 (33%) | 26.0 | 5.0 | 0/3 (0) | – | – |
| Females | 4/4 (100%) | 14.7 | 7.7 | 3/4 (75%) | 30.0 | 2.3 | 0/4 (0) | – | – |
| Total | 8/8 (100%) | 13.4 | 7.7 | 4/7 (57%) | 29.2 | 3.0 | 0/7 (0) | – | – |
| *4 months old* | | | | | | | | | |
| Males | 10/10 (100%) | 14.4 | 5.9 | 2/8 (25%) | 35.0 | 2.0 | 0/8 (0) | – | – |
| Females | 4/4 (100%) | 17.7 | 5.7 | 1/4 (15%) | 32.0 | 3.0 | 0/4 (0) | – | – |
| Total | 14/14 (100%) | 15.3 | 5.8 | 3/12 (25%) | 34.0 | 2.3 | 0/12 (0) | – | – |
| **Saline** | | | | | | | | | |
| *4 months old* | | | | | | | | | |
| Males | 5/5 (100%) | 13.0 | 8.4 | 1/3 (33%) | 31.0 | 2.0 | | | |
| **High dose CsA (until day 17)** | | | | | | | | | |
| *4 months old* | | | | | | | | | |
| Males | 5/5 (100%) | 44.6 | 2.4 | 0/4 (0) | – | – | 0/4 (0) | – | – |
| Females | 2/2 (100%) | 46.0 | 2.0 | 0/2 (0) | – | – | 0/2 (0) | – | – |
| Total | 7/7 (100%) | 45.0 | 2.3 | 0/6 (0) | – | – | 0/6 (0) | – | – |
| **High dose CsA (until day 31)** | | | | | | | | | |
| *4 months old* | | | | | | | | | |
| Males | 4/4 (100%) | 52.5 | 2.5 | 0/3 (0) | – | – | 0/3 (0) | – | – |
| Females | 3/3 (100%) | 55.6 | 2.0 | 0/3 (0) | – | – | 0/3 (0) | – | – |
| Total | 7/7 (100%) | 53.8 | 2.3 | 0/6 (0) | – | – | 0/6 (0) | – | – |
| **Low dose CsA (until day 29)** | | | | | | | | | |
| *2 months old* | | | | | | | | | |
| Males | 5/5 (100%) | 12.0 | 6.7 | 4/4 (100%) | 40.5 | 3.2 | 4/4 (100%) | 61.7 | 2.7 |
| Females | 3/3 (100%) | 15.0 | 7.0 | 3/3 (100%) | 33.3 | 4.6 | 3/3 (100%) | 57.3 | 2.7 |
| Total | 8/8 (100%) | 13.1 | 6.8 | 7/7 (100%) | 37.4 | 3.8 | 7/7 (100%) | 59.8 | 2.7 |
| *4 months old* | | | | | | | | | |
| Males | 15/15 (100%) | 17.3 | 4.4 | 13/13 (100%) | 36.6 | 3.3 | 5/10 (70%) | 47.8 | 3.1 |

[a] The total number in each group is reduced because some animals were sacrificed for histology.

[b] Means are calculated using data from affected animals only.

TABLE 2

HISTOLOGICAL FINDINGS

| Rat number | Day of sacrifice (after first inoculation) | Clinical status | Histologic findings in nerve roots and dorsal root ganglia |
|---|---|---|---|
| **Controls** | | | |
| *Control animals (no CsA)* | | | |
| 84 | 15 | 1st episode | Inflammation and demyelination |
| 125 | 15 | 1st episode | Inflammation and demyelination |
| *Control animals (saline)* | | | |
| 122 | 15 | 1st episode | Inflammation and demyelination |
| 125 | 15 | 1st episode | Inflammation and demyelination |
| 126 | 43 | Fully recovered after one episode | No demyelination or inflammation but evidence of remyelination |
| *Control animal (for re-challenge age 7 / 12)* | | | |
| 155 | 17 | 1st episode | Inflammation and demyelination |
| **High dose CsA** | | | |
| *High dose (to day 15)* | | | |
| 96 | 15 | No neurological signs | Some inflammatory cells and occasional demyelinated fibres |
| *High dose (to day 17)* | | | |
| 113 | 45 | 1st episode | Mild inflammation and demyelination |
| *High dose (to day 31)* | | | |
| 94 | 54 | 1st episode | Mild inflammation and demyelination |
| **Low dose CsA** | | | |
| *Low dose (to day 29)* | | | |
| 128 | 19 | 1st episode | Inflammation and demyelination |
| 129 | 19 | 1st episode | Inflammation and demyelination |
| 131 | 38 | 2nd episode | Severe inflammation and demyelination Evidence of remyelination |
| 140 | 43 | 2nd episode | Severe inflammation and demyelination Evidence of remyelination |
| 136 | 86 | Fully recovered after 3 episodes | Evidence of remyelination |

followed up to day 85, but no animals developed relapses. No animals from this treatment group developed neurological signs after re-challenge with antigen (Table 3).

TABLE 3
INOCULATION OF ANIMALS AT AGE 7 MONTHS (RE-CHALLENGE EXPERIMENTS)

| Number of animals in group | Previous inoculation | Previous treatment | Animals developing EAN after inoculation at age 7 months |
|---|---|---|---|
| 15 | None | None | 15/15 |
| 5 | At age 2 months | None | 0/5 |
| 5 | At age 4 months | None | 1/5 |
| 2 | At age 4 months | Saline | 0/2 |
| 5 | At age 4 months | High dose CsA (up to day 17) | 0/5 |
| 6 | At age 4 months | High dose CsA (up to day 31) | 0/6 |
| 5 | At age 2 months | Low dose CsA (up to day 29) | 0/5 |
| 4 | At age 4 months | Low dose CsA (up to day 29) | 0/4 |

*High dose CsA (until day 31)*
In this experiment, 4-month-old animals were inoculated with BIR myelin plus adjuvants. The animals were given prophylactic subcutaneous CsA (30 mg/kg 3 times per week) until day 31. By day 31 all control animals had recovered fully from EAN. No animal in this high dose CsA group developed signs of EAN during the period of CsA treatment. All animals developed signs of EAN 20-22 days after ceasing CsA (Fig. 1) although in three animals the signs were minimal. The histologic appearances were of inflammation and demyelination in the spinal roots and dorsal root ganglia. No animals had more than one episode of disease during a period of follow-up to day 85. No animals experienced a relapse after re-challenge with antigen.

*Low dose CsA (until day 29)*
All animals inoculated at 2 months and at 4 months of age developed EAN during the period of low dose CsA treatment (4 mg/kg 3 times per week) although the time of peak disability was later than for the untreated animals (Figs. 1 and 2). In the 2-month-old group the disease was as severe as in controls, but in the 4-month-old group the initial episode of disease was less severe than in controls. Histological examination during the first episode (while on low dose CsA) showed inflammation and demyelination. In the group inoculated at 2 months every animal had a second episode of weakness commencing on days 33-42 post-inoculation and a third episode of weakness commencing on days 57-64 post-inoculation. These episodes were less severe than the initial episode of EAN but were more severe than the relapses observed in the control animals. Animals were followed to day 87 but none of the 2-monthold animals had more than three episodes of EAN. Of the group inoculated at 4 months of age, every animal had a second episode of weakness at day 31-43. Seven of ten animals had a third episode at day 39-69 after inoculation and three animals had a mild fourth episode. Histological examination of tissue from 4-month-old animals perfused during the second episode of disease (at day 44 post-inoculation) showed severe inflammation and demyelination of the nerve roots, ganglia and spinal nerves (Fig. 4A and B). There was also evidence of remyelination (Fig. 4B). Tissue from an animal perfused after three episodes of disease showed extensive evidence of remyelination. No animal in this treatment group developed a further episode after re-challenge with antigen.

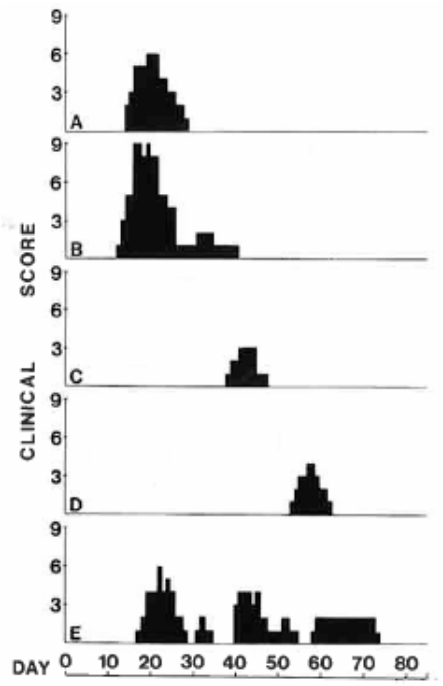


**Fig. 1.** Typical clinical courses of EAE in individual 4-month old animals given different doses of CsA. (A) received no treatment, (B) received subcutaneous saline injections, (C) received high dose CsA until day 17, (D) received high dose CsA until day 31, and (E) received low dose CsA until day 29. The figure shows that high dose CsA prevented development of signs of EAN during the period of treatment, although disease occurred after CsA withdrawal. It can also be seen that the animal treated with low dose CsA developed a chronic relapsing course.

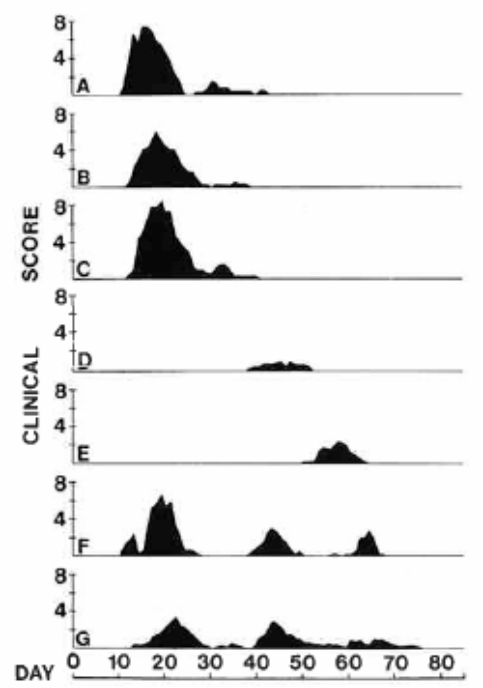


**Fig. 2**. Mean clinical scores of groups of 2-month- and 4-month-old male Lewis rats given different doses of CsA. The mean scores were obtained from animals which were observed until day 85 and do not include data on animals which were sacrificed for histological examination. (A) 2-month-old animals with no treatment, (B) 4-month-old animals with no treatment, (C) 4-month-old animals given saline treatment, (D) 4-month-old animals given high dose CsA until day 17, (E) 4-month-old animals given high dose CsA until day 31, (F) 2-month-old animals given low dose CsA until day 29, and (G) 4-month-old animals given low dose CsA until day 29. It can be seen that high dose CsA prevented clinical signs of EAN during the time of treatment, and that low dose CsA produced relapsing disease.

## Discussion

In the untreated control group all animals developed EAN and 36% had a mild relapse of weakness soon after recovery from the first episode. Brosnan et al. (1984) found that 30% of male Lewis rats developed chronic disease after BIR myelin-induced EAN. Adam et al. (1989) found that one of nine Lewis rats inoculated with 2.5 mg and eight of nine rats inoculated with 5.0 mg myelin developed an early relapse of EAN. In-creased incidence of relapses with higher doses of inoculum has also been shown by Harvey et al. (1987) in EAN in rabbits. Craggs et al. (1984b) described relapses after 100 days post-inoculation in all 4-month-old Lewis rats with EAN induced by inoculation with whole bovine dorsal roots. This difference from our studies may be due to the different inoculum used. In our study younger animals had more relapses, as described previously by Craggs et al. (1986). In the 2-month-old group but not the 4-month-old group the relapse rate was higher for females than for males, as has been observed by Keith (1978) in EAE.

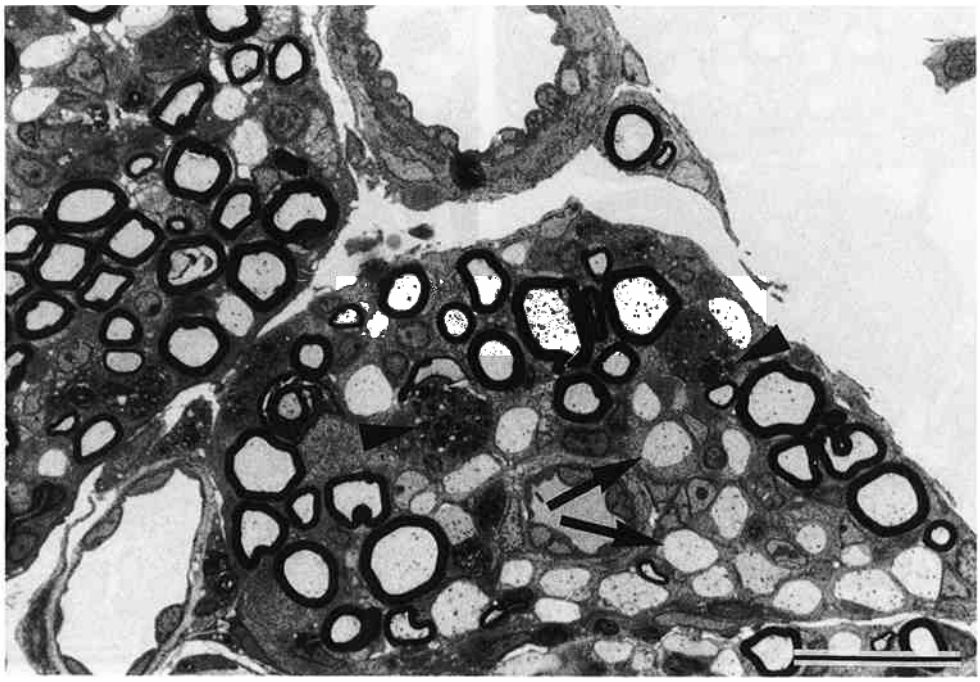

**Fig. 3.** Pathological findings in tissue from a control rat with EAN perfused on day 15 post-inoculation. Section from cauda equine showing demyelination (arrows) and macrophages containing myelin debris (arrowheads). Epoxy section stained with toluidine blue (bar = 25μm).

Our results for the saline-treated animals were similar to those for untreated controls, suggesting that the stress associated with repeated subcutaneous injections does not affect the relapse rate. When control animals were re-challenged with a further inoculum given in a different site, one of ten animals developed a further episode of acute EAN which was confirmed histologically (Table 3). This suggests that most animals acquired tolerance to the antigens, as occurs in EAE (Willenborg, 1979). Adam et al. (1987) using higher doses of myelin than in our study, found that most Lewis rats developed signs of EAN after re-challenge at day 86 post-inoculation. However, while some animals developed severe disease, in most animals the disease was less severe than in the first episode, indicating that most animals were relatively resistant to re-induction of disease. Pollard et al. (1975) also found with guinea pigs that while most became resistant to re-induction of EAN, in a minority this failed to occur. In animals treated with high doses of prophylactic CsA, the development of clinical signs of EAN was prevented as long as the treatment was continued. This has been found previously in Lewis rats with EAN induced by inoculation with whole dorsal roots (King et al.. 1983) and with EAE (Bolton et al., 1982a, b) and is probably due to the effects of CsA in preventing activation of helper T cells which are necessary for the induction of EAN (Linington et al., 1984; Rostami et al., 1985; Brosnan et al., 1987). The present study confirms the findings of King et al. (1983) that animals develop an episode of EAN after cessation of high dose CsA. Whether the animals were given high dose CsA up to day 17 or 31, disease occurred 20-30 days after ceasing treatment. Similar occurrence of disease after withdrawal of high dose CsA (50 mg/kg given orally on alternate days for 26 days) was seen with actively induced EAE (Bolton et al., 1982b). King et al. (1983) suggested that EAN occurred after cessation of CsA because antigen was

still present and able to elicit an immune response. The importance of persisting antigen in late episodes of disease was shown by Tabira et al. (1984) who found that an antigen depot is necessary for the production of chronic relapsing EAE in juvenile strain 13 guinea pigs. However, occurrence of EAN after CsA withdrawal could also be due to the persistence of sensitized cells as Raine et al. (1984) showed that chronic relapsing EAE could be passively transferred to SJL/J mice by lymph node cells alone and Bolton et al. (1982a) found that, with passively transferred EAE, disease is prevented during treatment but occurs after cessation of high dose CsA. In our study the EAN occurring after withdrawal of high dose CsA was clinically less severe than disease in controls. King et al. (1983) found that whole-dorsal-root-induced EAN after withdrawal of high dose oral CsA was clinically as severe as control disease although not as severe pathologically. This difference from our study may be related to differences in the inoculum used or in the dose and route of administration of CsA.

In contrast to our findings with high dose CsA,we found that low dose CsA permits development of EAN during administration and leads to the development of relapsing disease in 100% of animals. These findings in EAN are similar to those of Polman et al. (1988) and Stanley and Pender (1989) in whole-spinal-cord-induced EAE in the Lewis rat. The increased incidence of early relapses with low dose CsA compared to controls is presumably due to effects of CsA on immunoregulation. One possibility is that low dose CsA did not interfere with development of the effector T cells which produce EAN but did interfere with the development of acquired tolerance to BIR myelin so that new episodes of EAN occurred by continued stimulus from remaining antigen or continued activity of T cells, although the animals retained the ability to recover from each separate

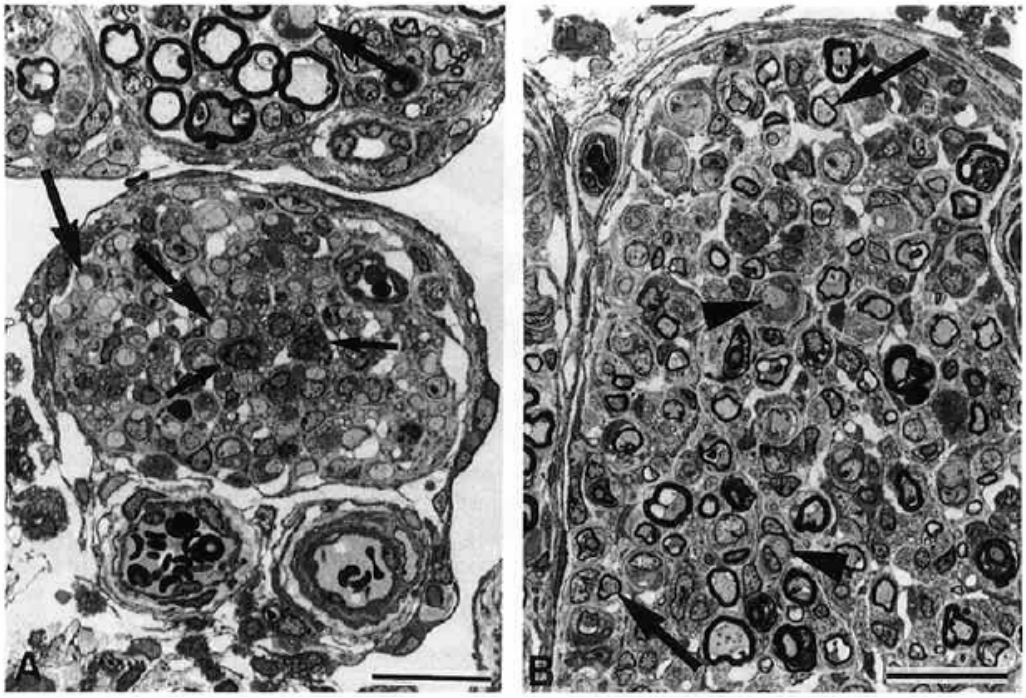


**Fig. 4.** Pathological findings in tissue from a rat treated with low dose CsA and perfused on day 38 post-inoculation during a second episode of EAN. (A) Cauda equina showing mononuclear inflammatory cells (small arrows) and demyelinated fibres (large arrows). (B) Another area of the same section as (A) showing many remyelinated fibres (arrows). Mononuclear inflammatory cells and demyelinated fibres (arrowheads) are also present. Epoxy section stained with toluidine blue (bar = 25 μm).

attack. High dose CsA would also affect these regulatory T cells but disease does not arise because of the effects of CsA on effector cells. These observations are consistent with the findings in EAE that different mechanisms control recovery from disease and resistance to disease re-induction (Willenborg, 1982) and that immunologically non-specific factors cause recovery from separate episodes of disease (MacPhee et al., 1989). CsA is known to affect immunoregulation because under certain circumstances CsA can induce autoimmune disease (Sakaguchi and Sakaguchi, 1989) and syngeneic graft-versus-host disease (Fischer et al., 1989). There are a range of different regulatory cells including suppressor-inducer cells (Morimoto et al., 1985), contrasuppressor cells (Ptak et al., 1988), anti-idiotypic cells (Lider et al., 1988) and anti-ergotypic cells (Lohse et al., 1989) which could

be affected by CsA. Different effects of low dose CsA on these cells compared to $CD4^+$ effector cells could interfere with suppression of disease. The observations of Strigard et al. (1989) that reduction of OX-19 ($CDS^+$) cells could allow non-OX-19 cells to cause a relapse of EAN is consistent with this explanation.

These studies may be extrapolated to the human disease, the Guillain-Barre syndromé (GBS), which has similarities to EAN, and may suggest that inadequate dose or duration of treatment of GBS with CsA could be associated with long-term problems. Treatment of GBS with corticosteroids or plasma exchange has been associated with an increased incidence of relapses (Hughes et al., 1978; Osterman et al., 1988) and in the case of plasma exchange has been related to ceasing treatment too soon. These clinical observations and our experimental evidence on the effects of CsA on EAN indicate that while treatment can interfere with the effectors of the pathological process, inadequate treatment may also interfere with the immunological mechanisms which prevent relapse of disease.

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