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ULTRASTRUCTURAL STUDY OF THE EFFECTS OF CYCLOSPORINE IN THE BRAINSTEM OF WISTAR RATS SUBMITTED TO THE ETHIDIUM BROMIDE DEMYELINATING MODEL

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Abstract – The ethidium bromide-demyelinating model (EB) was used to study remyelination in the brainstem under the use of cyclosporine (CsA). Wistar rats were submitted to intracisternal injection of 0.1% EB or 0.9% saline solution, and others were taken as histologic controls (group I). Within those injected with EB, some have not received immunosuppressive treatment (II); some were treated by intraperitonial route with CsA (III.E - 10 mg/kg/day). Rats from group III.C were injected with saline solution and treated with CsA. The animals were perfused from 15 to 31 days post-injection collecting brainstem sections for light and transmission electron microscopy studies. After EB injection it was noted the presence of macrophages and non-degraded myelin debris, demyelinated axons, oligodendrocyte or Schwann cell remyelinated axons, groups of infiltrating pial cells, hypertrophic astrocytes and few lymphocytes. Tissue repair of EB-induced lesions in group III.E was similar to that of group II, but with the presence of a higher density of oligodendrocytes near remyelinating areas.

KEY WORDS: central nervous system, myelin sheath, oligodendroglia, Schwann cells, cyclosporine, ethidium bromide.

Estudo ultra-estrutural dos efeitos da ciclosporina no tronco encefálico de ratos Wistar submetidos ao modelo desmielinizante do brometo de etídio

Resumo – Empregou-se o modelo desmielinizante do brometo de etídio (BE) com o objetivo de estudar a remielinização no tronco encefálico frente ao uso de ciclosporina (CsA). Foram utilizados ratos Wistar, submetidos à injeção de BE a 0,1% ou de solução salina na cisterna pontina, assim como controles histológicos (grupo I). Dos animais injetados com BE, alguns não receberam tratamento imunossupressor (II); outros foram tratados por via intraperitoneal com CsA (III.E - 10 mg/kg/dia). O grupo III.C incluiu animais injetados com salina e tratados com CsA. Os animais foram perfundidos dos 15 aos 31 dias pós-injeção, com colheita de material do tronco encefálico para estudos de microscopia de luz e eletrônica de transmissão. Após injeção de BE, foram observados macrófagos e restos de mielina não-degradada, axônios desmielinizados ou remielinizados por oligodendrócitos e por células de Schwann, grupos de células piais infiltrantes, astrócitos hipertróficos e poucos linfócitos. O processo de reparo das lesões no grupo III.E apresentou-se similar ao do grupo II, porém com maior densidade de oligodendrócitos próximos às áreas de remielinização.

PALAVRAS-CHAVE: sistema nervoso central, bainha de mielina, células de Schwann, oligodendróglia, ciclosporina, brometo de etídio.

Several studies on the biology of demyelination and remyelination in the central nervous system (CNS) have been made based on the use of the gliotoxic agent ethidium bromide (EB)[1-11]. In this experimental model, it was noted the presence of lymphocytes during the process of myelin loss and removal after EB injection, as well as an intense macrophagic activity in the damaged areas[2,3,6]. The presence of lymphocytes remains controversial, maybe just part of the inflammatory response induced by the agent. Despite the toxic nature of this model, it can not be ruled out the possibility of some participation of these lymphocytes as a feature of antigenic recognition or as effectors in some immune-mediated responses to the detached myelin sheaths[3]. Immunosuppressive agents have been broadly used in toxic and immune-mediated experimental demyelination, as well as in several demyelinating diseases char-

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acterized by immunologic involvement and expression of autoimmunity. Previous studies using cyclophosphamide[5] and dexamethasone[6] showed that their use after EB injection has delayed in general terms the repairing process, specially the reconstruction of the lost myelin sheaths.

In this context, the search for immunosuppressive therapies that do not promote delays in CNS myelin restoration reveals the necessity of further studies involving other immunomodulating drugs, observing their possible influence on the cellular events related to the repair of the nervous tissue and of the damaged myelin sheaths.

Thus the aim of this investigation was to study the cellular processes involved in CNS repair after local EB injection under an attempt of pharmacological interference with cyclosporine (CsA) in the rat brainstem remyelinating process.

METHOD

This experiment was approved by the Ethics Comission of the Universidade Paulista (UNIP). Thirty male Wistar rats, 4 to 6 months old, were used. They were divided into 4 groups - I (n=2), including animals taken as histologic controls; II (n=8), constituted of animals injected with EB into the cisterna pontis; III.E (n=16), animals equally injected with EB, but treated with CsA; and III.C (n=4), including animals injected with saline solution and treated with CsA.

The rats were anaesthetized with ketamine and xylazine (5:1, 0.1 mL/100g) and a burr hole was made on the right side of the skull, 8 mm rostral to the fronto-parietal suture. Injections were performed freehand using a Hamilton syringe, fitted with a 35º angled polished gauge needle into the cisterna pontis, an enlarged subarachnoid space below the ventral surface of the pons.

Ten microliters of 0.1 EB solution were injected into the cisterna pontis of rats from groups II and III.E and the same volume of 0.9% saline solution was injected in rats from group III.C.

Rats from groups III.E and III.C were daily treated with CsA using 10 mg/kg by intraperitoneal route in the first week and, thereafter, 3 times a week, with a minimal interval of 48 hours. A solution containing 10 mg/mL of CsA (Sandimmun®, Sandoz S/A Pharmaceutical Division. São Paulo SP, Brazil) was obtained by diluting the content for intravenous infusion (50 mg/mL) in sterile 0.9% saline solution.

The animals were anaesthetized and submitted to intracardiac perfusion with 4% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH 7.4) at the following post-injection periods - 15, 17, 21 and 31 days. From group II, 4 rats were perfused for each period; from group III.E, 2 rats; and 2 from group C, 1 rat.

Thin slices of the brainstem (pons, mesencephalon and trapezoid body) were collected and post-fixed in 1% osmium tetroxide, dehydrated with graded acetones and embedded in Araldite 502 resin, following transitional stages in acetone. Thick sections were stained with 0.25% alkaline toluidine blue. Selected areas were trimmed and thin sections were stained with 2% uranyl acetate and lead citrate and examined using a Philips EM-201 transmission electron microscope.

RESULTS

Observations on group II (EB injection and no CsA treatment)

The examination of semithin sections from rats of group II revealed the appearance of lesions of variable extent (from the mesencephalon into the trapezoid body), but affecting mostly the ventral surface of the pons, and allowed the establishment of 2 areas with very distinct morphological characteristics. The center of the lesion presented an extended extracellular space, with many foamy macrophages, demyelinated axons and some myelin debris (Fig 1). At the periphery, phagocytic cells were

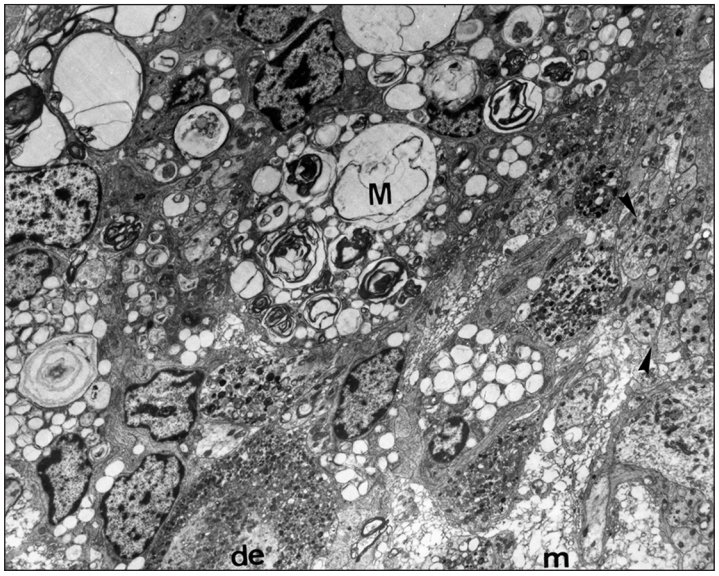

Fig 1. Center of the lesion, with phagocytic cells (M) containing myelin at several stages of degradation, demyelinated axons (arrowhead), axons in degeneration (de) and some myelin derived-membranes (m). Lesion of 17 days - Group II. Electron micrograph - 3.350x.

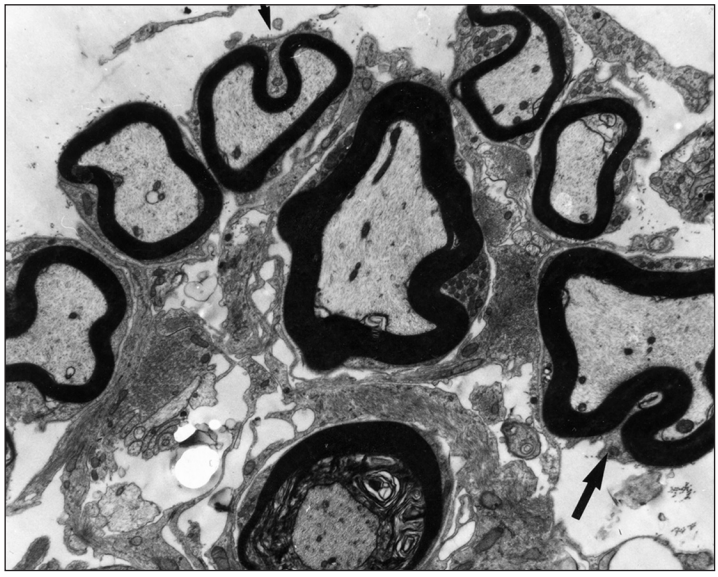

Fig 2. Redundant myelin loops (loops-arrows) formed by Schwann cells. Lesion of 15 days - Group III.E. Electron micrograph - 7.450x.

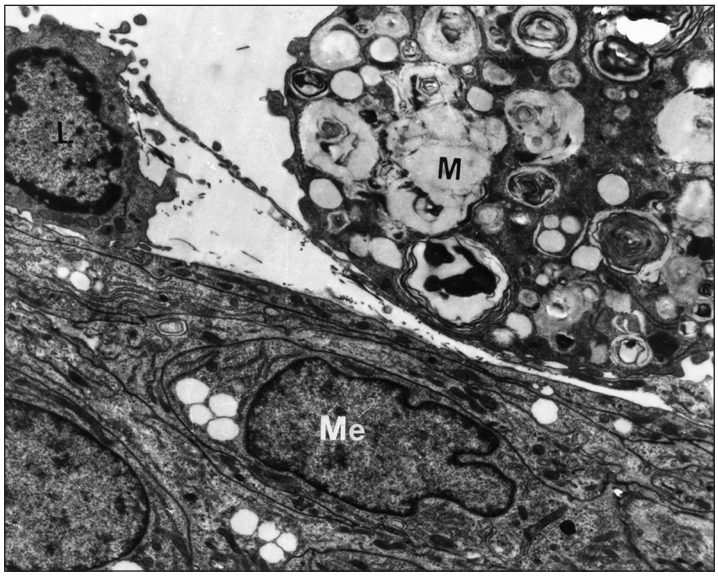

Fig 3. A lymphocyte (L) and a macrophage (M) contacting a group of infiltrating pial cells (Me). Lesion of 15 days - Group III.E. Electron micrograph - 7.450x.

less conspicuous and thinly remyelinated axons could be seen, some clearly associated with Schwann cells.

Ultrastructural analysis of the central area revealed the appearance of macrophages containing myelin in different stages of degradation, from loose lamellae to the accumulation of fat neutral droplets in the cytoplasm. In the vicinity of these macrophages, there were many demyelinated axons, some with clear signs of degeneration, as well as concentrations of myelin-derived membranes in the extracellular space of some areas.

At peripheral sites, it was found groups of demyelinated axons in association with astrocytic processes and axons exhibiting thin myelin sheaths of oligodendrocyte origin. Lymphocytes were commonly observed in perivascular locations and all over the neuropil. Infiltrating pial cells were also found as monotypical nests or as diffuse arrangements joined by desmossome-like juncions.

Around blood vessels and in areas of expanded extracellular space, where the glia limitans was disrupted and astrocytic processes had disappeared, Schwann cells were seen related to one or more naked axons or at initial remyelinating stages.

Axons related to Schwann cells presented faster remyelination, with thicker myelin sheaths than those produced by oligodendrocytes at the same period, although there was an evident preponderance of oligodendroglial remyelinated axons. Naked axons either clumped or separated by astrocytic prolongations were also noted.

Observations on group III.E
(EB injection and CsA treatment)

By 15-17 days after EB injection, light microscopy re-

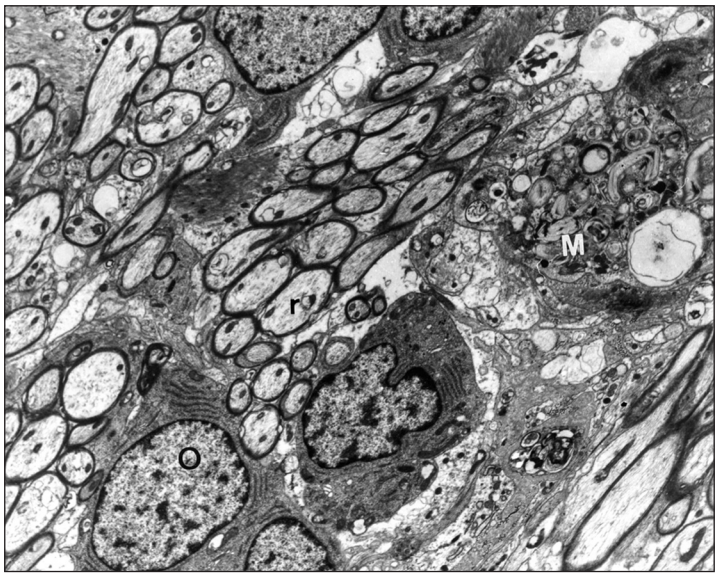

Fig 4. Oligodendrocytes (O) at the periphery of the lesion close to groups of remyelinating axons (r). Note the presence of a macrophage (M) containing myelin in different degrees of breakdown. Lesion of 21 days - Group III.E. Electron micrograph - 4.875x.

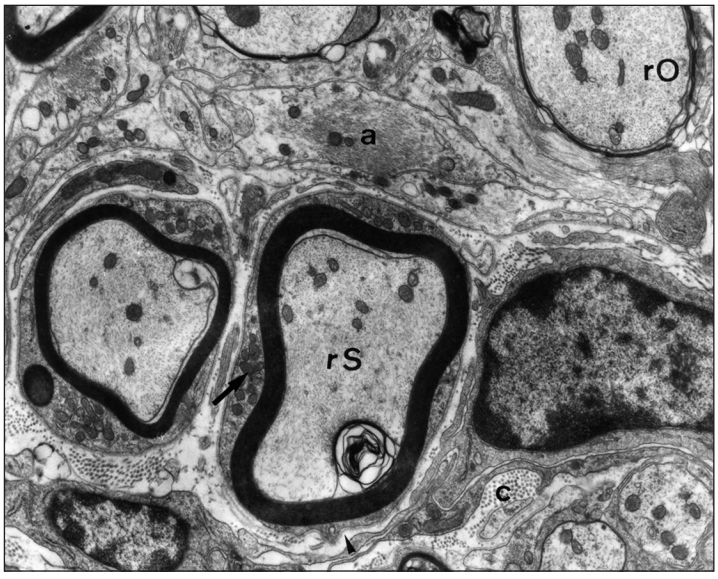

Fig 5. Area of oligodendrocyte remyelinated axons (rO) and of Schwann cell remyelinated axons (rS). Note that the first ones are associated with astrocytic processes (a) and thin myelin sheaths, while the last ones present thicker myelin sheaths and are covered by portions of Schwann cell cytoplasm (arrow). Collagen fibers (c) in the extracellular space and a basal lamina (arrowhead) on the surface of the Schwann cell can be seen. Lesion of 31 dias - Group III.E. Electron micrograph - 11.200x.

vealed lesions similar to those in group II and also presenting naked axons, macrophages and few myelin debris in the extracellular space of the central area. At the periphery, some dark cells of difficult identification by light microscopy could be noticed, as well as pial infiltration, mostly perivascular, demyelinated fibers and thinly remyelinated axons.

Ultrastructural examination showed as the most prominent aspect of the lesion (in relation to animals from group II) the high density of round cells identified as oligodendrocytes, close to small remyelinated axons and/or astrocytic processes and filled with long cisternae of endoplasmic reticulum and evident Golgi apparatus.

Other common peripheral features included areas of oligodendrocyte-remyelinated axons, the presence of hypertrofic astrocytic prolongations and of perivascular Schwann cells, associated with one or more naked axons and even forming redundant myelin loops (Fig 2). In addition, lymphocytes were seen around blood vessels and in the neuropil contacting macrophages, myelin debris and agglomerates of pial cells (Fig 3). Demyelinated axons, associated or not with astrocytic prolongations, were also found.

By 21-31 days post-injection, there was a massive predominance of oligodendroglial remyelination at peripheral sites in rats that received CsA. Oligodendrocytes continued to show a prominent endoplasmic reticulum and they appeared in groups or nests or were separated by astrocytic processes and axons with slim myelin sheaths (Fig 4).

Thin and thick astrocyte prolongations were associated to remyelinated fibers, oligodendrocytes and blood vessels. However, some blood vessels remained unrelated to astrocytic prolongations. In addition lymphocytes

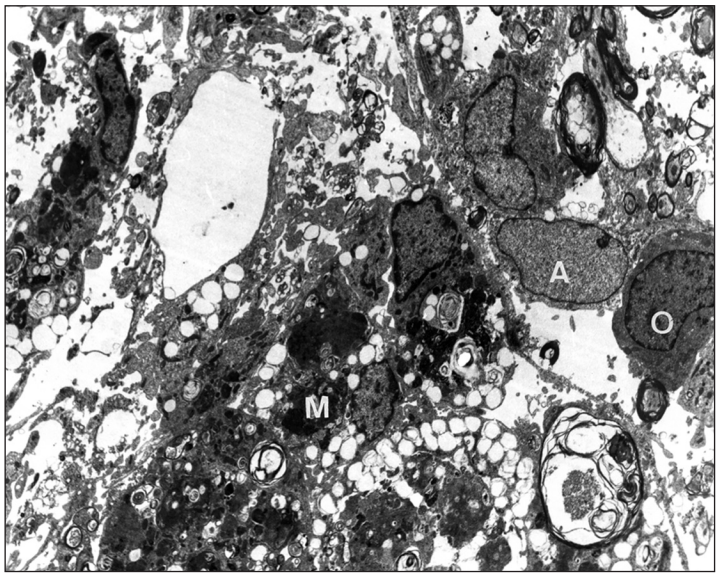

Fig 6. General aspect of the center of the lesion after injection of saline solution. Observe the presence of macrophages (M) containing phagocytosed myelin, as well as the persistence of glial cells (A - astrocyte;O - oligodendrocyte). Lesion of 15 days - Group III.C. Electron micrograph - 3.350x.

were observed in fewer numbers than those found at 15-17 days and they appeared frequently contacting astrocytes and lamellae of vesiculated myelin.

Many axons persisted demyelinated and some of them were already related to Schwann cells individually or in groups. Thicker myelin sheaths than those produced by oligodendrocytes were deposited by Schwann cells (Fig 5), mainly in perivascular areas, filled with collagen fibers and sometimes close to the nests of pial cells.

Observations on group III.C
(saline solution injection and CsA treatment)

From the 4 rats injected with saline solution, just 2 (those perfused at 15 and 21 days) presented a focal lesion due to injection procedure. This lesion was circumscribed to the pons and showed a light expansion of the extracellular space, containing some loose myelin lamellae and clearing of cellular debris by phagocytic cells, but with no evidence of either primary demyelination or loss of the neuroglia (Fig 6).

No mortality was recorded in the groups that received CsA treatment using the drug administration scheme previously mentioned.

DISCUSSION

In general terms, the results obtained in the present investigation confimed and complemented those described in former studies with the EB model in the brainstem[5-11].

The most important feature observed in rats injected with EB and treated with CsA was the higher density of oligodendrocytes on the edges of the lesions, in relation to the immunocompetent animals from group II and those treated with cyclophosphamide[5] or dexamethasone[6] from previous studies.

CsA has as its major known effect the capacity of reducing the synthesis and liberation of interleukin-2 (IL-2) from T CD_4^+ lymphocytes (Th - *helper*)[12], with consequent suppression in the generation of T cytotoxic lymphocytes (CD_8^+), although relatively sparing the suppressor ones and even the cytotoxic lymphocytes already formed[13]. Other components of the immune system, such as monocytes, macrophages, NK (*natural killer*) cells and B lymphocytes, can be equally affected, but by an indirect manner, due to the reduction of IL-2 and other cytokines, like GM-CSF, IL-3, IL-4 and IFN-γ, among others[14].

IL-2 may be present in the CNS in pathological states following disruption of the blood-brain barrier (BBB) and infiltration of activated T cells in this site, more precisely of those producing the referred cytokine such as Th1 cells and T cytotoxic cells[15,16].

Injection of IL-2 in the rat brainstem induces sleep, which seems to be mediated by receptors in the *locus coeruleus*. In addition IL-2 mRNA was localized in some areas of the murine encephalon, being found in neuronal bodies and astrocytes[17].

In the CNS there are no evidences that IL-2 may modulate the function or gene expression of any other glial cell excepting oligodendrocytes and their progenitors[15,16].

Human recombinant IL-2 appears to influence proliferation and differentiation of rat oligodendrocytes *in vitro*, increasing as far as in 3 times their numbers in cultures containing IL-2 and stimulating their maturation, as shown by the augmented expression of myelin basic protein (MBP) and of MBP mRNA[15,16]. Additionally two lineages of human glioblastoma with oligodendroglial phenotype proliferate in the presence of IL-2 and also presented receptors for this cytokine[15,16.]

However, IL-2 seems to show opposite effects on rat oligodendrocyte progenitor cells, as it is capable of inhibiting their proliferation[15,16,18]. So, IL-2 may have variable biological effects on oligodendrocytes depending on their stage of differentiation.

Detectable levels of IL-2 and clones of encephalitogenic T cells (with phenotype preferentially Th1) were found in serum and in the cerebrospinal fluid from patients with relapsing multiple sclerosis (MS), as well as in animals with experimental allergic encephalomyelitis (EAE)[16].

The fact that only activated T cells have the capacity to cross the BBB decreases the number of these patrolling cells in normal CNS, as just a minority of T circulating cells are in state of activation[19].

Lymphocytes are present in EB-induced lesions, many times contacting myelin debris in the extracellular space and activated macrophages containing phagocytosed myelin, in a relationship suggestive of antigenic recognition[3]. In the CsA-treated group, the number of lymphocytes did not differ from that found in non-immunosuppressed animals, which may indicate that the CsA dosage used in this investigation was not sufficient to cause adequate immunosuppression or maybe that lymphocytes present in these lesions belong to a CsA-unresponsive lymphocytic subpopulation. Besides, lymphocytes recruited to the CNS might escape from the immunosuppressive action of the drug, as, despite its high lipossolubility, Csa paradoxically seems to not pass through intact BBB in significant amounts[20].

The possibility of insuficient CsA plasmatic levels to cause immune depression in the CNS can be refuted by the fact that similar administration schemes have been widely used to avoid rejection of mice cerebral grafts in rat CNS[21] and of glial xenografts in myelin deficient rats[22].

Mechanical damage to the BBB is inevitable when microinjections are applied in the nervous tissue and, thus, it is believed that, in the present investigation, BBB disruption occurred as a result of injection trauma and of astrocytic disappearance induced by EB gliotoxic action. Astrocytes are recognized to play a key role in the induction and maintenance of BBB characteristics in the CNS[23].

Blood vessels devoided of nearby astrocytic prolongations were seen until the 31st day after EB injection, suggesting the lack of a completely developed BBB, as it is known that astrocyte processes must be in proximity to CNS endothelial cells for the expression of BBB tight junctions[23]. Although tight junctions could be easily visualized from the 15th to the 31st day post-injection, it is worthwhile mentioning that simple morphological observation of these junctions is not sufficiently conclusive to allow functional approaches on the permeability of CNS blood vessels. In addition, BBB breaches are reversible and may occur only in initial phases of CNS degenerative changes.

Areas of complete remyelination by Schwann cells following EB injection in the rat spinal cord, with consequent oligodendrocyte and astrocyte disappearance, have recovered BBB integrity even in the absence of astrocytes[24]. On the contrary, MS inactive chronic plaques have showed permanent changes in BBB selectivity[25].

Csa has showed to be capable of preventing the onset of EAE in several animal species when administered during the sensitization phase and of suppressing or reducing the incidence and severity of clinical signs in animals already sensitized, with usual recurrence of symptoms after the end of treatment[12]. In Theiler`s murine encephalomyelitis, the same drug, given during the infection period, was responsible for the suppression of inflammation and demyelination, although it did not decrease myelin loss if administered after the establishment of inflammatory reaction[26].

Even during inflammation, the total number of lymphocytes in the CNS is comparatively small in relation to other body sites. So the amount of cells capable of reacting to a given antigen is proportionally reduced and lymphocytic response is restricted to a relatively small number of clones (oligoclonal response)[27].

In the present study, there was no evidence that immunosuppression with CsA was prejudicial to macrophagic activity, because, on the contrary to that observed with the use of cyclophosphamide[5] or dexamethasone[6], it was seen much smaller amounts of myelin-derived membranes using CsA, in a pattern similar to group II.

Even though it was not seen a remarkable difference in the extension of oligodendrocyte remyelinated areas between animals from groups II and III.E, those treated with CsA presented a greater proportion of oligodendroglial cells on the edges of the lesions. These oligodendrocytes showed a cytoplasmic density that had resemblance with the appearance of cells referred by Mori and Leblond[28] as medium and dark oligodendrocytes. These authors attributed the notable morphological variation on oligodendroglia of young rats to different stages of cellular differentiation. The light ones would represent those of bigger size and greater mitotic activity; as maturation occurred they were progressively transforming themselves into smaller and darker cells with lesser proliferative capacity[28].

Nests of oligodendrocytes similar to those observed in our study were seen in attempts of remyelination in MS[29] and EAE[30]. Such distribution is not considered a normal aspect of the nervous tissue and may be related to the existence of oligodendroglial progenitor cells and to the action of trophic factors on them[30].

In the EB model, it was described a cell population with membrane immunoreactivity to ganglioside D_3 (GD_3) from 6 to 12 days after the gliotoxic injection. These cells were considered as probable oligodendroglial progenitor cells that came out in response to the demyelinating process[9].

A possible explanation for the high density of oligodendrocytes in rats submitted to CsA treatment could be the inhibition of IL-2 secretion induced by the drug. This requires that lymphocytes in the EB-induced lesions had the capacity of secreting the cytokine (such as the Th1 subpopulation) and were activated by antigenic stimulation. Since the referred cytokine inhibits *in vitro* proliferation of oligodendroglial progenitor cells of rats[15,16,18], it is possible that depletion of IL-2 in the lesion site facilitates division and migration of such cells. Paradoxically, mature oligodendrocytes are capable of accelerating their capacity of myelination or remyelination in the presence of IL-2[15,16]. This would be beneficial in demyelinating diseases, although it is not observed in immune-mediated conditions characterized by abundant lymphocytic infiltrates, specially of Th1 cells, as it is seen in MS and EAE[16]. It is important to note that studies connecting IL-2 and oligodendroglial cells are commonly performed *in vitro* and the environment *in vivo* is much more complex and unpredictable due to the intricate interactions between the different cell types involved and their secreted factors.

CsA could affect directly or indirectly the final balance between proliferative and antiproliferative factors related to oligodendroglial populations in the lesion microenvironment, maybe facilitating the first ones and by doing this resulting in greater numbers of oligodendrocytes.

Oligodendrocyte proliferation has been described in adult animals[28], as well as from bipotential progenitor cells (O-2A^{adult} cells). The latter have the capacity of presenting division, differentiation and migration under appropriate stimulus therefore creating the necessary oligodendrocytes for myelin repair following a demyelinating event[8].

On the other hand Schwann cells kept a similar behaviour and distribution on groups II and III.E. However, comparing to group II, it was more common the appearance of redundant myelin loops with the CsA treatment (already seen at 15 days), suggesting that somehow CsA could have some positive effect on the remyelinating activity of Schwann cells.

Although the use of CsA in this study changed the dynamics of the nervous tissue repair following EB injection, it is not possible to assume that these alterations were directly due to the suppression of lymphocytic activity.

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