

CYTOKINES AND INTRATHECAL IgG SYNTHESIS IN MULTIPLE SCLEROSIS PATIENTS DURING CLINICAL REMISSION

*Carlos Otávio Brandão¹, Heloísa Helena Ruocco²,
Alessandro dos Santos Farias¹, Celina Oliveira¹,
Dannie Eiko Maeda Hallal-Longo¹, Sandra Regina Mirandola¹,
Elaine Conceição Oliveira¹, Fernando Cendes²,
Benito Pereira Damasceno², Leonilda Maria Barbosa Santos¹*

ABSTRACT - Cytokines and intrathecal IgG synthesis were determined in the cerebrospinal fluid (CSF) and sera to evaluate inflammatory activity in multiple sclerosis (MS) patients during clinical remission. Although the disease was stable, there had been a significant increase of proinflammatory cytokines such as TNF α and IFN γ in the CSF and serum, with no significant changes of IL12 and IL10 production. The changes in the cytokine production patterns were associated with an increase of leukocytes in the CSF, as well as the presence of oligoclonal bands suggesting intrathecal IgG synthesis. These results suggest that even when the disease is clinically silent, one can observe inflammatory activity in these MS patients.

KEY WORDS: multiple sclerosis; cerebrospinal fluid; cytokines; intrathecal IgG synthesis.

Citocinas e síntese intratecal de IgG em pacientes com esclerose múltipla durante remissão clínica

RESUMO - Os níveis de citocinas e síntese intratecal de IgG foram dosados no líquido cefalorraquidiano (LCR) e soro, com o objetivo de avaliar a atividade inflamatória em pacientes com esclerose múltipla durante remissão clínica. Foram detectados níveis elevados de citocinas pró-inflamatórias (TNF α e IFN γ) no LCR e soro, sem alterações significativas na produção de IL12 e IL10. O perfil de produção das citocinas pró-inflamatórias estava associado ao aumento de leucócitos no LCR, assim como a presença de bandas oligoclonais IgG sugerindo síntese intratecal de IgG. Estes resultados sugerem que mesmo quando a doença está clinicamente silenciosa, a atividade inflamatória está presente nestes pacientes.

PALAVRAS-CHAVE: esclerose múltipla, líquido cefalorraquidiano, citocinas, IgG síntese intratecal.

Multiple sclerosis (MS) is a disorder of the central nervous system (CNS) characterized by perivascular inflammation and demyelination in the white matter. The etiology and pathogenesis of MS is unknown, although it is a complex phenomenon involving both genetic and environmental aspects. These forces interact to produce individual susceptibility to the disease and influence its course¹. Although MS is a disease of the CNS, the peripheral blood and cerebrospinal fluid (CSF) of patients contain activated autoreactive T cells recognizing myelin components such as myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein and myelin oligodendrocyte glycoprotein

(MOG)². Activated T lymphocytes also produce cytokines that modulate the immune response either positively or negatively. Some of these cytokines have proinflammatory effects enhancing inflammatory reactions; whereas others have anti-inflammatory properties. The cytokines produced by Th1 cells, such as tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ), may promote the progress of the disease^{3,4}, while those produced by the Th2/Th3 subsets, such as interleukin 10 (IL10) and transforming growth factor beta (TGF β), may limit it⁵. Some authors have also reported the important contribution of antibodies in the initiation of myelin sheath damage in MS⁶.

¹Department of Microbiology and Immunology; ²Department of Neurology, Medical School, University of Campinas, Campinas SP, Brazil (UNICAMP). Financial support: FAPESP (grant # 00/07703-7, 01/12827-0); CNPq (grant # 300375/84-87) and FAEP-UNICAMP.

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Dra. Leonilda M.B. Santos - Departamento de Microbiologia e Imunologia - Instituto de Biologia / UNICAMP - 13083-970 Campinas SP - Brazil. E-mail: leonilda@unicamp.br

For diagnosis, magnetic resonance imaging (MRI) has shown great potential as an indicator of disease activity in patients with MS⁷, with the use of CSF analysis receiving less attention. However, the CSF analysis is still a valuable tool and in association with imaging data provide reliable information about the inflammatory status of a patient.

In the present study, we assessed serum and CSF levels of cytokines, leukocytes count and intrathecal IgG synthesis to evaluate inflammatory activity in a subgroup of untreated MS Brazilian patients during clinical remission.

METHOD

Patients – Patients were recruited at the hospital of the University of Campinas, located in Campinas, SP, Brazil, during the period of 2002-2004. A total of 58 individuals (42 women and 16 men) were included in the study. At that time, the individuals were divided into three groups: I. 23 clinically definite MS patients (6 men, 17 women, the mean age was 30) with relapsing-remitting form of disease (RRMS) according to Poser criteria⁸; II. 16 patients with other neurologic diseases (3 men, 13 women, the mean age was 33): 4 with motor neuron disease, 2 with hereditary sensory-motor neuropathy, 2 with Parkinson's disease, 4 with epilepsy, 2 Alzheimer's disease, 2 with cerebrovascular disease; III. 19 individuals (7 men, 12 women, the mean age was 31 years) with backache or headache and who had no evidence of organic neurological disease, studied as a healthy control group. The patients with clinically definite MS who were seen in our MS clinic were studied. MRI and laboratory investigations were performed during the initial contact for diagnosis purposes and the determination that the patients were in remission, without treatment. The delay between first attack and first consultation was 4.2 ± 3.8 years. The Expanded Disability Status Scale (EDSS) score was determined at lumbar puncture. None of the patients had received corticosteroids or other immunosuppressive drugs during a period of at least 6 months prior to donating CSF and blood for the study. The study was approved by the Medical Ethics Committee of the hospital, and informed consent was obtained.

Samples – All specimens were obtained for the routine determination of CSF leukocyte count, IgG synthesis and oligoclonal IgG bands. Paired samples of serum and CSF were stored at -80°C until these exams were performed. The CSF (10 mL) was taken and cell counting done quickly before sampling.

Laboratory procedures – IgG and albumin in the CSF and serum were measured by nephelometry (BNII; Dade Behring, Marburg, Germany), and Link Indexes (LI) were calculated according to the literature⁹. Oligoclonal IgG bands were identified by isoelectrofocusing¹⁰.

Antigen, antibody and recombinant cytokines – The IL12, IFN γ , TNF α , and IL10 were quantified using commercial kits from Biosource International, Nivelles, Belgium. All measure was made at the single occasion in order to minimize the intra assay variability. Briefly, 96-well microtiter plates were coated with 1-2 $\mu\text{g/mL}$ of capture monoclonal antibody for each cytokine in 0.1M NaHCO₃ (pH= 8.5) and allowed to incubate overnight at 4°C. Following blocking with 3% dry milk in PBS at room temperature for 2 h, samples and standard recombinant IFN γ , TNF α , IL10 and IL12 were added again and incubated overnight at 4°C. Then, 0.5-2.0 $\mu\text{g/mL}$ of biotinylated detection monoclonal antibody for human IL12, IFN γ , TNF α and IL10 were added, followed by the addition of avidin-peroxidase 1/400 (Sigma Chem.- USA) and the peroxidase substrate. A stop solution was used to obtain OD determined at 492 nm.

Imaging – MRIs were performed, using a 2.0 Tesla system (Elsint, Prestige[®]). All exams covered the whole brain using 6.0 mm slice thick and 1.2 mm inter-slice gap. MRI acquisition parameters for images used for analysis were: T1 sagittal images and T2 axial.

Statistics – The statistical significance of the results was determined using a non-parametric analysis of variance, and a Kruskal-Wallis test and Spearman Rank test. A *p* value smaller than 0.05 was considered to be significant.

RESULTS

Twenty three patients with MS were evaluated (Table 1). Brain MRI showed abnormalities with demyelinating lesions in 23 (100 %) and presence of gadolinium-enhancing lesions in 5 (21 %) of the 23 MS patients. These patients showed CSF hypercytosis and intrathecal IgG synthesis. Twenty-one (91.3 %) MS patients were oligoclonal bands (OCB) positive.

CSF data and patients characteristics of the groups are shown in Table 2. The percentage of patients with hypercytosis was increased in the MS group when compared with the group II (other neurologic diseases; *p*= 0.004) and group III (healthy controls; *p*= 0.0001).

Production of TNF α in CSF and serum of patients with MS, other neurological disorders, as well as healthy controls – The means for MS patients were 137.3 ± 119.7 and 116.0 ± 115 pg/ml in serum and CSF, respectively; whereas for the OND group these were 7.6 ± 24.1 and 8.6 ± 28.4 pg/ml for serum and CSF, respectively; for the healthy controls, these values were 15.3 ± 19.8 and 4.3 ± 8.2 pg/ml. A significant increase (*p*<0.001) in TNF α was observed in the CSF, and serum of patients with MS.

Table 1. Clinical and laboratory data of relapsing-remitting multiple sclerosis group.

Patient	Age, y	Sex	DD	N.rel.	EDSS1	Cells	Alb- I	IgG- I	OB IgG
1	44	M	1	2	1	8	2.6	0.65	+
2	32	M	3	4	2.5	15	5.23	0.51	-
3	32	F	3	3	1.5	20	3.64	3.35	+
4	37	M	3	5	2	2	2.82	1	+
5	30	F	4	4	3	3	2.25	0.82	+
6	22	F	4	5	2	16	2.4	0.98	+
7	10	F	8	2	2	14	5.84	0.84	+
8	25	F	7	4	1.5	13	2.7	1.49	+
9	43	M	9	6	2	1	7.97	1.12	+
10	24	F	2	4	2	15	3.25	0.76	+
11	46	F	9	3	3	13	6.4	1.73	+
12	43	F	12	6	2.5	3	4	0.88	+
13	33	F	5	4	1.5	1	5.13	0.67	+
14	35	F	13	5	2	3	1.97	1.52	+
15	29	F	11	3	1	16	5.9	0.81	+
16	43	M	8	2	2	1	1.66	0.59	-
17	25	F	5	4	2	5	3.44	0.83	+
18	13	M	3	9	2	24	4	0.74	+
19	21	M	7	3	1	5	6.17	0.6	+
20	37	F	1	2	1.5	18	5.1	2.37	+
21	21	F	7	6	1.5	5	5.35	1.66	+
22	39	F	6	2	1	4	5.37	1.03	+
23	40	F	4	3	2.5	5	4	0.8	+

DD, duration of disease; EDSS1, Expanded Disability Status Scale at the time of the study; Alb-I, albumin index; IgG-I, IgG index; OB IgG, oligoclonal IgG bands; N.rel., number of relapses; y, years.

Table 2. Patients characteristics, number of CSF leukocytes, intrathecal IgG synthesis index and oligoclonal bands.

Patients	Age, y (mean± SD)	Te, y	Sex, M/F	Cells	IgG-index	OB IgG+
MS (23)	30± 9	5 ± 3	6/ 17	9.13 ± 7.05	1.12 ± 0.66	21/ 23
ODN (16)	31± 10	6 ± 3	3/ 13	4.19 ± 5.55	0.53 ± 0.13	1/ 16
HC (19)	37 ± 16	-	6/ 13	1.47 ± 0.77	0.45 ± 0.12	0/ 19

MS, multiple sclerosis; ODN, other neurologic diseases; HC, healthy controls; OB, oligoclonal bands; Te, time of evolution; y, years; SD, standard deviation.

TNF α levels in the CSF exceeded those in the serum for 10/23 patients, suggesting an intrathecal synthesis (Fig 1A). There was a positive correlation between the number of leukocytes in the CSF and the level of TNF α ($R^2 = 0.6874$, $p = 0.001$).

Production of IL12p40 in CSF and serum – The means for MS patients were 49.1 ± 18.3 and 39.4 ± 13.2 pg/mL for serum and CSF, respectively; whereas for the ODN group they were 76.2 ± 32.2 and 58.1 ± 35.1 pg/mL; for the healthy controls these means were 57.4 ± 18.8 and 45.4 ± 15.3 pg/mL. No significant difference ($p > 0.05$) were observed between the three groups (Fig 1 B).

Production of IFN γ in CSF and serum patients with MS and, other neurological disorders, as well as healthy controls – The means for MS patients were 500.9 ± 169.5 and 359.0 ± 85.1 pg/mL in serum and CSF respectively; whereas for the ODN group, these were 418.6 ± 67.0 and 325.2 ± 70.5 pg/mL; for the healthy controls these mean were 102.6 ± 102.7 and 54.1 ± 39.6 pg/mL. A significant increase ($p < 0.001$) in the IFN γ was observed in both CSF and serum of patients with both MS and ODN (Fig 1C).

Production of IL-10 in CSF and serum – The means for MS patients were 304.6 ± 112.0 and $287.6 \pm$

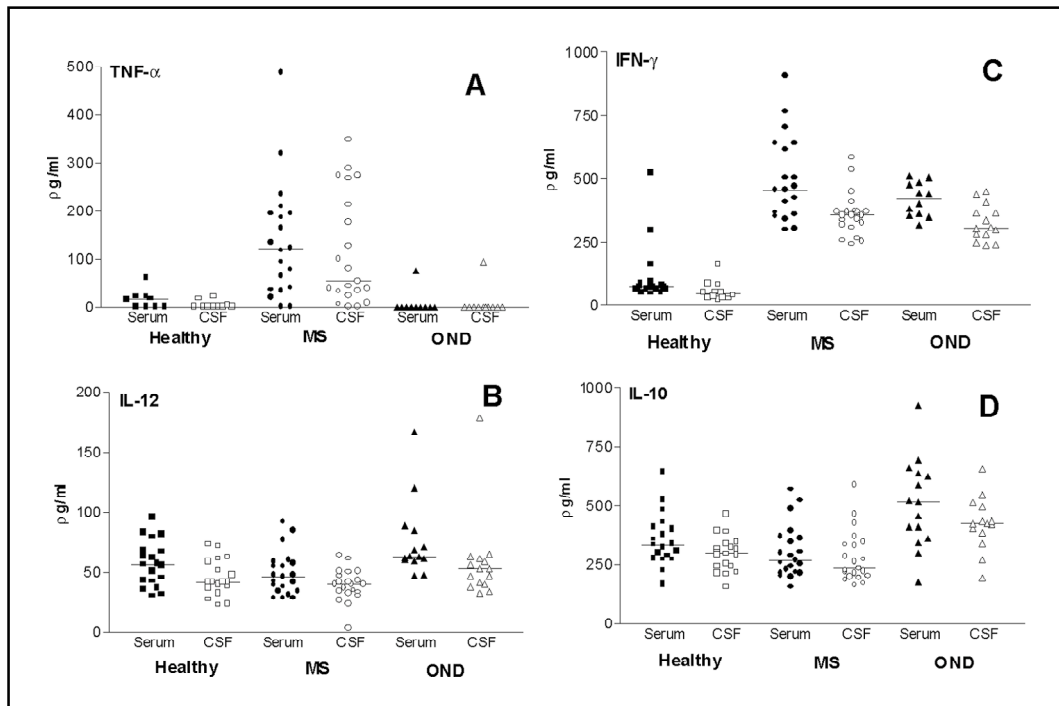


Fig 1. Production of cytokines ($TNF\alpha$, IL 12, $IFN\gamma$ and IL 10) in the CSF and sera from MS patients, OND (other neurologic diseases) and healthy controls, detected by ELISA.

113.7 pg/mL for serum and CSF, respectively; whereas for the OND group they were 507.6 ± 187.1 and 424.3 ± 113.7 pg/mL for serum and CSF, respectively; for the healthy controls, these means were 354.6 ± 111.4 and 296.2 ± 74.8 pg/mL. No significant differences were found between the patients with MS and healthy controls ($p > 0.05$), whereas the OND group of patients revealed significantly higher levels of IL 10 ($p = 0.006$) (Fig 1D).

DISCUSSION

The purpose of this investigation was to evaluate inflammatory activity in MS patients during clinical remission. Our results showed that patients with MS which is identified stable phase of the disease reveal increase in the secretion of pro-inflammatory cytokines in both serum and CSF associated with the increase in the intrathecal synthesis of IgG and the number of leukocytes in the CSF. This study demonstrated that patients with stable RRMS have an elevated number of leukocytes in the CSF when compared with patients with other non-inflammatory diseases of CNS and healthy controls. Other studies suggested that the presence of white blood cells in the CSF is a good predictor of the activity of MS, since after two years, patients with a high number of white blood cells in the CSF had more relapses than those in control group¹¹.

Parallel to the increase of the number of cells there is also the presence of oligoclonal bands in 91% of studied MS patients. These results are in agreement with previous Brazilian reports^{12,13}. Although the antibody specificity in the oligoclonal bands is still enigmatic, as suggested by reports in the literature, a lack of intrathecal synthesis of oligoclonal IgG bands is related to short lasting and benign course of MS¹⁴.

The B-cell proliferation, differentiation and antibody production is coordinated by the helper T cells and the cytokines they produce. Although Th1 and Th2 cells are the major sources of their respective cytokines, many others cells within and outside the immune system also produce these cytokines, contributing to an overall Th1 and Th2 cytokine pattern. It was thus decided to quantify both pro and anti-inflammatory cytokines in the CSF and the serum, independent of their source.

The cytokines produced in the early phase of inflammatory response, such as IL12, contribute to the development of Th1 immune response. Conflicting results regarding IL12 production have, however, been observed in MS. An increase in the production of this cytokine has been observed in progressive MS¹⁵ and increased frequencies of IL-12 secreting monocytes appear to correlate with

the presence of active brain lesions detected by MRI¹⁶. Serum levels of IL12, however, have been found to be similar in MS patients and controls¹⁷. In the present study, as well, no change in the production of this cytokine was observed in the serum or CSF of MS patients. It seems that, blood monocytes must be stimulated in order to produce detectable amounts of IL12, but since we did not use activated cells, the production of IL12 may have been too low to be detected in the ELISA assay.

The IFN γ of MS patients, on the other hand, showed significant increase in the CSF and serum over that of healthy controls, although no difference was observed in relation to the OND group. Initially, interferons were tested as therapeutic agents for MS because of their antiviral properties and it was felt that MS might be due to persistent viral infection. However, a pathogenic role for MS patients who received recombinant IFN γ treatment in MS has been reported¹⁸. Our data support previous ones which demonstrated that IFN γ has potent proinflammatory response, including the ability to induce the production of other proinflammatory cytokines¹⁹. In the animal model, the production of isotypes of IgG, such as IgG2a is induced by the IFN γ . In our findings, no information is given about the subclass of IgG, which is increased in the CSF of MS patients, but it is possible that the increase in intrathecal IgG production is due to the increase in intrathecal synthesis of IFN γ .

In addition to the production of IFN γ , there was a parallel increase of TNF α in the serum and CSF. Approximately 40 % of the MS patients presented levels of TNF α in the CSF equal to or greater than that in the serum, suggesting the intrathecal synthesis of this cytokine. TNF α has been described as a major cytokine in this demyelinating disease, since it has been demonstrated to be myelinotoxic²⁰⁻²³. TNF α is also a major inducer of endothelial adhesion molecules and chemokines, hence, the upregulation TNF α may have a major effect on the recruitment of leukocytes to the CNS²⁴. In this study it was possible to show a positive correlation between the number of leukocytes in the CSF and the level of TNF α . This property may explain, at least in part, the increased number of inflammation-perpetuating leukocytes observed in the CSF in MS patients. Elevation in CSF concentrations of soluble ICAM-1 and soluble TNF α receptor were demonstrated previously in Brazilian MS patients with acute relapsing form of MS during exacerbation²⁵.

As the inflammatory response develops, the cytokine products of Th1 or Th2 lymphocytes provide mutually inhibitory functions for the differentiation and effector effect of the reciprocal phenotype. IFN γ prevent Th2 cell proliferation, whereas IL10 profoundly inhibits the synthesis of Th1 cytokines²⁶. Reports on the IL-10 production showed that patients with high IL-10 production had significantly lower disability scores and lower T2 lesion load²⁷. In the present study, we did not observe significant changes in IL10 levels in MS patients. These data agree with that of studies demonstrating a reduction in IL10 levels in the serum, as well as in the number of IL10-secreting cells in MS patients^{19,28-30}, reinforcing the fact that the proinflammatory response prevails in this group of patients, despite the absence of clinical manifestation.

This study provides evidence of a significant increase in inflammatory activity in patients with stable MS over that in the control groups. These observations suggest that an investigation of the inflammatory parameters in the CSF may provide a valuable tool, which would be useful in the indication of activity of the disease, thus helping understand the damages caused by the inflammatory response.

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