



Tumor necrosis factor- α and interleukin-4 in cerebrospinal fluid and plasma in different clinical forms of multiple sclerosis

Vrednosti faktora nekroze tumora alfa i interleukina 4 u likvoru i plazmi bolesnika sa različitim formama multiple skleroze

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Abstract

Background/Aim. Multiple sclerosis (MS) is an immune-mediated central nervous system disease characterized by inflammation, demyelination and axonal degeneration. Cytokines are proven mediators of immunological process in MS. The aim of this study was to investigate whether there is a difference in the production of the tumor necrosis factor alpha (TNF- α) and interleukin-4 (IL-4) in cerebrospinal fluid (CSF) and plasma in the MS patients and the controls (other neurological non-inflammatory diseases) and to determine a possible difference in these cytokines in plasma and CSF in different clinical forms of MS. **Methods.** This study involved 60 consecutive MS patients – 48 patients with relapsing-remitting MS (RRMS) and 12 patients with secondary progressive MS (SPMS). The control group consisted of 20, age and sex matched, non-immunological, neurological patients. According to the clinical presentation of MS at the time of this investigation, 34 (56.7%) patients had relapse (RRMS), 14 (23.3%) were in remission (RRMS), while the rest of the patients, 12 (20.0%), were SPMS. TNF- α and IL-4 concentrations were measured in the same time in CSF and plasma in the MS patients and the controls. Extended disability status score (EDSS), albumin ratio and IgG index were determined in all MS patients. **Results.** The MS patients had significantly higher CSF and plasma levels of TNF- α than

the controls ($p < 0.001$ for both samples). IL-4 CSF levels were significantly lower in the MS patients than in the controls ($p < 0.001$), however plasma levels were similar. The patients in relapse (RRMS) and with progressive disease (SPMS) had higher concentrations of CSF TNF- α levels than the patients in remission ($p < 0.001$). IL-4 CSF levels in relapse (RRMS) and SPMS groups were lower than in the patients in remission. The patients in remission had an unmeasurable plasma TNF- α level and the patients with SPMS had significantly lower IL-4 levels in plasma than the patients in relapse and remission ($p < 0.001$). The only significant correlation between cytokine level with either EDSS, or albumin ratio, or IgG index, was found between CSF TNF- α levels and albumin ratio in the patients with relapse (R square = 0.431, $p < 0.001$). **Conclusion.** According to the obtained data MS relapse was characterized by high concentrations of TNF- α in CSF and plasma and low concentrations of IL-4 in CSF. Remission was characterized by high concentrations of IL-4 and low concentrations of TNF- α both in CSF and plasma. SPMS was characterized with lower concentrations of TNF- α and IL-4 compared to relapse, both in CSF and plasma.

Key words:

multiple sclerosis; tumor necrosis factor- α ; interleukin-4; plasma; cerebrospinal fluid; disease progression; treatment outcome.

Apstrakt

Uvod/Cilj. Multipla skleroza (MS) je imunološki posredovana bolest centralnog nervnog sistema koju karakterišu inflamacija, demijelinizacija, degeneracija aksona i glijoza. Citokini su važni medijatori imunoloških procesa kod MS. Cilj ove studije bio je da se ispita postojanje razlike u produkciji inflamacijskog citokina faktora nekroze tumora alfa (TNF- α) i antiinflamacijskog citokina interleukina 4 (IL-4) u likvoru i plazmi bolesnika sa različitim kliničkim faktorima MS i kod bolesnika sa drugim

neurološkim neinflamacijskim oboljenjima (kontrolna grupa). **Metode.** U studiju je bilo uključeno 60 bolesnika sa MS, 48 sa relapsno-remitentnom MS (RRMS) i 12 bolesnika sa sekundarno progresivnom MS (SPMS). Kontrolnu grupu je sačinjavalo 20 bolesnika sa neurološkim, neimunološkim bolestima. U vreme ispitivanja, 34 (56,7%) bolesnika bilo je u fazi pogoršanja, 14 (23,3%) u fazi remisije, dok je 12 (20%) bolesnika imalo SPMS. Citokini TNF- α i IL-4 određivani su istovremeno u plazmi i likvoru bolesnika sa MS i kontrolne grupe. Nivo neurološkog poremećaja, izmeren korišćenjem *The Expanded*

Disability Status Scale (EDSS), albuminski koeficijent i IgG indeks određivani su kod svih bolesnika sa MS. **Rezultati.** Bolesnici sa MS imali su značajno više koncentracije TNF-alfa i u likvoru i u plazmi, u poređenju sa bolesnicima kontrolne grupe ($p < 0,01$). Koncentracija IL-4 u likvoru bila je značajno niža kod bolesnika sa MS, nego kod bolesnika kontrolne grupe ($p < 0,01$), dok su koncentracije plazmatskih vrednosti bile slične. Bolesnici u fazi pogoršanja (RRMS) i sa SPMS imali su više koncentracije TNF-alfa u likvoru, nego bolesnici u fazi remisije bolesti (RRMS), ($p < 0,01$). Koncentracija IL-4 u likvoru bila je niža kod bolesnika sa RRMS u fazi pogoršanja i SPMS, nego kod bolesnika koji su bili u remisiji bolesti ($p < 0,01$). Kod bolesnika u remisiji nije nađen TNF-alfa u plazmi, dok su bolesnici sa SPMS imali značajno niže koncentracije IL-4 u plazmi u poređenju sa bolesnicima u fazi pogoršanja i u remisiji ($p < 0,01$).

Introduction

Multiple sclerosis (MS) is a central nervous system (CNS) disease characterized by inflammation, demyelination and axonal degeneration. The etiology of MS is still unknown. The results of numerous experimental and clinical studies support the thesis of MS being immunologically mediated disease¹⁻⁴. According to currently accepted theory, autoreactive Th1 cells (CD4+) directed at myelin or oligodendrocyte antigens, initiate aberrant immune response in peripheral blood, away from immunologically protected and privileged CNS. Through interaction with adhesive molecules Th1 cells cross the damaged blood-brain barrier, inside the CNS they have to be reactivated and in further interaction between Th1 cells, macrophages, residential CNS cells and B cells, numerous inflammatory, anti-inflammatory cytokines, auto-antibodies, oxidative species and enzymes are produced¹. Recently, the presence of Th17 cells has been acknowledged, characterized by interleukin (IL)-17A production, another potent proinflammatory cytokine, which causes upregulation of several inflammatory cytokines such as the tumor necrosis factor-alpha (TNF-alpha), IL-1beta, IL-6, IL-8³. They have a potential to damage myelin, directly or indirectly with consequent axonal damage. So, cytokines are important mediators of immune response in MS⁵, and possible shifting toward Th1 and Th17, with the down regulation of Th2 cytokine response, might be one of the important causes of ongoing inflammatory damage in MS. However, an interplay between immune mediated inflammation and neurodegeneration seems to play crucial role in MS development and progression⁶.

Clinically, MS is characterized by the phases of remissions and relapses in the majority of patients, relapsing-remitting MS (RRMS). In so called the time course, most of these patients develop secondary progressive form of MS – secondary progressive MS (SPMS), while the smallest proportion of patients is characterized by the progressive course from the onset – primary progressive MS (PPMS). Some studies^{7,8} suggest that different immunopathological mechanisms might be involved in the development of different MS types. That might explain different course and prognosis of the disease, necessity of different therapeutic approach in

Jedina statistički značajna korelacija nađena je između koncentracije TNF-alfa u likvoru i albuminskog koeficijenta i to kod bolesnika u fazi pogoršanja bolesti ($R_{square} = 0,431$, $p < 0,01$). **Zaključak.** Prema rezultatima našeg istraživanja, pogoršanje MS karakterišu visoke koncentracije TNF-alfa u likvoru i plazmi i niske koncentracije IL-4 u likvoru. Remisiju bolesti karakterišu visoke koncentracije IL-4 i niske koncentracije TNF-alfa i u plazmi i u likvoru. Progresivnu formu (SPMS) karakterišu niže koncentracije TNF-alfa i IL-4 u poređenju sa relapsom, i to i u plazmi i u likvoru.

Ključne reči:

multipla skleroza; faktor nekroze tumora; interleukin-4; plazma; cerebrospinalna tečnost; bolest, progresija; lečenje, ishod.

different MS types and unresponsiveness of SPMS and PPMS to current immunomodulatory treatment⁹.

There are few studies that compare concentrations of cytokines both in cerebrospinal fluid (CSF) and plasma in MS patients^{7,10} and to the best of our knowledge none that compares concentrations of cytokines in both compartments, in clinically different stages of MS. TNF-alpha is one of the most potent inflammatory cytokines with the confirmed role in direct myelin damage¹¹. It enhances expression of adhesion molecules on endothelial cells and lymphocytes¹², induces secretion of interferon gamma and other inflammatory cytokines and chemokines¹³, and indirectly through activation of macrophages and microglial cells, stimulates the production of reactive oxidative species, nitric oxide and lytic enzymes¹⁴. On the other hand, IL-4 is an anti-inflammatory Th2 cytokine with the proposed protective role in MS¹⁵⁻¹⁸.

Hence, the aim of our study was to investigate whether there is a difference in the production of TNF-alfa and IL-4 in CSF and plasma in MS patients and patients with other non-inflammatory neurological diseases, to determine a possible difference in the production of these cytokines both in plasma and CSF in different stages of MS – relapse, remission, progressive phase, and to evaluate a correlation between cytokine concentrations in CSF and plasma and clinical impairment, albumin ratio, intrathecal IgG synthesis.

Methods

Sixty consecutive patients from the Department of Neurology, Military Medical Academy, Belgrade were enrolled in the study after obtaining a permission of the Ethical Committee of the Military Medical Academy in Belgrade. All of them had clinically definite diagnosis of MS according to Poser criteria¹⁹ and had either exacerbation (relapse), or SPMS or clinically stable disease (remission). None of them were on immunosuppressive treatment at least 3 months prior the study. Clinical assessment was performed by using an expanded disability status scale (EDSS) score²⁰. The control group consisted of 20 age and gender matched patients with non-inflammatory neurological dis-

eases (epilepsy, spasmodic torticollis and hereditary neuropathy).

In all the patients, CSF and blood samples were obtained and after spinning stored at -70°C for cytokine analysis. Cytokine analysis was done by the ELISA method (Genzyme, Predicta), according to the written instructions. Apart from cytokine analysis, blood and CSF samples were used for evaluation of albumin ratio and IgG index. Albumin ratio was calculated according to the formula: CSF albumin/serum albumin, while IgG index was calculated by using the formula – IgG CSF / IgG serum : albumin CSF / albumin serum (normal values: < 5.7 and < 0.7 respectively)²¹.

Discrete variables are shown as counts and percentages. Continuous variables are presented as median with the interquartile range (iqr) (25-75 percentile). TNF-alpha and IL-4 CSF and plasma levels were compared between the MS patients and the control group by the Mann-Whitney test. Comparison of TNF-alpha and IL-4 CSF and plasma levels within the MS subgroups were done by using Kruskal-Wallis test. A correlation between TNF-alpha CSF levels and albumin ratio was presented as a linear regression curve. A *p*-value of less than 0.05 was accepted as statistically significant.

Results

The majority of the patients were classified as RRMS – 48 (80.0%) and 12 patients (20.0%) had secondary progressive disease (SPMS). According to the clinical presentation at the time of this investigation, 34 (56.7%) patients had relapse, 14 (23.3%) were in remission, while the rest of the patients were in secondary progressive phase of MS 12 (20.0%). In our study group female predominance was present (60.0%), average age was 43.5 ± 3.2 the disease duration was 5.6 ± 2. Average EDSS in our group was 3.8 ± 0.7.

Concentrations of TNF-alpha and IL-4 in CSF and plasma for MS patients and controls analyzed in this study are shown in Table 1.

TNF-alpha was detected in CSF of all MS patients and in none of the patients of the control group. In plasma, it was detected in 38 (63.3%) of MS patients, and in 7 patients (35.0%) of the control group, with significantly higher values in the MS group (*p* < 0.001). There was no significant difference in TNF-alpha concentrations in CSF and plasma in the MS group.

In relation to clinical presentation of MS, higher concentration of TNF-alpha in CSF was found in the relapse group than in SPMS (without a statistical significance between these two groups), and the lowest in the remission phase of the disease (*p* < 0.001). TNF-alpha was not detected in plasma of the patients in remission. However, TNF-alpha was detected in plasma in the majority of relapse patients, 30 (88.2%), and in all the patients with SPMS, without a significant difference between these two groups.

IL-4 was detected in plasma of 37 (61.7%) and in CSF of 54 (90%) of MS patients and in all the patients in the control group, in both CSF and plasma (Table 1). IL-4 was significantly lower in CSF of the MS group compared with the controls (*p* < 0.001), while there was no difference between its' plasma concentrations in the two groups (*p* = 0.154). CSF concentration of IL-4 was significantly lower as compared with plasma concentration in MS group (*p* < 0.05).

All the patients with progressive MS (SPMS), those in remission and 70% of the relapse patients had detectable CSF levels of IL-4. The highest CSF concentration of IL-4 was found in the remission group and it was significantly higher than in the relapse and progressive MS groups.

We did not find a correlation between either of the cytokine and EDSS.

On the other hand, we found a significant linear correlation between albumin ratio and TNF-alpha concentration in CSF of the relapse MS group (Figure 1). No correlation was found between IgG index and cytokine concentrations in the CSF and plasma of the MS patients.

Table 1
Concentrations of TNF-alpha and IL-4 in cerebrospinal fluid (CSF) and plasma of the multiple sclerosis (MS) patients and the control group

Body fluid	TNF-alpha				IL-4			
	MS group		Control group		MS group		Control group	
	Median (IQR) (pg/mL)	Detectability (%)	Median (IQR) (pg/mL)	Detectability (%)	Median (IQR) (pg/mL)	Detectability (%)	Median (IQR) (pg/mL)	Detectability (%)
CSF								
All patients	198.8 (88.9–330.5)	100.0	Not detected	0.0	60.8 (23.1–106.1)	90.0	650.0 (543.1–769.3)	100.0
Relapse	197.8 (81.6–246.9)	100.0	/	/	77.5 (56.0–90.1)	70.0	/	/
SPMS	165.2 (93.2–226.4)	100.0	/	/	64.6 (48.2–74.7)	100.0	/	/
Remission	9.8 (4.3–15.1)	100.0	/	/	253.0 (202.1–294.6)	100.0	/	/
<i>p</i>	ns ¹	< 0.001 ²			ns ⁵	< 0.001 ⁶		
Plasma								
All patients	263.3 (133.2–444.2)	63.3	47.7 (28.5–63.4)	35.0	200.3 (64.3–421.2)	61.0	370.6 (268.2–490.6)	100.0
Relapse	300.4 (210.0–360.2)	88.2	/	/	244.5 (211.6–288.2)	62.0	/	/
SPMS	231.1 (152.1–299.1)	100.0	/	/	26.0 (19.5–33.1)	50.0	/	/
Remission	Not detected	0.0	/	/	144.8 (122.7–164.7)	50.0	/	/
<i>p</i>	ns ³ , 0.001 ⁴				<i>p</i> < 0.001 ⁷			

¹ Non-significant difference in CSF TNF-alpha levels for the relapse and the SPMS group.
² Significant difference in CSF TNF-alpha levels for both the relapse and the SPMS group and the remission group.
³ Non-significant difference in plasma TNF-alpha levels for the relapse and the SPMS group.
⁴ Significant difference in plasma TNF-alpha levels for both the relapse and the SPMS group and the remission group.
⁵ Non-significant difference in CSF IL-4 levels for the relapse and the SPMS group.
⁶ Significant difference in CSF IL-4 levels for both the relapse and the SPMS group and remission group.
⁷ Significant difference in plasma IL-4 levels for all three groups.
 SPMS – secondary progressive multiple sclerosis; IQR – the interquartile range.

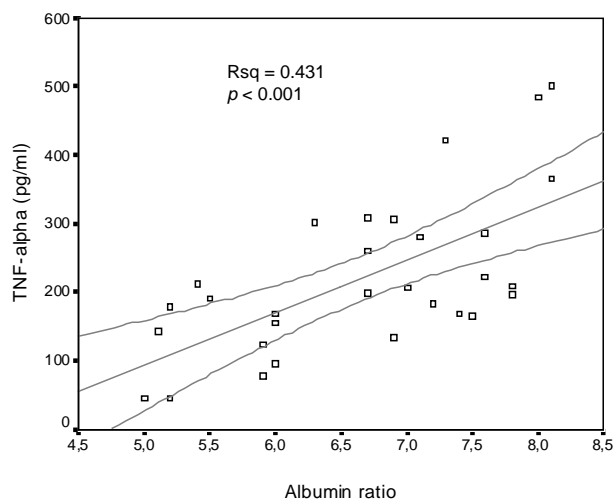


Fig. 1 – Correlation between cerebrospinal fluid TNF-alpha levels and albumin ratio in the relapse multiple sclerosis patients

Discussion

The role of TNF-alpha in MS pathogenesis is implicated by several studies – it has been identified in acute and chronic MS lesions²², TNF-alpha secretion from monocyte has been shown to be higher before²³ and during MS relapse²⁴ and, recently, it has been reported that increased TNF-alpha concentrations is related to fatigue in MS²⁵.

We detected TNF-alpha in CSF of all MS patients, but it was not detected in CSF of the patients with other neurological non-inflammatory diseases. Our findings are in concordance with the results of Tsukada et al.¹¹ and Baraczka et al.²⁶. Other authors⁸ detected TNF-alpha in a smaller percentage of MS patients, though the studies were performed in a significantly smaller patient group. In relation to clinical stage of MS, TNF-alpha concentrations differ among the groups. The highest concentration was found during the relapse phase, as were noted in other studies^{7,27}. Rather high values of TNF-alpha were detected in CSF of SPMS in our study, though lower compared to relapse values. Other authors reported high concentration of TNF-alpha in progressive MS, form as well^{27,28}. In relation to cytokine production from peripheral mononuclear blood cells derived from MS patients and stimulated *in vitro*, no difference in TNF-alpha production was reported in progressive and stable MS²⁹. However, there is no data related to TNF-alpha concentrations in progressive and relapse group of MS patients within the same study and using the same methodology. Increased TNF-alpha concentrations in the CSF potentiate inflammatory response, by augmenting macrophage functions, production of other inflammatory cytokines, lytic enzymes and reactive oxidative species (ROS). The importance of ROS in MS is confirmed in our previously published data^{30,31}. We reported increased index of lipid peroxidation both in CSF and plasma, increased concentrations of superoxid anion radical in plasma and increased activity of two anti-oxidative enzymes, superoxide dismutase and glutathione reductase³¹.

On the other hand, in remission TNF-alpha level was significantly lower, though still present in CSF. Shaw et al.³² did not find a significant difference in TNF-alpha concentrations in the stable MS patients and the control group both in CSF and sera, while Sharief et al.²⁷ did not detect TNF-alpha in CSF of the stable MS patients. Our findings support the thesis of continuous inflammatory response within the CNS during the MS course^{6,28,32}. One could speculate that among other inflammatory factors, high concentrations of TNF-alpha in CSF is needed for clinical presentation of MS relapse and/or progression of the disease.

A very recent report emphasizes the role of TNF-alpha in relapse by finding that suppressive effect of glucocorticoids on TNF-alpha production is associated with its clinical effect in MS³³. Similar pattern of TNF-alpha production was found in plasma of our MS patients group. High levels were found in the relapse and SPMS group, higher than in CSF though not significantly, while in the stable MS patients TNF-alpha was not detected in plasma. Detectability of TNF-alpha in plasma varies in different studies. It was detected in rather small percentage¹⁰, or in one third of MS patients⁷. Only Sharief et al.²⁷ found significantly higher concentrations of TNF-alpha in CSF compared to plasma. In our group of MS patients detectability of TNF-alpha in CSF was 100%, while in plasma it was detected in 63.3% of the MS patients and in the control group in only 35.0% of patients. Having in mind autocrine function of cytokine, it would not be expected to find such high levels of TNF-alpha in plasma. Although pathological process is within the CNS, immunologically privileged and protected compartment, systemic reaction is still present. High levels of TNF-alpha in plasma might be explained by systemic reaction to stress, MS relapse being powerful stressful event, especially since TNF-alpha was not detected in plasma of stable MS patients. In other words, during the MS course inflammatory reaction and immunological response are not limited within the CNS, but widespread. The results of recent studies^{34,35} are consistent with this hypothesis. Significant and sustained increase of serum TNF-alpha was found in healthy subjects a day after a psychologically stressful event³⁴. We did not find a correlation between CSF values of TNF-alpha and EDSS as others did²³. In our study, albumin ratio, a marker of the blood-brain barrier damage correlated with the CSF values of TNF-alpha in the patients with relapse. No relationship was found between the intrathecal IgG synthesis and TNF-alpha values in plasma and CSF in our group of patients.

However, it seems that immunoregulatory role of TNF-alpha is far more complex, since MS treatment with anti-TNF-alpha antibodies resulted in further demyelination and disease progression³⁶.

Some studies have shown that production of IL-4 and IL-10, both Th2 cytokines, is increased in stable¹⁷ and interferon beta treated MS patients³⁷ and reduced in clinically active phase of MS³⁸. That is in concordance with our results. In the patients with relapse and those with SPMS, IL-4 concentration in CSF was low and even undetectable in one third of the relapse patients. On the contrary, in CSF of stable patients IL-4 concentration was significantly higher and

detected in all. However, IL-4 showed a different secretion pattern in plasma. The highest concentration was detected in relapse patients, than in stable patients, and the lowest in SPMS. The secretion pattern of both cytokines, pro-inflammatory TNF-alpha and anti-inflammatory IL-4 appears to be the same as far as plasma concentrations are concerned. Relapse is characterized by high concentrations of TNF-alpha and other inflammatory cytokines in plasma, while high concentrations of IL-4 might be a consequence, an effort to dampen systemic inflammatory response. According to our results, systemic cytokine response and cytokine secretion within the CNS may not be in concordance with the cytokine dominant function in a case of relapse or remission, as some experimental data has shown³⁹. IL-4 shows inhibitory effect on TNF-alpha secretion and augments TNF-alpha sR production *in vitro*⁴⁰, however in our study no negative correlation was found between IL-4 and TNF-alpha concentrations neither in CSF nor in plasma. Our data confirm previous reports of lack of correlation between CSF and plasma values of IL-4 and EDSS¹⁵. No correlation was found either for intrathecal IgG synthesis, nor for albumin ratio and IL-4 plasma and CSF values, as previously reported³⁸.

A misbalance found between inflammatory and anti-inflammatory cytokines, is one of many immune dysregulations in MS and one should be aware of oversimplified interpretation⁴¹. However, these results support the thesis that modification of cytokine profiles could be associated with prevention of disease relapse and maintaining remission of MS. Present therapeutic strategies in MS are shifted from immunomodulation to more aggressive immunosuppressive treatments with monoclonal antibodies⁴². Clinical improvement in those studies has been shown to correlate with decrease of inflammatory cytokines and increase of anti-inflammatory cytokine production⁴³⁻⁴⁵.

Conclusion

According to our data MS relapse is characterized by high concentration of TNF-alpha in CSF and plasma and low concentration of IL-4 in CSF and high in plasma. Remission is characterized by high concentration of IL-4 and low concentrations of TNF-alpha both in CSF and plasma. SPMS is characterized with the same cytokine pattern as relapse, though both cytokines, inflammatory TNF-alpha and anti-inflammatory IL-4, have been detected in lower concentrations.

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