ORIGINAL ARTICLE

CONTENTS

Cytokine profile in multiple sclerosis patients with and without Covid-19

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ABSTRACT

Aim: To investigate the possible effect of COVID-19 disease on cytokine profile and some circulating growth factors in patients with multiple sclerosis (MS).

Materials and Methods: Serum cytokine levels as well as growth factors content were assessed be means of a solid phase enzyme linked-immunosorbent assay in 97 MS patients of which 41 had and 56 did not have confirmed COVID-19 in the past 4-6-month period, and 30 healthy individuals who were age-, and gender-matched.

Results: Some proinflammatory cytokine (such as TNFα, IFNγ) levels were higher while anti-inflammatory cytokine, namely IL-4, was lower in MS patients compared to controls indicating Th1/Th2 imbalance. Our findings revealed that the imbalance of circulating Th1/Th2 cytokines in MS patients after SARS-CoV-2 infection became even more pronounced, thus, might be a reason for the disease deterioration. Furthermore, nuclear factor κB level in MS patients after COVID-19 was found significantly elevated from that with no history of SARS-CoV-2 infection, and could be the cause of proinflammatory cytokines overexpression.

Conclusions: Our findings revealed that immunopathology of MS is associated with a Th1/Th2 imbalance, furthermore, SARS-CoV-2 infection can lead to the deterioration of this condition in MS patients, causing even more pronounced overexpression of proinflammatory cytokines and decrease in anti-inflammatory cytokines. Our results also indicated that studied growth factors can be involved in MS development but exact mechanism is not clearly understood and requires further research.

KEY WORDS: multiple sclerosis, coronavirus disease, SARS-CoV-2, Th1/Th2 cytokines, growth factors, NFκB, HIF-1α

Wiad Lek. 2024;77(4):640-645. doi: 10.36740/WLek202404104 DOI

INTRODUCTION

Multiple sclerosis (MS) is one of the most frequent disabling neurological diseases affecting millions worldwide. Since the global prevalence of MS is rising every year and today 2.8 million people are living with this diagnosis around the globe [1], it has become a trending research topic and area of considerable interest to the science community. MS is characterized by multifocal and scattered lesions through the grey and white matter of the central nervous system (CNS) originating from an autoimmune disturbance [2]. In MS, a person's immune system attacks the protective covering (myelin sheath) of nerve fibers in the brain and spinal cord often leading to spasms, pain, muscle weakness, different organs disfunction, and sometimes, decline in cognitive ability, coordination and vision loss [3].

It has been generally accepted that MS is a chronic immune-mediated disorder in which demyelination and tissue injury is driven and amplified by the inflammatory process throughout all stages of the disease [4]. Since pathogenesis of MS is accompanied by the blood brain barrier (BBB) disruption [5], macrophages and lymphocytes can migrate freely into the CNS consequently initiates an inflammatory cascade followed by sclerotic plaques formation, demyelination, and neurodegeneration [2, 6]. During the past few decades, major progress has been made in understanding the relationship between inflammatory process and MS pathogenesis [7]. Autoreactive T cells, namely, T helper (Th)-1 CD4+ T cells and Th17 cells, are involved into the MS pathogenesis, primarily, through their secretion of pro-inflammatory cytokines and chemokines that trigger microglia activation and chronic oxidative injury [8]. Macrophages and microglial cells also release different immunomodulators which may harm oligodendrocytes and are crucial for recruitment of inflammatory cells into the CNS [9].

Due to the multi-faceted autoimmune nature of MS,

immunomodulating therapy are crucial for patients with MS [4, 10]. Because of that it has been speculated that patients with MS are at higher risk for a severe course of infection diseases [11]. One the other hand, infections can result in an increased severity of MS-related symptoms and may trigger clinical relapses [12, 13]. Given the rapid rate of spread of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [14, 15], it is important to focus the current studies on the identification of the clinical characteristics and outcomes of COVID-19 among patients with MS. Preliminary results from recent studies have shown that pathogenetic mechanisms of COVID-19 and MS largely overlap [16]. In particular, there are several potential crossroads of MS and COVID-19 immunological pathways: the type-1 IFN (IFN-I) response, the T-helper 17 (TH-17) axis, and the inflammasome pathway. Thus, pinpointing the biomodulators of neurodegenerative and inflammatory events associated with both COVID-19 and MS could help extend knowledge on the etiopathogenesis of these conditions.

AIM

Taking into consideration the important role of cytokines in the neuroinflammatory processes, the aim of the present study was to evaluate the pro-inflammatory (including tumor necrosis factor (TNF)-α and interleukin (IL)-1β, -6 and -12), Th 1 (interferon (IFN)-γ), and Th 2 (IL-4 and -10) cytokine serum levels in MS patients without or with COVID-19 disease confirmed in the period of the past 4-6 months. Additionally, the levels of some important growth factors such as fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) as well as hypoxia-inducible factor 1alpha (HIF-1α) and *nuclear factor kappa B* (*NF-κB*) were also estimated in the serum of patients with MS previously suffered or did not from COVID-19.

MATERIALS AND METHODS

The study was conducted by means of collaboration between ESC "Institute of biology and medicine" of Taras Shevchenko National University of Kyiv (Kyiv, Ukraine) and the University Clinic of the Bogomolets National Medical University (Kyiv, Ukraine) from January 2021 to August 2022. Ethical approval was obtained from the both Institutional Ethical Committees. The written informed consent was acquired from all patients and healthy volunteers.

The study comprised a group of 127 people; 97 were MS patients of which: 41 were diagnosed with COVID-19 disease in the past 3-6-month period (MS, Covid-19$^+$ group) and 56 did not suffer from COVID-19 previously (MS, Covid-19$^-$ group), and 30 were healthy volunteers (control group) who were well matched to the group of MS patients by age and gender. A diagnosis of MS was confirmed if individuals have evidence of CNS damage that is disseminating in space and in time according to the 2017 McDonald criteria [17]. We excluded all those MC patients who had an active malignancy (either metastatic or nonmetastatic); were on immunosuppressive medications; had one of the following: advanced renal failure, chronic lung disease or liver cirrhosis. At the time of blood sampling, all participants did not have any symptoms of infection disease. The recruitment was also limited to patients aged from 35 to 45 years.

Blood samples were taken, and serum was obtained by centrifugation at +4°C and 1300 g for 20 min. Serum was collected and frozen at –20 °C until analysis. The serum samples of all participants were also used to determine anti-SARS-CoV-2 IgG titers. To measure the titers of anti-SARS-CoV-2 IgG in samples, a chemiluminescent microparticle immunoassay (Abbott Laboratories, USA, Cat. No 06R86-20) was performed according to the manufacturer's instructions. Results from the anti-SARS-CoV-2 IgG assay are given as index values (S/C). The S/C value less than 1.40 was considered negative while the S/C value of 1.40 or greater was classified as positive per the manufacturer's recommendation.

The levels of cytokines (TGFα, IFNγ, *IL*-1β, -4, -6, -8, -10, and -12) and growth factors (FGF-2, EGF, VEGF, PDGF) as well as factors such as HIF-1α and *NF-κB* were measured in serum by enzyme-linked immunosorbent assay (ELISA), following the standard protocols. Primary antibodies were purchased from *Santa Cruz Biotechnology,* Inc., USA (Dalas, TX, USA). Corresponding secondary antibodies conjugated to horseradish peroxidase were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Substrate solution (0.04 % *o*-phenylenediamine and 3.5 mM H_2O_2 in 100 mM *acetate buffer*, pH 5) was used for detection. The reaction was stopped after 20 min by the addition of 100 µL of a stop solution and the absorbance was determined at 492 nm with ELISA reader (BioTek Instruments, USA). All the values were recorded in duplicate. The concentrations of cytokines as well as growth and transcription factors in the serum of healthy volunteers were set at 100%, and *changes* in their concentrations were given as *percentage* of *controls*.

Statistical analysis was performed with STATISTICA Package version 12.0 (StatSoft. Inc.). The arithmetic mean (M) and mean squared deviation (SD) indicators were calculated. To clarify the normal distribution of quantitative data, the Kolmogorov-Smirnov test or

Table 1. Basic characteristics of the MS patients and healthy controls

Indicator (unit of measurement)	Patients' groups		
	Control (n = 30)	MS, Covid-19$^+$ (n = 41)	MS, Covid-19$^-$ (n = 56)
Age (years)	41 ± 4	40 ±5	41 ± 5
Sex (male/female)	10/20	14/27	20/36
Time since MS diagnosis (years)	-	4.6 ± 1.3	4.4 ± 1.5
Current anti-SARS-CoV-2 IgG titer (S/C index)	< 1.40 (negative)	70 ± 8 (positive)	< 1.40 (negative)

Shapiro-Wilk test was used. Data between two groups were compared using the Mann-Whitney U-test. Kruskal-Wallis H-test was performed to compare data among multiple groups. A $p<0.05$ was considered statistically significant.

RESULTS

Some basic characteristics of the MS patients and healthy individuals are shown in Table 1. MS patients consisted of 34 males and 63 females, and their age ranged from 35 to 46 years. In the aggregate, 42% of MS patients had COVID-19 disease in the past 3-6-month period, while the others had no COVID-19 previous history. MS patients were age and gender matched to a control group (Table 1).

Since MS is a progressive immune-mediated disorder, and cytokines play an important role in the disease progression, we wondered whether pro- and anti-inflammatory cytokine levels were changed in MS patients after COVID-19. Thuse, serum cytokine levels were compared between both groups of MS patients (with or without confirmed COVID-19) and healthy controls (Fig. 1).

MS is considered a Th 1 lymphocyte-mediated disorder, and it was quite expected that serum levels of IFNγ, Th1-related molecules, were increased in MS patients, however, it should be noted that IFNγ level was significantly higher in the group of MS patients after COVID-19 compared with MS patients with no COVID-19 history (Fig. 1).

With regards to the pro-inflammatory cytokine profile, TNF-α levels did not differ significantly among both groups of MS patients but were higher compared with the control. Serum IL-12 level was higher in MS patients after COVID-19 compared with the control value but in the group of MS patients with no COVID-19 history it was at the control level. At the same time, serum IL-6 levels observed among both MS groups did not differ from that of the control group. Finally, IL-1β levels were higher among healthy subjects compared with MS patients (Fig. 1).

In the Th 2 anti-inflammatory cytokine profile, IL-4 levels were higher in control subjects compared with both groups of MS patients. Although, the IL-10 levels did not differ between MS patients with no history of COVID-19 and controls, its level in MS patients after COVID-19 was significantly elevated (Fig. 1).

Since growth factors comprised an important group of cytokines playing a crucial role in the pathways of cell proliferation, differentiation and activation, we hypothesized that among them also could be risk factors for the initiation and progression of MS. Thus, in the present study, we also examined the circulating levels of some important growth factors, namely FGF-2, VEGF, EGF, PDGF.

As can be seen from the Figure 2, no difference was detected in FGF-2 levels between MS patients with no COVID-19 history and controls while in the group of MS patients after COVID-19 the level FGF-2 was significantly elevated. The levels of EGF and PDGF were remarkedly lower in the serum of both MS patients' groups compared with control subjects, however, there were no difference between their values in MS patients with or without COVID-19 history. The serum VEGF levels were higher among MS patients of both groups than those of controls but they did not differ between MS patients with or without COVID-19 history (Fig. 2).

In this study we also estimated the circulating levels of hypoxia-inducible factor 1alpha (HIF-1α) as it is a key regulator in hypoxic and ischemic brain injury. Higher HIF-1α level was observed among MS patients with no COVID-19 history compared with control subjects. However, no difference was detected between HIF-1α levels of MS patients after COVID-19 and control individuals.

The transcription factor NF-κB is a central mediator of inflammation with strong contribution to inflammasome pathway during MS development. In this study, we compared serum levels of NF-κB in MS patients with or without COVID-19 history (Fig. 2). Our results revealed that NF-κB levels were increased in MS patients either with or without COVID-19 history compared with controls. However, it should be noted that NF-κB level was significantly higher in MS patients after COVID-19 compared with value of MS patients with no COVID-19 (Fig. 2).

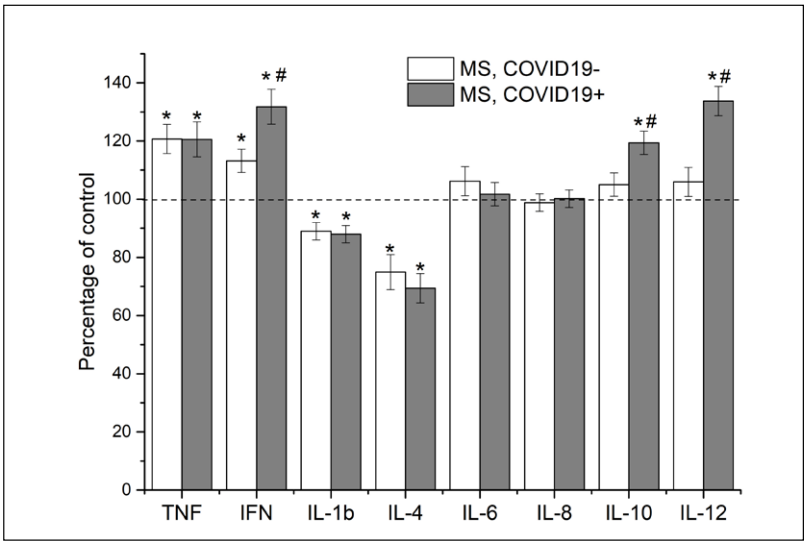

Fig. 1. The serum cytokine profile of MS patients with or without COVID-19 history. Cytokine TNFα, IFNγ, IL-1β, IL-4, IL-6, IL-8, IL-10, and IL-12 contents were expressed as percentage of control (100 %).
*p<0.05 significantly different from the control group; # p<0.05 significantly different from the MS, COVID19- group.

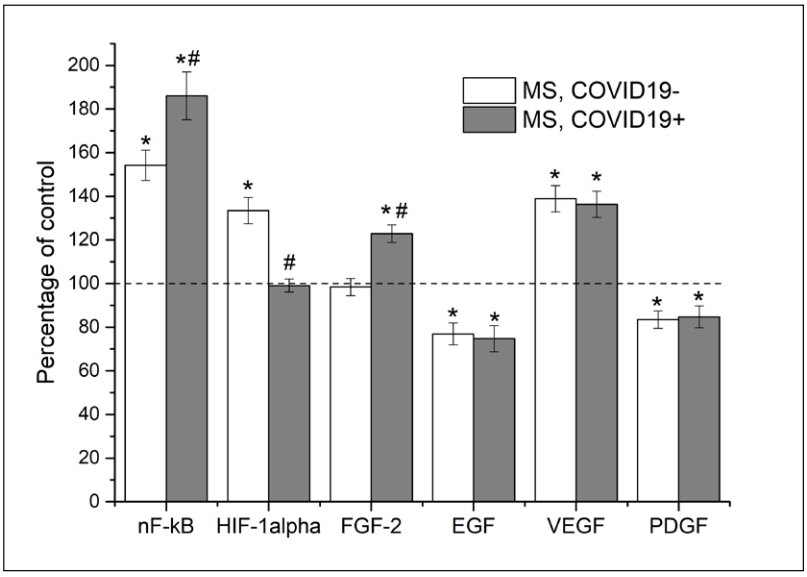

Fig. 2. The serum growth factor profile of MS patients with or without COVID-19 history. The contents of growth factors (FGF-2, EGF, VEGF, PDGF) as well as nF-κB and HIF-1α were expressed as percentage of control (100 %).
*p<0.05 significantly different from the control group; # p<0.05 significantly different from the MS, COVID19- group.

DISCUSSION

Previously, it has been shown that infections can trigger MS exacerbations [12, 13, 18] but an association between COVID-19 and increased risk of infection-related MS exacerbations has not been conclusively improved. Since immune mediators have a crucial role in the development of MS lesions, and pro- and anti-inflammatory cytokine levels have been found to correlate with MS progression [7, 8, 19], in the current study we aimed not only to evaluate the changes in serum cytokine profile of MS patients but also to elucidate the impact of COVID-19 on the circulatory cytokine levels. The study enrolled 97 MS patients from the University Clinic of the Bogomolets National Medical University (Kyiv, Ukraine). Among them there were 41 individuals who had been diagnosed with COVID-19 in the past 4-6-month period. To understand the impact of COVID-19 on serum cytokine profile laboratory data from MS patients with COVID-19 were compared with corresponding data from patients with MS but without COVID-19 history.

With regards to the serum cytokine profile in MS patients with no COVID-19 history, the findings from the current study are consistent with results of previous studies [19, 20] and supported the generally accepted immune-mediated mechanism underpinning MS disease [2, 4]. Hence, MS is considered a Th1 lymphocyte-mediated disease, and it was expected to find enhanced levels of Th1-related molecules, namely IFN-γ and TNF-α, in MS patients. On the other hand, anti-inflammatory cytokines produced by Th2 cells (IL-4, IL-10, etc.) act as negative feedback regulators on proliferation and differentiation of Th1 cells, and have anti-inflammatory effect, thereby, inhibiting MS progression. Our results revealed that immunopathology of MS is associated with a Th1/Th2 imbalance related to overexpression of proinflammatory cytokines and decrease in anti-inflammatory cytokines. According to our findings, SARS-CoV-2 infection in MS patients can lead to the deterioration of the condition of patients. As could be seen from the results obtained (Fig. 1), Th1/Th2 imbalance in MS patients after COVID-19 became even more pronounced. Overproduction of proinflammatory cytokines while decreasing the anti-in-

flammatory cytokines could lead to acute inflammatory lesions in MS patients, and, consequently, to more severe course of the disease.

It is well known that the nuclear factor NF-κB pathway enhances the expression of proinflammatory genes and is involved in MS development [21]. Our results revealed increased level of circulating NF-κB in MS patients (Fig. 2) which might be one of the reasons of proinflammatory cytokines overproduction under the disease development. Furthermore, NFκB level in MS patients after COVID-19 was found significantly different from that with no history of SARS-CoV-2 infection (Fig. 2) and might indicate a more severe disease prognosis.

Previously, it was shown that HIF-1α could be implicated in neuronal apoptosis, and disruption of BBB [22], and, contrarily, some other studies demonstrated protective effect of HIF-1α against neurologic dysfunction and neuronal apoptosis [23]. Since, HIF-1α seems to be involved in the cell's response to brain injury, in a current study, we investigate its levels in MS patients with and without COVID-19. The elevated serum HIF-1α level was shown in MS patients with no COVID-19 history, while in MS individuals after SARS-CoV-2 infection its level was decreased and did not defer from control (Fig. 2).

Here, we also hypothesized that growth factors may also have an essential role in the MS development, thus establishing the causal relationship between circulating levels of growth factors in MS patients before and after COVID-19 could be important from clinical perspective. Our results highlight that MS patients either with or without COVID-19 history have elevated levels of VEGF and, simultaneously, decreased EGF and PDGF content compared to controls. However, no differences were observed among MS patients of both groups (Fig. 2). We hypothesized that VEGF, which is also known as vascular permeability factor, could be involved in the MS development due to its ability to regulate vascular permeability, and to contribute to BBB breakdown [24]. Overall, growth factors might be important mediators linked to MS lesion progression however, exact relation and mechanism are not clearly understood and require further research.

CONCLUSIONS

In summary, the results of the present study suggest a Th1/Th2 balance shift in favor of a Th1 cytokine profile in patients suffering MS. Furthermore, SARS-CoV-2 infection in MP patients associated with even more pronounced overexpression of proinflammatory cytokines and decrease in anti-inflammatory cytokines. The increase of circulating Th1 cytokines can lead to the deterioration of symptoms in MS patients. Our results also indicated that studied growth factors may be involved in MS development but exact mechanism is not clearly understood and requires further research.

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The research was carried out within the framework of the topic «Thrombosis as a risk factor for complications in patients with COVID-19», all-Ukrainian, MES/ Taras Shevchenko Kyiv National University (2021-2023, № state registration 0121U109854).

CONFLICT OF INTEREST
The Authors declare no conflict of interest

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RECEIVED: 12.12.2023
ACCEPTED: 28.03.2024