ORIGINAL ARTICLE

PRODUCTION OF INTERLEUKINS 1B, 2, 4, 10 AND C-REACTIVE PROTEIN IN ISCHEMIC STROKE

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ABSTRACT

The aim: The aim of this study was to determine the content of interleukins (IL) 1β, 2, 4, and 10, as well as the generally accepted marker of inflammation - C-reactive protein (CRP) - in the peripheral blood on the first and tenth days of ischemic stroke (IS).

Materials and methods: The study involved 25 patients with IS (including 8 people with mild case of neurological disorders, 9 – moderate case and 8 – severe case) and 14 people of the control group. The levels of IL-1β, IL-2, IL-4 and IL-10 in the blood were determined by the immunoenzyme method.

Results: It was found that on the first day in patients with IS an increase in the concentration of CRP and all the studied cytokines, especially pro-inflammatory cytokines IL-1β and IL-2, is marked. On the tenth day, the content of pro-inflammatory cytokines and CRP significantly decreases compared to the first day, but remains higher than in the control, but the concentration of anti-inflammatory cytokines (IL-4 and IL-10) continues to increase.

Conclusions: The results obtained on the first day of IS indicate the development of neuroinflammation. On the tenth day the severity of the inflammatory process is significantly reduced, but it still occurs. It was also shown that the outcome of IS depends on the concentration of cytokines in the blood: the higher the level of pro-inflammatory interleukins on the first day, the lower the content of anti-inflammatory interleukins and the higher the amount of pro-inflammatory interleukins on the tenth day, the more pronounced the neurological deficit.

KEY WORDS: C-reactive protein, cytokines, ischemic stroke, neuroinflammation, innate and adaptive immunity

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INTRODUCTION

Ischemic cerebrovascular accidents (ICA) are an important medical and social problem. They make up 13-15% of all causes of death and disability in these diseases consists of 20%. Acute and chronic ICA lead to severe neurological deficit, which greatly affects the quality of life. In the future, according to the conclusion of WHO experts, the number of patients with this pathology will increase, as recently, there has been a tendency to the spread in the population of the causes leading to ICA, namely: arterial hypertension, obesity, atherosclerosis, diabetes mellitus, metabolic syndrome, etc. [1].

At present, inflammatory and neuroimmune processes are of great importance in the pathogenesis of ICA [2]. First of all, the aforementioned diseases are the causes of ICA, as well as the pre-stroke hypoxic brain damage arising on their background are characterized by the so-called chronic low-grade inflammation. It is believed that this inflammation is the basis of the majority of the chronic non-infectious diseases [3]. By itself, it may not manifest itself clinically for a long time (the so-called "silent" inflammation), persists for years, gradually damaging organs and tissues, but is characterized by an increase in the level of cytokines in the blood and infiltration of peripheral tissues by macrophages [4, 5].

With the development of ischemic stroke (IS) as well as any other tissue damage acute inflammation occurs, which among other common inflammatory manifestations [or so-called systemic inflammatory response syndrome (SIRS)] also manifested by increased level of cytokines in the blood [6].

Thus, inflammation in IS appears to be a combination of pre-stroke chronic low-grade inflammation and post-stroke acute inflammation.

It is known that in the early period after having IS the increase in the level of cytokines in the blood, in particular IL-1β and IL-10, is determined [7, 8]. The experiment has shown that during cerebral hypoxia an increase in the concentration of IL-1β in the blood is determined, and, with its prolonged presence in high doses in blood plasma and cerebrospinal fluid, more pronounced neurological symptoms are observed [9].

Of considerable interest is a comprehensive study of the cytokines production - markers of various cells of the immune response and inflammation – monocytes/macrophages, T-helper lymphocytes of the 1st and 2nd types (Th1 and Th2) - IL-1β, IL-2, IL-4, IL-10 - and analysis of the relative role of the phenomena mediated by them with IS.

IL-1β is produced mainly by macrophages, that is, it is a marker of within named cells and innate (nonspecific) *innate immunity* and the major pro-inflammatory cytokine. Being one of the first mediators of inflammation, in partic-

ular, in the ischemic zone, it also stimulates the synthesis of other cytokines, including IL-2 [10, 11].

IL-2 is produced mainly by Th1, is a marker of Th1 and adaptive (specific) cellular immunity, plays a key role in the development of a rapid immune response, induces T-lymphocyte proliferation and activates cytotoxic T-lymphocytes, and also belongs to pro-inflammatory cytokines [11, 12].

IL-4 is produced mainly by Th2, that is, it is a marker of Th2 and adaptive humoral immunity, regulates the growth and differentiation of B-lymphocytes, biosynthesis and secretion of antibodies [13]. IL-10 is produced mainly by monocytes and Th2 and increases the survival rate of B-lymphocytes, their proliferation and antibody production [14].

IL-4 and IL-10 are anti-inflammatory cytokines that control the strength and shape of the immune response and inflammation. IL-4 prevents Th1 differentiation, arrests most functions of macrophages; IL-10 suppresses antigen presentation by macrophages and Th1 activation; both of them inhibit the production of pro-inflammatory cytokines by Th1 cells and macrophages [14].

THE AIM

The aim of this study was to determine the content of interleukins (IL) 1β, 2, 4, and 10, as well as the generally accepted marker of inflammation - C-reactive protein (CRP) - in the peripheral blood on the first and tenth days of ischemic stroke (IS).

MATERIALS AND METHODS

All procedures were performed with the informed consent of the patients and in compliance with the principles of medical bioethics and deontology.

The study did not participate persons with acute inflammatory, autoimmune, neurodegenerative diseases, diabetes mellitus, neoplastic processes and repeated acute ischemic cerebrovascular accidents.

25 patients with IS (12 women and 13 men) were examined in the age range 41-73 years, and 14 people from the control group (7 women and 7 men). The patients were divided into 3 groups, depending on the clinical symptoms' severity. 1st group - 8 people (32%) - with minor case of neurological disorders, 2nd group - 9 people (36%) - with moderate case and 3rd group - 8 people (32%) - with severe case. Patients with neurological disorders of extreme severity and being in coma did not participate in the study. The severity of the course was assessed by the National Institutes of Health Stroke Scale (NIHSS). This scale assesses the level of consciousness, answers to questions, commands execution, eye movements, visual field, facial paralysis, movements of the upper and lower limbs, limb ataxia, sensitivity, aphasia, dysarthria, agnosia. The severity level is estimated within the range from 0 to 5 scores. The sum of scores is determined after evaluating individual functions, and the higher it is, the worse the patient's condition is. The diagnosis of IS was made on the basis of clinical neurological and *instrumental apparatus* studies. From the moment of admission to in-patient department all patients took medicamentous therapy in accordance with the protocols of the Ministry of Health of Ukraine, aimed at combating cerebral edema, improving cerebral circulation and correcting the work of the respiratory and cardiovascular systems.

The level of IL-1β, IL-2, IL-4 and IL-10 in the blood was determined using ELISA-BEST reagent kits A-8766, A-8772, A-8754 and A-8774, respectively, designed to determine in serum blood and urine cytokine concentrations by the immunoenzyme method. To investigate the venous blood it was taken on the first and tenth days of the disease and centrifuged at 3000 rpm for 10-15 minutes. The obtaining serum samples results were frozen at a temperature of -20 ° C and thawed immediately before analysis. The results were recorded by measuring the values of the optical density of liquids in the wells on a vertical-type scanning spectrophotometer in a dual wavelength mode. Outcome evaluation was carried out by such method as constructing in a linear calibration curve graph of optical density on the concentration of interleukins in the calibration samples and determining the content of cytokines in the control sample and analyzed samples according to the calibration graph. The level of CRP in the blood was also studied on the 1st and 10th days of the disease by the method of determining the highly sensitive hsCRP, allowing to estimate the degree of risk of the onset and outcome time of acute ischemic stroke [15].

The data obtained in this study was validated using Student`s t-criterion, a posteriori test of the Bonferroni correction, Pearson's correlation.

RESULTS

In the control, in the blood IL-1β and IL-10 far outweigh, as products of monocytes-macrophages and mediators of innate immunity, over products of lymphocytes and mediators of adaptive immunity IL-2 and IL-4, and between IL-1β and IL-10 - anti-inflammatory IL-10 over pro-inflammatory IL-1β (Table I). This is probably due to the fact that normally adaptive immunity is not yet involved and inflammation is absent.

With IS the content of all investigated cytokines in blood considerably increases: IL-1β - in 8,9 times, IL-2 - in 26,5 times, IL-4 - in 1,5 times, IL-10 - also in 1,5 times. As one can see, the content of IL-2 increases the most, which indicates the greatest activation of Th1, in other words adaptive cellular immunity, as well as IL-1β - a marker of macrophages and innate cellular immunity. Th2, that is adaptive humoral immunity, is less involved.

It is also seen that the production of pro-inflammatory cytokines increases rather, than anti-inflammatory, which indicates the development of inflammation. This is confirmed by a significant increase of the content of CRP in the blood - 3.8 times.

On the 10th day of treatment the content of IL-1β and IL-2 is significantly reduced compared to that one that

Table I. The content of cytokines and CRP in the blood of patients with ischemic stroke both in pretreatment and posttreatment time

Value	Control	Ischemic stroke	
		pretreatment time (the 1st day)	posttreatment time (the 10th day)
IL-1β, pg/ml	1,445 ±0,010	12,917 ±0,453 p_1< 0,001	3,797 ±0,433 p_1< 0,001 p_2< 0,001
IL-2, pg/ml	0,074±0,014	1,964±0,138 p_1< 0,001	0,266±0,065 p_1< 0,001 p_2< 0,05
IL-4, pg/ml	0,110±0,023	0,167±0,010 p_1< 0,05	0,357±0,023 p_1< 0,05 p_2< 0,001
IL-10, pg/ml	3,235±0,224	4,851±0,150 p_1< 0,001	16,459±0,517 p_1< 0,001 p_2< 0,001
CRP, pg/ml	1,354±0,068	5,081±0,176 p_1< 0,001	4,363±0,142 p_1< 0,001 p_2< 0,001

Note. p_1 - compared with control, p_2 - compared with pretreatment value.

Table II. The content of cytokines and CRP in the blood of patients with ischemic stroke in pretreatment time (on the 1st day) depending on the severity evaluation scale

Value	Stroke scale		
	minor	moderate	severe
IL-1β,pg/ml	10,449 ± 0,436 p_1< 0,001	13,065 ± 0,444 p_1< 0,001 p_2< 0,001	15,219 ± 0,353 p_1< 0,001 p_2< 0,001 p_3< 0,01
IL-2, pg/ml	1,575 ± 0,106 p_1< 0,001	1,740 ± 0,170 p_1< 0,001 p_2>0,05< 0,1	2,604 ± 0,262 p_1< 0,001 p_2< 0,01 p_3< 0,05
IL-4, pg/ml	0,193 ± 0,012 p_1< 0,01	0,174 ± 0,013 p_1< 0,05 p_2>0,05< 0,1	0,133 ± 0,020 p_1>0,05 p_2< 0,05 p_3>0,05
IL-10, pg/ml	4,998 ± 0,135 p_1< 0,001	5,278 ± 0,205 p_1< 0,001 p_2>0,05< 0,1	4,222 ± 0,283 p_1< 0,05 p_2>0,05< 0,1 p_3< 0,01
CRP, mg/l	4,306 ± 0,159 p_1< 0,001	5,013 ± 0,192 p_1< 0,001 p_2>0,05< 0,1	5,931 ± 0,276 p_1< 0,001 p_2< 0,001 p_3< 0,05

Note. p_1 - compared with the control (see table 1), p_2 - compared with minor case, p_3 – compared with moderate case.

was at pretreatment time - 3.4 times and 7.4 times respectively. At the same time, it still remains significantly larger than the control - 2.6 times and 3.6 times, respectively. In contrast, the content of IL-4 and IL-10 continues to increase. It significantly increases compared to that one that was at pretreatment time - 2.1 times and 3.4 times, respectively - and becomes greater than the control by 3.2 times and 5.1 times, respectively. Thus, on the 10th day of treatment, the production of pro-inflammatory cytokines is already significantly reduced compared to that that was at pretreatment time, but has not yet returned to the control, and the production of anti-inflammatory cytokines continues to increase. These data indicate that on the 10th day of treatment for IS, the inflammatory process subsides significantly, but is still quite pronounced. This is confirmed by the fact that the content of CRP in the blood on the 10th day of treatment, although significantly is reduced compared to that one that was at pretreatment

Table III. The content of cytokines and CRP in the blood of patients with ischemic stroke in posttreatment time (on the 10th day) depending on the severity evaluation scale

Value	Stroke scale		
	minor	moderate	severe
IL-1β, pg/ml	2,275 ± 0,184 p_1< 0,01	3,669 ± 0,470 p_1< 0,001 p_2 >0,05	5,463±0,979 p_1<0,01 p_2<0,01 p_3>0,05
IL-2, pg/ml	0,126 ± 0,036 p_1>0,05	0,210±0,098 p_1>0,05 p_2>0,05	0,468±0,151 p_1<0,05 p_2>0,05 p_3>0,05
IL-4, pg/ml	0,288 ± 0,005 p_1< 0,001	0,296±0,023 p_1< 0,001 p_2>0,05	0,496±0,029 p_1<0,001 p_2<0,001 p_3<0,001
IL-10, pg/ml	16,585 ± 0,579 p_1< 0,001	18,731±0,545 p_1< 0,001 p_2<0,05	13,777±0,536 p_1<0,001 p_2<0,01 p_3<0,001
CRP, mg/l	3,863 ± 0,141 p_1< 0,001	4,374±0,216 p_1< 0,001 p_2>0,05	4,850±0,258 p_1<0,001 p_2<0,05 p_3>0,05

Note. p_1 - compared with control (see table 1), p_2 - compared with minor level, p_3 – compared with moderate level.

time - 1.2 times, but remains much higher than the control (3.2 times).

Analyzing the content of cytokines in the blood depending on the Stroke scale it is seen that at minor severity the content of all studied cytokines is significantly higher than the control: IL-1β - 7.2 times, IL-2 - 21.3 times, IL-4 - 1.8 times, IL-10 - 1.5 times (Table II).

At the moderate case the content of IL-1β is significantly higher than at the mild one and at the severe case is higher than at the moderate one. Thereafter, in both cases, it becomes higher than the control. The content of IL-2 at the moderate case tends to increase compared to the minor case, but at the severe case is significantly higher than at the moderate one. Accordingly, in both cases, it is also becoming more than the control. The content of IL-4 at the moderate case tends to decrease compared to the minor case, but it remains significantly higher than the control, and at the severe case decreases even more and, although in adequately compared to the moderate case, but adequately compared to the minor case and so it significantly has no difference from the control. The content of IL-10 at the moderate case tends to increase compared to the minor case, and at the severe case is significantly decreasing compared to the moderate case, so that it significantly does not exceed that one at the minor case, but still remains significantly higher than the control.

Thus, the level of pro-inflammatory cytokines in the blood is increasing with increasing the severity of IS and the content of anti-inflammatory cytokines is decreasing. Apparently, this is due to the increased activity of the inflammatory process, as evidenced by changes in CRP production. At the minor severity of IS the level of CRP in the blood is significantly increasing compared with the control - 3.2 times (see Table II). At the moderate severity it tends to increase further but at the severe case it is increasing significantly compared to the moderate case, so it is significantly higher than one that was at the minor case. Of course that at the last two levels it remains significantly higher than the control (3.7 times and 4.4 times, respectively).

On the 10th day of treatment at the minor severity of IS the content of IL-1β, IL-4 and IL-10 in the blood increases significantly (1.6 times, 3.9 times and 5.1 times, respectively) and IL-2 does not differ with assurance from the control (Table III).

At moderate case the level of IL-1β has significantly no difference from that one at the minor case, and at the severe case - from that one at the moderate case, but becomes significantly higher than at the minor case. At the moderate and severe case it remains significantly greater than control.

The content of IL-2 at the moderate case does not differ with assurance from that one at the minor level and control, and at the severe case - from that one at the moderate and minor case, but becomes significantly higher than control.

The level of IL-4 at the moderate case is not significantly different from that one at the mild case, and at the severe case is significantly higher than both at the moderate and mild cases. At the moderate and severe cases, it remains significantly greater than the control.

The content of IL-10 at the moderate case is significantly higher than at the minor one and, accordingly, remains significantly higher than the control, and at the severe case

- significantly less than at the both moderate and minor case, but remains significantly higher than the control (4.3 times).

Thus, on the 10th day of treatment with increasing severity of IS the levels of IL-1β and IL-4 are increasing slightly, IL-2 - does not change significantly, and IL-10 - first is increasing and then decreasing, but remains significantly higher than the control.

Compared with the first day of IS, there is a less pronounced in terms of control increase in the production of pro-inflammatory cytokines and decrease - anti-inflammatory. Apparently, this is due to a decrease in activity of the inflammatory process, which is confirmed by changes in CRP production. Thus, on the 10th day of IS at its minor level, the level of CRP in the blood is 2.9 times higher than the control. At the moderate case it does not significantly differ from that one at the minor level, and at the severe level - significantly does not differ from that one at the moderate case, but becomes significantly bigger than at the minor level. Both at the moderate and severe cases it remains significantly bigger than the control.

In calculating the Pearson correlation coefficient on the first day of disease was found a moderate positive correlation between the concentrations of IL-1β and IL-2 (r = 0.613), IL-1β and CRP (r = 0.643), and a negative correlation - between the content of IL-2 and IL -10 (r = -0.611); on the tenth day - a moderate positive correlation between the concentrations of IL-1β and IL-2 (r = 0.667), IL-1β and IL-4 (r = 0.463), and negative - between the levels of IL-1β and IL-10 (r = -0.408), IL-4 and IL-10 (r = -0.687). The positive relationship between the content of IL-1β and IL-2 confirms the above mentioned that IL-1β stimulates the synthesis of IL-2. More broadly, there is a synergistic effect between IL-1β and IL-2, macrophages and lymphocytes in the pathogenesis of inflammation: they stimulate each other's production or activity [16]. The negative correlation between the levels of IL-2 and IL-10 and IL-1β and IL-10 confirms the above about the antagonism between pro- and anti-inflammatory mediators. The positive relationship between IL-1β and CRP confirms that pro-inflammatory cytokines may be, along with acute-phase proteins, markers of inflammation [17]. The decrease in the strength of the negative link between IL-1β and IL-10 after treatment, the appearance of a positive correlation between IL-1β and IL-4 and a negative correlation between IL-4 and IL-10 show that in inflammatory processes treatment the correlations between the indicators begin to change to the opposite, in particular, in this case the strength of the negative relationships decreases and there are appearing positive links between pro- and anti-inflammatory cytokines, as well as negative links between anti-inflammatory cytokines.

DISCUSSION

Analyzing the mechanisms of the results obtained, it should be noted that with IS, neurons and neuroglia cells - astrocytes - are primarily involved in the pathological process. Within the first minutes after carrying out the hypoxic damage of neurocytes their cytotoxic edema develops, which occurs as a result of stopping the work of the sodium-potassium pump, leading to sodium retention. Raised hyperosmolarity promotes the flow of water into the brain cells and causes their osmotic death. Hypoxia also contributes to the inhibition of mitochondrial oxidation, which leads to progressive ATP deficiency. This deficiency is partially compensated by glycolysis, but its activation causes the rapid development of acidosis. Due to the dysfunction of the membrane during energy deficiency Ca^{2+} accumulates in the cell. This leads to the activation of defense mechanisms, consisting in the capture of Ca^{2+} by the energy stations of the cell. Mitochondria, which are deficient in ATP under conditions of hypoxia, have to work actively to maintain a constant inner mitochondrial membrane charge, which in turn further disrupts energy metabolism [18]. In parallel with cytotoxic edema ionic edema occurs, where there is a flow of Na^+ and Cl^- from the vascular bed followed by water. The development of vasogenic edema aggravates the pathological process. At this stage the intercellular spaces increase due to the contraction of endothelial cells, but still do not let the blood corpuscle. The reason for the contraction of endotheliocytes, apparently, is the action of the formed and released in increased amounts of biologically active substances which are mediators, when inflammation happens, they are histamine, bradykinin, derivatives of arachidonic acid, free radicals, thrombin, etc. [19]. The next phase in the pathogenesis of cerebral edema is observed with the progression of endothelial dysfunction. Here, necrosis of endotheliocytes occurs, that means the complete destruction of contacts between them, which contributes to the passage of the blood corpuscle, above all of erythrocytes, and the development of diapedesis. Hemorrhagic transformation leads to severe disruption of homeostasis and, as a consequence, neuronal necrosis develops [20].

All of the above factors lead to the development of inflammation. In the nervous system, microglia, represented by resident macrophages, have protective functions [21]. In addition to microglia, astrocytes are actively involved in the process of neuroinflammation, regulating the functional activity of neurons and the blood-brain barrier permeability. They possess pro- and anti-inflammatory effects, producing gliotransmitters and cytokines [22].

It is known that the brain is "behind a barrier" body, i.e. the blood cells are unable to penetrate the hematoencephalic, hematoliquor and hematoleptomeningeal barriers [23]. However, modern imaging methods have made it possible to detect the migration of monocytes from the circulating blood into the central nervous system through these barriers in pathology [24, 25]. This, in turn, leads to the accumulation of immunocompetent blood cells in the inflammation focus, which, along with resident macrophages, can release and initiate the formation of inflammatory mediators. Thus, the appearance of interleukins not only in the cerebrospinal fluid, but also in the peripheral blood is the result of the activation of both local cells and those that have emigrated from the blood. It should also be kept

in mind that the increase in the content of mediators in the blood occurs not only due to their incoming from the focus of inflammation, but also due to the activation of blood leukocytes [26].

The obtained results are consistent with a number of literature data that in ischemic stroke there is an increase in the level of circulating IL-1β [27]. At the same time, literature data on the production and role of IL-2 in ischemic stroke are ambiguous and contradictory [28]. In addition, the literature data in general indicate mainly a deficiency of anti-inflammatory cytokines – IL4 and IL10 – in ischemic stroke [29, 30].

CONCLUSIONS

1. At an ischemic stroke on the 1st day/in pretreatment time the content in blood of all investigated cytokines - IL-1β, IL-2 IL-4, IL-10, and also CRP considerably increases. At the same time the level of pro-inflammatory cytokines increases rather than anti-inflammatory. All this indicates the development of inflammation. The most activated is the production of IL-2, a marker of Th1 lymphocytes - effectors of adaptive cellular immunity.
2. On the 10th day of treatment for ischemic stroke the production of pro-inflammatory cytokines and CRP is significantly reduced compared to that before treatment, but still significantly exceeds the control, and the production of anti-inflammatory cytokines continues to increase. This indicates that at this time the inflammatory process is significantly reduced, but it is still quite pronounced.
3. On the 1st day of ischemic stroke with increasing severity of the disease the level of pro-inflammatory cytokines and CRP in the blood increases and the content of anti-inflammatory cytokines decreases, which is associated with an increase in the severity of the inflammatory process.
4. On the 10th day of treatment with an increased severity of ischemic stroke compared with the first day there is less pronounced in terms of control of increased production of pro-inflammatory cytokines and CRP and decrease - anti-inflammatory cytokines, which is associated with reduced severity of inflammation.

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