IMPACT OF NITRIC OXIDE SYNTHESIS MODULATORS ON THE CYTOKINES PROFILE IN EXPERIMENTAL ANTIPHOSPHOLIPID SYNDROME

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Background. Antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of specific antibodies.

Objective. The aim of the study was to investigate the effect of combined use of L-arginine and aminoguanidine on cytokine profile (IL-1β, IL-6, TNF-α, IL-4, IL-10) in experimental APS.

Methods. The study was performed on BALB/c female mice. L-arginine (25 mg/kg) and aminoguanidine (10 mg/kg) were used for correction. Serum cytokines concentrations were assessed using an ELISA test.

Results. It was found that in APS the concentration of proinflammatory cytokines IL-1β, IL-6 and TNF-α increases in 3.2, 2.3 and 4.5 times respectively, compared to the control. At the same time a decrease of the IL-4 and IL-10 in 1.9 and 2.2 times was evidenced.

Aminoguanidine, a selective iNOS inhibitor, caused a significant decrease of TNF-α by 57% (p<0.001), but there were no changes in IL-1β, IL-6, IL-4 and IL-10 compared to the APS-group. L-arginine combined with aminoguanidine caused a significant decrease in the concentration of IL-1β by 30% (p<0.01), IL-6 – by 16% (p<0.05), TNF-α – by 59% (p<0.01) compared to the control. At the same time, the concentration of IL-4 increased by 35% (p <0.01), IL-10 – by 25% (p<0.005).

Conclusions. Combined use of the precursor of the NO synthesis L-arginine and aminoguanidine, a selective iNOS inhibitor, leads to a decrease in the concentrations of IL-1β, IL-6, TNF-α and an increase of IL-4 and IL-10 compared to the group of the BALB/c mice with APS and the group of animals administered with aminoguanidine.

KEY WORDS: antiphospholipid syndrome; cytokines; nitric oxide; L-arginine; aminoguanidine.

Introduction

Antiphospholipid antibody syndrome (APS) is an autoimmune condition characterized by the presence of antiphospholipid antibodies (aPL) [1], encompassing primary APS, secondary APS, seronegative APS (SNAPS) and catastrophic APS (CAPS) [2]. Secondary APS can be found in association with other autoimmune conditions such as systemic lupus erythematosus, rheumatoid arthritis, autoimmune thyroid disease, Crohn’s disease, Sjogren syndrome, systemic sclerosis, lymphoma or leukemia, malignancies of the ovary and cervix, drug induced as with oral contraceptive pills or in infectious disease such as HIV or syphilis [1]. In CAPS a systemic inflammatory response, systemic endothelial dysfunction and DIC develop. These processes are the pathogenetic basis for development of multiple organ failure [3, 4]. SNAPS is negative for lupus anticoagulant and antiphospholipid antibodies [2].

The diagnostic APS criteria are anticardiolipin (aCL), antiβ2-glycoprotein-1 (aβ2GPI) and lupus anticoagulant (LA) [1, 5]. APS can be classified only in the presence of thrombotic (non-inflammatory arterial, venous or small vessel thrombosis) obstetric complications (death of one or more morphologically normal fetus at or beyond the 10th week of gestation; one or more premature birth of normal fetus before the 34th week due to eclampsia, pre-eclampsia or placental insufficiency), or increased aPL level [2]. The mechanisms of thrombosis in APS have not been fully studied yet [6].

aβ2GPI antibodies are central in pathogenic APS mechanisms and, although the full pathogenesis of APS is not clear yet, the binding of these aPL antibodies to the antigens on the cell surface of platelets, monocytes, endothelial cells and trophoblasts triggers intracellular signaling with subsequent activation and alteration...
of diverse cell functions. Cellular activation starts after the binding of the complex aβ2GPI antibody/β2GPI [5, 7]. β2GPI is the most important antigenic target [2]. Platelet activation and the subsequent release of thromboxane favor their aggregation. Thrombosis at the fine vasculature of the target organ is thought to be more dependent from antibodies against the anticoagulant AnV. Endothelial cells and monocytes activation determine a pro-aggregation status due to up-regulated expression of adhesion molecules, such as E-selectin, and release of tissue factor (TF) and proinflammatory cytokines [5]. Many patients with aPL antibodies remain asymptomatic [2].

An important factor in APS immunopathogenesis is dysregulation of cytokine balance with increased synthesis of proinflammatory cytokines [8, 9].

Cytokines are the most versatile system of regulation. Cytokines, being synthesized at the inflammation site, affect virtually all cells involved in the inflammation development, as well as granulocytes, macrophages, fibroblasts, endothelial cells, epithelium cells, T and B lymphocytes [10]. The inflammatory processes are controlled by the proinflammatory (IL-1, IL-2, IL-6, IL-8, IL-12, TNF-α, IFN) and anti-inflammatory (IL-4, IL-10, TGF) cytokines [11]. Therefore, the study of pathobiochemical mechanisms of APS development, particularly establishment of the role of the cytokine system in development of this pathology, and search for effective methods of its treatment is an urgent and social issue [1, 6, 11, 12].

One of the links that are involved in the mechanisms of APS development is the nitric oxide (NO) system. In obstetric APS, the synthesis and bioavailability of nitric oxide (NO), which is involved in the regulation of vascular tone and blood coagulation properties, are impaired in the endothelium [4]. According to Cella M [13], a decrease in NO levels causes abortion and premature birth. On the other hand, NO overproduction mediated with inducible NO synthase (iNOS) increases uterine contractions and the risk of miscarriage [13]. Contradictions of the existing information on the involvement of the NO system in APS development as well as on the efficacy of NO precursors in reducing the manifestations of this pathology necessitates further study of the role of this system in APS.

The objective of research is to investigate the effect of combined use of L-arginine and aminoguanidine on cytokine profile (concentration of IL-1β, IL-6, TNF-α, IL-4, IL-10) in experimental antiphospholipid syndrome.

**Methods**

Female BALB/c mice, which were kept on a standard vivarium diet, were used in the research. The experiments were carried out following the principles of bioethics according to the “General Ethical Principles of Animal Experiments”, approved by the First National Congress on Bioethics (Kyiv, 2001) and in accordance with the provisions of the “European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes” (Strasbourg, 1986) and EU Directive 2010/63 EU for animal experiments.

APS was modeled using cardiolipin (Sigma, USA), which was injected intramuscularly four times (30 μg per 1 injection, the injection interval was 14 days) [14]. To enhance the effectiveness of the immune response, cardiolipin was emulsified in 75 μl of complete Freund’s adjuvant (first injection); subsequent injections were performed with incomplete Freund’s adjuvant. APS was developing for 2 weeks after the last cardiolipin injection.

The experimental animals were divided into 4 groups: the 1st – the intact; the 2nd – the BALB/c mice with APS; the 3rd – the animals with APS administered with aminoguanidine, the 4th – the animals with APS administered with L-arginine in combination with aminoguanidine. L-arginine (Sigma, USA, 25 mg/kg) and aminoguanidine (Khimlaboratorreaktiv, Ukraine, 10 mg/kg) were administered intraperitoneally once a day for 10 days after APS development. The animals of the control group were managed with the same volumes of the solvent intraperitoneally. In 10 days after confirmation of APS the animals were taken out of the experiment by thiopental sodium anesthesia (intraperitoneal administration of 1% solution at a dose of 50 mg/kg of animal body weight).

The concentration of cytokines IL-1β, IL-6, TNF-α, IL-10, IL-4 in the serum of BALB/c mice was determined by enzyme immunoassay using standard kits adapted for mice of Express Biotech International, USA (Mouse IL-1β ELISA Assay, Mouse IL-6 ELISA Assay, Mouse TNF-α ELISA Assay, Mouse IL-10 ELISA Assay, Mouse IL-4 ELISA Assay). The concentration of cytokines was expressed in pg/ml.

Statistical processing of digital data was performed by means of Excel software (Microsoft, USA) and STATISTICA 6.0 (Statsoft, USA) using non-parametric methods of estimation.
for the attained data. The arithmetic mean (M), its variance and standard error of the mean (m) were assessed for all parameters. The significant difference between the independent quantitative values was determined using the Mann-Whitney test. The changes were statistically significant at p≤0.05.

**Results**

According to the attained results, an increase of the concentration of IL-1β in 3.2 times (p<0.001) was proved in the BALB/c mice with APS compare to the control (Fig. 1).

An increase in the concentration of IL-6 in 2.3 times (p<0.001) in the serum of the animals with APS was evidenced compare to the intact animals (Fig. 2).

TNF-α concentration increased in 4.5 times (p<0.001) in the serum of the BALB/c mice with APS compare to the control (Fig. 3). At the same time, the anti-inflammatory cytokine IL-4 concentration reduced in 1.9 times (p<0.05) compare to the control group; IL-10 – in 2.2 times (p<0.001) compare to the control (Fig. 4-5).

The administration of aminoguanidine, a selective iNOS inhibitor, did not cause significant changes concentrations of IL-1β and IL-6 in the serum of the BALB/c mice with APS compare to the control (Fig. 1-2). In the case of aminoguanidine and aminoguanidine (M±m, n=10).

Fig. 1. IL-1β content in the blood serum of the BALB/c mice with APS in the case of administration of L-arginine and aminoguanidine (M±m, n=10).

**Conventional name of animal groups:** 1 – Control; 2 – Antiphospholipid syndrome (APS); 3 – APS + aminoguanidine; 4 – APS+L-arginine+aminoguanidine.

* – p<0.05 compare to the control group;

** – p<0.05 compare to the group of animals with APS.

Fig. 2. IL-6 content in the blood serum of the BALB/c mice with APS in the case of administration of L-arginine and aminoguanidine (M±m, n=10).

According to the attained results, an increase of the concentration of IL-6 in 2.3 times (p<0.001) in the serum of the animals with APS was evidenced compare to the intact animals (Fig. 2).

Fig. 3. TNF-α content in the blood serum of the BALB/c mice with APS in the case of administration of L-arginine and aminoguanidine (M±m, n=10).

Fig. 4. IL-4 content in the blood serum of the BALB/c mice with APS in the case of administration of L-arginine and aminoguanidine (M±m, n=10).

Fig. 5. IL-10 content in the blood serum of the BALB/c mice with APS in combined administration of L-arginine and aminoguanidine (M±m, n=10).
nidadine use, a significant decrease in the concentration of TNF-α by 57% (p<0.001) was evidenced compared to the intact animals (Fig. 3). It was found out that, under the influence of aminoguanidine, the concentrations of IL-6 and IL-10 did not change significantly compared to the control group of animals (Fig. 4-5).

In the case of administration of the predecessor of the synthesis of NO L-arginine in combination with aminoguanidine, a significant decrease in the concentration of IL-1β by 30% (p<0.01), IL-6 by 16% (p<0.05), TNF-α by 59% (p<0.001) was established compared to the control (Fig. 1-3). At the same time, the concentration of anti-inflammatory cytokines IL-4 increased by 35% (p<0.01) and IL-10 – by 25% (p<0.005) compared to the control animals (Fig. 4-5).

The results of the study proved that a significant decrease in the concentration of IL-1β by 22% (p<0.05), IL-6 by 23% (p<0.005) was evidenced in the case of combined administration of L-arginine and aminoguanidine compared to the indicators of the 3rd group of animals, which were administered with aminoguanidine (Fig. 1-2). An increase of the anti-inflammatory cytokine IL-4 concentration by 32% (p<0.05) and IL-10 by 19% (p<0.05) was proved compared to the 3rd group of the BALB/c mice administered with aminoguanidine (Fig. 4-5).

**Discussion**

Besides the pathogenic role of the aPL, pro-inflammatory cytokines and chemokines are significant in the pathogenesis of APS [12]. IL-1, TNF-α and endotoxins induce tissue factor (TF) expression in endothelial cells, monocytes, macrophages promoting blood clotting [10, 15]. The inhibitors of IL-1 production are IL-4, IL-10, IL-12, TNF-α [16]. IL-6 is involved in regulation of T and B cell interactions, macrophage, endotheliocys activity. IL-6 induces production of acute-phase proteins, stimulates hematopoiesis and platelet formation [16].

The attained results on increased concentrations of proinflammatory cytokines (IL-1β, IL-6, TNF-α) in the serum of the experimental animals with APS conform with the literature [6, 17, 18]. According to N.V. Seredavkina [6], an increased concentration of IL-6 and TNF-α in the patients with APS compared to the control group was established. It is not clear whether aPL affect endothelial cells directly or through TNF-α. Regardless of the mechanism, the prothrombotic condition, typical of APS, is associated with both significantly increased aPL levels as well as high TNF-α concentration [11]. According to J. Swadzba et al. [15] TNF-α is one of the main proinflammatory cytokines in APS; its level is increased and reflects pathological processes in endothelial cells. According to the literature, aPL and TNF-α can activate the endothelium and induce prothrombotic phenotype of endothelial cells, leading to increased thrombin production. Activation of endothelial cells causes upregulation of TF, which has been suggested to be a major potential mechanism of APS-related thrombosis. Once endothelial cells are activated, TF regulation can be more enhanced by a synergizing effect of TNF-α and factor Xa, thus expression of adhesion molecules (ICAM-1, VCAM-1, E and P selectins) and formation of endothelial microparticles take place [15].

According to A. Farzaneh-Far et al. [17], who investigated the levels of CRP IL-6, ISAM-1, pTNF α-P2, pTNF α-P2 in the patients with SLE, only increased pTNF α-P1 and pTNF α-P2 were associated with aPL positivity. According to NV Seredavkina a negative correlation between CRP and IgG β2GP1 levels was established [6]. R.R. Forastiero et al. [18] established that IL 6 levels were greater in the patients with APS and aPA carriers than in the control group. TNF concentration was the same in the patients with APS and aPA carriers but higher than in the control group. In the patients with positive aPA, a direct correlation between IL 6 and TNF α was proved [18]. Under the experimental conditions it has been established that TNF-α may manifest antiplatelet and antithrombotic activity [15].

According to the literature, IL-1β activates the synthesis of IL-6, S100B, α1-antitymoptrysin, inducible nitric oxide synthase (iNOS) causing increased NO synthesis [19, 20]. The iNOS is crucial in the primary proinflammatory response in macrophages [21]. AG is a nucelophilic hydrazine compound, structurally similar to L-arginine in that these compounds contain two chemically equivalent guanidino nitrogen groups and to L-arginine analogues that competitively inhibit NO synthase. AG completely prevents inflammatory stimuli induced formation of NO, and it is a potent inhibitor of the cytokine-inducible isofrom NOS [22].

The results of our studies proved that introduction of aminoguanidine, a selective iNOS inhibitor, did not cause significant changes in the concentration of proinflammatory cytokines (IL-1β and IL-6) and anti-inflammatory cytokines (IL-4 and IL-10) in the serum of the BALB/c mice.
with APS compare to the control group of animals. At the same time, in the case of aminoguanidine administration a significant decrease in TNF-α concentration was proved compare to the intact animals. According to EI Ferreira et al. [23] aminoguanidine decreases TNFα levels, oxidative stress indicators, and NO metabolites.

It is established that increased concentrations of TNF-α are associated with pregnancy miscarriage in APS [6, 8], endothelioocytes activation, and chemokine amplification that leads to subendothelial leukocyte accumulation, endothelial dysfunction, microcirculation disturbances [16].

Early endothelial dysfunction was observed in APS [24]. Patients with APS displaying thrombosis exhibited low plasma levels of nitrites and nitrates, which are the stable metabolites of NO breakdown. aPL can act as antagonists of endothelial nitric oxide synthase (eNOS) through β2GPI, and this interaction may impair NO synthesis. In particular, attenuation of eNOS activation by aPL was mediated by reduced phosphorylation of eNOS serine. This inhibition of eNOS phosphorylation was shown to be dependent upon protein phosphatase 2A, β2GPI, and apolipoprotein E receptor 2. aPL inhibition of eNOS activity contributes to thrombus formation, increased leukocyte adhesion, and alterations in vascular tone [4].

It is established that violation of the bioavailability of NO may be one of the causes of endothelial dysfunction. This may be associated with both the lack of substrate for NO L-arginine synthesis as well as formation of superoxide anion which rapidly binds and inactivates NO [24].

NO synthesis is not dependent on L-arginine concentration in physiological states. In pathological conditions, the availability of L-arginine may determine production of NO. It is proved that L-arginine is necessary for adequate translation of iNOS. When iNOS is being activated, superoxide anion is produced, which forms a highly reactive peroxynitrite, which in turn produces nitrosylation of amino acid residues sensitive to it, especially tyrosine, that leads to conformational changes in the structure of protein molecules. With administration of L-arginine the functional characteristics of T-cells enhance, production of antibodies increases as well. NO-dependent effect of L-arginine on the immune system may be hormone-mediated [25].

The next objective of our study was to investigate the effect of combined use of L-arginine and aminoguanidine on cytokine profile in APS.

In the case of the use of the precursor of NO L-arginine synthesis in combination with aminoguanidine, a selective iNOS inhibitor, a significant decrease in the concentration of proinflammatory cytokines (IL-1β, IL-6, TNF-α) and an increase in the concentration of anti-inflammatory cytokines (IL-4, IL-10) was established compare to the control group of animals. The attained results are consistent with the literature [25, 26]. These effects can be explained by the fact that glutamine formed from L-arginine is a conditionally essential amino acid and reduces the level of TNF-α soluble receptors [26]. According to VM Sheibak et al. [25] administration of L-arginine decreases the level of IL-6.

According to P. Soltesz et al. [12], besides the conventional Th1 pathway, Th2 cytokines are crucial in the mechanisms of APS development, i.e. IL-4 and IL-10. Various immunocompetent cells regulate the proinflammatory cascade that leads to cytokine imbalance and activated circulating lymphocytic pool in APS. This proinflammatory process leads to endothelial dysfunction, development of arterial and venous thrombosis [12]. As a result of the research, a decrease in anti-inflammatory cytokines (IL-4, IL-10) in APS was established; the results are consistent with the literature [11, 12]. According to P. Soltesz et al. [12], the markers of endothelial dysfunction positively correlate with IL-4 levels in APS. It allows suggesting that by activation of the humoral and cellular immune responses, IL-4 is crucial in development of endothelial dysfunction, atherosclerosis, arterial and venous thrombosis. IL-4 stimulates B and T cell proliferation as well as differentiation of CD4 + T cells into Th2 cells [12].

According to A. Menachem et al. [11] cytosolic and secreted IL-10 and IFN-γ levels in eAPS mice were lower at 6 and 15 weeks and higher at 24 weeks after immunization compared to adjuvant mice. IL-10 is significant in autoimmune diseases. As a result of other studies, IL-10 level was decreased in the serum of the patients with APS [12].

IL-10 inhibits secretion of IL-4, IL-5 and IFN-γ, growth factors and chemokines, and therefore acts as a key counter-regulator of autoimmune processes [12]. One of the functions of IL-10 is inhibition of the synthesis of proinflammatory cytokines: IL-1, IL-6, IL-12 and TNFa via a STAT3-dependent mechanism [27] and enhancement of IL-1 receptor antagonist
expression [19]. Decreased level of IL-10 in the serum in cases of APS compare to the control confirms the fact that IL10-mediated processes are impaired in APS that is why it leads to vascular damage [12].

Low IL-10 levels enable TNF-α unregulated production, resulting in procoagulant state. Decreased IL-10 levels can be associated with lymphocyte activation, which leads to the continuation of the autoimmune response. During the B-cell activation, IL-10 delivers signals that promote the apoptosis of B cells [11].

**Conclusions**

Thus, that in the serum of BALB/c mice with APS, an increase in the concentrations of proinflammatory cytokines (IL-1β, IL-6, TNF-α) and a decrease in the concentrations of anti-inflammatory cytokines (IL-4 and IL-10) was established compare to the control parameters. With the introduction of aminoguanidine, a selective iNOS inhibitor, a decrease in the concentration of TNF-α was proved compare to that of the animals with APS. In the case of the use of the precursor of NO synthesis L-arginine in combination with aminoguanidine, a significant decrease in concentrations of IL-1β, IL-6, TNF-α and an increase of IL-4 and IL-10 was evidenced compare to the group of BALB/c mice with APS and the group of animals administered with aminoguanidine.

**Funding**

This research received no external funding.

**Conflict of Interests**

The authors declare no conflict of interest.

**Authors Contributions**

Висновки. Встановлено, що комбіноване застосування попередника синтезу NO L-аргініну та селективного інгібітора іNOS аміногуаніду призводить до зниження концентрації IL-1β, IL-6, TNF-α та зростання IL-4 та IL-10, порівняно з показниками групи миші BALB/c з АФС та групи тварин, яким вводили аміногуанідин.

КЛЮЧОВІ СЛОВА: антифосфоліпідний синдром; цитокіни; оксид азоту; L-аргінін; аміногуанідин

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doi: 10.14412/1995-4484-2010-729


doi: 10.1016/j.jimmunol.174.1.485


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doi: 10.4049/jimmunol.1501923

Received 04 September 2019; revised 14 October 2019; accepted 25 October 2019.

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