

Results of Complex Immunopharmacological Analysis of the Mechanisms of Action of Bioregulation Agents

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Elucidation of the pharmacodynamic mechanisms of drugs capable of potentiating the effects of non-steroidal anti-inflammatory drugs is an important task. In this *in vitro* study, the ability of Traumeel S to influence the innate and acquired immunity was evaluated. Traumeel S was found to reduce activities of NADPH oxidase and neutrophil extracellular traps, as well as to evoke anti-inflammatory activity of lymphocyte subpopulations.

Key Words: *neutrophil extracellular traps; lymphocytes; inflammation; Traumeel S; non-steroidal anti-inflammatory drugs*

Until now, the problem of rational pharmacotherapy for infectious and non-infectious inflammatory diseases is highly relevant [1-5]. Non-steroidal anti-inflammatory drugs (NSAIDs) are most often used to treat inflammatory diseases; in the immunological terms, NSAIDs act by suppressing inflammatory mediators. In this mechanism, NSAIDs interact directly with the inflammatory mediator molecule rather than with the immune cells that produce these mediators [2,3,6].

In light of this, identification of pharmacological agents capable of modulating activity of mediator-producing immune cells and showing no drug-drug interactions with NSAIDs, without strict restriction of the duration of application and with a wide spectrum of indication seems important [7-9]. Such immunomodulatory drugs are suggested to act synergistically with NSAIDs and improve their effectiveness [10,11].

In this scenario, the pharmacological target is neutrophils, the most numerous white blood cells. In the inflammation focus, neutrophils destroy microorganisms using a number of mechanisms, mainly through phagocytosis, release of antimicrobial substances, and formation of neutrophilic extracellular traps (NET) [10,12,13]. Though the mechanism of NET formation

is not fully understood, their function is known to depend on neutrophilic NADPH oxidase activity [10,13]. Therefore, this study was aimed at evaluation of activity of neutrophilic NADPH oxidase in the presence of Traumeel S.

Other important components of the inflammatory process are lymphocytes, the main cells of adaptive (acquired) immunity. Analysis of the expression of lymphocyte surface receptors allows detecting the inflammation-induced immunological changes and their modification under the influence of the selected pharmacological agents.

Here we studied NADPH oxidase activity in inflammation-activated neutrophils and formation of NETs under the influence of Traumeel S and diclofenac (control) and evaluated the effect of Traumeel S on the population and subpopulation composition of cultured blood lymphocytes taken from patients with acute infectious inflammation.

MATERIALS AND METHODS

Neutrophil and leukocyte fractions isolated from patients with infectious inflammation were used in the study. Venous blood (10 ml) taken from patients was placed in siliconized test tubes with EDTA (anticoagulant).

Neutrophilic granulocytes were isolated from the venous blood of 12 patients with acute inflam-

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matory processes characterized by pronounced leukocytosis. The cells were isolated by centrifugation (30 min at 1600 rpm) in double Ficoll gradient (1.077 and 1.095 g/ml). The interphase ring containing neutrophilic granulocytes was collected, transferred to centrifugation tubes, and twice washed from Ficoll with sodium-phosphate buffer solution (15 min at 1200 rpm). Neutrophils were aseptically placed in RPMI-1640 medium (final cell concentrations 2×10^5 /ml) and incubated with the test agents at 5% CO₂ at 37°C.

NADPH oxidase activity was evaluated using the NBT test. Fluorescence microscopy was used to detect and count the NETs. The technique was developed in our laboratory (application for the Patent of the Russian Federation No. 2021104936). The results were expressed as the percent ratio of the number of NETs to the total number of neutrophils.

For *in vitro* experiments with neutrophils, commercial preparation Traumeel S (sterile solution; Biologische Heilmittel Heel) was diluted by factors of 10³, 10⁴, 10⁵, and 10⁶. Diclofenac served as a reference drug (final concentration 3 µg/ml). Neutrophils incubated in the absence of the test drugs served as the control.

Lymphocyte fractions were isolated from the venous blood of 20 healthy donors and 6 patients with acute inflammation. The cells were isolated by density gradient centrifugation according to A. Böyum (1968) under sterile conditions. The content of lymphocytes expressing surface antigens CD3, CD4, CD8, CD16, CD20, CD72, CD38, CD25, CD71, HLA-DR, CD95, and CD54 in samples of peripheral blood or *in vitro* cultures was determined in the reaction of indirect immunofluorescence using appropriate monoclonal antibodies.

Lymphocyte suspensions (2×10^5 cells/ml) were incubated with Traumeel S (dilution 10³) for 2 h at 5% CO₂ at 37°C.

The results were processed statistically, significance of differences was evaluated using the Student's *t* test (for big samples) or Wilcoxon and Mann—Whitney test (small samples and the data that did not fit normal distributions, as well as for pairwise comparisons. The results are presented as $M \pm m$; the difference from the control were considered significant at $p < 0.05$.

RESULTS

Innate immunity system. The incubation with Traumeel S in all specified doses significantly reduced the content of NBT⁺ neutrophils and NETs in comparison with the control (Table 1). Thus, Traumeel S treatment reduced the number of NETs, which from a clinical point of view, indicates a direct anti-in-

flammatory effect and control over the overreacting immune system.

Acquired immunity system. Analysis of changes in the population and subpopulation composition of peripheral blood lymphocytes from patients with acute infectious processes under the effect of Traumeel S revealed significant ($p < 0.05$) differences from the control.

The proportion of T lymphocytes with cytotoxic function (CD8⁺ cells) in patients with infectious inflammation was reduced to $23.63 \pm 1.18\%$ (vs $26.18 \pm 0.96\%$ in healthy donors). Traumeel S had practically no effect on this parameter: the number of CD8⁺ cells was $22.99 \pm 1.26\%$.

In healthy subjects, the subpopulation of activated NK cells (CD16⁺ lymphocytes) was relatively small ($4.78 \pm 0.52\%$). In patients with infectious inflammation, the number of CD16⁺ cells increased to $8.99 \pm 1.30\%$, while under the influence of Traumeel S, this parameter practically returned to normal ($4.26 \pm 0.41\%$).

The number of cells expressing HLA-DR antigen (marker of mature cells) was not changed in patients with acute infection ($11.85 \pm 1.10\%$) in comparison with healthy subjects ($11.68 \pm 0.52\%$). However, in the presence of Traumeel S, the number of HLA-DR⁺ cells increased to $16.33 \pm 0.65\%$. These findings suggest that the drug can stimulate lymphocyte differentiation.

In patients with acute infection, the number of cells expressing CD95 receptor (marker of pre-apoptosis) 2-fold surpassed the corresponding level in healthy subjects (9.33 ± 0.69 and $4.66 \pm 0.38\%$, respectively). In the presence of Traumeel S, this indicator tended to decrease ($7.06 \pm 0.68\%$).

The number of activated B cells (CD23⁺ cells) in patients with infection increased from 5.41 ± 0.61 to $9.16 \pm 1.12\%$, which could indicate the involvement of the B-cell immune system into the development of inflammation. In the presence of Traumeel S, the fraction of activated B cells increased to $11.33 \pm 1.09\%$, *i.e.*

TABLE 1. Effect of Traumeel S in Different Dilutions on the Number of NBT⁺ Cells and NET ($M \pm m$)

Group	Number of NBT ⁺ cells, %	Number of NET, %
Control	31.11 ± 3.40	24.02 ± 2.05
Diclofenac	N.d.	$16.55 \pm 1.13^*$
Traumeel S 10 ³	$23.45 \pm 1.56^*$	$6.15 \pm 0.82^*$
10 ⁴	$12.45 \pm 0.95^*$	$5.03 \pm 0.67^*$
10 ⁵	$8.02 \pm 0.84^*$	$2.98 \pm 0.75^*$
10 ⁶	$10.52 \pm 1.47^*$	$4.18 \pm 0.85^*$

Note. N.d. — nor determined. * $p < 0.05$ in comparison with the control.

almost 2-fold surpassed the level observed in healthy controls.

Another functional lymphocyte marker CD54 (ICAM-1) correlated with the severity of the inflammatory process, because CD54 expression is known to be induced by such cytokines as interleukin IL-1 β and TNF α [14]. In patients with infectious inflammatory process, the number of CD54⁺ cells was consistently increased by almost 2-fold in comparison with the reference values (to 10.37 \pm 0.42% vs 5.49 \pm 0.98% in controls). Traumeel S suppressed the expression of the CD54 receptor and practically normalized this parameter. These data lead us to a conclude that Traumeel S can reduce the severity of inflammation presumably by inhibiting the action of inflammatory cytokines.

The HLA-DR/CD95 ratio after incubation with Traumeel S was 2.31, *i.e.* close to the value typical of healthy subjects (2.50), which indicates normalization of the activation processes in the immune system.

The revealed effect of Traumeel S on the cells of innate and adaptive immunity reflects the clinical role of Traumeel S pharmacotherapy in various types of inflammatory process.

In chronic inflammation (aseptic or infectious), the control of innate immune cells is more important, because they can induce more potent (in comparison with adaptive immunity cells) toxic effect on healthy tissues, which ultimately can lead to degenerative processes in tissues and dysfunction.

In acute aseptic inflammation, controlling the behavior of innate immune cells is also important. Similar to chronic inflammation, anti-inflammatory drugs reducing activity of neutrophils should be used.

In acute infectious inflammation, the role of acquired immunity cells becomes more important than cells of innate immunity, because the reactivity of cellular components of the immune system, namely T helpers, NK cells, and B lymphocytes against pathogens is a decisive factor. Our experiments showed that Traumeel S stimulates the antigen-presenting function of T cells (which can facilitate the recognition and neutralization of the pathogen) and reduces activity of NK cells associated with undesirable toxic effects on healthy tissue. Thus, the use of Traumeel S protects intact cells in the focus of inflammation and prevents tissue damage and dysfunction. It is known that the decrease in the level of CD54 (ICAM) indicates the end of acute inflammation. B lymphocytes are a key factor in resolution of prolonged acute inflammation. An increase in the level of immunoglobulins is a marker of B lymphocyte activity. The trend to an increase in the level of CD72 in the presence of Traumeel S indicates a shift from the proinflammatory to anti-inflammatory profile at late stages of acute inflammation.

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