EFFECT OF $\beta$-1,3/1,6-D-GLUCAN ON THE PHAGOCYTIC ACTIVITY AND OXIDATIVE METABOLISM OF PERIPHERAL BLOOD GRANULOCYTES AND MONOCYTES IN RATS

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Abstract

The experiment was performed on 20 adult Wistar rats aged 12 weeks, divided into two equal groups (control and experimental), each comprised of five males and five females. From the first day of the experiment, the experimental group rats were fed Murigran feed supplemented with $\beta$-1,3/1,6-D-glucan at a dosage of 12-19 mg/rat/d, subject to body weight, while the control-group was administered the same feed without any additives. At the beginning of the experiment and then after 14 days, arterial blood samples were collected from the rats and diluted with heparin to measure and compare the phagocytic activity and oxidative metabolism of peripheral blood granulocytes and monocytes by flow cytometry. Statistically higher levels of the activity were observed in the group of rats administered glucan than in controls, expressed in terms of the percentage of phagocytic cells as well as average fluorescence intensity. $\beta$-1,3/1,6-D-glucan also had a positive effect on the oxidative metabolism of both granulocytes and monocytes after stimulation with $E$. coli, and on the oxidative metabolism of granulocytes after stimulation with PMA.

Key words: rats, granulocytes, monocytes, $\beta$-1,3/1,6-D-glucan, phagocytic activity, oxidative metabolism.

Immunomodulation is a process, which stimulates or suppresses the immune system's response to the administered drugs or chemical compounds (14). The above applies to all pharmacological products and xenobiotics affecting the immune system. Subject to the induced effect, immunomodulators are divided into immunostimulators, which augment or restore the immune response, and immunosuppressants that suppress or inhibit the immune response (10, 22, 28). In view of their biological origin, immunomodulators are further divided into standard animal proteins and isolated substances purified of microbes and post-culture bacterial liquid (33).

Synthetic and natural immunomodulators are widely applied in the prevention and treatment of infections, non-infectious diseases, and neoplastic diseases, while some immunomodulators that suppress immune system reactivity (pharmacological immunosuppression) are used in transplantology, thus furthering the development of this medicinal science (9, 28). These types of drugs are also used in the treatment of autoimmunological diseases.

The significance of immunostimulation is growing, due to the immunosuppressive impact of environmental pollution and to the suppressive effect of popular drugs on immunity factors in animals (9). Immunostimulators have a stimulating effect on non-specific cellular and humoral immune mechanisms and a specific immune response. There is a very broad range of known and applied immunostimulators, but the search for new ones continues. Researchers aim to obtain compounds with a stronger effect that eliminate or significantly minimise the resulting side effects. The intensity of the desired effect is largely dependent on the length of the immunomodulator's retention in the body. For this reason, scientists are searching for derivatives of known modulators, which are not quickly eliminated (17).

The objective of this study was to determine the effect of $\beta$-1,3/1,6-D-glucan on the phagocytic activity and oxidative metabolism of peripheral blood granulocytes and monocytes in rats.
Material and Methods

The experiments were carried out in conformance with the Animal Protection Law and the recommendations of the Animal Ethics Committee of the University of Warmia and Mazury in Olsztyn. During the experiments, animals were kept on Faculty premises with the observance of adequate experimental conditions.

Experimental design. The experimental material comprised 20 adult Wistar rats aged 12 weeks, divided into two equal groups (control and experimental), consisting of five males and five females each. Over a period of two weeks starting on the first day of the experiment, the experimental group rats were fed Murigran feed supplemented with β-1,3/1,6-D-glucan (Biolex Beta-HP) at a dosage of 12-19 mg/rat/d, subject to body weight, while the control group animals were administered the same feed without any additives. At the beginning of the experiment and then after 14 days, arterial blood samples were collected from rats and diluted with heparin.

Measurement of phagocytic activity. The phagocytic activity of granulocytes and monocytes was determined with the use of Phagotest (Orpegen Pharma). All test reagents were prepared in accordance with the manufacturer’s recommendations. One hundred microlitres of whole blood chilled to 0ºC and 20 µl of chilled bacteria were added to each of the two 5 ml test tubes (control and experimental) and shaken for around 3 s at low speed. The experimental sample was incubated for 10 min at 37ºC, and the control sample in an ice bath at 0ºC. After incubation, 100 µl of quenching solution was added to each sample and the samples were shaken. Three millilitres of washing solution chilled to 0ºC were added, the samples were centrifuged for 5 min at 4ºC (250 x g), and the supernatant was removed. The rinsing procedure was performed twice, and 2 ml of lysing solution at room temperature were added. Test tubes were shaken and incubated at room temperature for 20 min. All samples were centrifuged for 5 min at 4ºC (250 x g), and the supernatant was removed. All test tubes were rinsed once with 3 ml of washing solution, centrifuged for 5 min at 4ºC (250 x g), and then the supernatant was removed. Two hundred microlitres of staining solution chilled to 0ºC was added to each sample, and test tubes were shaken and incubated for 10 min in an ice bath. Intracellular killing activity of phagocytes was determined in a cytometer (Beckmann Coulter, Epics XL) in less than 30 min after the last reagent had been added. Three activators were used for cell stimulation: E. coli strains, PMA (4-phorbol-12-β-myristate-13-acetate) as the strong activator, and fMLP as the weak activator. The added dihydrodorodamine (123-DHR) was oxidised in mitochondria by H₂O₂ resulting from cell stimulation and was converted to cation rhodamine 123 (R123), the fluorescent emitter.

The obtained results were processed statistically in a one-factorial analysis of variance in an orthogonal design. The significance of differences between groups was verified by the Student’s t-test with the use of GraphPad Prism 5 software.

Results

The analysis of the obtained results has shown that the phagocytic activity of granulocytes was significantly higher (P<0.05) in the group of rats administered β-1,3/1,6-D-glucan than in control, both in terms of the percentage of phagocytising cells and average fluorescence intensity. The percentage of phagocytic monocytes and their phagocytic activity, expressed in terms of average fluorescence intensity, was also statistically higher in the group of rats stimulated with the glucan than in controls (Table 1).

β-1,3/1,6-D-glucan also had a positive effect on the oxidative metabolism of granulocytes after stimulation with E. coli, where the percentage of stimulated cells was significantly higher in this group than in the control, while average fluorescence intensity, although higher in the group of rats stimulated with β-1,3/1,6-D-glucan than in control, did not produce statistically significant differences (Table 2).
## Table 1
Percentage of phagocytic granulocytes and monocytes and average fluorescence intensity of granulocytes and monocytes in each rat group measured in the Phagotest

<table>
<thead>
<tr>
<th></th>
<th>Granulocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phagocytic cells (%)</td>
<td>Average fluorescence intensity</td>
</tr>
<tr>
<td>Control</td>
<td>82.22 ±0.54</td>
<td>31.22 ±2.15</td>
</tr>
<tr>
<td>β-glucan</td>
<td>93.64 ±0.95**</td>
<td>46.55 ±1.55***</td>
</tr>
</tbody>
</table>

** - P≤0.01; *** - P≤0.001

## Table 2
Average intracellular killing activity of granulocytes and average fluorescence intensity in each rat group after stimulation with fMLP, PMA, and *E. coli* as determined in the Bursttest

<table>
<thead>
<tr>
<th></th>
<th>Granulocytes</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fMLP</td>
<td>PMA</td>
<td>E. coli</td>
</tr>
<tr>
<td>% stimulated</td>
<td>% stimulated</td>
<td>% stimulated</td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>average</td>
<td>average</td>
<td></td>
</tr>
<tr>
<td>average</td>
<td>fluorescence intensity</td>
<td>fluorescence intensity</td>
<td>fluorescence intensity</td>
</tr>
<tr>
<td>Control</td>
<td>21.51 ±1.26</td>
<td>1.53 ±0.44</td>
<td>83.32 ±1.90</td>
</tr>
<tr>
<td></td>
<td>82.58 ±2.80</td>
<td>13.22 ±0.43</td>
<td></td>
</tr>
<tr>
<td>β-glucan</td>
<td>19.57 ±0.84</td>
<td>2.04 ±0.40</td>
<td>90.71 ±1.10</td>
</tr>
<tr>
<td></td>
<td>89.99 ±0.46</td>
<td>11.92 ±0.44</td>
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</table>

* - P<0.05; ** - P<0.01

## Table 3
Average intracellular killing activity of monocytes and average fluorescence intensity in each rat group after stimulation with fMLP, PMA, and *E. coli* as determined in the Bursttest

<table>
<thead>
<tr>
<th></th>
<th>Monocytes</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>fMLP</td>
<td>PMA</td>
<td>E. coli</td>
</tr>
<tr>
<td>% stimulated</td>
<td>% stimulated</td>
<td>% stimulated</td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td>average</td>
<td>average</td>
<td></td>
</tr>
<tr>
<td>average</td>
<td>fluorescence intensity</td>
<td>fluorescence intensity</td>
<td>fluorescence intensity</td>
</tr>
<tr>
<td>Control</td>
<td>8.6 ±1.20</td>
<td>7.83 ±1.10</td>
<td>45.78 ±1.24</td>
</tr>
<tr>
<td></td>
<td>42.57 ±1.30</td>
<td>7.98 ±0.72</td>
<td></td>
</tr>
<tr>
<td>β-glucan</td>
<td>9.23 ±0.90</td>
<td>9.12 ±0.52</td>
<td>47.56 ±1.40</td>
</tr>
<tr>
<td></td>
<td>51.79***±0.72</td>
<td>10.75**±0.40</td>
<td></td>
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</tbody>
</table>

** - P<0.01; *** - P<0.001

Following monocyte stimulation with *E. coli*, oxidative metabolism, expressed as the percentage of stimulated cells and average fluorescence intensity, was significantly higher in the group of rats stimulated with β-glucan than in control (Table 3). A similar effect to that noted after granulocyte and monocyte stimulation with *E. coli* was observed after PMA stimulation (strong respiratory-burst activator). Although the level of oxidative metabolism of granulocytes and monocytes was higher in the group of rats administered β-1,3/1,6-D-glucan than in the control (Tables 2 and 3), in both cases a statistically-significant increase was reported only in reference to the percentage of stimulated granulocytes in the group of animals receiving β-glucans (Table 2).

Although the investigated parameters were higher in the group stimulated with β-1,3/1,6-D-glucan than in the control group after granulocyte and monocyte stimulation with fMLP (weaker respiratory-burst activator), statistically significant differences were not observed in the percentage of stimulated granulocytes and monocytes or the intensity of those cells' oxidative metabolism (Tables 2 and 3).

### Discussion

The preventive and therapeutic effectiveness of drugs modulating the immune system of various animal species has been investigated in numerous laboratory studies and clinical trials for many years. A special
attention should be paid to biostimulants (18, 28), immunostimulants of natural origin, which may be effectively used in organic animal farms where the animals' diets are not supplemented with synthetic stimulants. Organic farming shows a preference for non-invasive treatment methods, including the administration of immunity stimulators, wherever possible, in the most convenient form of feed supplements. One of the most popular feed supplements are β-glucans (23, 28, 29, 38), including β-1,3/1,6-D-glucan, which is found in the cell wall of Saccharomyces cerevisiae yeast species. The branched β-1,3/1,6-glucan polysaccharide found in the Biolex-Beta HP product has the structure of a β-1,3 main chain connected to a β-1,6 side chain. This polysaccharide is isolated from mannoproteins without the use of aggressive alkalines or acids in the hydrolysis process, and it occurs in an unmodified, natural form the use of aggressive alkalines or acids in the hydrolysis process, and it occurs in an unmodified, natural form.

β-glucan has the structure of a Biolex-Beta HP product has the structure of a β-1,3 main chain connected to a β-1,6 side chain. This polysaccharide is isolated from mannoproteins without the use of aggressive alkalines or acids in the hydrolysis process, and it occurs in an unmodified, natural form that guarantees the highest level of biological activity (36). Research results have shown that β-glucans significantly stimulate the immune system to viral, bacterial, fungal, and parasitic infections of many animal species (2, 16, 27, 35, 37). β-1,3/1,6-glucan's ability to activate the immune system was investigated in detail. It was found to offer effective protection of animals against experiment conducted on mice. When β-glucan was supplied intravenously, macrophages increased the production of H2O2 and interleukin 1 (IL-1). The above was not reported in the group of mice injected with β-glucan intraperitoneally. According to Suzuki et al. (30) oral administration of β-glucan induced the production of H2O2 and IL-1 in mouse macrophages, stimulating their phagocytic activity in comparison with control. Other studies have confirmed that the above methods of β-glucan administration are also capable of stimulating respiratory-burst activity of phagocytes in fish. Intraperitoneal administration increased phagocyte RBA in different fish species, including the Atlantic salmon (Salmo salar) (1, 5), the rainbow trout (Oncorhynchus mykiss) (13), and the turbot (Psetta maxima) (25). When applied orally, yeast β-glucans increased the respiratory-burst activity of leukocytes in the rainbow trout (26), turbot (34), and North African catfish (Clarias gariepinus) (39). The results of in vivo experiments have shown that β-glucans stimulate the production of superoxide anions by macrophages in the Atlantic salmon (6, 12), the rainbow trout (19), and the turbot (8).

In this experiment, a significant increase in the percentage of phagocytic cells and average fluorescence intensity of monocytes and granulocytes (Phagotest) was observed in comparison with the control in the experimental group of rats fed a diet supplemented with β-1,3/1,6-D-glucan (Table 1). Similar results were reported by other authors in studies of mice (24), shrimps (4), and lambs (36), and they noted a 30%-50% increase in intracellular killing activity of blood phagocytes in the group of animals fed diets supplemented with β-1,3/1,6-D-glucan. In in vitro and in vivo experiments, Estrada et al. (7) have demonstrated that β-glucan isolated from oats had an immunostimulatory effect on immunocompetent cells in mice, activating, among others, mouse macrophages and boosting the animals' resistance to diseases caused by selected pathogens, including Staphylococcus aureus and Eimeria vermiformis. According to the results of the experiment conducted on mice by Szymańska-Czerwińska et al. (31) the highest level of macrophage phagocytic activity was observed after oral as well as intravenous administration of β-glucans. The exact mechanism of β-glucan's effect on the functioning of phagocytic cells has not yet been fully explained, but researchers have demonstrated that by binding to, for example, CR3 receptors or TLR (Toll-like receptors), β-glucans activate phagocytes.

In this study, β-glucan also had a positive effect on the intracellular killing activity of granulocytes and monocytes stimulated with E. coli as well as granulocytes stimulated with PMA, expressed as a higher percentage of stimulated cells and higher average fluorescence intensity (Tables 2-3). An increase in the above parameters is indicative of higher activity of phagocytes against bacteria and more effective elimination of pathogens. Similar results were reported by Wójcik et al. (36) in a study of sheep, where the respiratory-burst activity (RBA) of phagocytes increased significantly in the experimental group receiving β-glucan in comparison with the control. Research findings reported by Suzuki et al. (30) coincide with the results of this study due to increased production of hydrogen peroxide (H2O2) and increased activity of lysosomal enzymes in mouse macrophages after oral administration of β-glucan. In a study conducted by Brattgjerd et al. (1) macrophages isolated from the renal cortex and stimulated with both β-glucan and PMA increased hydrogen peroxide levels. As noted by Ohno et al. (20) and Tikunaka et al. (33) β-glucan also stimulated the production of nitric oxide (NO), another RBA factor, and activated lysosomal enzymes (29). Similar in vivo experiments carried out by Brattgjerd et al. (1) have shown that β-glucan induces and increases the phagocytic ability of macrophages to kill bacteria by raising H2O2 levels in the Atlantic salmon after stimulation with Vibrio salmonicida.

β-glucan has a varied effect on the activity of phagocytic cells, subject to the manner of administration, dosage and the period of treatment, as observed by Sakurai et al. (24) in an experiment conducted on mice. When β-glucan was supplied intravenously, macrophages increased the production of H2O2 and interleukin 1 (IL-1). The above was not reported in the group of mice injected with β-glucan intraperitoneally. According to Suzuki et al. (30) oral administration of β-glucan induced the production of H2O2 and IL-1 in mouse macrophages, stimulating their phagocytic activity in comparison with control. Other studies have confirmed that the above methods of β-glucan administration are also capable of stimulating respiratory-burst activity of phagocytes in fish. Intraperitoneal administration increased phagocyte RBA in different fish species, including the Atlantic salmon (Salmo salar) (1, 5), the rainbow trout (Oncorhynchus mykiss) (13), and the turbot (Psetta maxima) (25). When applied orally, yeast β-glucans increased the respiratory-burst activity of leukocytes in the rainbow trout (26), turbot (34), and North African catfish (Clarias gariepinus) (39). The results of in vivo experiments have shown that β-glucans stimulate the production of superoxide anions by macrophages in the Atlantic salmon (6, 12), the rainbow trout (19), and the turbot (8).
were marked by similar or even lower levels of respiratory-burst activity in comparison with the control, which was not treated with β-glucan. The response to PMA stimulation could be related to the place of its activity, *i.e.* the preserved or modified phosphorylation capacity of NADPH oxidase cytosolic factor (15). Tahir *et al.* (32) discovered that β-glucan doses lower than 1 mg/ml optimise the level of respiratory-burst activity of macrophages (*Limanda limanda, L.*) already after 24-48 h of incubation.

The results of this study pave the way for follow-up research into practical applications of β-glucans in the immunophrophylaxis in animals and humans, particularly in the restoration of impaired immunity in periods of increased incidence of bacterial and viral infections.

**References**


