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Immunomodulatory activity of exopolysaccharide from the rhizobacterium *Paenibacillus polymyxa* CCM 1465

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Abstract

Bacterial polysaccharides are promising stimulants of protective functions in humans and animals. We investigated the ability of exopolysaccharide from the rhizobacterium *Paenibacillus polymyxa* CCM 1465 to induce nonspecific resistance factors in the macroorganism. We examined in vitro the effect of the exopolysaccharide, produced with different carbon sources, on the phagocytic activity of murine macrophages, on the generation of reactive oxygen species and of enzymes (acid phosphatase and myeloperoxidase), on the proliferation of murine splenocytes, and on the synthesis of proinflammatory cytokines [interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α)] by human mononuclear cells. The exopolysaccharide promoted the phagocytosis of bacterial cells, activated metabolic processes in human and animal leukocytes, and moderately affected the production of TNF-α and IL-1β. The exopolysaccharides produced on media with glucose and sucrose differed in their effect on the immune cells, possibly owing to their different compositions, structures, and properties. The results validly indicate that the exopolysaccharide of *P. polymyxa* CCM 1465 promotes nonspecific immunity. Therefore, it can find application as a biologically active immunomodulatory substance.

Keywords *Paenibacillus polymyxa* · Exopolysaccharide properties · Immunomodulatory activity · Proinflammatory cytokines

Introduction

In the past few decades, interest in immunostimulatory therapy has increased sharply. This increase is due to problems in infection pathology and in oncology. An infection proceeds much worse if the immune system and the nonspecific protection mechanisms are damaged. Therefore, the search is ongoing for nontoxic and cheap immunomodulatory biopolymers. Bacterial polysaccharides are just such biopolymers,

as they excel in being able to affect the immunobiological reactivity of a macroorganism. Polysaccharides induce a multicomponent protective reaction, changing the level of resistivity (Moscovici 2015).

Natural polysaccharides are highly susceptible to biodegradation and are less harmful than synthetic polymers (Liang and Wang 2015). In recent years, substantial progress has been made in searching for microbial exopolysaccharides (EPSs) that possess novel and highly functional properties (Shih 2010). The surface localization of the EPSs makes them mediators of intercellular interactions with micro- and macroorganisms. Forming a dense layer on the bacterial surface, the EPSs can screen the cellular structures beneath and can determine the immunological properties of bacteria (Vasilyev et al. 1984).

There is a strong interest in *Paenibacillus* EPSs, which have diverse physiological and biotechnological functions (Jung et al. 2007; Liu et al. 2010, 2012; Wang et al. 2011; Raza et al. 2011; Rafigh et al. 2014; Liang and Wang 2015). The genus *Paenibacillus* consists of more than 200 species of facultative anaerobic, endospore-forming, neutrophilic, periflagellated heterotrophic and low G+C gram-positive

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bacilli with the type species *Paenibacillus polymyxa* (Grady et al. 2016). *P. polymyxa* has been found in various milieus, including soil, water, the rhizosphere, plant substances, fodder, insect larvae, and clinical samples (Raza et al. 2011). This bacterium is a strong producer of neutral and acidic EPSs, which have diverse structures and physicochemical properties and which are mostly low toxic or nontoxic (Jung et al. 2007; Liang and Wang 2015). The numerous reports of the production and use of *P. polymyxa* EPSs are evidence of the growing interest in these polymers as biomaterials (Jung et al. 2007; Liu et al. 2009, 2010, 2012; Raza et al. 2011; Wang et al. 2011; Rafigh et al. 2014; Liang and Wang 2015).

The soil bacterium *P. polymyxa* JB115 produces a linear glucan with a β-(1→3), (1→6)-structure, which can be used as an animal food additive to increase immunity (Jung et al. 2007). In a study of EPS activation of murine splenocytes (Chang et al. 2009), this glucan induced, in a concentration-dependent way, the synthesis of nitric oxide (NO)—an essential factor of the macroorganism's nonspecific resistance to intracellular infections. In addition, the glucan promoted the production of interleukin-6 (IL-6) and of inducible NO synthase (iNOS), an enzyme that may play a key part in NO synthesis (Chang et al. 2009). The β-glucan of *P. polymyxa* JB115 activates macrophages by way of the MAPKs and NF-κB signaling pathway (Chang et al. 2010) and can be used as an immunostimulant or an adjuvant in some animal vaccines (Chang et al. 2009).

Microbial levans, including those from *Pseudomonas* sp., *Bacillus* sp., *Zymomonas* sp., *Microbacterium* sp., *Streptococcus* sp., and *P. polymyxa*, have different biological activities and can be applied widely. They can be used in medicine as hypocholesterol, antitumor, immune modulatory, antiinflammatory, and plasma substitute agents (Yoo et al. 2004). A levan-type EPS from the endophytic bacterium *P. polymyxa* EJS-3 has scavenging effects on superoxide and hydroxyl radicals (Liu et al. 2009), and its acetylated, phosphorylated, and benzylated derivatives are promising antioxidants and antitumor agents (Liu et al. 2012).

The degree of activation of the immune system and the specific action on its elements depend on the composition and structure of the glucan administered to the organism. The interaction of such glucans with the immune system is governed by the molecule's conformation at the time of action. The biological activity of glucans also depends on many other factors, including the type and configuration of the linkages between the component sugar residues, the degree of branching of the side chains, and the molecular mass and water solubility of the polysaccharides (Cleary et al. 1999; Besednova et al. 2000; Falch et al. 2000; Leung et al. 2006).

Previously, we had isolated the EPS of batch-cultivated *P. polymyxa* CCM 1465 (EPS$_{1465}$), had characterized it, and had prepared rabbit polyclonal antibodies against total EPS$_{1465}$ (Yegorenkova et al. 2008). In contrast to an earlier report suggesting that such compounds were weakly immunogenic (Glukhova et al. 1986), we had achieved high immunogenicity of EPS$_{1465}$ (Yegorenkova et al. 2008). A later study of the biological activity of the EPS toward plant cells had shown that EPS$_{1465}$ significantly increased the number of root hair deformations in wheat seedlings (sevenfold, as compared with the control) and that it was more active than the EPSs of the other *P. polymyxa* strains tested (Yegorenkova et al. 2013).

Here we investigated in vitro the immunomodulatory properties of EPS isolated from *P. polymyxa* CCM 1465. For this purpose, we examined the effect of EPS$_{1465}$, produced with different carbon sources, on the phagocytic activity of macrophages, on the generation of reactive oxygen species (ROS) and of enzymes (acid phosphatase and myeloperoxidase), on the level of proliferation of murine splenocytes, and on the synthesis of proinflammatory cytokines (TNF-α and IL-1β) by human leukocytes. Finally, we assessed the possible interrelation between the composition, structure, and properties of EPS$_{1465}$ on the one hand and its biological activity on the other.

Materials and methods

Strain, culture conditions, and isolation of EPS

Paenibacillus polymyxa CCM 1465, obtained from the Czech Collection of Microorganisms (Brno, Czech Republic), was grown with rotary shaking (220 rpm) at 30 °C for 48 h in a liquid nutrient medium of the following composition (g l^{-1}): yeast extract, 4; Na$_2$HPO$_4$, 1.1; KH$_2$PO$_4$, 0.5; MgSO$_4$·7H$_2$O, 0.2; (NH$_4$)$_2$SO$_4$, 0.1; CaCO$_3$, 0.2; glucose or sucrose, 30; distilled water, up to 1 l (pH 7.2–7.5). The total EPS$_{1465}$ was isolated as follows. The culture liquid was diluted two to threefold with distilled water to decrease viscosity. The cells were separated by centrifugation at 15,000*g* for 30 min, the supernatant liquid was concentrated to the original volume of the culture liquid by rotary vacuum evaporation (40 °C), and the EPS was precipitated with 3 volumes of acetone. The precipitate was separated by centrifugation at 3000*g* for 20 min, washed repeatedly with acetone, and lyophilized in a BENCH-TOP 2K lyophilizer (VirTis, NY, USA). EPS activity was examined with preparations obtained from bacterial growth with glucose (EPS$_{GL}$) and sucrose (EPS$_{SUC}$). For comparison, we used commercial lipopolysaccharide (LPS) of *Escherichia coli* O55:B5, prodigiosan (LPS of *Bacterium prodigiosum*), pyrogenal (LPS of *Pseudomonas aeruginosa*), and zymosan A (polysaccharide of *Saccharomyces cerevisiae*).

Isolation of splenocytes and estimation of their proliferation

Splenocytes were isolated from murine spleens by a standard procedure (Klaus 1987), and their EPS-induced proliferation and the production of NO were estimated. Splenocyte proliferation was estimated by the blast transformation test, based on the incorporation of [3H]-thymidine in the DNA of splenocytes. Ninety-six-well round-bottomed plates were used, and each well received (i) 100 µl of culture liquid consisting of 20% calf embryo serum and a suspension of murine splenocytes (5×10^6 cells ml^{-1} of RPMI-1640 medium) and (ii) 100 µl of EPS (final concentrations, 0.1, 1, and 10 µg ml^{-1}). The plates were incubated at 37 °C for 48 h in a CO_2 incubator. Next, each well received 20 µl of [3H]-thymidine (activity, 0.5 mCi ml^{-1}) and the plates were incubated at 37 °C for a further 24 h. The contents of the wells were then transferred to special filter plates. The absorption of 3H was measured with a β-plate reader (Wallac Oy, Finland). The proliferative response was expressed as pulses per min (ppm; Klaus 1987).

Estimation of NO production by splenocytes

The intensity of NO production by splenocytes was determined by Griess's method by the accumulation of NO_2^- ions in the incubation medium (Green et al. 1982). The method is based on the development of a coloration in the diazotization by sulfanilamide nitrate, which is part of Griess's reagent. To the wells of immunological plates were added 180 µl of a suspension of splenocytes (concentration, 10^7 cells ml^{-1}) and 20 µl of EPS or LPS (concentrations, 0.1, 1, and 10 µg ml^{-1}). The plates were incubated at 37 °C for 48 h in an atmosphere containing 5% CO_2. Then, 100 µl of the incubated culture liquid and 100 µl of Griess's reagent were applied to the wells of 96-well flat-bottomed plates. The mixture was left to stand for 15 min. The absorbance of the samples (A_{540}) was measured with an AIF-Ts-01S immunoenzyme analyzer. The content of NO_2^- was calculated from a calibration graph constructed by use of different concentrations of $NaNO_2$ (up to 200 mM; Green et al. 1982).

Obtainment of peritoneal macrophages (PMPs) and modeling in vitro of phagocytosis

PMPs were obtained from male mice by a conventional method, with modifications (Hogg and Parish 1980; Rudik and Tikhomirova 2006). For phagocytosis, we used a 24-h-old culture of *E. coli* Ca53, which was added to a suspension of macrophages (50:1) in petri plates containing PMPs and EPS (1 µg ml^{-1}). Phagocytic activity was determined at 1, 2, 4, 6, and 24 h. After the plates were incubated at 37 °C, the monolayer of macrophages was fixed with alcohol and stained

by the method of Romanovsky–Gimze. The results were followed microscopically with a Biolar PI polarizing interference microscope (Poland; 15×90), and phagocytic indices (PIs) and phagocytosis completeness indices (PCIs) were calculated. According to Rudik and Tikhomirova (2006), phagocytosis is complete if $PCI \geq 1$, partial if $0 < PCI < 1$, and incomplete if $PCI < 0$.

Determination of myeloperoxidase (MPO) activity in macrophages

The activity of MPO in a lysate of peritoneal macrophages was estimated as described by Saidov and Pinegin (1991). To Eppendorf tubes were added 450 µl of a suspension of PMPs ($0.5–1 \times 10^6$ cells ml^{-1}) and 50 µl of EPS (final concentrations, 0.01, 0.1, 1.0, and 10 µg ml^{-1}), and the mixture was incubated at 37 °C for 1 h. Next, an equal amount of distilled H_2O was added, and the mixture was left to stand for 20 min until cell lysis was complete. To the wells of flat-bottomed plates were added 25 µl of cell lysates and 100 µl of a substrate composed of 0.04% *o*-phenylenediamine solution in citrate buffer (pH 5.0) and 0.33% H_2O_2 solution. The enzymatic reaction was stopped at 5 min by adding 100 µl of 1 N H_2SO_4 to each well. The absorbance (A_{495}) of the samples was measured with the AIF-Ts-01S analyzer. The content of myeloperoxidase was found from a calibration graph constructed by using horseradish peroxidase (concentration, 0.015–2.00 µg/ml). For convenience, the value found was multiplied by 100 and MPO activity was obtained in arbitrary units (a. u.; Saidov and Pinegin 1991).

Nitroblue tetrazolium (NBT) test

The oxygen-dependent killing of bacteria was assessed with a spectrophotometric version of the NBT test (Gentle and Thompson 1990). To the wells of 96-well flat-bottomed plates were added 50 µl of a suspension of PMPs ($0.5–1 \times 10^6$ cells ml^{-1}), 20 µl of NBT, and 20 µl of EPS (final concentrations, 0.01, 0.1, and 1 µg ml^{-1}). The plates were left to stand in a thermostat at 37 °C for 1 h. Next, the cells were washed three times with 0.1 M phosphate-buffered saline (pH 7.2) and fixed for 20 min by adding 100 µl of 96% ethyl alcohol to each well. Excess ethyl alcohol was removed, and the plates were dried at room temperature. The resultant precipitate was dissolved by adding 200 µl of dimethyl sulfoxide (Gentle and Thompson 1990). The absorbance (A_{640}) of the samples was measured with the AIF-Ts-01S analyzer.

Isolation of human peripheral blood mononuclear cells and neutrophils

Mononuclear cells and neutrophils were isolated from blood of 28 arbitrarily healthy volunteers by using a double density

gradient of ficoll–urografin (Kheifets and Abalakina 1973). Freshly drawn heparinized vein blood was left to stand at 37 °C for 15–20 min with 3% polyglucin solution at a 10:1 ratio to sediment erythrocytes and granulocytes. The upper ring (plasma with mononuclear cells) was removed with a pipet and transferred to a sterile test tube. The granulocyte and erythrocyte sediment was used to isolate neutrophils. A gradient solution of ficoll–urografin (density, 1.092 g cm^{-3}) was placed into centrifuge test tubes, then another gradient solution (density, 1.077 g cm^{-3}) was placed as the next layer, and finally the sediment containing granulocytes and erythrocytes was placed as the top layer. The contents of the test tubes were centrifuged at 4000 rpm for 45 min. Six fractions formed, of which we chose the one that contained neutrophils. This fraction was transferred to a centrifuge test tube and washed with nutrient medium 199. The upper fraction, containing mononuclear cells, was added to a sterile test tube containing the previously obtained mononuclear cells, and the mixture was centrifuged at 1000 rpm for 10 min. The sediment was resuspended in 3–5 ml of medium 199 (Totolian et al. 2001). The number of cells was counted in a Goryaev chamber, and cell viability was calculated. The mononuclear cells and neutrophils were used to examine the effect of *P. polymyxa* EPS on the induction of inflammation mediators (ROS, acid phosphatase, and cytokines).

Production of reactive oxygen species (ROS) by whole blood cells

The generation of ROS was assessed by chemiluminescence by using human peripheral blood. To the wells of 96-well flat-bottomed plates were added 120 µl of Hanks's solution, 20 µl of EPS (final concentrations, 1, 10, 100, and 1000 µg ml^{-1}), 20 µl of whole heparinized blood (50 U of heparin/ml of whole blood), and 40 µl of a 0.177% solution of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Serva, Germany). The intensity of luminol-dependent chemiluminescence, in mV, was measured automatically within 1 and 2 h after the addition of luminol, by using an LKB-Wallac 1251 luminometer (Finland). For comparison, zymosan (1000 µg ml^{-1}) was used.

Measurement of acid phosphatase activity in human peripheral blood neutrophils

A commercial reagent kit (Acid/Prostate Phosphatase; Biozyme, Russia) was used. Acid phosphatase activity was measured spectrophotometrically by the ability to hydrolyze 1-naphthylphosphate to phosphate and 1-naphthol, which under the effect of Fast Red (2-amino-5-toluene) yield a colored diazo dye complex. The rate of 1-naphthol generation, directly proportional to the activity of acid phosphatase

in the sample, was measured at 405 nm with a Specord 40 instrument (Analytic Jena, Germany).

Determination of the production of cytokines (IL-1β and TNF-α)

The induction of cytokines by human peripheral blood mononuclear cells during phagocytosis of *E. coli* Ca53 was detected by placing in Eppendorf tubes a suspension of phagocytes (10^6 cells ml^{-1}), EPS (1 µg ml^{-1}), and cells of a 24-h-old culture of *E. coli* Ca53 (50 cells per phagocyte). The test tubes were incubated in a thermostat at 37 °C for 1, 2, 4, 6, and 24 h. The contents of IL-1β and TNF-α in the supernatant liquid were found immunoenzymatically with assay systems based on monoclonal antibodies (OOO Tsitokin, St. Petersburg, Russia). The absorbance (A_{495}) of the samples was measured with the AIF-Ts-01S analyzer.

Statistics

Data were processed with Excel 2007 software (Microsoft Corp., USA). Results are expressed as mean ± standard error of the mean (SEM) and are given with 95% confidence limits. Means were compared between treatments by Student's *t* test, and differences were considered significant at $p < 0.05$.

Results and discussion

Effect of *P. polymyxa* EPS on the activity and completeness of phagocytosis

In phagocytosis, a complex of defense and adaptation mechanisms operates. These "switch on" not only cytotoxic or bactericidal action but also the production and secretion of inflammation mediators, the activation of the energy metabolism of phagocytes, and the processing of antigens and their presentation to lymphocytes (Ernst and Stendahl 2006). The administration of translam (1→3; 1→6-β-D-glucan) considerably increased the ability of macrophages of intact and irradiated animals to engulf and digest both gram-negative and gram-positive microorganisms (Besednova et al. 2000).

Here bacterial phagocytosis, a principal early mechanism of natural immunity, was used to examine the activity of EPS$_{1465}$ toward the immune system. The phagocytosis was investigated for 1, 2, 4, 6, and 24 h. The addition of EPS$_{1465}$ (1 µg ml^{-1}) to PMPs before phagocytosis increased the number of activated macrophages, as compared with the control (Table 1). At all stages of *E. coli* phagocytosis, the PIs were higher than the control values by 17–30%. Within 4 h of phagocytosis, the activation by EPS$_{SUC}$ was greater than that by EPS$_{GL}$ and also that by prodigiosan, the positive control. By 6 h, EPS$_{GL}$ had strongly promoted *E. coli* phagocytosis,

Table 1 Change in the phagocytic indices (PIs) during phagocytosis of *E. coli* Ca53, as affected by EPS from *P. polymyxa* CCM 1465 ($1 \ \mu g \ ml^{-1}$)

Preparation	PI (%)				
	1 h	2 h	4 h	6 h	24 h
Control (no PS)	34±2.1	32±5.7	37±0.7	40±3.5	33±2.8
EPS$_{GL}$	51±0.7*	58±1.4*	62±0.7*	70±2.1*	58±2.1*
EPS$_{SUC}$	55±2.1*	67±1.4*	67±2.8*	57±1.4*	57±2.8*
Prodigiosan	51±2.8*	53±0.7*	63±4.9*	39±1.7	38±2.1

Results are expressed as mean±standard error of the mean (SEM) and are given with 95% confidence limits

*Significant differences as compared with the control ($p < 0.05$)

but the PCIs obtained on the addition of EPS$_{1465}$ to the macrophages were negative, unlike those observed with the control and with prodigiosan. The PCIs for EPS$_{GL}$ and EPS$_{SUC}$ were -0.14 and -0.04, respectively. These data show that the phagocytosis of *E. coli* Ca53 was incomplete and that EPS$_{1465}$ did not affect the ability of the cells to completely digest the bacteria.

Glycopolymer influence on reactive oxygen species (ROS) synthesis in leukocytes

The microorganisms engulfed by both neutrophils and macrophage monocytes are killed through oxygen-dependent and -independent mechanisms (Roitt et al. 2000). In a study of the effect of various substances on the macroorganism, it is important to estimate the production of ROS. We examined the functional activity of cells in ROS production by using human peripheral blood and white laboratory mouse macrophages, objects most commonly used in such experiments (Dolgushin and Bukharin 2001).

We assessed by chemiluminescence the effect of polysaccharide-containing biopolymers (1–1000 $\mu g \ ml^{-1}$) on the generation of ROS by whole peripheral blood cells. We found that EPS$_{1465}$ did not have a significant effect on the chemiluminescence activity of the peripheral blood cells (Table 2). We also assessed the intracellular oxygen-dependent metabolism of murine macrophages with a spectrophotometric version of the NBT test. The data showed that EPS$_{GL}$ and EPS$_{SUC}$ did not promote ROS production by peritoneal macrophages (Table 3). On the contrary, EPS$_{SUC}$ inhibited ROS generation by 15–24% at all concentrations tested. This finding may indicate that these polysaccharides have antioxidant properties. The inhibitory action of EPS$_{SUC}$ was not concentration-dependent. Prodigiosan, used for comparison, enhanced the production of ROS by 1.2–1.3 times at all concentrations tested, as compared with the control.

The in vitro antioxidant ability of *P. polymyxa* EPS was evaluated with various assays and activity indices (Liu et al. 2009, 2010, 2012; Raza et al. 2011; Wang et al. 2011). Liu et al. (2009, 2010) studied the in vitro and in vivo antioxidant activities of the levan-type EPSs from the endophytic bacterium *P. polymyxa* EJS-3. In antioxidant assays in vitro, both crude EPS and its purified fractions (EPS-1 and EPS-2) had a moderate 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, hydrogen peroxide scavenging activity, lipid peroxidation inhibition effects, and a strong ferrous ion chelating activity (Liu et al. 2009, 2010).

Table 3 Effect of EPS from *P. polymyxa* CCM 1465 on reactive oxygen species (ROS) generation in whole blood cells, as examined by the NBT test

Preparation	ROS generation (μM)			
$\mu g \ ml^{-1}$	0.01	0.1	1.0	10
EPS$_{GL}$	75±4.0*	88±3.2	100±7.3	99±5.5
EPS$_{SUC}$	75±3.1*	66±3.3*	70±1.7*	74±3.0*
Prodigiosan	113±6.1*	106±5.8*	116±3.1*	
Control (no PS)	90±6.0			

Results are expressed as mean±standard error of the mean (SEM) and are given with 95% confidence limits

*Significant differences as compared with the control ($p < 0.05$)

Table 2 Effect of EPS from *P. polymyxa* CCM 1465 on reactive oxygen species (ROS) generation in whole blood cells, as examined by chemiluminescence

Preparation	Light sum of chemiluminescence (mV)							
	After 1 h of incubation				After 2 h of incubation			
$\mu g \ ml^{-1}$	1.0	10	100	1000	1.0	10	100	1000
EPS$_{GL}$	6.4±0.2	6.3±0.2	6.5±0.3	6.6±0.5	6.4±0.3	5.4±0.4	6.0±0.1	6.3±0.2
EPS$_{SUC}$	6.1±0.3	6.5±0.2	6.6±0.1	6.0±0.2	5.9±0.4	6.3±0.3	6.1±0.3	6.7±0.6
Zymosan	–	–	–	16.4±1.1*	–	–	–	85.5±3.0*
Control	6.3±0.2				6.0±0.2			

Results are expressed as mean±standard error of the mean (SEM) and are given with 95% confidence limits

*Significant differences as compared with the control ($p < 0.05$)

Glycopolymer effect on myeloperoxidase (MPO) activity in murine peritoneal macrophages

Myeloperoxidase belongs to heme-containing mammalian peroxidases; is present in azurophilic granules of neutrophils, monocytes, and certain types of tissue macrophages; and is secreted into the phagosome during phagocytosis. MPO is an indicator of neutrophil activity and can serve as a marker of inflammation intensity (Ruleva et al. 2007). Table 4 reports on the effect of EPS_{GL} and EPS_{SUC} on MPO activity in PMPs. MPO, which ensures the operation of an alternative mechanism of oxygen-dependent killing, was activated with statistical significance only by EPS_{GL} at the highest dose of 10 µg ml^{-1}.

EPS-affected NO production by splenocytes

NO is a very important messenger in diverse biological functions, which include neuron transmission, regulation of blood vessel tonicity, immunomodulatory action, and cytotoxicity against tumors. Nonetheless, an excess of NO in the cell may act in two ways: on the one hand, it may damage the DNA, and on the other hand, it may have a proinflammatory effect (Moncada et al. 1991; Granik 2002).

The production of NO by murine spleen macrophages (splenocytes) was measured by Griess's method by the accumulation of NO_2^- ions in the incubation medium. A study of the effect of EPS_{1465} on NO production by splenocytes showed that NO synthesis was enhanced only by EPS_{SUC} at 0.01 µg ml^{-1} (Table 5). This finding may indicate that the EPS had activated the microbicidal processes inside the cells. Of note, this promotion of NO synthesis was similar to the effect exerted by the same concentration of pyrogenal. These results agree with those in the literature. Specifically, Chang et al. (2009) reported for *P. polymyxa* JB115 EPS that the β-glucan induction of NO synthesis was concentration-dependent but that the effect was not as great as that of the LPS. At physiological concentrations, ROS and nitrogen

Table 5 Effect of EPS_{1465} on NO production by murine splenocytes

Preparation	NO production (µM)			
µg ml^{-1}	0.01	0.1	1.0	10
EPS_{GL}	12.9 ± 0.8	12.6 ± 0.7	12.6 ± 0.3	13.3 ± 1.2
EPS_{SUC}	16.2 ± 0.3*	13.4 ± 2.1	13.3 ± 0.5	14.9 ± 2.8
Prodigiosan	18.0 ± 0.5*	19.5 ± 0.4*	20.1 ± 0.3*	–
Pyrogenal	15.5 ± 0.6*	20.1 ± 1.0*	24.0 ± 2.0*	–
Control (no PS)	12.4 ± 1.2			

Results are expressed as mean ± standard error of the mean (SEM) and are given with 95% confidence limits

*Significant differences as compared with the control ($p < 0.05$)

have regulatory functions, so if their generation is impaired, immunity is decreased: an excess of these species causes oxidative stress, which induces alterations in cells (Kulinsky 2007).

Glycopolymer effect on acid phosphatase activity

In oxygen-independent killing, the microbicidal effect is related to a rapid and deep shift of the pH toward acidic values in phagolysosomes and to the action of lysozyme, lactoferrin, cationic (basic) proteins, hydrolytic enzymes, and defensins (Totolian and Freidlin 2000). The oxygen-independent mechanism of bacterial killing was assessed by the activity of acid phosphatase in the peripheral blood neutrophils during phagocytosis of *E. coli* Ca53 (Table 6). Both EPS_{GL} and EPS_{SUC} activated the phosphatase, with EPS_{GL} being the better activator (2.0- and 1.8-fold at 0.01 and 1 µg ml^{-1}, respectively). The promotive effects of prodigiosan and pyrogenal, nearly at all concentrations tested, were about the same level as those of the EPSs.

Thus, our experiments to examine the effect of *P. polymyxa* EPSs on the functional and metabolic state of human and murine leukocytes have shown that these polysaccharides are active toward human and murine immune cells.

Table 4 Effect of EPS_{1465} on myeloperoxidase activity in murine macrophages

Preparation	Myeloperoxidase activity (a. u.)			
µg ml^{-1}	0.01	0.1	1.0	10
EPS_{GL}	6.5 ± 1.5	7.0 ± 1.5	6.8 ± 1.3	7.9 ± 0.9*
EPS_{SUC}	5.8 ± 0.1	5.9 ± 0.2	5.9 ± 0.4	6.5 ± 0.5
Prodigiosan	8.5 ± 0.06*	8.2 ± 0.1*	8.8 ± 0.2*	9.6 ± 0.3*
Pyrogenal	8.3 ± 0.32*	9.9 ± 0.4*	9.6 ± 0.19*	–
Control (no PS)	6.1 ± 0.3			

Results are expressed as mean ± standard error of the mean (SEM) and are given with 95% confidence limits

*Significant differences as compared with the control ($p < 0.05$)

Table 6 Acid phosphatase activity in neutrophils during phagocytosis of *E. coli* Ca53, as affected by EPS_{1465}

Preparation	Acid phosphatase activity (U l^{-1})		
µg ml^{-1}	0.01	0.1	1.0
EPS_{GL}	19.1 ± 2.0*	11.9 ± 1.5	17.3 ± 0.9*
EPS_{SUC}	9.4 ± 1.6	13.4 ± 1.6*	12.5 ± 1.1*
Prodigiosan	16.6 ± 1.5*	13.8 ± 0.2*	15.0 ± 2.0*
Pyrogenal	16.1 ± 1.2*	17.2 ± 1.1*	10.1 ± 0.7
Control (no PS)	9.7 ± 2.3		

Results are expressed as mean ± standard error of the mean (SEM) and are given with 95% confidence limits

*Significant differences as compared with the control ($p < 0.05$)

EPS_{SUC} may have antioxidant properties, as it inhibits ROS generation in murine peritoneal macrophages. The highest concentration of EPS_{1465} significantly activates MPO, which ensures the operation of an alternative mechanism of oxygen-dependent killing. The EPS either does not affect at all or affects only weakly the nitrogenic mechanisms inside the cells, yet it increases the activity of acid phosphatase when used at certain concentrations—an effect comparable to those of pyrogenal and prodigiosan.

EPS effect on murine splenocyte proliferation

Increased splenocyte proliferation can be used to judge the immunomodulatory, mitogenic, and even allergenic properties of research preparations (Khaitov et al. 2000). Splenocyte proliferation was assessed by the blast transformation test, based on the incorporation of [^{3}H]-thymidine in the DNA of splenocytes. Both EPS_{GL} and EPS_{SUC} promoted cell proliferation only slightly and not in a concentration-dependent way. But the values obtained from the EPS treatments differed significantly from the control ones (Table 7). When EPS_{SUC} was added at 0.1 µg ml^{-1}, the cellular synthesis of DNA was enhanced the most (1.9-fold, as compared with the control). Nonetheless, this value was much lower than that obtained with the classic mitogen, E. coli O55:B5 LPS (Table 7). The mitogenic activity of murine splenocytes changed from 47,000 ppm at 0.01 µg/ml of LPS to 90,000 ppm at 1 µg ml^{-1} of LPS. Analysis of the literature data showed that the activity of EPS_{SUC} was at the level of that of the β-glucan translam, which, however, at 10 µg ml^{-1} was as active as EPS_{SUC} (Besednova et al. 2000).

EPS-affected production of proinflammatory cytokines

Cytokines are a large group of protein, glycoprotein, or peptide compounds that affect intercellular transmission of

signals in an immune response between various cells of a macroorganism. Monokines (cytokines released by activated monocytes and macrophages) are very important regulators of inflammatory and immune processes. Determination of cytokines, particularly the proinflammatory interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and interferon-γ (INF-γ) in biological fluids has become a useful diagnostic test owing to the large role these compounds play in patho- and immunogenesis (Ryzikova et al. 2011; Simbirtsev 2013).

We examined how EPS induced the synthesis of the main proinflammatory cytokines TNF-α and IL-1β by human peripheral blood mononuclear cells during phagocytosis of E. coli Ca53. Both EPS_{GL} and EPS_{SUC} induced IL-1β synthesis, but they differed greatly in their action (Fig. 1). EPS_{GL} promoted IL-1β production as early as an hour into phagocytosis, and the cytokine concentration values were almost twofold greater than those obtained with prodigiosan. The concentration of IL-1β increased for up to 6 h of phagocytosis and then decreased to the control values by 24 h. On the contrary, with EPS_{SUC}, the content of IL-1β began to increase after 4 h of phagocytosis, greatly exceeded the control values after 6 h, and had become maximal by 24 h (127 ± 10 pg ml^{-1}). Prodigiosan, in turn, promoted the production of IL-1β from 2 h of phagocytosis onward, and the cytokine concentration increased throughout the phagocytosis (Fig. 1).

In parallel with the estimation of the IL-1β content, we recorded the production of TNF-α. Early in phagocytosis, EPS_{GL} and EPS_{SUC} did not affect TNF-α production, as the values did not differ significantly from the control ones (Fig. 2). By 4 and 6 h of phagocytosis, the EPSs had promoted TNF-α production by mononuclear cells. The cytokine concentration was two- to threefold higher than that in the control. By 24 h of phagocytosis, the content of TNF-α, as affected by EPS_{SUC}, had become tenfold greater
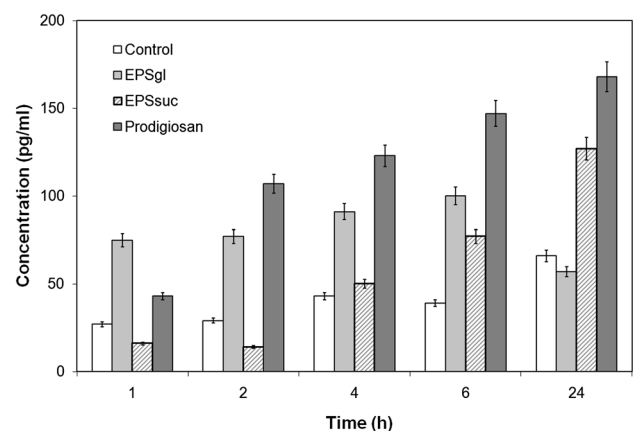
Table 7 Effect of glycopolymers on murine splenocyte proliferation

Preparation	Activity of DNA synthesis upon incorporation of [^{3}H]-thymidine (ppm)		
µg ml^{-1}	0.01	0.1	1.0
EPS_{GL}	16,028 ± 1772*	14,635 ± 2970	14,686 ± 1105*
EPS_{SUC}	18,820 ± 588*	21,878 ± 2791*	15,442 ± 1908*
Prodigiosan	–	34,621 ± 3609*	67,717 ± 5408*
LPS of E. coli 055:B5	46,717 ± 4623*	58,654 ± 6956*	89,679 ± 10,446*
Control (no PS)	11,520 ± 1024		

Results are expressed as mean ± standard error of the mean (SEM) and are given with 95% confidence limits

*Significant differences as compared with the control ($p < 0.05$)

Fig. 1 Induction of IL-1β production by human peripheral blood mononuclear cells as affected by EPS_{1465} (1 µg ml^{-1}) during phagocytosis of E. coli Ca53

than the control values. With prodigiosan, TNF-α production increased substantially (Fig. 2).

Thus, EPS_{1465} moderately stimulates the production of both proinflammatory cytokines. Both EPS_{GL} and EPS_{SUC} promoted mostly the production of IL-1β and only slightly the production of TNF-α late in phagocytosis. An excess of TNF-α, produced by activated macrophages, may play a negative role in sepsis, "burning" the organism with an intense inflammation (like IL-1, TNF-α is regarded as a universal inflammatory mediator; Dinarello 1997). IL-1 and TNF-α are particularly toxic when they act in concert; a combined effect of these cytokines, if they are released in large quantities into the bloodstream and persist there for a long time, may be lethal (Dinarello 1997).

Effect of differences in composition, structure, and properties between EPS_{GL} and EPS_{SUC} on EPS activity

The differences in the effect on the immune cells that are observed between EPS_{GL} and EPS_{SUC} could be explained as follows. The degree of activation of the immune system and the specific action on this or other elements of it depend on several factors, including the composition, structure, and molecular mass (M_r) of the biopolymer given to the organism (Cleary et al. 1999; Falch et al. 2000; Leung et al. 2006). For example, linear 1→3-β-D-glucans (e.g., curdlan) promote the cytotoxicity of polymorphonuclear leukocytes in vitro. By contrast, 1→3-β-D-glucans such as lentinan, which apart from β-1→3-linkages also has β-1→6-linked glucose residues as its side chains, do not have this activity; this also applies to yeast glucans. In other cases, the highest manifestation of biological activity of β-D-glucans is related to the optimal content (most often 20–30%) of β-1→6-linked glucose residues in their molecules. The presence and content of β-1→6 linkages define the

Fig. 2 Induction of TNF-α synthesis by human peripheral blood mononuclear cells as affected by EPS_{1465} (1 μg ml^{-1}) during phagocytosis of *E. coli* Ca53

spatial structure of β-D-glucans, which is associated with the biological action of these polysaccharides (Besednova et al. 2000). 1,3;1,6-β-D-glucans can regulate metabolic processes and can express immunomodulatory properties in both animals and plants (Eliakova 1995). We have shown previously that treatment of wheat (*Triticum aestivum* L. cv. Saratovskaya 29) seeds with EPS_{1465} promoted plant growth, development, and protective reactions. Of note, EPS_{GL} and EPS_{SUC} differed in the magnitude of their promoting effect (Yegorenkova et al. 2016).

The conditions used to grow *P. polymyxa* CCM 1465 (in particular, the source of carbon) affect the monosaccharide composition of its EPSs, the ratio between their constituent polysaccharide fractions (differing in M_r and charge), and their rheological and antigenic properties (Yegorenkova et al. 2008). In EPS_{GL}, high-molecular-weight acid fractions were predominant. Their aqueous solutions were more viscous, and they were composed mainly of mannose, glucose, and galactose (2:2.5:1), uronic acids, and galactosamine (Yegorenkova et al. 2008). EPS_{SUC} showed a predominance of neutral fractions and a threefold decrease in the solutions' kinematic viscosity. It had the same monosaccharide constituents as EPS_{GL} but much less galactose and uronic acids (Yegorenkova et al. 2008).

The complex of methods we used to examine the EPS data included nuclear magnetic resonance, Smith degradation, and gas–liquid chromatography in combination with mass spectrometry. The data suggest that EPS_{1465} is a structurally irregular branched heteroglycan whose backbone is formed from (1→4)- and (1→6)-linked hexose residues in the pyranose form. The EPS structure was also investigated by IR spectroscopy, a method used widely to decipher the structures of polysaccharide molecules, detect functional groups and their mutual arrangement, determine linkage types, and establish the identity of compounds. The IR absorption spectra of EPS_{GL} and EPS_{SUC}, despite being very similar in their total profile and position of the main characteristic bands, had some differences, attesting that the EPSs had structural peculiarities. Specifically, the EPS_{SUC} spectra had absorption bands at 920 cm^{-1}, characteristic, according to some authors, of the α-glycosidic bond. The EPS_{GL} spectra had more intense absorption bands at 885–890 cm^{-1}, possibly indicating that β-glycosidic linkages were predominant (Yegorenkova et al. 2018). Our present data justify the assumption that the inter-EPS differences found in composition, M_r, charge, and rheological and antigenic properties affect the biological activity of these polymers.
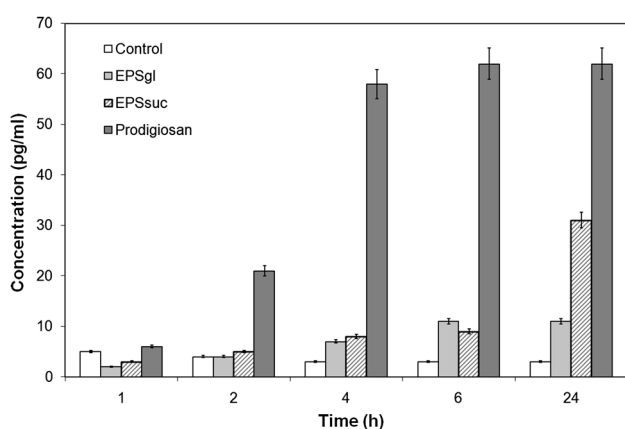
Conclusion

In vitro, the EPSs of *P. polymyxa* CCM 1465 promoted the phagocytosis of bacterial cells, activated metabolic processes in human and animal leukocytes, and moderately affected the production of the proinflammatory cytokines TNF-α and IL-1β by human mononuclear cells. EPS_{1465} has been found to have antioxidant properties, because it inhibited ROS generation in peritoneal macrophages. Overall, the results attest that EPS_{1465} can activate immune cells. This, together with the low (or absent) toxicity of *P. polymyxa* EPSs, open the prospect for their further study to develop, on this basis, preparations for medical–biological practice. Currently, there are no criteria for the selection of an immunostimulant (and a dose thereof) that would match the immune status of a specific patient. Therefore, the need still exists to expand the range of immune system correctors and investigate their structure and action mechanisms (Besednova et al. 2000).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics This study was approved by the Committee of Experts of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (IBPPM RAS; record no. 1049). Animals were cared for and handled in accordance with the Guide for the Care and Use of Laboratory Animals, the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the legislation of the Russian Federation. Informed consent was obtained from all human participants.

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