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Carbohydrate Research 338 (2003) 2835–2840
CARBOHYDRATE RESEARCH
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The effect of (1→3)-β-D-glucans, carboxymethylglucan and schizophyllan on human leukocytes in vitro

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Received 23 April 2003; received in revised form 3 September 2003; accepted 8 September 2003

Abstract

(1→3)-β-D-glucans are known as potent inductors of humoral and cell-mediated immunity in humans and animals. (1→3)-β-D-glucans isolated from various sources differ in their chemical structure and physical parameters and consequently in their immunomodulatory potential. In this study the immunomodulatory activity of two (1→3)-β-D-glucans schizophyllan (SPG) and carboxymethylglucan (CMG) was determined and compared on human blood leukocytes in vitro. Both SPG and CMG activated blood phagocytes and lymphocytes as demonstrated by increased whole blood production of reactive oxygen species, by increased production of pro-inflammatory cytokines IL-6, IL-8, and TNF-α, by increased surface expression of CD69 on lymphocytes, and by altered expression of CD11b and CD62L on polymorphonuclear leukocytes and monocytes. SPG demonstrated a significantly higher potential to stimulate blood phagocytes and production of selected pro-inflammatory cytokines than CMG. The higher potency of SPG to stimulate human blood phagocytes in vitro could be caused by factors such as higher branching frequencies or neutral polymer charge of SPG or different conformation in solution if compared with CMG.

Keywords: Carboxymethylglucan; Schizophyllan; Lymphocytes; Phagocytes

1. Introduction

Glucans are (1→3)-β-linked glucose polymers that are produced as cell wall constituents of fungi, algae, lichens and plants. These glucose polymers can exist as a non-branched (1→3)-β-linked backbone or as a (1→3)-β-linked backbone with (1→6)-β-branches.[1] (1→3)-β-D-glucans exhibit various immunomodulating activities such as decreasing infectious complications and inhibiting tumour growth.[2–5] The enhancement or potentiation of host defence mechanisms has been suggested as a possible means of (1→3)-β-D-glucans tumour growth inhibition rather than direct cytotoxic effects on tumour cells.[5,6]

Schizophyllan (SPG) is one of the (1→3)-β-D-glucans, which is known to have immunomodulating potential and antitumour activity.[2,3,5] SPG is now clinically used as an immunopotentiator against some types of cancer or leukocytopenia. Another (1→3)-β-D-glucan, carboxymethylglucan (CMG), was also proved to be a potent immunomodulator and an agent enhancing hematopoiesis.[7–9]

In contrast to the proved considerable immunostimulatory effect of (1→3)-β-D-glucans, the cellular and molecular mechanisms by which (1→3)-β-D-glucans affect immunological functions have yet not been clearly defined.[10] The first step in the modulation of cellular activity by (1→3)-β-D-glucans seems to be binding to specific cell surface receptor on polymorphonuclear leukocytes (PMNL), macrophages or lymphocytes.[1,11] However, the structural variability of polysaccharides obtained from various natural sources profoundly influences their biological activity.[10,12] Many authors

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doi:10.1016/j.carres.2003.09.007

suggest that parameters, such as primary structure—degree of branching, solution conformation, solubility, molecular weight and/or polymer charge may play a role in determination of (1→3)-β-D-glucans activity and strongly modulate immune functions.[1,3,5,11,13] However, the relationships between the structure of (1→3)-β-D-glucans and their stimulatory activities are still controversial and investigations of immunological potential of (1→3)-β-D-glucans with different structures are needed.[4,10,14–16]

In the present study, we investigated and compared the immunomodulatory effects of two different polysaccharides in vitro: SPG isolated from *Schizophyllum communae* and CMG prepared from *Saccharomyces cerevisiae* cell wall.

2. Results

2.1. Blood phagocyte production of reactive oxygen species (ROS)

Both SPG and CMG induced an increase in spontaneous chemiluminescence (CL) as well as *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated CL in comparison with untreated control (Table 1). However, SPG enhanced both spontaneous and FMLP-activated CL response significantly more than CMG. Phorbol myristate acetate (PMA)-activated CL was significantly increased only by SPG in comparison with untreated control (Table 1).

2.2. Expression of CD11b and CD62L on PMNL and monocytes

SPG and CMG significantly increased the expression of CD11b and decreased the expression of CD62L on both PMNL and monocytes when compared with control (Fig. 1). However, SPG increased the expression of CD11b to a significantly higher extent than CMG.

Table 1
The effect of SPG and CMG on the production of ROS by phagocytes measured as a whole blood CL

	Spontaneous CL [RLU *s]	PMA activated CL [RLU *s]	FMLP activated CL [RLU *s]
Control	57.4±0.4	866.9±179.7	97.4±23.3
SPG	140.9±3.5 *	1056.4±122.7 *	231.0±33.3 *
CMG	94.4±13.1 *,+	934.6±79.7	187.7±19.8 *,+

The data represent mean±S.E.M. ($n=10$).
* Statistically significant differences against control.
\+ Statistically significant differences between SPG and CMG effects.

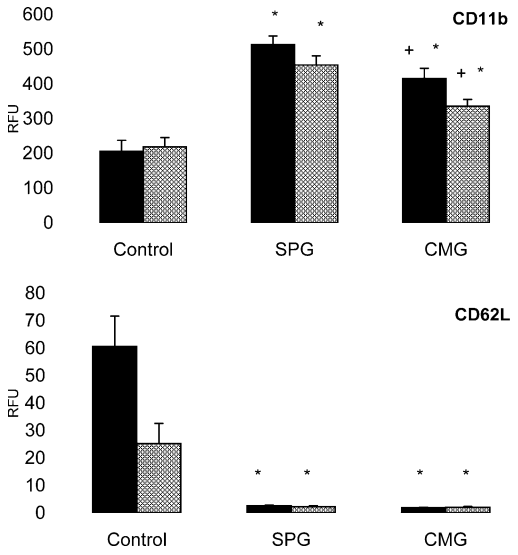

Fig. 1. Expression of CD11b and CD62L on PMNL (black bars) and monocytes (grey bars) incubated with SPG or CMG (100 μg/mL both) or with HBSS as a control for 3 h prior of surface antigen determination. Data are expressed as RFU (mean±S.E.M.; $n=10$). Asterisks indicate statistically significant differences ($P<0.05$) compared with controls. Crosses indicate statistically significant differences ($P<0.05$) comparing the effects of SPG and CMG.

2.3. Expression of surface molecule CD69 on lymphocytes

Both SPG and CMG significantly increased the expression of CD69 on blood lymphocytes when compared with control without significant differences between SPG and CMG (Fig. 2).

2.4. Production of IL-6, IL-8, and TNF-α

Both SPG and CMG significantly increased leukocyte production of IL-6 and IL-8 when compared with

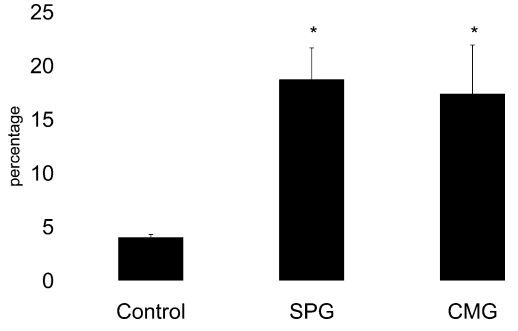

Fig. 2. Expression of surface molecule CD69 on lymphocytes incubated with SPG or CMG (100 μg/mL both) or with HBSS as a control for 3 h prior of surface antigen determination. Data are expressed as % of positive lymphocytes (mean± S.E.M.; $n=10$). Asterisks indicate statistically significant differences ($P<0.05$) compared with controls.

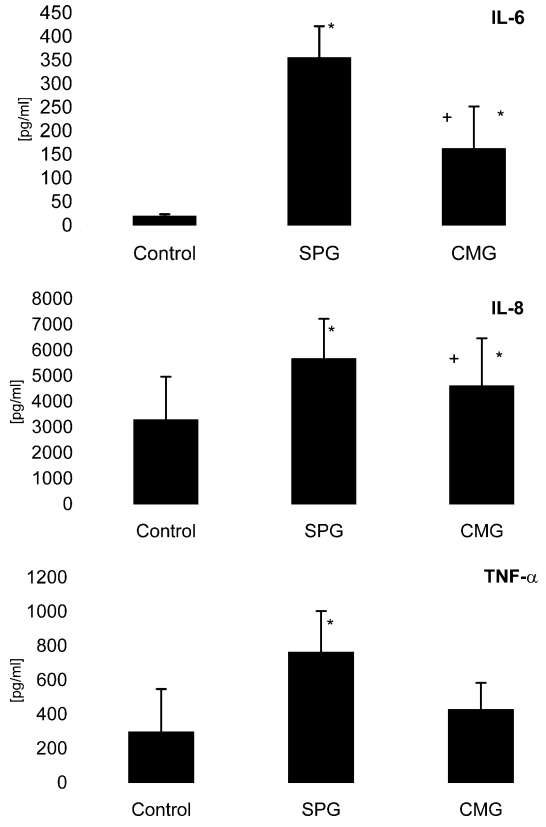

Fig. 3. Production of IL-6 (A), IL-8 (B) and TNF-α (C) by isolated leukocytes incubated with SPG and CMG (100 μg/mL) and HBSS as a control after 18 h. Data are expressed as concentration of cytokines in supernatant at the end of incubation (mean ± S.E.M.; $n = 10$). Asterisks indicate statistically significant differences ($P < 0.05$) compared with controls. Crosses indicate statistically significant differences ($P < 0.05$) comparing the effects of SPG and CMG.

control (Fig. 3a and 3b). However, the production of IL-6 induced by SPG was significantly higher than the increase induced by CMG. TNF-α production was significantly increased only by SPG when compared with control (Fig. 3c).

3. Discussion

It has been claimed that the most probable mode of (1→3)-β-D-glucans action is through the activation of macrophages, dendritic cells, endothelial cells, neutrophils and monocytes. Activation of lymphocytes and NK cells also plays a key role in (1→3)-β-D-glucan action.[2,10] Polysaccharides cannot penetrate cells due to their large molecular mass, so the first step in the modulation of cellular activity by (1→3)-β-D-glucans is binding to B-cell, T-cell, PMNL and macrophage receptors. However (1→3)-β-D-glucan receptors are not precisely described and they may include multiple glucan binding sites on macrophages, neutrophils and NK-cells.[1,3,11] Complement receptor CR3 (CD11b/CD18), expressed on the surface of neutrophils, monocytes, macrophages and NK-cells has been identified as the key receptor of β-glucans.[17] However, the existence of other non-CR3 receptors has also been reported.[1,18] Binding of (1→3)-β-D-glucans to their receptors stimulates intracellular signalling pathways, which culminate in the activation, translocation and nuclear binding of immunoregulatory and pro-inflammatory transcriptional activator proteins.[18]

Production of free radicals during oxidative burst of blood phagocytes plays one of the major roles in antimicrobial, anti-tumour, and inflammatory response of the human body and is a sensitive marker of phagocyte activation.[19] Further, activation of blood phagocytes, both PMNL and monocytes, is also associated with a rapid increase in CD11b surface expression and a rapid decrease in CD62L surface expression.[20] Lymphocyte activation is linked to a fast rise in CD69 surface expression. Determination of CD69 surface expression is a very early marker of lymphocyte activation and correlates well with the [^{3}H]thymidine incorporation assay.[21] In our study, both tested (1→3)-β-D-glucans activated blood phagocytes (PMNL and monocytes) and also lymphocytes as demonstrated by increased whole blood ROS production, by increased production of selected pro-inflammatory cytokines, and by the quantitative changes of selected surface antigen expression. Production of selected pro-inflammatory cytokines IL-6, IL-8 and TNF-α by human blood leukocytes represents an important factor indicative of an immune system response and is a sensitive marker of leukocyte activation.[22] The capability of SPG to stimulate the synthesis of pro-inflammatory cytokines by various cell lines has already been described by other authors.[10,11,16,23,24]. In our study, the SPG tested demonstrated a significantly higher potential for blood phagocytes stimulation and production of selected pro-inflammatory cytokines in comparison with CMG. In contrast to our findings, Vetvicka et al. did not find regulation of neutrophil CD11b expression or superoxide production by SPG.[6] They suggested that soluble β-glucans prime neutrophils without causing non-specific pro-inflammatory action of neutrophils. However, in contrast to our study they used solubilised zymosan.

The higher potency of SPG than CMG to stimulate human blood phagocytes in vitro could be caused by several factors. A number of reports suggest that the bioactivity of (1→3)-β-D-glucans is related to the degree of side chain branching.[1,3,5] The ratio of (1→3)/(1→6)-linkages and the architecture of β-D-glucans networks varies significantly depending on species.[11] We observed that SPG with the higher branching frequency (1/3) exhibited more intensive biological activity than CMG with lower frequency of branching (1/8). In agreement, Williams et al. showed that SPG exhibited a fivefold

increase in affinity for human macrophage (1→3)-β-D-glucan receptor compared to non-branched glucan phosphate.[4] Other authors also concluded that the higher branching frequency might enhance the affinity of (1→3)-β-D-glucans to leukocytes and extend their biological activity.[1,5] However, significant differences between binding of SPG and scleroglucan were observed even though their branching frequency was very similar.[1]

According to various authors biological activity of these polysaccharides is also dependent on their size, so polymers with the greater molecular weight exhibit higher binding affinities and biological activity than lower molecular weight (1→3)-β-D-glucans.[1,3,5] In contrast, Kulicke et al. reported that low molecular mass glucans (around 550,000) increased TNF-α release and superoxide anion release by human blood monocytes more intensively than high molecular mass glucans (over 2,000,000).[14] We observed higher stimulation activity to blood phagocytes with SPG than with CMG regardless of their similar molecular weight.

The observed higher potential of SPG than CMG can be due to their different conformations, triple helix respectively random coil. Solution conformation has been suggested as an important factor for the binding of glucans to their receptors and therefore for their biological activity.[1,3,5,10,14,16] Generally, glucans can exist as a random coil, as a single polymer strand with a helical conformation (single helix) or as a stable complex of three polymer strands forming a triple helix.[4,5,14,15] Contradictory data exist concerning the effect of the specific molecular structure on biological activity of (1→3)-β-D-glucans. There are data describing both lentinan and SPG are active only when they exist in a single helical structure.[25] Similarly the single helix and random structures of curdlan were more active then double- or triple-stranded helices[10] and the single helical conformer of SPG had a higher ability than triple helical conformer of SPG to produce nitric oxide and pro-inflammatory cytokines.[15,16,26] In contrast, Kulicke et al. observed that helical structure was not essential or advantageous for induction of immunological activity.[14] Tsuzuki et al. did not observe dependence of hematopoietic response and IL-6 production in mice in vivo on single or triple helix conformation of SPG.[23] Neither did Suzuki observe a difference in IL-8 production by human leukocytes or in platelets activation in vitro induced by single or triple helix conformation of SPG.[11]

However, interestingly only high molecular weight (1→3)-β-D-glucans appear to form triple helical structures, therefore the molecular weight of tested polysaccharides could play a role in these contradictory observations.[25]

Another important factor influencing the potency of (1→3)-β-D-glucans to bind and consequently stimulate leukocytes is the presence of charged species.[1,5,10] SPG belongs to neutral polysaccharides whereas CMG is a polyelectrolyte glucan. In agreement with our results Mueller et al. showed that neutral polysaccharides exhibit a higher affinity binding than polyelectrolyte glucans.[1] However, in this study these neutral polysaccharides also exhibited higher branching frequency. Kataoka et al. showed that carboxymethylated curdlan was inactive when compared with untreated curdlan.[10]

In conclusion, we have proven that the tested (1→3)-β-D-glucans SPG and CMG exhibited immunostimulatory activity on human blood leukocytes in vitro. The observed higher potency of SPG than CMG to stimulate human blood phagocytes in vitro could be caused by different chemical structure and physical properties of tested (1→3)-β-D-glucans as was discussed above.

4. Experimental

4.1. Reagents

SPG and CMG were prepared in the laboratories of CPN (Czech Republic). SPG was produced as an extracellular product by *Schizophyllum communae* cultured under standard cultivation conditions. All post-fermentation processes were done under a pH between 5 and 6 and room temperature. CMG was isolated from cell walls of *Saccharomyces cerevisiae* by alkali digestion. Obtained β-D-glucans were treated by monochloracetic acid to substitute free carboxyl groups. This modification of chemical structure allowed the extraction of CMG. The presence of endotoxin was tested by Limulus amebocyte lysate (LAL) coatest (Chromogenix, US) and all tested samples did not have the content of endotoxins higher than 500 IU/mg. Basic chemical properties of tested SPG and CMG are summarised in Table 2.

PMA, FMLP, RPMI-1640 medium and gentamycin sulphate were obtained from Sigma-Aldrich (USA). Luminol was obtained from Molecular Probes (USA), Dextran-T500 from Pharmacia (Sweden) and Telebrix N 300 from Leciva (Czech Republic). Heat-inactivated human AB serum was obtained from a local hospital. Lysing solution Cal-Lyse, fluorescein isothiocyanate (FITC)-labelled anti-human CD11b murine monoclonal antibody, phycoerythrin labelled anti-human CD62L murine monoclonal antibody, FITC labelled anti-hu-

Table 2
Basic chemical characteristics of tested (1–3)-β-D-glucans

	SPG	CMG
Degree of branching	1/3	1/8
Polymer charge	neutral	negative
Solution conformation	triple helix	random coil

man CD69 murine monoclonal antibody and appropriate control isotype murine antibodies were purchased from Caltag Laboratories (USA). Phycoerythrin–cyanin 5.1 labelled anti-human CD14 murine monoclonal antibody was purchased from Beckman Coulter (USA). Enzyme-linked immunosorbent assays (ELISA) Modul Sets for human IL-6, IL-8 and TNF-α were obtained from BenderMedSystems (Austria). All other chemicals were purchased in the highest grade p.a. from local distributors or Sigma-Aldrich (USA).

4.2. Blood sampling and leukocyte isolation

Heparinised (50 IU/mL) blood samples were obtained from the cubital vein of ten healthy volunteers after overnight fasting. The number of leukocytes in the blood and their relative differentiation counts were determined using Coulter counter STKS (Coulter, England) and stained blood smears, respectively. Isolation of leukocytes was performed as described previously.[27] Blood was layered in ratio 1:1 over the separation mixture (4% Dextran-T500 in saline and 60% telebrix N 300 in saline in ratio 3.7:1; final density 1.08 g/cm^3). Erythrocytes were removed after 1 h sedimentation at room temperature and leukocytes with plasma were obtained. Then the leukocytes were washed in RPMI-1640 (200 g, 5 min) and resuspended to reach final density 1×10^5 mL in RPMI-1640 supplemented with 10% heat inactivated human AB serum.

4.3. Experimental protocol

Whole blood samples were incubated with SPG and CMG (100 μg/mL) at 37 °C for 1 h prior to the determination of oxidative burst by CL or for 3 h prior determination of expression of surface antigens on PMNL by flow cytometry. The tested concentration of polysaccharides and incubation times were selected based on our previous unpublished results and they are in agreement with literature as the most efficient concentration for in vitro test.[10,11]

Isolated leukocytes were incubated with SPG and CMG (100 μg/mL) in RPMI-1640 medium supplemented with 10% of heat inactivated human AB serum and gentamycin sulphate (45 mg) at 37 °C for 18 h. This time period was chosen based on results of Suzuki et al.[11] Hanks balanced salt solution, pH 7.4 (HBSS) was used instead of polysaccharides as a control. Concentrations of selected cytokines were determined in supernatant at the end of incubation.

4.4. Measurement of oxidative burst of blood phagocytes

Luminol-enhanced CL of whole blood phagocytes was measured using microplate luminometer LM-01T (Immunotech, Czech Republic) as described previously.[28] Briefly, the reaction mixture consisted of 10 μl whole blood, 1 mM luminol (stock solution of 10 mM luminol in 0.2 M borate buffer) and one of the activators (PMA-0.5 μM or FMLP-3 μM). Stock solutions of 10 mM activators were prepared in Me_2SO. Final concentration of Me_2SO did not exceed 0.1%, which was proved not to effect CL reaction. The total reaction volume of 200 μL was adjusted with Hanks balanced salt solution, pH 7.4 (HBSS). The assays were run in duplicates. Spontaneous CL measurements in samples containing 10 μL of whole blood and other substances except any activator were included in each assay. The CL emission expressed as relative light units (RLU) was recorded continuously for 90 min at 37 °C. The integral value of the CL reaction, which represents the total ROS production by blood phagocytes, was corrected to the number of PMNL.

4.5. Determination of the expression of cell surface molecules

The measurements were performed according to the manufacturer's protocol using unfixed whole blood (Caltag Laboratories, USA) with minor modifications.[20] Briefly, 100 μL of blood were incubated with anti-CD11b and anti-CD62L or anti-CD14 and anti-CD69 monoclonal antibodies at room temperature for 15 min. 100μL of blood incubated with FITC- or PE-conjugated murine immunoglobulins of the same isotype were used as the negative controls. Then the samples were fixed by Cal-lyse and the red blood cells were lysed by distilled water in the case of whole blood. The remaining cells were resuspended in PBS, placed on ice and analysed within 2 h. At least 10,000 PMNL, 1000 monocytes or 10,000 lymphocytes selected on the basis of their typical scattering characteristics and CD14 antigen expression were analysed by flow cytometer FACSCalibur (Becton Dickinson, USA). The geometric mean of relative fluorescence units (RFU) was quantified and corrected to the background fluorescence of the isotype control in the case of CD11b and CD62L determination or percentage of CD69 positive lymphocytes were determined.

4.6. Cytokine determination

The determination of cytokines in supernatant was performed according to the manufacturer's protocol for IL-6 Modul Set, IL-8 Modul Set, and TNFα Modul Set (BenderMedSystems, Austria).

4.7. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) of ten experiments. Results were analysed by the Student t-test for dependent samples and significances were verified by the non-parametric Wil-

coxon test using software Statistica for Windows 5.0 (Statsoft, USA).

Acknowledgements

This study was elaborated in the frame of research plan Z 5004920 and supported by grants GA CR 524/02/0395 and IGA AS CR S5004009.

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