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Characterization of the novel chemically modified fungal polysaccharides as the macrophage stimulators

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ABSTRACT

By means of carboxymethylation, a novel water-soluble carboxymethyl chitin–glucan (CM-CG) was prepared from the mycelium of *Aspergillus niger*, and its ability to stimulate macrophages was assessed and compared to that of the previously studied carboxymethylated glucan (CMG) from the yeast *Saccharomyces cerevisiae*. It was demonstrated that single intraperitoneal (i.p.) administration of CMG and CM-CG to the CBA mice led to a significant increase of leukocyte number. At the same time, the number of monocytes in the bone marrow was increased to more than two-fold. Application of both polysaccharides also resulted in the augmented number of liver macrophages and to the rise of their content of the secondary lysosomes. A markedly enhanced carbon clearance was observed as well as the increased release of tumor necrosis factor-α by the peritoneal macrophages indicating their amplified phagocytic activity. The effect of CM-CG in these experiments was ca. 1.7 times higher than that of CMG. Administration of both polysaccharides also led to the elevated level of free acid phosphatase in liver homogenate, implying labilization of the lysosomes. Increased serum chitotriosidase also indicated increased macrophage activity. The results obtained indicate similar *in vivo* macrophage stimulation activity of both applied fungal polysaccharides and suggest their potential clinical use as non-toxic natural compounds.

1. Introduction

Compounds that are capable of interacting with the immune system by upregulation or downregulation of the specific pathways of immune response are known as immunomodulators or biological response modifiers (BRMs) [1]. In the family of immunomodulatory compounds that have been increasingly used in the prevention and therapy of various infectious and chronic diseases a special position is occupied by fungal (1→3)-β-D-glucans, non-specific immunomodulators that also act as lysosomotropic agents [2–6]. In yeast cell walls, (1→3)-β-D-glucans represent the most abundant polysaccharide and play role of a skeletal carcass defining rigidity and stability of the cell and its morphological shape [7,8]. Glucans comprising a backbone built of (1→3)-β-glycosidically linked D-glucose units with variable (1→6)-β-D-glucosyl branching have been isolated from various fungal, bacterial and algal sources. Numerous studies have shown that (1→3)-β-D-glucans enhance the functional status of macrophages and neutrophils [9], modify immunosuppression [10], increase, as well as exert anti-tumor activity [11].

Stimulation of the functional activity of the leukocytes and macrophages is accompanied by the enhanced generation of reactive oxygen and nitrogen species [12], release of the pro-inflammatory cytokines [3,13], and increased activity of lysosomal enzymes in serum and leukocytes [14]. (1→3)-β-D-Glucans stimulate T-cell immunity and their application leads to increased release of the interleukins IL-6, IL-8, and IL-12 by macrophages [15,16], neutrophils [17,18], and natural killer (NK) cells [19,13]. Recently obtained data strongly support the assumption that (1→3)-β-D-glucans mediate their protective and immunomodulating effects by binding to specific sites (receptors) on monocytes/macrophages and granulocytes triggering a cascade of immunological events. It is now established that β-D-glucan receptors include CR3 [20], lactosylceramide [21], scavenger receptors [22], and Dectin-1 [23].

Since being rigid skeletal molecules (1→3)-β-D-glucans are often insoluble in water, their biological application necessarily precludes preparation of the water-soluble derivatives as parenteral administration of insoluble (particulate) preparations might be associated with granuloma formation, micro-embolization, hepatosplenomegaly, and enhanced endotoxin sensitivity [24]. Previously we have described the preparation of the water-soluble derivatives from the cell wall β-D-glucan of the baker's yeast *Saccharomyces cerevisiae* and reported their antioxidant, antimutagenic, and antitumor activities [25–29].

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Table 1
Effect of CMG and CM-CG administration (25 mg/kg body mass, i.v.) on the cellular composition of the bone marrow from murine femur.

Animal group	Total number of myelokaryocytes ($\times 10^6$)	Total number of neutrophils ($\times 10^6$)	Number of proliferating neutrophils ($\times 10^6$)	Maturation index	Total number of monocytes ($\times 10^6$)	Total number of lymphocytes ($\times 10^6$)	Total number of eosinophils ($\times 10^6$)
Control	21.8 ± 1.29	8.22 ± 0.56	2.73 ± 0.3	3.01 ± 0.28	0.51 ± 0.01	2.01 ± 0.12	1.25 ± 0.07
CMG, on the 2nd day	42.12 ± 3.24*	10.92 ± 0.47	4.79 ± 0.06*	2.24 ± 0.12	1.1 ± 0.04*	2.4 ± 0.39	1.2 ± 0.09
CMG, on the 7th day	38.52 ± 1.61*	10.75 ± 0.29	4.56 ± 0.42*	2.39 ± 0.24	1.08 ± 0.09*	2.2 ± 0.21	1.08 ± 0.01
CM-CG, on the 2nd day	44.5 ± 3.14*	12.73 ± 0.98*	5.56 ± 0.21*	2.09 ± 0.51	1.28 ± 0.12*	2.9 ± 0.39	1.28 ± 0.03
CM-CG, on the 7th day	38.82 ± 2.41*	12.57 ± 0.74*	5.68 ± 0.39*	2.21 ± 0.17	1.09 ± 0.08*	2.7 ± 0.26	1.09 ± 0.05

* $P<0.01$ relative to control. Number of animals in control group—10, number of animals in the treated groups—8–10.

In filamentous fungi, cell wall (1→3)-β-D-glucan is covalently linked to chitin, another skeletal polysaccharide, which content reaches in filamentous fungi up to 40% in contrast to the yeast cell walls, where chitin amounts to 1–2% of all polysaccharides [8]. Thus, fungal mycelium represents another source of a valuable polysaccharide complex—chitin–glucan (CG). Moreover, since CG can be prepared from the mycelium of *Aspergillus niger* that remains as waste upon the industrial production of citric acid, its preparation is inexpensive and contributes to environment-friendly valorization of the waste products. Previously we have described the preparation of carboxymethyl chitin–glucan and its antimutagenic and antioxidant activities [26,30,31], however a systematic investigation of its biological properties has not yet been carried out. In the present work we evaluated macrophage-stimulating activity of the prepared water-soluble CM-CG and compared the results with those obtained at the administration of the established macrophage stimulator—CMG.

2. Materials and methods

2.1. Polysaccharide preparation

The water-insoluble (1→3)-β-D-glucan was isolated from the commercial baker's yeast biomass purchased from Slovlik (Trenčín, Slovakia). Yeast cells were treated with 6% NaOH at 60 °C followed by 4% phosphoric acid extraction at room temperature as previously described [32]. After the removal of all soluble material, β-D-glucan was left as the insoluble residue. Preparation of CMG by means of carboxymethylation of the insoluble β-D-glucan was performed according to Machová et al. [33]. The degree of carboxymethylation determined by potentiometric titration was 0.56, and the molar mass established by HPLC was 346,000. The analyses of the prepared CMG were performed as previously described [33].

The crude chitin–glucan complex was prepared from the mycelium of the industrial strain of the filamentous fungus *A. niger* used for the commercial production of citric acid (Biopo, Leopoldov, Slovakia). Isolation, carboxymethylation, and characterization of molecular parameters of the prepared CM-CG were carried out as previously reported [34]. The degree of carboxymethylation was established by potentiometric titration to be 0.43, the molar mass of the used fraction of CM-CG was ca. 60,000, and the content of chitin in the complex determined by means of ^{13}C NMR was ca. 14%.

Zymosan was purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Animals

The experiments were performed using male mice of the CBA line having body mass of 20–25 g (obtained from the breeding station of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Medical Sciences, Novosibirsk). All experiments followed the official "Rules for the work involving experimental animals" (Addendum to the Instruction of the Ministry of Health of the USSR # 755 of August 12, 1977).

2.3. Administration of the polysaccharides and the experimental outline

CMG and CM-CG were administered intravenously (i.v.) to animals as a single dose of 25 mg/kg body mass and mice were sacrificed after 48 h or 7 days after the polysaccharide application. Control animals received a corresponding volume of physiological solution. After the sacrifice, the excised liver was homogenized in 0.25 M sucrose solution containing 0.001 M EDTA and this homogenate was used for the determination of enzymatic activity. Calculation of the total number of the myelokaryocytes and preparation of the bone marrow

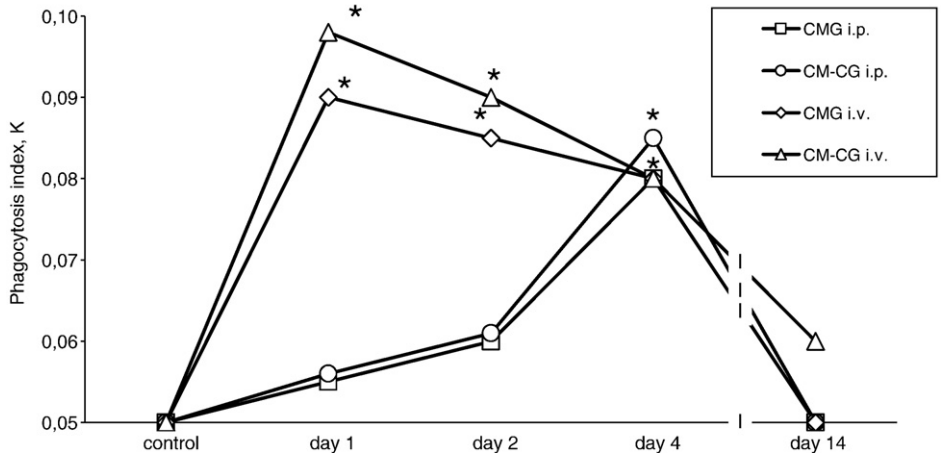

Fig. 1. Macrophage stimulation with CMG and CM-CG administered i.v. and i.p., 25 mg/kg body mass (*$P<0.05$ relatively to control, number of animals in each group = 5). Values of phagocytosis index *K* were calculated as described in Materials and methods.