Stimulation of TNF-α Release by Fungal Cell Wall Polysaccharides

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Carboxymethylated derivatives were prepared from the (1→3)-β-D-glucan isolated from the cell wall of baker's yeast *Saccharomyces cerevisiae* and from the chitin-glucan complex of the mycelium of the industrial filamentous fungus *Aspergillus niger*. The polysaccharides were applied to peritoneal mouse macrophages and after a 2 h incubation the release of TNF-α by the stimulated macrophages was measured using an enzyme-linked immunosorbent assay. As the third polysaccharide stimulant, a water-soluble derivative of chitin was assayed and the observed cytokine release was compared with the control experiment. In three concentrations of the

polysaccharides applied, carboxymethyl glucan revealed a dramatic increase in the TNF-α release, while addition of carboxymethylated chitin-glucan resulted only in a moderate enhancement, and carboxymethyl chitin was inactive. The results indicate that fungal polysaccharides, especially (1→3)-β-D-glucan, are potent macrophage stimulators and activators of TNF-α release, which implies their potential application in antitumor therapy.

Keywords: Tumor necrosis factor-α, glucan, chitin

Introduction

Fungal cell walls consist predominantly of polysaccharides (up to 90%), the most abundant of which is β-D-glucan (50 - 60% of all cell wall polysaccharides), which plays role of a skeletal carcass defining rigidity and stability of the cell and its morphological shape (Bartnicki-Garcia, 1968; Farkaš, 1979). Glucans having 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 and in the recent decades increased attention has been paid to these compounds due to their ability to act as non-specific modulators of the immune system (Williams, 1997; Kogan, 2000). Glucans belong to the class of drugs known as biological response modifiers (Bohn and BeMiller, 1995) and numerous studies have shown that (1→3)-β-D-glucans enhance the functional status of macrophages and neutrophils (Williams *et al*., 1996), modify immunosuppression (Browder *et al*., 1990), increase resistance to infections by Gram-negative bacteria (Pretus *et al*., 1991), as well as exert antitumor activity (Sherwood *et al*., 1986, 1987).

In our previous work we have reported on antibacterial (Kogan *et al*., 1989), antimutagenic (Čipák *et al*., 2001), antioxidant (Babincová *et al*., 1999; Slameňová *et al*., 2003, Kogan *et al*., 2005), and antitumor activities (Kogan *et al*., 2002; Khalikova *et al*., 2005) of the prepared water-soluble derivatives of (1→3)-β-D-glucan isolated from the cell walls of baker's yeast *Saccharomyces cerevisiae*. Now we have demonstrated that at least some of its immunomodulatory activities could be explained by an increased release of specific cytokines from the activated immunocompetent cells, *e.g.*, macrophages. At the same time, we compared its activity with that of the carboxymethylated derivative of the chitin-glucan complex from the mycelium of *Aspergillus niger*, in which β-D-glucan constitutes about 80%, and with carboxymethyl chitin (CM-C), the second polysaccharide component of chitin-glucan complex.

Materials and Methods

Preparation of the polysaccharides

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 ^{o}C followed by 4% phosphoric acid extraction at room temperature as previously described (Kogan *et al*., 1988). After the removal of all soluble material, β-D-glucan was left as the insoluble residue. Solubilization of the insoluble glucan was performed by means of carboxymethylation with monochloroacetic acid and aqueous NaOH in *iso*-propyl alcohol as previously described (Machová *et al*., 1995). The degree of carboxymethylation determined by potentiometric titration was 0.56, and the molecular mass established by HPLC was 346,000. The analyses of the prepared carboxymethyl glucan (CM-G) were performed as previously described (Machová *et al*., 1995).

The crude chitin-glucan complex was prepared from the mycelium of the industrial strain of the filamentous fungus *Aspergillus niger* used for the commercial production of citric acid (Biopo, Leopoldov, Slovakia). Isolation, carboxymethylation and characterization of molecular parameters of the prepared carboxymethyl chitin-glucan (CM-CG) were carried out as previously reported (Machová *et al*., 1999). The degree of carboxymethylation was established to be 0.43 by potentiometric titration, the molecular 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%.

The sample of chitin was purchased from Primex ehf (Siglufjordur, Iceland) and carboxymethylated using the conditions similar to those applied for carboxymethylation of chitin-glucan complex (Machová *et al*., 1999). The degree of carboxymethylation determined by potentiometric titration was 0.35 and the molecular mass was 150,000 as established using HPLC in the previously reported conditions (Machová *et al*., 1999).

Animals

Male ICR mice aged 8 - 12 weeks were obtained from the breeding facility of the Institute of Experimental Pharmacology, Slovak Academy of Sciences (Dobrá voda, Slovakia). All animals were housed in microisolator cages in a temperature-controlled room. Food and water were provided *ad libitum*. All animal experiments were conducted according to the ethical guidelines issued by the Institute of Virology, Slovak Academy of Sciences.

Cell cultures and their activation

Peritoneal mouse macrophages were prepared according to Park and Rikihisha (1991) by means of elicitation using intraperitoneal injection of 2 ml sterile 5% thioglycolate broth (Difco

Laboratories, Detroit, MI). Upon 5 d, the mice were sacrificed by applying diethyl ether and peritoneal exudates cells were collected by lavage. Cells were washed by centrifugation, resuspended [5×10^6 cells in 0.5 ml RPMI 1640 medium containing L-glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% heat-inactivated fetal bovine serum (FCS, Gibco-BRL, Life Technologies, Eggenstein, Germany)], and placed into every well of the 24-well plates (Sarstedt AG & Co., Nümbrecht, Germany). Upon 2 h incubation at 37 ^{o}C in a humidified atmosphere of 5% carbon dioxide, non-adherent cells were removed by rinsing and subsequently 0.5 ml of complete RPMI 1640 medium (containing 10% FCS) supplemented with the tested polysaccharide stimulants was added to each well. After the cultivation period, the supernatants were collected and stored at –80 ^{o}C.

Enzyme-linked immunosorbent assay (ELISA)

The content of TNF-α was determined in the cell culture supernatants collected after 3, 6, and 24 h of cultivation using an ELISA kit (DUO Set, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. A recombinant mouse TNF-α was used as reference. All assays were carried out in triplicate.

Application of the polysaccharides

Solutions of CM-G, CM-CG, and CM-C were prepared in 12.5, 25, and 50 μg/ml concentrations in complete RPMI 1640 medium and rendered sterile by filtration through a 0.22 μm membrane (Millipore, Bedford, MA).

Statistical analysis

The results are presented as mean ± standard deviation (SD). All data were statistically analyzed by a one-way analysis of variance ANOVA and a Bonferroni test to determine whether there were differences within the groups. *P* values smaller than 0.01 were considered to be significant. The analyses were performed using OriginPro 7.5 software (OriginLab Corporation, Northampton, MA).

Results and Discussion

Immunomodulatory substances can be subdivided into two groups basing on their source and type of interaction with the immune system. One group contains mammalian cell products (mainly, proteins) that selectively augment activities of specific cells of the lymphohematopoietic system. Another group consists of the compounds isolated from microorganisms or microbial culture fluids. The latter type of immunomodulators, also known as biological response modifiers (BRMs), usually non-specifically potentiate the host immune system by interacting with its multiple components involving innate and adaptive mechanisms. (1→3)-β-D-Glucans are probably the most extensively studied BRMs (Bohn and BeMiller, 1995; Williams, 1997; Kogan, 2000) and some of them have been used clinically in cancer therapy in Japan (Maeda *et al*., 1984; Sakagami *et al.*, 1988) and have been involved in preclinical trials of antiseptic effect in trauma and surgical patients (Browder et al., 1990; Babineau et al., 1994).

Recently obtained data strongly support the assumption that (1→3)-β-D-glucans mediate their protective and immunopotentiating effect by binding to specific sites (receptors) on monocytes/macrophages and granulocytes triggering a cascade of immunological events. Among the elicited effects are: bone marrow colony stimulating activity leading to augmented production of monocytes and granulocytes, increased antibody titers, boosted cytokine release (including IL-1, IL-

2, IL-6, and TNF-α), prostaglandin E_2 production, activation of alternative complement pathway, and release of lysosomal enzymes (Thornton *et al*., 1996; Vetvicka *et al*., 1996). In support of this concept, Czop and Austen (1985) have reported observation of (1→3)-β-D-glucan receptors on human monocytes, Goldman (1988) has reported the presence of (1→3)-β-D-glucan receptor on P388D1 cells, a mouse macrophage-like tumor cell line, and Williams *et al.* (1986) have reported a (1→3)-β-D-glucan receptor on human polymorphonuclear lymphocytes. All these early studies were carried out using particulate, water-insoluble β-D-glucans and the researchers did not elucidate the nature of the receptors, which limited the *in vivo* significance of these studies. On the other hand, recent in-depth investigations performed with different water-soluble derivatives of (1→3)-β-D-glucans and their low-molecular-weight fragments corroborated the fact that β-D-glucan receptors belong to the class of pattern recognition receptors (PRR), by which innate immune system recognizes conserved microbial structures called pathogen-associated molecular patterns (PAMPs), which include lipoteichoic acid for Gram-positive bacteria, lipopolysaccharide (LPS) for Gram-negative microorganisms, and β-D-glucan on fungi (Herre et al., 2004). It is now established that β-D-glucan receptors include CR3 (Ross *et al*., 1987), lactosylceramide (Zimmerman *et al*., 1998), scavenger receptors (Rice *et al*., 2002), and Dectin-1 (Brown and Gordon, 2001).

As mentioned above, one of the mechanisms, by which binding of β-D-glucan to the macrophage receptors results in the increased immune protection is enhancement of the cytokine release. Cytokines are an important contributing factor in immunological and inflammatory reactions. Tumor necrosis factor (TNF) is the major mediator of biophylaxis reaction against Gram-negative bacteria. TNF-α is a cytokine produced mainly by the activated macrophages and shows an array of antibacterial, antiviral, and tumoricidal activities (Havell, 1989; Watanabe and Niitsu, 1991). Previously it has been demonstrated that some β-D-glucans can induce the release of TNF-α

from macrophages *in vivo* and *in vitro* (Abel and Czop, 1992; Adachi et al., 1994). However, most of the investigated glucans enhanced TNF-α production stimulated with LPS (priming effect), but did not directly induce TNF-α release (Ohno *et al*., 1995; Tokunaka *et al*., 2000). All β-D-glucans tested for stimulation of TNF-α release from macrophages were non-derivatized sometimes semi-soluble samples (Tabata *et al.*, 1981; Maeda *et al.*, 1988) and the conclusion has been made that native triple helical conformation is required for the most effective immunological stimulation (Hirata *et al*., 1998). Oxidation of neutral β-D-glucans (introduction of carboxylate groups) led to destruction of the ordered helical conformation and in the loss of macrophage-stimulating activity (Tokunaka *et al*., 2000).

In contrast to these data, our observations revealed that CM-G exerted a potent macrophage-stimulating effect resulting in a dramatic increase of the released TNF-α in comparison to the control (Table I). The eliciting effect was concentration and time-dependent, increasing (although not proportionally) with the increased concentration of the applied polysaccharide and declining with the prolongation of the cultivation period (Table I). The eliciting effect of CM-CG was much less pronounced, however time- and concentration-dependence patterns were similar to those observed at the application of CM-G. The observed significant diminishment in the stimulation of TNF-α release was due to the presence of chitin component in the chitin-glucan complex. In agreement with this assumption, CM-C revealed almost no stimulating activity on TNF-α release, and the observed values were very close to that of the control. There is no data in the literature reporting cytokine eliciting activity of chitin, the second skeletal polysaccharide of the fungal cell walls. Recently, Feng *et al*., (2004) reported *in vitro* stimulation of TNF-α and interleukin-1β release from macrophages elicited by application of oligochitosan – de-*N*-acetylated derivatives of chitin with low degree of polymerization. However, contrary to β-D-glucan that binds to several

glucan-specific receptors, the authors claimed that oligochitosan was bound with a macrophage lectin receptor with D-mannose specificity. Thus, contrary to the macrophage-stimulatory function observed for oligochitosan and high-molecular-weight chitosan (Peluso *et al*., 1994), we did not observe similar activity by a soluble derivative of chitin and, moreover, presence of chitin in fungal chitin-glucan complex led to a significant reduction of the major immunostimulating polysaccharide - β-D-glucan. Our observations indicate that antitumor effect reported by some authors for chitin or chitooligosaccharides (Suzuki *et al*., 1987; Saiki *et al*., 1990) should be attributed to other mechanisms than macrophage activation and increased cytokine release.

The documented ability of β-D-glucan to significantly stimulate TNF-α release and the observed differences in the physiological activity of the two fungal polysaccharides open new possibilities for the study of the mechanism of signal transduction in the model systems including mammalian and insect organisms.

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Table I**.** Stimulation of release of TNF-α by carboxymethyl glucan (CM-G), carboxymethyl chitin-glucan (CM-CG), and carboxymethyl chitin (CM-C) in three concentrations:12.5 μg/ml, 25.0 μg/ml, and 50.0 μg/ml. Data are expressed as mean ± SD from at least three independent experiments analyzed by ANOVA and Benferroni´s pair-wise tests. $^{*}P < 0.001$ was calculated *vs*. non-stimulated cells (control) and *vs*. experiments involving CM-CG and CM-C. $^{**}P < 0.001$ was calculated *vs*. control.

Concentration of stimulant (μg/ml)	TNF-α release (pg/ml)		
	3 h	6 h	24 h
CM-G			
12.5	1390.4 ± 56.6*	1322.8 ± 56.4*	1145.2 ± 64.9*
25.0	1929.0 ± 136.1*	1585.4 ± 69.4*	1328.1 ± 84.1*
50.0	2653.8 ± 42.6*	2504.9 ± 63.1*	2114.9 ± 108.6*
CM-CG			
12.5	34.5 ± 10.8	22.0 ± 6.6	16.4 ± 2.0
25.0	113.9 ± 17.9	90.0 ± 9.7	73.7 ± 7.8
50.0	216.8 ± 13.9**	175.8 ± 17.7**	160.9 ± 12.9**
CM-C			
12.5	13.5 ± 1.8	7.2 ± 1.8	6.4 ± 1.6
25.0	8.11 ± 4.3	6.7 ± 1.6	7.8 ± 3.0
50.0	13.3 ± 2.2	6.3 ± 1.5	5.4 ± 1.1
Non-stimulated cells	9.5 ± 3.8	12.1 ± 4.4	9.0 ± 2.8