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β-1,3-Glucan reduces growth of *Mycobacterium tuberculosis* in macrophage cultures

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Abstract

The effect of β-1,3-D-glucans SSG, from *Sclerotinia sclerotiorum*, or soluble (sMG) or particulate (pMG) MacroGard® from baker's yeast on growth of *Mycobacterium tuberculosis* H37Rv in cultures of peritoneal macrophages from BALB/c mice was examined. After 24 h intracellular bacteria from lysed macrophages were cultured and the number of cfu counted. SSG given with challenge, but not 24 h after, reduced the number of *M. tuberculosis* cfu significantly. pMG, but not sMG, given with challenge had an even stronger inhibitory effect, which was enhanced after serum opsonization of the particles. The effect of serum-treated pMG was abrogated by addition of a monoclonal antibody to CD11b. The results indicate that β-glucans inhibit growth of *M. tuberculosis* in host cells in vitro, probably due to cellular stimulation and/or competitive inhibition of uptake of bacteria via CR3 (CD11b/18). © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: β-1,3-D-Glucan; Antibacterial effect; *Mycobacterium tuberculosis*; Macrophage

1. Introduction

β-1,3-D-Glucan is a glucose polymer and a major structural constituent of the cell wall of yeasts and moulds. It is a known immunomodulator that stimulates the innate immune system [1], activates complement [2], and has anti-infection [3–5] and anti-tumor [6] effects in rodent models. β-1,3-Glucan is believed to function by binding to the lectin-binding site in CR3 (CD11b/18) on polymorphonuclear leukocytes, mononuclear phagocytes and natural killer cells [7,8]. SSG is a gel-forming, but soluble β-1,3-D-glucan from the fungus *Sclerotinia sclerotiorum* [6]. MacroGard® (MG) is a β-1,3-D-glucan extracted from baker's yeast (US patent 5,401,727; EP patent 0466037) and can be produced in a soluble (sMG) and a particulate (pMG) form.

In vivo, macrophages are host cells for the obligate intracellular parasite *Mycobacterium tuberculosis*. In addition to several other uptake mechanisms, mycobacteria may enter mononuclear phagocytes via CR3 (CD11b/18) [10], which seems to be a preferred route of entry into the cells [11]. Tuberculosis is the leading cause of death among infectious diseases [12] and the incidence of multidrug-resistant tuberculosis is also increasing worldwide [13], calling for alternative pharmaceuticals. Since we have found that β-glucan protects against *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) infection in mice [14], the aim of the present investigation was to study whether β-1,3-D-glucan also has a protective effect against *M. tuberculosis* infection. However, since animal work with *M. tuberculosis* is more difficult to perform and requires P3 facilities, we have chosen first to examine whether β-glucan reduces growth of *M. tuberculosis* in cultures of macrophages from susceptible BALB/c mice. Peritoneal macrophages from the animals were cultured with the highly virulent *M. tuberculosis* strain H37Rv and with or without the β-1,3-glucans SSG, sMG or pMG. The effect was determined by growth of intracellular *M. tuberculosis* from the cultures.

2. Materials and methods

2.1. Mice

All animal experiments were approved by the local rep-

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resentative for the National Animal Research Committee and performed in accordance with standards published by the Norwegian Ministry of Agriculture. Female inbred, specific-pathogen-free mycobacteria-susceptible BALB/c mice (Gl. Bomholt gård Ltd., Ry, Denmark) were 6 weeks of age at arrival and were rested for more than 1 week before two or three animals were killed per experiment. Macrophages were harvested by peritoneal lavage with 2.5 ml of Hanks' balanced salt solution (Gibco BRL, Paisley, UK) and collected in heparinized tubes.

2.2. Bacteria

The bacterium used was *M. tuberculosis* strain H37Rv.

2.3. β-Glucans

A soluble, highly branched and gel-forming β-1,3-glucan with MW $>5\times10^6$ obtained from the culture broth of the fungus *S. sclerotiorum* IFO 9395 [6,9] was a highly appreciated gift from Dr. Naohito Ohno, Tokyo, Japan. Another β-1,3-glucan, MacroGard® (Biotec ASA, Tromsø, Norway) [15], from baker's yeast was used either in soluble form (sMG) or as particles (pMG). pMG was also treated with BALB/c serum or normal human serum (75%) for 1 h at 37°C under rotation, washed five times and resuspended in phosphate-buffered serum (PBS).

2.4. Experimental procedure

Peritoneal macrophages were obtained from BALB/c mice by peritoneal lavage, pelleted and washed twice with PBS and resuspended in RPMI 1640 supplemented with L-glutamine, 25 mM HEPES, 10% fetal calf serum, 100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin (Gibco BRL, Life Technologies, Paisley, UK). 10^5 cells in 100 μl were seeded per well into 96-well plates (Nunc, Roskilde, Denmark) and incubated at 37°C in 5% CO_2. Non-attached cells were removed by washing twice after 1 h. Then 5×10^6 *M. tuberculosis* bacteria were added per well with or without 10–500 μg ml^{-1} of SSG or 1–200 μg ml^{-1} of sMG or pMG for 24 h. On some occasions serum-treated pMG was used and on other occasions SSG was added 24 h after challenge and further incubated with cells and bacteria for another 24 h. Before adding pMG, the cultures were incubated with or without 1 mg ml^{-1} γ-Kabi (Kabi Pharmacia, Stockholm, Sweden) for 10 min and then 5 μg ml^{-1} of rat anti-mouse CD11b (cat. no. 01710D, BD Pharmingen, Heidelberg, Germany) for 20 min. The experiments were terminated by washing the cell cultures three times to remove extracellular bacteria and lysing the macrophages with 0.25 M NaOH for 15 min. The lysates were further cultured or prepared for fluorescence microscopy.

2.5. Preparations for fluorescence microscopy and bacterial cultures

The cell lysates were diluted 1:10 and 1:100 in PBS and either applied onto customized object slides (Cel-line Associates, New Field, NJ, USA) or seeded onto Løwenstein–Jensen egg medium in tubes and cultured for 3–4 weeks. The fixing and auramin staining of the slides and counting of acid-fast and stained *M. tuberculosis* bacteria by fluorescence microscopy is described in detail elsewhere [14]. *M. tuberculosis* colony-forming units (cfu), characterized by their small size, white color and easy detachment from the medium surface by a spatula, were counted after 3 or 4 weeks of cultivation.

2.6. Statistics

Student's paired *t*-test was used when the data were normally distributed, otherwise the Mann–Whitney rank sum test. *P* values <0.05 were considered significant.

3. Results

3.1. Effect of SSG on growth of M. tuberculosis bacteria in macrophage cultures

There was a significant 40% reduction ($P<0.05$) in the number of intracellular *M. tuberculosis* cfu from peritoneal macrophages incubated with 0.5 mg ml^{-1} of soluble SSG for 24 h as compared to cells incubated with PBS (Fig. 1). Lower concentrations of SSG did not have a significant effect. Growth of bacteria was also determined when SSG was added to the cultures 24 h after challenge and further

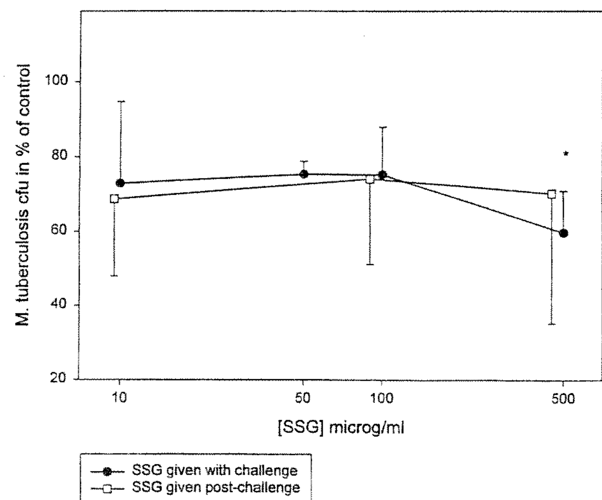

Fig. 1. Effect on number of *M. tuberculosis* H37rv cfu in cell lysates of SSG given to mouse peritoneal macrophage cultures with (●) or 24 h after (□) challenge. Control (100%) is cells cultured with bacteria only. Data points represent mean values of four experiments ± S.E.M. $*P<0.05$.

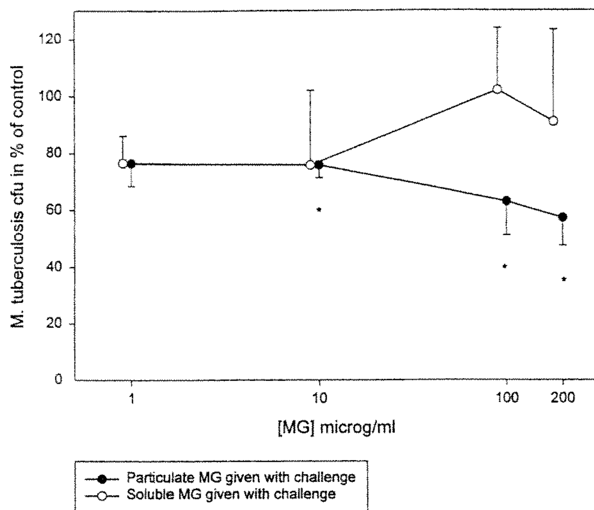

Fig. 2. Effect of pMG (•) and sMG (○) given with challenge on growth of *M. tuberculosis* cfu in cultures of mouse peritoneal macrophages. Data points are means of three to six experiments ± S.E.M. $*P<0.05$.

incubated for another 24 h. Although the cfu levels were approximately 70% of the control in the presence of SSG, the reductions were not statistically significant (Fig. 1).

3.2. *Effect of MacroGard® on growth of M. tuberculosis in macrophage cultures*

The next experiments were performed with sMG and pMG incubated with macrophages and bacteria for 24 h. No effect on the number of intracellular *M. tuberculosis* cfu was observed with sMG. On the other hand, pMG given with challenge was even more effective than SSG and inhibited growth of *M. tuberculosis* in a concentration-dependent manner at pMG concentrations of 10–200 μg ml^{-1} ($P<0.05$) (Fig. 2).

3.3. *Effect of treatment of pMG with serum and addition of anti-CD11b antibodies on M. tuberculosis number and growth in the cell cultures*

In another set of experiments pMG (0.1 mg ml^{-1}) was pre-incubated with serum and added to *M. tuberculosis*-infected macrophage cultures to examine the combined stimulatory effect of β-1,3-glucan and complement (iC3b) on the cells' anti-mycobacterial defense. However, although there was such a trend (Fig. 3), treatment of pMG with mouse or human serum did not significantly enhance the inhibitory effect of pMG alone on growth of the bacteria. Interestingly, when anti-CD11b against CR3 was added, the inhibitory effect on bacterial growth of serum-treated pMG was abolished, but not that of pMG alone (Fig. 3). Anti-CD11b alone had no effect.

Similarly, when the number of intracellular bacteria was counted there was a significant reduction in the number of bacteria after addition of pMG (> 50%) and even lower (> 80%) ($P<0.05$) after addition of serum-treated pMG (Fig. 4). The latter effect disappeared in the presence of anti-CD11b in the co-cultures.

4. Discussion

Our results show the following. (1) The β-1,3-glucans SSG and pMG reduced the number of live intracellular *M. tuberculosis* in macrophage cultures when incubated together with the bacteria. This effect was not observed

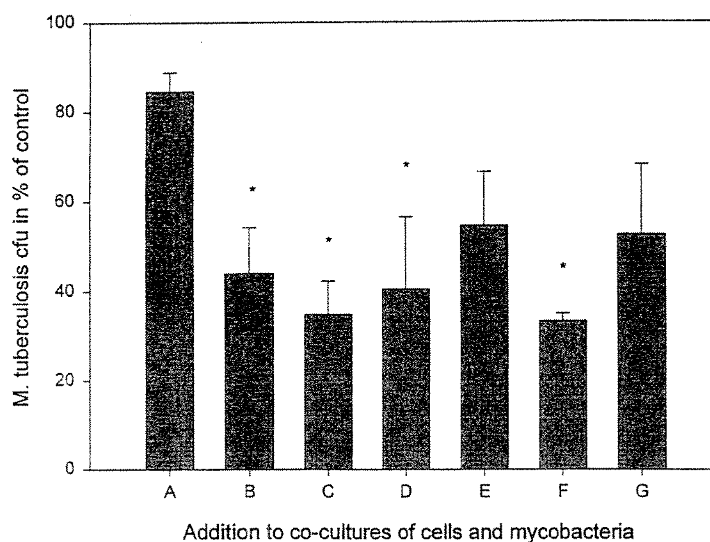

Fig. 3. Effect of serum-opsonized pMG and anti-CD11b given with challenge on growth of *M. tuberculosis* cfu in macrophage cultures. Addition to co-cultures of: anti-CD11b only (A), pMG only (B), pMG+anti-CD11b (C), pMG+mouse serum (D), pMG+mouse serum+anti-CD11b (E), pMG+human serum (F), and pMG+human serum+anti-CD11b (G). Columns represent mean values of three experiments+S.E.M. $*P<0.05$.

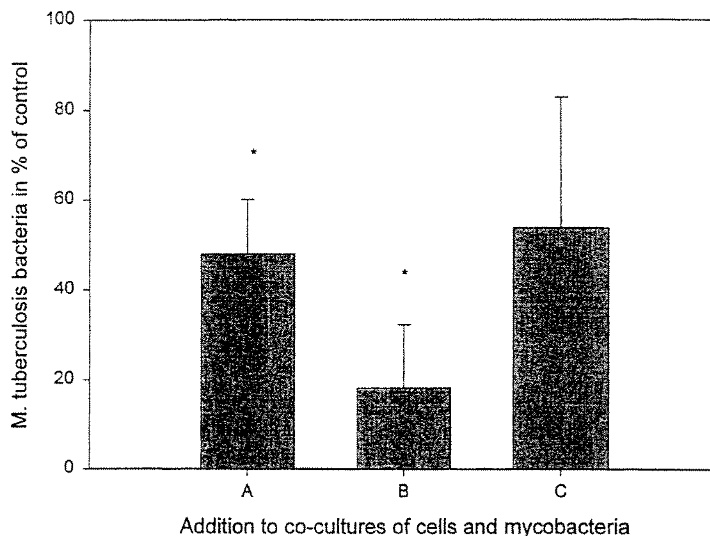

Fig. 4. Effect of serum-opsonized pMG and anti-CD11b given with challenge on number of acid-fast *M. tuberculosis* bacilli in macrophage cultures. Addition to co-cultures of: pMG only (A), pMG+serum (B), and pMG+serum+anti-CD11b (C). Columns are means of three experiments+S.E.M. $*P<0.05$.

when β-glucan was added after *M. tuberculosis*. (2) pMG given with challenge was more effective than SSG and the effect seemed to increase after serum treatment of the particles. (3) Anti-CD11b to CR3 abolished the reductive effect on bacterial number and growth of serum-treated, but not native pMG.

The number of live intracellular *M. tuberculosis* was determined by culturing the macrophage lysate and counting cfu. Although the number of auramin-stained bacteria counted by fluorescence microscopy in principle includes both live and dead non-disintegrated bacteria, these counts confirmed the cfu data. β-Glucan activates the alternative pathway of complement [2] and serum treatment of pMG particles was used to opsonize them with complement activation products and increase their effect. Although the results only suggested an increased efficacy after serum treatment, the finding agrees with known enhanced effects on monocytes of β-glucan opsonized with iC3b [16,17] and our own observation that serum-treated pMG induced interleukin (IL) 8 synthesis in differentiated U937 cells (Hetland, unpublished results).

In contrast to unstimulated mononuclear phagocytes, cells in the activated state may kill intracellular mycobacteria. The mechanisms for the observed β-glucan-induced reduction in the load of live *M. tuberculosis* bacteria when added simultaneously to macrophage cultures may be several. Firstly, β-glucans are known immunomodulators and stimulate general phagocytic defense mechanisms in macrophages [18–20], enhance release of lysosomal enzymes [21] and the pro-inflammatory cytokines tumor necrosis factor α (TNFα), IL-1 [22], and IL-6 [23]. TNFα has also been shown to promote killing of *M. tuberculosis* bacteria in mouse models [24]. Secondly, because CR3 contains a binding site for β-glucan [8] and there may be cross-reactive epitopes on *M. tuberculosis* cell wall and β-glucan [14], β-glucans may block or competitively inhibit mycobacterial entry via CR3 [10]. Such a mechanism could explain the lack of effect of soluble in contrast with particulate MG and SSG (se below). The lack of effect of β-glucan (SSG) added 24 h after challenge in vitro contrasts with the curative post-challenge effect of β-glucan observed against both established BCG and pneumococcal type 6B infection in mice [14,15]. This is probably due to additional mechanisms taking place in vivo with the whole organism reacting to the invading bacteria.

The reason for the proposed higher effect of pMG than SSG, which again was more effective than sMG, is probably the following. Whereas SSG has only one monoglucosyl unit in the side chains, MacroGard has three, which is believed to be a prerequisite for an optimal engagement of the β-glucan receptors [15]. However, the three-dimensional structure of the β-glucan, e.g. particle or viscous large molecule (SSG molecular mass $>5\times10^6$) [6] versus soluble form, may be more important for the effect than the composition of the side chains, at least in the current experiments. MG particles (4 μm in diameter) and SSG per se can cross-bind CR3 and stimulate the macrophages. Serum treatment of pMG and subsequent opsonization of the particles with iC3b after activation of the alternative complement pathway [2] may induce further enhanced stimulation of the cells. Moreover, because of their sizes the latter β-glucans could physically prevent binding of the mycobacteria to the common CR3 receptor.

Since CR3 (CD11b/18) both mediates a possible preferred mechanism of uptake for mycobacteria in macrophages [10,11], due to lack of toxic oxidative burst in response to engagement of CR3 [25], and contains a binding site for β-glucans [8], anti-CD11b was used to block the observed effect. However, the antibody blocked the effect of opsonized pMG, but not that of native pMG.

Recently, a novel receptor for β-glucan, the dectin-1 receptor [26,27], has been discovered on macrophages. Serum proteins, including complement activation product iC3b, may adhere to and cover up the β-glucan side chains on serum-treated pMG. This may prevent binding of the pMG to the dectin-1 receptor or CR3's lectin-binding site and promote binding to CR3's complement-binding site. Hence, anti-CD11b will affect the action of opsonized but not unopsonized pMG. The dectin-1 receptor may be the specific macrophage β-glucan receptor described by Czop and Kay [7] revisited, but disputed by Ross et al. [17].

In conclusion, the observed effect of β-glucans on *M. tuberculosis* growth may be due to both macrophage stimulation and competitive inhibition of uptake of bacilli via CR3 (CD11b/18). Particulate and gel-forming β-glucans may be more effective at this than easily soluble ones. Whether β-glucans have a protective effect against tuberculosis should be studied in rodent models in P3 facilities.

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