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Induction of IFN-γ by a highly branched 1,3-β-D-glucan from *Aureobasidium pullulans* in mouse-derived splenocytes via dectin-1-independent pathways

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ABSTRACT

We have previously elucidated the precise structure of a unique type of 1,3-β-D-glucan, AP–FBG (*Aureobasidium pullulans*-fermented β-D-glucan), from the fungus *A. pullulans* and found that AP–FBG strongly induced the production of various cytokines in DBA/2 mouse-derived splenocytes *in vitro*. However, the mechanism(s) of action of AP–FBG on *in vitro* mouse primary cells have not been characterized in detail. Herein, we report that the production of IFN-γ in DBA/2 mouse-derived splenocytes by AP–FBG was not inhibited following treatment with an anti-dectin-1 neutralizing antibody. In addition, AP–FBG not only failed to activate dectin-1-mediated signaling pathways, examined by a reporter gene assay but also failed to bind to dectin-1, a pivotal receptor for 1,3-β-D-glucan. Taken together, AP–FBG induced cell activation via dectin-1-independent pathways.

1. Introduction

β-Glucan is a well-known biological response modifier that is widely distributed in nature. A variety of β-glucans have been isolated from different sources, e.g., fungi, plants, and seaweed. The physicochemical properties of β-glucans differ depending on their structural features, i.e., their linkage type, degree of branching, degree of polymerization, conformation (triple helix, single helix, or random coil), and molecular weight [1–3]. Recent reports have highlighted the fact that β-glucans play a significant role in the treatment of cancer and infectious diseases and can be used in both modern medicine and traditional oriental therapies. β-Glucans are also important dietary substances; they lower the level of plasma cholesterol, enhance hematopoietic response, and possess anti-tumor and immunomodulating properties [4–6]. In Japan, lentinan extracted from *Lentinus edodes* [7] and sonifilan (SPG) from *Schizophyllum commune* [8] have been clinically used in cancer therapy.

We recently isolated a unique type of β-D-glucan, AP–FBG (*Aureobasidium pullulans*-fermented β-D-glucan), from the polymorphic fungus *A. pullulans*. Nuclear magnetic resonance (NMR) spectroscopy revealed that this β-D-glucan had a mixed structure comprising a 1,3-β-D-glucan backbone with single 1,6-β-D-glucopyranosyl branching units at every 2nd residue as the major structure and a 1,3-β-D-glucan backbone with single 1,6-β-D-glucopyranosyl-branching units at every 3rd residue as the minor structure (the ratio of the major and minor units was approximately 7:3) and found that AP–FBG strongly induced the production of various cytokines in DBA/2 mouse-derived splenocytes *in vitro* [9–12]. However, the mechanism(s) of action of AP–FBG, which possesses a characteristic high branching structure, on immune cells have not been characterized in depth.

Recent studies have proposed that the pivotal mechanism(s) underlying the immunostimulatory effects of β-D-glucans involves their ability to induce innate immune responses via specific receptors [13–16]. Many studies have confirmed that dectin-1 is a pivotal receptor for 1,3-β-D-glucan because it is essential for the following biological effects: (1) the anti-tumor effects of SPG, a 1,3-β-D-glucan [17]; (2) the production of cytokines and reactive oxygen species by both dendritic cells (DCs) and macrophages after stimulation with 1,3-β-D-glucans; (3) DC maturation induced by a soluble, mushroom-derived β-D-glucan, i.e., *Sparassis crispa* glucan (SCG); (4) the host defense response against β-D-glucan hold fungi [18–20]; and (5) the activation of immune cells by a barley-derived β-D-glucan, which is a linear mixed-linkage β-D-glucan composed of 1,3- and 1,4-β-D-glucopyranose polymers [21,22].

Although the relationship between the structure and biological activities of β-D-glucans is well characterized, the ligand specifici-

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ties of β-D-glucan receptors, e.g., dectin-1, and their relationship with biological effects are still obscure [16,21,23,24]. To address this issue, it is useful to determine the binding ability of a unique type of β-D-glucan, AP–FBG, to 1,3-β-D-glucan receptors such as dectin-1 and ascertain its biological activities. This information may provide novel insights into the development of more effective β-D-glucan agents, especially immunostimulatory agents.

Determining the effects and mechanism(s) of action of AP–FBG on immune cells is important not only to promote its use in the clinical setting, as a health food, and/or in immunotherapy but also to reveal the underlying mechanism(s). Therefore, the aim of our study was to investigate whether the induction of interferon-γ (IFN-γ) production by AP–FBG was dependent on dectin-1, which is most likely to be a typical 1,3-β-D-glucan, despite differences in their primary structures with respect to the frequency of 6-branched side chain units. In this study, we examined the effects of AP–FBG on splenocytes derived from the DBA/2 mouse strain that are highly responsive to 1,3-β-D-glucan [25]. We also investigated the possible involvement of the pivotal β-D-glucan receptor, dectin-1, in the production of IFN-γ in these DBA/2 mouse-derived splenocytes.

2. Materials and methods

2.1. Animals and materials

Male DBA/2 mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in a specific-pathogen-free environment and were used at 8–10 weeks for all experiments. All animal experiments were conducted in accordance with the Guidelines for Laboratory Animal Experiments established by the Tokyo University of Pharmacy and Life Sciences, and all the experimental protocols were approved.

2.2. Carbohydrates

AP–FBG was prepared according to a method described in a previous report [9]. Elemental analysis (C:H:N = 41.01:6.36:0.09) revealed that AP–FBG was a highly pure carbohydrate and that it did not contain contaminants such as nucleic acids, proteins, chitins, and peptidoglycans. SCG was prepared as previously described [25,26]. Zymosan particles were purchased (Sigma, St. Luis, USA); they were washed thrice with sterilized water and autoclaved at 121 °C for 20 min before use in order to remove endotoxin-like substances [27]. Dextran was purchased from GE Healthcare (Buckinghamshire, UK). Lipopolysaccharide (LPS) was extracted from *Escherichia coli* O111:B4 cells (Sigma, St. Luis, USA) by the phenol-water method.

2.3. Preparation of splenocytes for cell culture

Splenocytes were prepared as previously described [25]. Briefly, the spleens from male DBA/2 mice were teased apart in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma, St. Louis, USA), and after centrifugation, the single cell suspension was treated with ACK lysis buffer to lyse the red blood cells. After centrifugation, splenocytes were maintained in RPMI-1640 supplemented with 50 μg/mL of gentamicin sulfate and 10% heat-inactivated fetal bovine serum (FBS) (EQUITECH-BIO, Texas, USA). Cells were cultured in 24-well flat bottomed plates (SUMILON, Tokyo, Japan) and stimulated with glucans for 48 h at 37 °C under a 5% CO_2 atmosphere.

2.4. Examination of the effect of polymyxin B

We examined the effect of polymyxin B (PMB) (Sigma, St. Louis, USA) on the production of cytokines in splenocytes stimulated with glucans in order to rule out possible endotoxin contamination. Briefly, cells were cultured in 24-well flat bottomed plates (SUMILON, Tokyo, Japan) and stimulated with pre-mixed glucans (100 μg/mL) and PMB (10 μg/mL) before stimulation, and cultured for 48 h at 37 °C under a 5% CO_2 atmosphere.

2.5. Examination of the effect of an anti-dectin-1 monoclonal antibody

We examined the effect of an anti-dectin-1 monoclonal antibody, 2A11, (Hycult Biotechnology b.v., Frontstraat, Netherlands) on the production of cytokines in splenocytes stimulated with glucans in order to examine the possible contribution of dectin-1. Briefly, cells were cultured in 48-well flat bottomed plates (SUMILON, Tokyo, Japan) and before stimulation with glucans (100 μg/mL), splenocytes were preincubated with 2A11 (0.2–5 μg/mL) for 1 h, and cultured for 48 h at 37 °C under a 5% CO_2 atmosphere.

2.6. Cytokine assay

The cytokine concentrations in the samples were determined using the OptiEIA kit (BD Biosciences, New Jersey, USA).

2.7. Preparation of soluble dectin-1 molecules

We prepared two different forms of recombinant dectin-1 molecules, namely, soluble dectin-1 (sDectin-1) and soluble dectin-1-Fc (sDectin-1-Fc). sDectin-1 was isolated from the culture supernatant of CHO cells transfected with mouse dectin-1 cDNA encoding the carbohydrate recognition domain (CRD) and a polyhistidine tag sequence. The glycosylated portion of sDectin-1 was subjected to periodate oxidation and cyanoborotritide reduction for biotin hydrazide conjugation. Biotin-conjugated sDectin-1 was desalted by dialysis against phosphate-buffered saline (PBS). sDectin-1-Fc was prepared as a chimeric protein of the mDectin-1 CRD and the Fc portion of human immunoglobulin (Ig)G1. The recombinant Fc-chimeric protein was isolated on a Hi-trap Protein A column (GE Healthcare, Buckinghamshire, UK) from the culture supernatant.

2.8. sDectin-1-binding assay

The binding specificity of various β-glucan preparations was assessed by competitive enzyme-linked immunosorbent assay (ELISA). Briefly, an ELISA plate (Nunc, Roskilde, Denmark) was coated with 1,6-β-monoglucopyranosyl branched 1,3-β-D-glucan, obtained from *S. commune* (SPG, 20 μg/mL), dissolved in bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. The unbound SPG was washed with PBS containing 0.05% Tween 20 (PBST), and the plate was blocked with PBS containing 0.5% bovine serum albumin (BSA) (BPBS). The various glucan samples were diluted with BPBS to achieve concentrations of 0–500 μg/mL and mixed with biotin-labeled sDectin-1 (100 ng/mL) for 1 h before addition to the SPG-coated ELISA plate. The plate containing sDectin-1 was incubated for 1 h at room temperature, washed with PBST, and further incubated with peroxidase-conjugated streptavidin (Pharmingen). The binding of sDectin-1 to solid-phase SPG was monitored using the peroxidase substrate TMB (KPL Inc., MD, USA), and color development was stopped by adding 1 M phosphoric acid; the optical density was measured at 450 nm. To exclude the possibility that these glucan preparations nonspecifically inhibited the binding of sDectin-1 molecules to solid-phase SPG, the ELISA plate was coated with each of the various glucans, and the ability of sDectin-1 to bind the glucan preparations was tested. The ELISA plate was coated with various concentrations of glucan samples by an overnight incubation at 4 °C; the unbound glucans were removed by washing, and the plate was blocked with BPBS. The glucans on the ELISA plate were incubated with sDectin-1-Fc,