Inhibitory Effects of Water-Soluble Low-Molecular-Weight β-(1,3-1,6) D-Glucan Isolated from Aureobasidium pullulans 1A1 Strain Black Yeast on Mast Cell Degranulation and Passive Cutaneous Anaphylaxis

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Received July 19, 2011; Accepted October 4, 2011; Online Publication, January 7, 2012
[doi:10.1271/bbb.110536]

We investigated the effects of water-soluble low-molecular-weight β-(1,3-1,6) D-glucan isolated from Aureobasidium pullulans 1A1 strain black yeast (LMW-β-glucan) on mast cell-mediated anaphylactic reactions. Although it is known that LMW-β-glucan has anti-tumor, anti-metastatic and anti-stress effects, the roles of LMW-β-glucan in immediate-type allergic reactions have not been fully investigated. We examined whether LMW-β-glucan could inhibit mast cell degranulation and passive cutaneous anaphylaxis (PCA). LMW-β-glucan dose-dependently inhibited the degranulation of both rat basophilic leukemia (RBL-2H3) and cultured mast cells (CMCs) activated by calcium ionophore A23187 or IgE. However, LMW-β-glucan had no cytotoxicity towards RBL-2H3 cells and CMCs. Furthermore, orally administered LMW-β-glucan inhibited the IgE-induced PCA reaction in mice. These results show LMW-β-glucan to be a possible compound for the effective therapeutic treatment of allergic diseases.

Key words: β-glucan; mast cell degranulation; passive cutaneous anaphylaxis; anti-allergic effect

The prevalence of such allergic diseases as allergic rhinitis, atopic dermatitis, asthma and food allergies has increased in most countries.¹³) Mast cells and basophils play critical roles in various biological processes relating to allergic diseases.²³) These cells express the high-affinity receptor for IgE on their surface. The interaction of multivalent antigens with surface-bound IgE causes the secretion of granule-stored mediators, as well as the de novo synthesis of cytokines.³⁵) Those mediators and cytokines activate the migration of neutrophils and macrophages, and the reactions brought about by these cells cause tissue inflammation⁶ and an allergic reaction.

Beta-glucan consists of many glucose molecules joined together with β-1,3- and/or β-1,6-linkages and is synthesized by many species of mushroom, black yeast (Aureobasidium pullulans) and fungi. Beta-glucan is a substrate known to stimulate immune reactions.⁸ Beta-1,3 or β-1,6-glucan isolated from mushrooms has high viscosity, a high molecular weight (over 2,000 kDa) and is insoluble.⁹ Beta-glucan can also easily form a gel and higher-order structures. The purification of these compounds is consequently extremely difficult, and crude β-glucan fractions are used in many experiments.¹⁰ A water-soluble low-molecular-weight (approximately 100 kDa) β-(1,3-1,6) D-glucan (LMW-β-glucan) has recently been prepared from the Aureobasidium pullulans 1A1 strain of black yeast.¹¹ It has been reported that the LMW-β-glucan exerted anti-tumor, anti-metastatic¹² and anti-stress actions,¹³ although the effects of LMW-β-glucan on anaphylactic reactions have not previously been fully reported.

We investigated in the present study the anti-allergic effects of LMW-β-glucan in vitro and in vivo. We evaluated the anti-degranulation effects of LMW-β-glucan by using an in vitro culture of rat basophilic leukemia (RBL-2H3) and bone marrow-derived cultured mast cells (CMCs). To evaluate its action in vivo, we investigated the effects of orally administered LMW-β-glucan on the increase in vascular permeability induced by stimulating ICR mice with IgE.

Materials and Methods

Reagents. Dulbecco’s modified Eagle’s medium (DMEM), an antibiotic solution (100×), pokeweed mitogen (PWM), p-nitrophenyl-N-acetyl-β-d-glucopyranoside (PNAG), the mouse monoclonal anti-dinitrophenyl (DNP) IgE antibody (clone SPE-7), DNP-labeled human serum albumin (HSA), calcium ionophore A23187 and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Alpha-MEM was obtained from ICN Biochemicals (Costa Mesa, CA, USA), and fetal bovine serum (FBS) was purchased from Nippon Bio-Supply Center (Nagoya, Japan). Evans blue dye and Triton X-100 were purchased from Wako Pure Chemical Industries (Osaka, Japan), and Rizaben, which contained 10% tranilast, was obtained from Kissui Pharmaceutical Co. (Nagano, Japan).

Preparation of LMW-β-glucan from A. pullulans 1A1. The A. pullulans 1A1 strain was obtained from strain K-1 by a general mutation treatment¹¹,¹⁴ and was cultured for 5–10 d at 25–30°C in a medium supplemented with 0.3% sodium ascorbate, 3% sucrose, 0.001% Rizaben, which contained 10% tranilast, was obtained from Kissui Pharmaceutical Co. (Nagano, Japan).

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Abbreviations: LMW-β-glucan, low-molecular-weight β-(1,3-1,6) D-glucan; PCA, passive cutaneous anaphylaxis; DNP, dinitrophenyl; HSA, human serum albumin; CMC, cultured mast cell
FeSO₄·7H₂O, 0.05% MgSO₄·7H₂O, 0.1% KCl, 0.1% K₂HPO₄ and 0.2% NaClO₃ (3L). The resulting culture was treated under alkaline conditions by adding NaOH (pH 13) to lower its viscosity. After removing the cells by centrifugation, the culture supernatant was filtered and condensed by ultrafiltration through a UF membrane (Nitto Denko Co., Tokyo, Japan) to remove the low-molecular-weight substances and salts, and the ultrafiltered supernatant was adjusted to pH 3.5 with citric acid. LMW-β-glucan was finally precipitated with 70% ethanol, freeze-dried and dissolved in a sterile 0.9% NaCl solution.

### Animals
Female 6-week-old ICR mice (25–27 g) and male 9-week-old ddY mice (35–37 g) were purchased from Japan SLC (Hamamatsu, Japan). All animals were housed at 23 ± 2 °C and 55 ± 10% humidity and had access to standard laboratory rodent feed (Oriental Yeast, Tokyo, Japan) and water ad libitum. All animal experiments were performed according to the Guidelines Relating to the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animal Science and were approved by the Institutional Animal Care and Use Committee of Senri Kinran University.

### Cells
Rat basophilic leukemia RBL-2H3 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS. A PWM-stimulated spleen cell-conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al. The ddY strain mice were used to obtain CMCs. The mice were killed by decapitation after ether anesthesia, and the bone marrow cells were taken. These bone marrow cells were cultured in α-MEM supplemented with 10% PWM-SCM and 10% heat-inactivated FBS. Half of the medium was replaced every 7 d. More than 95% of the cells were CMCs 4 weeks after initiating the culture.

### Degranulation of mast cells
It has been reported that the release of β-hexosaminidase was well correlated with that of histamine, a major component of mast cell granules, so the degranulation of mast cells was determined by using a β-hexosaminidase release assay previously described. Briefly, the mast cells were washed with Tyrode’s buffer (137 mM NaCl, 5.6 mM glucose, 11.9 mM NaHCO₃, 2.7 mM KCl and 0.32 mM NaH₂PO₄) containing 1 mM CaCl₂ and 0.5 mM MgCl₂, and then various concentrations of LMW-β-glucan (0, 250, 500, 750, and 1000 μg/mL) were added. RBL-2H3 cells (9.6 × 10⁴) were treated with 10 μM A23187 for 1 h, and CMCs (5 × 10⁴) were treated with 0.5 μM A23187 for 30 min at 37 °C immediately after adding LMW-β-glucan. The cell supernatant and total cell lysate dissolved in 2% Triton X-100 were collected, and β-hexosaminidase in the supernatant and cell lysate was quantified by spectrophotometrically measuring the hydrolysis of PNAG in a 0.1 M sodium citrate buffer (pH 4.5). The reaction was terminated after 90 min by adding 0.2 M glycine (pH 11.0).

In the case of IgE-dependent degranulation, RBL-2H3 cells (9.6 × 10⁴) or CMCs (5 × 10⁴) were sensitized overnight at 37 °C with anti-DNP IgE (0.5 μg/mL) for the RBL-2H3 cells and 1 μg/mL for CMCs. The cells were washed with Tyrode’s buffer containing 1 mM CaCl₂ and 0.5 mM MgCl₂ and then various concentrations of LMW-β-glucan (0, 250, 500, 750, and 1000 μg/mL) were added. The cells were stimulated for 1 h at 37 °C with DNP-HSA (10 μg/mL for the RBL-2H3 cells and 1 μg/mL for CMCs) immediately after adding LMW-β-glucan. Beta-hexosaminidase in the supernatant and cell lysate was quantified in the same manner as that for stimulation with A23187.

The cells stimulated with A23187 showed a degranulation rate of more than 60% in the case of RBL-2H3 and more than 80% in the case of CMCs. When the cells were stimulated with IgE, the degranulation rate was more than 40% in the case of RBL-2H3 and more than 70% in the case of CMCs. The percentage inhibition of β-hexosaminidase release was calculated by using the following formula: percentage inhibition = (β-hexosaminidase release without LMW-β-glucan − β-hexosaminidase release with LMW-β-glucan)/β-hexosaminidase release without LMW-β-glucan × 100. To confirm whether LMW-β-glucan did not itself inhibit the activity of β-hexosaminidase, we sonicated RBL-2H3 cells or CMCs, used this as a solution containing β-hexosaminidase, added LMW-β-glucan, incubated for 1 h, and measured the activity of β-hexosaminidase.

### MTT assay
Cell viability was determined by applying an MTT assay. Briefly, 100 μL of RBL-2H3 cells (5 × 10⁴) or CMCs (5 × 10⁴) was cultured in 96-well plates for 24 or 48 h after treatment at each concentration of LMW-β-glucan. A volume of 10 μL of the MTT solution (5 mg/mL) was added, and the cells were incubated for an additional 4 h at 37 °C. After removing the supernatant, the insoluble formazan product was dissolved in 100 μL of 0.04 N HCl in isopropanol alcohol, and the optical density was measured at 570 nm. Viability is quantified as the percentage of the mean ± standard error (SE) for three independent experiments. The control group was assigned a value of 100%, and the LMW-β-glucan-treated groups are presented as a percentage of control value.

### Passive cutaneous anaphylaxis (PCA) reaction
The mice were intradermally injected into the ear with 0.1 μg of anti-DNP IgE. The mice were intravenously challenged with 0.2 mL (1 mg/mL) of DNP-HSA containing 2% Evans blue dye 4 h after sensitization with IgE. LMW-β-glucan (50, 100, 125, or 150 mg/kg) or saline was administered orally 2 h before the antigen challenge in a series of experiments, saline being used in the control group. Tranilast (400 mg/kg), a chemical mediator release suppressor, was orally administered 2 h before the antigen challenge, and the mice were sacrificed and the ears removed and weighed 30 min after the antigen challenge. The ears were dissolved in 200 μL of 1 N KOH and incubated overnight at 37 °C to measure the amount of Evans blue dye present in the exudate. The dissolved tissue solution was then added to 400 μL of a mixture of acetone and 0.6 N phosphoric acid (5:13 v/v), and the optical density was measured at 620 nm. The amount of dye in the exudate was calculated from a standard curve for a known concentration of Evans blue. The results are expressed as a percentage of the mean amount of dye in the exudate from the administered test mice compared with the control mice that had received saline.

### Statistical analysis
The results obtained are expressed as the mean ± SE. Statistical significance was tested by Dunnett’s multiple-comparison test following a one-way analysis of variance and Student’s t-test for two-sample comparison. Results with a p < 0.05 value are considered statistically significant.

### Results

#### Structure of the LMW-β-glucan
The structure of the LMW-β-glucan consisted of a main chain of β-1,3-linked β-D-glucan with β-1,6-linked side-chains (Fig. 1. The integral ratio of the branches of β-1,6 to β-1,3 was estimated to be 50–80% from the results of NMR and enzymatic analyses. The average molecular weight of LMW-β-glucan was determined to be approximately 100 kDa by a direct comparison with a water-soluble pullulan standard marker with molecular weight of 5,900–1,600,000 (Shodex, Standard P-82, Showa Denko Co., Tokyo, Japan) by gel chromatography. The purity of LMW-β-glucan was finally found to be 95% or more for all the formed polysaccharides based on the results of the NMR analysis.

#### Effects of LMW-β-glucan on mast cell degranulation
We investigated the effects of LMW-β-glucan on mast cell degranulation to examine its in vitro anti-allergic activity. Various concentrations of LMW-β-glucan were added to mast cells before A23187 stimulation as already described. The β-hexosaminidase release was markedly and dose dependently decreased in RBL-2H3 cells (Fig. 2A) and CMCs (Fig. 2B) by adding 500–1000 μg/mL of LMW-β-glucan. This indicates the suppressive effect of LMW-β-glucan on the degranulation of both RBL-2H3 cells and CMCs induced by A23187. Various concentrations of LMW-β-glucan were next added to RBL-2H3 cells after IgE
sensitization as already described, and the cells were then stimulated with the antigen. Figure 2C shows that the β-hexosaminidase release was markedly decreased in a dose-dependent manner by adding 500–1000 μg/mL of LMW-β-glucan. We also examined the effects of LMW-β-glucan on the degranulation of CMCs. Figure 2D shows the dose-dependent inhibition of β-hexosaminidase release by adding 250–1000 μg/mL of LMW-β-glucan. This indicates the suppressive effect of LMW-β-glucan on the degranulation of both RBL-2H3 cells and CMCs induced by IgE. We examined whether LMW-β-glucan did not itself inhibit the activity of β-hexosaminidase. This was confirmed since the activity of β-hexosaminidase did not change after adding LMW-β-glucan. These findings indicate that LMW-β-glucan exerted its anti-allergic effect by inhibiting the release of chemical mediators from the mast cells. The rate of degranulation was measured when mast cells had not been stimulated to investigate whether LMW-β-glucan induced degranulation in the RBL-2H3 cells or CMCs. There was no difference between the rate of degranulation with and without LMW-β-glucan in both the RBL-2H3 cells and CMCs, indicating that LMW-β-glucan did not affect the spontaneous release of mast cells.

Effect of LMW-β-glucan on the viability of RBL-2H3 cells and CMCs

We next examined the effects of LMW-β-glucan on the viability of RBL-2H3 cells. The RBL-2H3 cells were incubated with LMW-β-glucan at 37°C for 24 or 48 h, before the viability of the cells was determined by an MTT assay. The viability of RBL-2H3 cells had not decreased after incubating with 250–1000 μg/mL of LMW-β-glucan for either 24 h (Fig. 3A) or 48 h (Fig. 3B). The effect of LMW-β-glucan on the viability of CMCs was also determined. Treating with 250–1000 μg/mL of LMW-β-glucan had no cytotoxic effect on cell viability after either 24 h (Fig. 3C) or 48 h (Fig. 3D) of incubation.

Effect on the PCA reaction of an oral administration of LMW-β-glucan

We measured the effect of LMW-β-glucan on the IgE-dependent PCA reaction, one of the most important in vivo models of anaphylaxis in local allergic reactions.21,22 A high dose of LMW-β-glucan (125 or 150 mg/kg) orally administered to the ICR mice significantly reduced the amount of dye in the exudate (Fig. 4), indicating that LMW-β-glucan inhibited the PCA reaction. Tranilast (400 mg/kg), an anti-allergic drug often used as a positive control for anti-allergic reactions targeting mast cell degranulation, also inhibited the PCA reaction.

Discussion

The anti-allergic effect of LMW-β-glucan has been examined in this study. We found that LMW-β-glucan inhibited the A23187- and IgE-induced β-hexosaminidase release in both RBL-2H3 cells and CMCs. The
value for released β-hexosaminidase has frequently been used as an indicator for evaluating the extent of degranulation by mast cells. The release of histamine and other chemical mediators from mast cells is an important process in initiating the immediate type of anaphylactic reaction.5) LMW-β-glucan therefore exerted an anti-allergic effect by inhibiting the release of chemical mediators from mast cells.

The concentration of LMW-β-glucan for its inhibitory effect of degranulation was more than 750 µg/mL in RBL-2H3 cells that had been stimulated with both A23187 and IgE. It was also more than 750 µg/mL in CMCs stimulated with A23187. In contrast, LMW-β-glucan has induced interleukin (IL)-6 production at 100 µg/mL in macrophages. 12) These results indicate that the effective concentration of LMW-β-glucan was dependent on the type of cell.

Examining the effects of LMW-β-glucan on the viability of both RBL-2H3 cells and CMCs indicated that LMW-β-glucan up to 1000 µg/mL had no cytotoxicity. This result indicates that β-hexosaminidase released from both RBL-2H3 cells and CMCs was reduced after they had been treated with LMW-β-glucan without affecting the proliferation and viability of the cells.

The IgE-mediated PCA reaction in vivo is a method used for studying the mechanism of the immediate allergic reaction. This present study is the first to show the inhibitory effect of LMW-β-glucan by the IgE-mediated PCA reaction. Tranilast, an anti-allergic drug often used as a positive control for anti-allergic reactions targeting mast cell degranulation, inhibited the PCA reaction. The inhibitory effect of LMW-β-glucan on the PCA reaction may therefore have been due to reduced degranulation. Although the inhibitory effect of LMW-β-glucan on the PCA reaction was detected at both 125 mg/kg and 150 mg/kg, the extent of inhibition was significantly greater at 150 mg/kg than at 125 mg/kg. Further investigation will be required for confirming this finding.

Various allergic effects occur via IgE-mediated hypersensitivity reactions.4,5) The production of IgE is promoted by T helper (Th)2 cells and their cytokines such as IL-4 and IL-5, while Th1 cells and their cytokines such as interferon (IFN)-γ and IL-12 can inhibit IgE production.23) Orally administered LMW-β-glucan has signi-
We have demonstrated here that production of IFN-γ significantly inhibited the ova-albumin (OVA)-induced allergic reaction in mice, and this might have been due to the inhibition of OVA-specific IgE elevation through the production of IFN-γ and IL-12. Moreover, treatment with β-glucan from zymosan was effective for adjusting the Th1/Th2 balance in patients with allergic rhinitis. We have demonstrated here that β-glucan had an inhibitory effect on mast cell activation, in addition to its anti-allergic properties by influencing the Th1/Th2 balance and suppressing the IgE production.

We investigated in the present study the anti-allergic effect of LMW-β-glucan on IgE-stimulated RBL-2H3 cells and CMCs. LMW-β-glucan inhibited the degranulation of mast cells. Mast cells initiate exocytosis of the contents of secretory granules in response to IgE receptor cross-linking. There is a possibility that mast cells are unable to bind to IgE due to a lack of or weak expression of IgE receptors in the presence of LMW-β-glucan. Furthermore, aggregation of the IgE receptor activates such cytosolic Src protein-tyrosine kinases as Lyn which phosphorylates the immunoreceptor tyrosine-based activation motif (ITAM) and activates Syk after ITAM binding. Lyn and Syk phosphorylate several adaptor molecules and enzymes to regulate mast cell activation.

LMW-β-glucan may affect the phosphorylation of those proteins which regulate signal transduction in mast cells. LMW-β-glucan also inhibited the degranulation of A23187-stimulated RBL-2H3 cells and CMCs. Since A23187 induces Ca\(^{2+}\) flux and activates protein kinase C (PKC), which is essential for mast cell degranulation, LMW-β-glucan may have affected the activities of PKC. Further studies are necessary to explain the inhibitory mechanism of LMW-β-glucan for mast cell degranulation.

In conclusion, LMW-β-glucan inhibited the release of β-hexosaminidase from mast cells elicited by both the A23187- and IgE-mediated pathways. It also inhibited the anti-DNP IgE-induced PCA reaction when orally administered. These results indicate that LMW-β-glucan had an inhibitory effect on an allergic reaction. Furthermore, the in vivo and in vitro anti-allergic effects of LMW-β-glucan suggest possible therapeutic applications of this component in inflammatory allergic diseases.

Acknowledgments

This work was supported by research funding from IGA Bio Research Co., Ltd., and by a grant from Senri Kinran University.

References