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Effects of water-soluble low-molecular-weight β-1, 3-D-glucan (branch β-1, 6) isolated from *Aureobasidium pullulans* 1A1 strain black yeast on restraint stress in mice

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Abstract

It is well known that different stress paradigms are able to rapidly induce corticosterone production and immune function through the activation of the hypothalamic–pituitary–adrenal axis. It has been reported that glucocorticoids suppress natural killer (NK) activity and interleukin (IL)-1 production and, on the other hand, that IL-1 and IL-6 stimulate the release of corticotrophin-releasing-hormone from the rat hypothalamus. Moreover, it has been reported that IL-12 plays a central role in the initiation of cell-mediated immunity, directly and via its induction of interferon (IFN)-γ and activation of NK cells. In this study, we examined the effects of water-soluble low-molecular-weight β-glucan isolated from *Aureobasidium pullulans* 1A1 strain on the corticosterone levels and immune function, such as NK activity and IL-6 and IL-12 production, using a restraint stress-induced mouse model. The water-soluble low-molecular-weight β-glucan at a dose of 50 or 100 mg kg^{-1} inhibited the increases in the blood corticosterone level and the reduction of NK activity induced by restraint stress. Furthermore, the water-soluble low-molecular-weight β-glucan (100 mg kg^{-1}) prevented the reduction of IL-6 and IL-12 production by splenocytes caused by restraint stress. These findings suggest that the inhibitory actions of water-soluble low-molecular-weight β-glucan on the increase in corticosterone level and reduction of NK activity induced by restraint stress may be associated with the abrogation of the IL-6 and IL-12 reduction caused by the stress. Thus, water-soluble low-molecular-weight β-glucan may be an effective dietary supplement for the prevention of stress.

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Introduction

It has been established that stress can affect immune function through the activation of the hypothalamic–pituitary–adrenal axis resulting in the production of a number of neuroendocrine mediators (Riley 1981; Zwilling et al 1993). Some of these mediators, such as corticosterone in rodents or cortisol in man, have been shown to be immunosuppressive in both rodents and man (Dhabhar et al 1994). It is well known that glucocorticoids are major mediators of the stress response and modulate many signalling events in the immune response. Glucocorticoids modulate antigen presentation, cytokine production, T-cell expansion and natural killer cell activity (Belsito et al 1982; Synder & Unanue 1982; Chrousos & Gold 1992; Bonneau et al 1997; Maes et al 1998; Steer et al 1998; Wiegers & Reul 1998). Plasma corticosterone levels have been used for many years as an indicator of stress in mice. Thus, stress causes various disorders in relation to the bio-regulatory, autonomic nervous, endocrine and immune systems. In general, (1→3) or (1→6) β-glucans isolated from basidiomycetes mushrooms have high viscosity and high molecular weight (over 2000 kDa) and are water-insoluble. In addition, β-glucan easily forms gels containing high-order structures of single spirals or triplet spirals due to its unique primary structure; therefore, its purification is extremely difficult, and consequently crude β-glucan fractions have been used in many reported studies rather than purified β-glucan. We succeeded in the isolation and industrial-scale production of water-soluble low-molecular-weight β-(1,3-1,6) D-glucan from *Aureobasidium pullulans* GM-NH-1A1 strain (black yeast, a mutant of the strain K-1)

(Suzuki et al 2005). *A. pullulans* is a dematiaceous fungus, characterized by the presence of melanin pigment in the cell wall (Rinaldi 1996). It is a saprophyte distributed widely throughout the environment, commonly isolated from outdoor plant debris, soil and wood, and indoor textiles and shower curtains (Lewetin & Horowitz 1978; Sneller et al 1979; Al-Doory et al 1982; Giarardi et al 1993). It has been reported that exposure of the airway and skin to this black yeast potentiates allergic responses (Taylor et al 2006; Niedoszytko et al 2007) and causes various infections (Redondo-Bellon et al 1997; Hawkes et al 2005; Panda et al 2006). It seems likely that the causative substance(s) may be β-(1,3) D-glucan, and it has been reported that exposure of the airway to β-(1,3) D-glucan contained in house dust, indoor molds and some bacteria potentiates airways allergic responses (Wan et al 1999; Rylander & Lin 2000; Instanes et al 2004; Douwes 2005; Schram-Bijkerk et al 2005; Taylor et al 2006). Thus, there are a number of reports that the inhalation of β-glucan into the airway in house dust and indoor molds causes allergic reactions with elevation of IgE (Wan et al 1999; Rylander & Lin 2000; Instanes et al 2004). However, Instanes et al (2004) reported that mold extracts contained less than 2% β-(1,3) D-glucan; therefore, the potentiation of the ova-albumin-specific immune response in a Th_2-dependent manner (ova-albumin-specific IgE elevation) suggests that mold also contains other, more potent, adjuvants than β-(1,3) D-glucan that are active at lower concentrations. Conversely, Miyamoto et al (2002) reported that the oral administration of polysaccharide fractions prepared from the basidiomycete *Agaricus blazei* might clinically improve the symptoms of bronchitis through elevation of the interferon-γ level. Xie et al (2006) reported that the oral administration of a polysaccharide isolated from the fruiting body of *Cryptoporus volvatus* prevented ova-albumin-induced allergic rhinitis through the inhibition of eotaxin mRNA expression in nasal mucosa and lung tissues. Thus, the experimental findings about β-glucan suggest that it may have opposite effects on allergic reactions, which probably depend on variations in dosage, route of administration, average molecular weight, purity and water-solubility of β-glucan that may contribute to the immune response, although this has not been proven yet. It has also been reported that particulate β-(1,3) D-glucan derived from *Saccharomyces cerevisiae* activated both the classical pathway and the alternative pathways of the complement system in normal human sera (Glovsky et al 1983). Williams et al (1991) reported that a water soluble β-(1,3) D-glucan sulfate derived from *S. cerevisiae* exerted anti-tumour activity through the activation of macrophages and stimulation of bone marrow. We also reported that water-soluble low-molecular-weight β-(1,3–1,6) D-glucan purified from *A. pullulans* 1A1 strain exerted anti-tumour and anti-metastatic actions through the stimulation of the immune system in the small intestine (Kimura et al 2006) and prevented the ova-albumin-induced allergic reaction through the inhibition of IL-12 and interferon-γ reduction in spleen of the ova-albumin-sensitized mice (Kimura et al 2007). Although the crude β-glucan fraction prepared from basidiomycetes mushrooms has anti-stress activity, whether purified β-glucan possesses such activity has not been proven yet, especially on the relationship between immune responses (cytokine production and NK activity) and endocrine system (e.g. the secretion of corticosterone) under the restraint stress in mice. In this study, we examined the anti-stress effect of water-soluble low-molecular-weight β-(1,3–1,6) D-glucan isolated from *A. pullulans* 1A1 strain in mice with restraint-induced stress.

Materials and Methods

Materials

RPMI 1640 medium was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) and antibiotic and antimycotic solution (× 100) were purchased from Gibco BRL (Aukland, New Zealand) and Sigma Chemical Co. (St Louis, MO), respectively. 3′-*O*-Acetyl-2′,7′-bis(carboxyethyl)-4- or 5-carboxylfluoresein acetoxymethylester (BCECF-AM) was purchased from Dojin Co. (Kumamoto, Japan). Six- and ninety-six-well plates were purchased from Corning Glass Works (NY) and Nalge Nunc International (Denmark), respectively. Other chemicals were of reagent grade.

Cells

YAC-1 cells (natural killer cell-sensitive target cells) were obtained from Riken Gene Bank (Tsukuba, Japan) and maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}) and amphotericin (0.25 μg mL^{-1}).

Preparation of low-molecular-weight β-glucan from *A. pullulans* 1A1

The *A. pullulans* 1A1 strain was obtained from the strain K-1 by a general mutation treatment (Suzuki et al 2005) and was then cultured in medium (0.3% ascorbate sodium, 3% sucrose, 0.001% $FeSO_4 \cdot 7H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% KCl, 0.1% K_2HPO_4 and 0.2% $NaNO_3$) (3L) for 10 days at 25–30°C. The culture-conditioned medium was filtered by ultrafitration (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. The obtained β-glucan was precipitated with 70% ethanol, freeze-dried and dissolved in sterile 0.9% NaCl solution.

Determination of molecular weight and structural analysis of β-glucan

The molecular weight was measured by Toyoperl-gel chromatography (HW-650; TOSO, Tokyo, Japan) with 0.1 M NaOH (pH 12) according to the method of Suzuki et al (2005). ^1H NMR (499.83 MHz) and ^{13}C NMR (125.68 Hz) spectra were recorded in D_2O using a Varian Unity Inova 500 spectrometer (TOSO, Tokyo, Japan). The structure and purity of isolated β-glucan were determined based on ^1H and ^{13}C NMR spectral analysis.

Animals

Male Balb/c strain mice, 5 weeks old, were obtained from SLC Japan (Shizuoka, Japan). The mice were housed for 1 week

before they were used in experiments in a room maintained at 25 ± 1°C with 60% relative humidity and given free access to laboratory standard diet (Oriental Yeast Co, Tokyo, Japan) and water. The room lights were on for 12 h per day starting at 0700 h. Mice were treated according to the ethical guidelines of the Animal Center, Graduate School of Medicine, Ehime University. The experimental protocol was approved by the Animal Studies Committee of Ehime University.

Restraint stress protocol

Mice were subjected to restraint stress according to a modification of the methods of Manfredi et al (1998) and Zhang et al (1998). Briefly, mice were restrained for 12 h (1900–0700 h) on days 3, 5 and 7 in a 50-mL conical polypropylene centrifuge tube in which holes had been drilled. Since the restrained mice could not access food and water during the restraint stress period, a food- and water-deprived group of mice without restraint stress was used as a control. Furthermore, a group of mice supplied with food and water for a whole day without restraint stress were used as a non-treated control (normal). Low-molecular-weight β-glucan was orally administered once daily (0730 h) at a dose of 25, 50 or 100 mg kg^{-1} for 7 days during the experimental period. Thus, the 3 doses of water-soluble low-molecular-weight β-(1,3–1,6) D-glucan were used to clarify the efficacy on the restraint stress.

Measurement of serum corticosterone

On day 8, blood samples were obtained by venipuncture from mice under pentobarbital anaesthesia, and then the spleens were removed and weighed under sterile conditions. The obtained sera were collected and stored at −80°C before assay. Serum corticosterone was measured using an ELISA kit (Diagnostic Systems Lab. Inc. TX) according to the manufacturer's instructions.

Measurement of cytokine production in splenocytes

The spleen was gently teased to release cells in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}) and amphotericin B (0.25 μg mL^{-1}). The cell suspension was passed through a glass-wool column to remove cell debris and adherent cells, and then the isolated splenocytes were adjusted to 1×10^8 cells per mL. Isolated splenocytes were placed in RPMI 1640 medium containing 5% FBS, penicillin, streptomycin and amphotericin B at 1×10^6 cells per well in 48-well culture plates (Corning, NY) and incubated for 1 h. Then concanavalin A (Con A) (10 μg mL^{-1}) was added to the splenocytes and they were cultured for 48 h. After the incubation period, the culture medium was centrifuged at 4°C and 1500 *g* for 10 min to remove the cells. The supernatants from the centrifugation were stored at −80°C until they were analysed for the content of interleukin (IL)-6 and IL-12 using mouse IL-6 (R & D Systems Inc., MN) and IL-12 (Pierce Biotechnology Inc., IL) ELISA kits.

Measurement of natural killer (NK) activity

Splenic lymphocytes were isolated using methods described previously (Kimura & Okuda 2001). Briefly, the splenocyte suspension (5 mL) was layered onto 5 mL of Lymphocytes-Mouse (Dainippon Pharm. Co. Osaka, Japan) and centrifuged at 1500 *g* for 30 min at room temperature. The lymphocyte band at the interface was recovered, and the cells were rinsed three times with the above medium. Preparation of BCECF-labelled YAC-1 cells (natural killer cell-sensitive target cells) was performed using a modification of the method described previously (Kimura 2002). Isolated splenic lymphocytes (effector cells) were placed in RPMI 1640 medium containing 10% FBS, penicillin, streptomycin and amphotericin B, at 4×10^5 cells per well in 96-well culture plates, and then BCECF-labelled YAC-1 cells (Target cells: 4×10^3 cells) were added to the effector cells and incubated with them for 2 h, after which the cell mixture was centrifuged at 410 *g* for 10 min. The fluorescence intensity of the supernatant was measured by fluorimetry (FP-777; JASCO, Tokyo, Japan) with excitation at 500 nm and emission at 540 nm. The total fluorescence intensity of the target cells (BCECF-labelled YAC-cells) was determined after solubilizing the cells by adding 0.25% Triton X-100. The specific cytotoxicity activity was calculated as follows: percent specific cytotoxicity = [(fluorescence intensity of target cells treated with splenic lymphocytes isolated from experimental group minus fluorescence intensity of spontaneous release of target cells)/(total fluorescence intensity of target cells minus fluorescence intensity of spontaneous release of target cells)] × 100.

Statistical analysis

All values are expressed as means ± s.e. Data were analysed by one-way analysis of variance, and then differences among means were analysed using Fisher's protected least-significant differences (LSD) multi-comparison test. $P < 0.05$ denoted significant difference.

Results

Structure and characteristics of β-glucan produced by *A. pullulans* 1A1 strain

The ^1H NMR spectrum (in D_2O, ppm) of β-glucan isolated from *A. pullulans* 1A1 strain exhibited signals of C-1 position corresponding to β(1,3)- and β(1,6)-linkage at 4.73 (broad singlet) and 4.47 (broad singlet), respectively. In the ^{13}C NMR spectrum (in D_2O, δ ppm) of β-glucan, the signals at 103.94 and 104.46 corresponded to the β(1,3)- and β(1,6)-linkage at C-1 position, respectively. The signals at 71.43 and 62.16 ppm in the ^{13}C-NMR spectrum were assigned to the β(1,6)-linkage at C-6 position and free C-6 position, respectively. Furthermore, when β-glucan isolated from *A. pullulans* 1A1 strain was incubated with the exo-β-(1,3)-glucanase, glucose and gentiobiose were produced. Therefore, the structure of β-glucan isolated from *A. pullulans* 1A1 strain (Figure 1) was elucidated to be β (1→3) D-glucan with 50–80% branches β (1→6) by analysis of the ^1H and ^{13}C NMR spectra

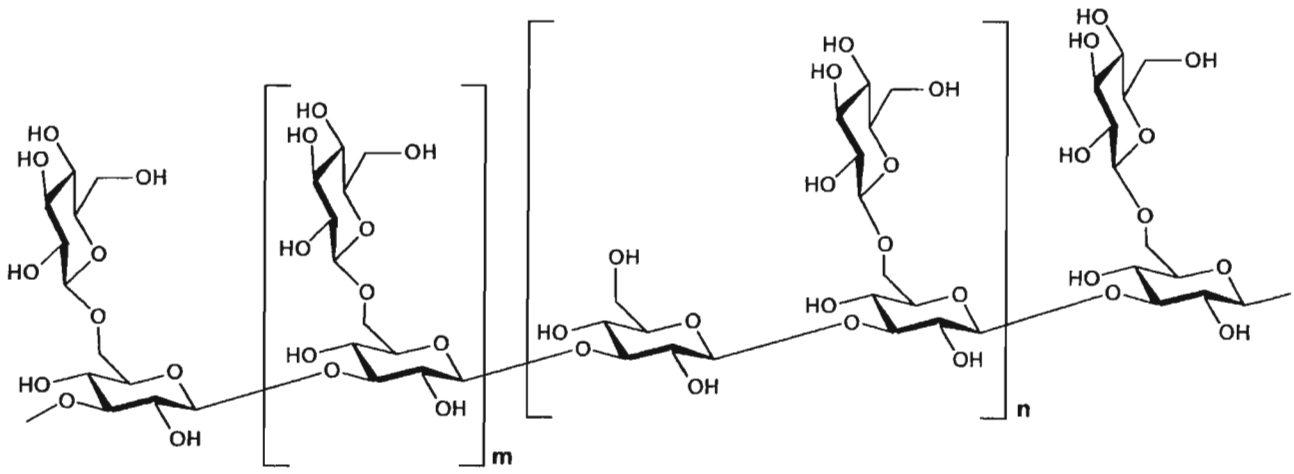

Figure 1 The structure of water-soluble low-molecular-weight β-glucan isolated from *Aurobasidium pullulans* 1A1 strain.

and enzymatic (exo-β-(1,3)-glucanase) reaction. The average molecular weight of β-glucan was determined to be approximately 100 kDa by the direct comparison of water-soluble standard marker Pullulan with molecular weight of 5900–1 600 000 (Shodex, STANDARD P-82; Showa Denko Co., Tokyo, Japan) by gel chromatography. The viscosity of β-glucan (2 m mL^{-1}) was less than 20 mPa s (cp) at 30°C using the rotary viscometer.

Effect of low-molecular-weight β-glucan (LMW-β-glucan) on spleen weight and blood corticosterone level in restraint-stressed mice

The spleen weight in food- and water-deprived mice was significantly lower than that in non-treated mice (normal). Furthermore, the spleen weight in restraint-stressed mice was significantly reduced compared with that in normal mice and food- and water-deprived mice (Table 1). The blood corticosterone level in food- and water-deprived mice tended to be increased ($P < 0.078$) compared with that in normal mice. The corticosterone level in restraint-stressed mice was significantly higher than that in normal and food- and water-deprived mice. The increase in the blood corticosterone level caused by restraint was significantly reduced by the orally administered LMW-β-glucan at the doses of 50 and 100 mg kg^{-1} (Figure 2).

Table 1 Effect of LMW-β-glucan on the weights of body and spleen in restraint-stressed mice

Treatment	Body weight at day 8 (g)	Spleen weight (mg)
Normal	26.0 ± 0.52	133.95 ± 4.36*
Food and water-deprived mice	24.3 ± 0.51	108.67 ± 4.73*
Restraint-stressed mice	23.0 ± 0.27	81.15 ± 2.77
Restraint + LMW-β-glucan		
(25 mg kg^{-1}, 7 days)	22.1 ± 0.40	78.52 ± 2.42
(50 mg kg^{-1}, 7 days)	23.2 ± 0.39	86.22 ± 3.79
(100 mg kg^{-1}, 7 days)	23.1 ± 0.37	83.72 ± 4.04

Values are means ± s.e. of 6 mice. *$P < 0.05$ vs restraint control.

Effect of LMW-β-glucan on cytokine production in restraint-stressed mice

The basal and Con A-stimulated levels of IL-12 and IL-6 production by splenocytes in restraint-stressed mice were significantly reduced compared with those in normal and food- and water-deprived mice (Figures 3 and 4). Thus,

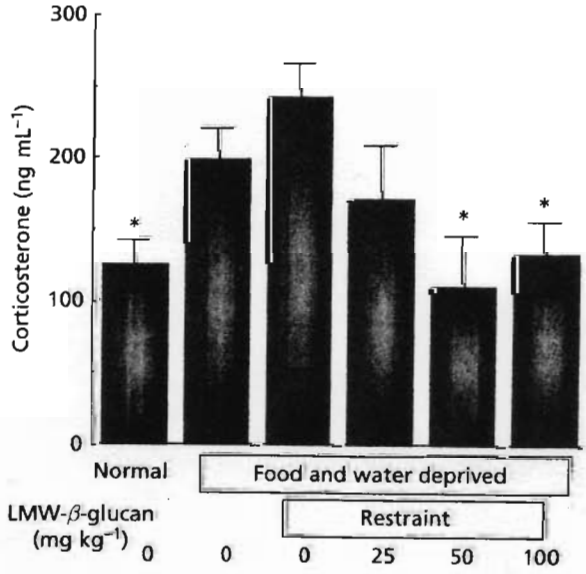

Figure 2 Effect of water-soluble low-molecular-weight β-glucan (LMW-β-glucan) on the plasma corticosterone level in mice subjected to restraint stress. Values are means ± s.e. of 6 mice. *$P < 0.05$ vs restraint control.

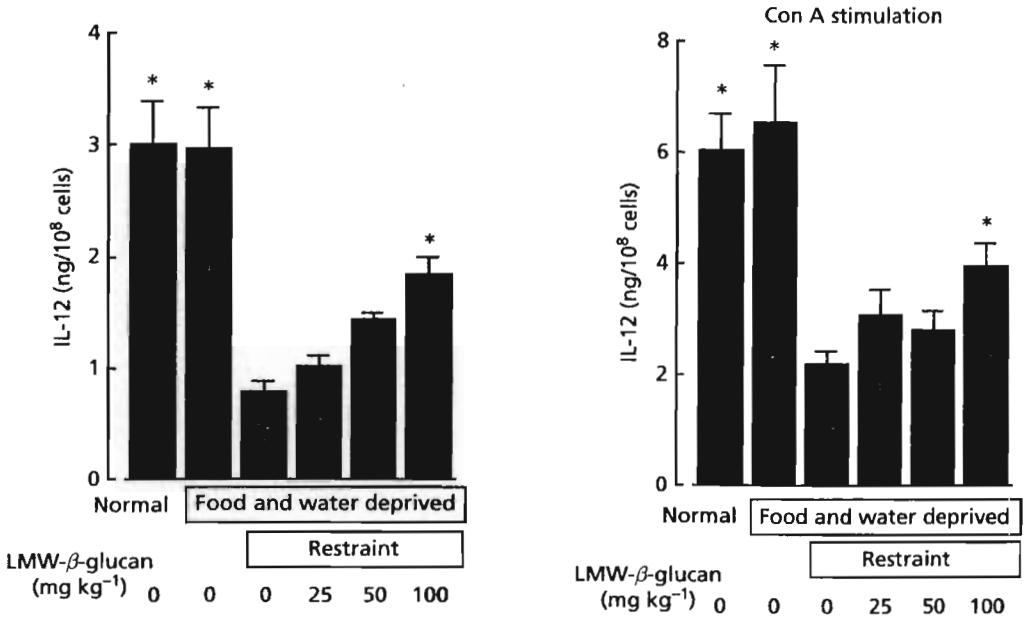

Figure 3 Effect of water-soluble low-molecular-weight β-glucan (LMW-β-glucan) on IL-12 production by splenocytes in mice subjected to restraint stress. Isolated splenocytes were cultured with or without concanvalin A (Con A) (10 μg mL^{-1}) for 48 h in RPMI 1640 medium containing 5% FBS. Values are means ± s.e. of 6 mice. *$P < 0.05$ vs restraint control.

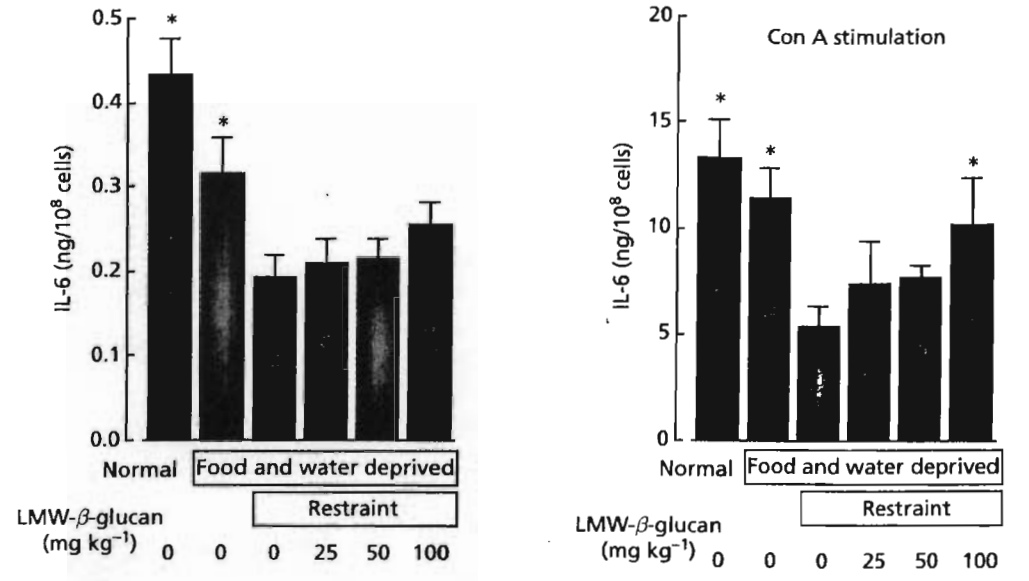

Figure 4 Effect of water-soluble low-molecular-weight β-glucan (LMW-β-glucan) on IL-6 production by splenocytes in mice subjected to restraint stress. Isolated splenocytes were cultured with or without concanvalin A (Con A) (10 μg mL^{-1}) for 48 h in RPMI 1640 medium containing 5% FBS. Values are means ± s.e. of 6 mice. *$P < 0.05$ vs restraint control.

reduction of cytokine production was caused by restraint stress. The reduction of spontaneous and Con A-stimulated IL-12 production caused by restraint stress was significantly attenuated by orally administered LMW-β-glucan at a dose of 100 mg kg^{-1} (Figure 3). The reduction of Con A-stimulated IL-6 production in restraint stress mice was also inhibited by orally administered LMW-β-glucan at a dose of 100 mg kg^{-1} (Figure 4) but the reduction of the basal IL-6 production was not affected by LMW-β-glucan.

Table 2 Effect of LMW-β-glucan on NK activity in restraint-stressed mice

Treatment	NK activity (% specific lysis)[a]
Normal	8.47 ± 0.268*
Food and water-deprived mice	6.14 ± 0.509
Restraint-stressed mice	4.01 ± 0.666
Restraint + LMW-β-glucan	
(25 mg kg^{-1}, 7 days)	5.12 ± 0.978
(50 mg kg^{-1}, 7 days)	6.64 ± 1.147*
(100 mg kg^{-1}, 7 days)	7.25 ± 0.990*

Values are means ± s.e. of 6 mice. [a]Effector/target ratio 100:1. *$P < 0.05$ vs restraint control.

Effect of LMW-β-glucan on NK activity in restraint-stressed mice

NK activity was significantly reduced by restraint stress (Table 2). The reduction of NK activity in restraint-stressed mice was attenuated by orally administered LMW-β-glucan at doses of 50 and 100 m kg^{-1}.

Discussion

It is well known that polysaccharides, especially β-(1,3) D-glucans with β-(1,6) branches, have immune-stimulatory actions (Demleitner 1992; Kulicke et al 1997). The immunomodulatory effects of β-glucans are influenced by the molecular mass, chain length, degree of branching, tertiary structure and solubility of the polymer. Although no consensus could be reached regarding the structure–activity relationship (Kulicke et al 1997), the (1→3)-β-linkage has been described as an explicit requirement for biological activity (Demleitner et al 1992). Furthermore, it has been reported that β-glucan isolated from yeast protects against anthrax (*Bacillus anthracis*) infection in a mouse model (Kournikakis et al 2003). Recently, we reported that water-soluble low-molecular-weight (100 kDa) β-(1,3) D-glucan with 50–80% branches of β-(1,6) isolated from *Aureobasidium pullulans* 1A1 strain inhibited tumour growth and liver metastasis in colon 26-bearing mice (Kimura et al 2006). We also found that water-soluble low-molecular-weight β-glucan isolated from *A. pullulans* 1A1 strain induced greater IL-6 production by macrophages than shyzophyllan and lentinan with an average molecular weight of 2000 kDa (Kimura et al 2006). It has been reported that α-D-glucans also have immune-stimulating properties (e.g., stimulation of NK activity and increased production of various cytokines) (Bao et al 2001; Nair et al 2004). IL-12 is primarily secreted by macrophages, monocytes, dendritic cells and splenocytes in response to a variety of microbical factors (Hsieh et al 1993; Macatonia et al 1995; Shida et al 2002). IL-12 plays a central role in the initiation of cell-mediated immunity directly and via its induction of interferon (IFN)-γ and NK cells (Chan et al 1992; Seder et al 1993). Glucocorticoids are major mediators of the stress response and directly suppress NK activity (Shakhar & Blumenfeld 2003). IL-6 is a pleiotropic cytokine that is not only produced by cells of immune tissues (Akira et al 1990), but also by cells in neuronal and endocrine tissues, such as the hypothalamus, the anterior pituitary and the adrenal cortex (Spangelo et al 1990; Judd & MacLeod 1992; Murakami et al 1993). It has been reported that IL-6 can regulate the secretion of hormones from the hypothalamus, the pituitary and the adrenal (Lyson & McCann 1991; Navarra et al 1991; Perlstein et al 1991). It is well known that different stress paradigms are able to rapidly induce corticosterone production through activation of the hypothalamic–pituitary–adrenal axis, and the induction of ACTH. In this study, we found that an increase in the blood corticosterone level and reduction of NK activity and IL-6 (derived from Th_2 cells) and IL-12 (derived from Th_1 cells) production from splenocytes were caused by restraint stress in mice. Thus, it was possible that restraint stress induced increases in corticoid hormones through the regulation of cytokines, such as IL-6 and IL-12, and consequently caused the reduction of NK activity. The water-soluble low-molecular-weight β-(1,3) D-glucan with β-(1,6) branches isolated from *A. pullulans* 1A1 strain inhibited the increase in blood corticosterone level and the reduction of NK activity, IL-12 and IL-6 production at a dose of 50 or 100 mg kg^{-1} in restraint-stressed mice. Recently, we reported that water-soluble low-molecular weight β-glucan stimulated IL-6 production in macrophage cell line RAW 264.6 cells in-vitro, and that the intraperitoneal injection of water-soluble low-molecular-weight β-glucan (5 or 15 mg kg^{-1}) elevated plasma IL-12 to levels comparable with those in non-treated tumour-bearing mice (Kimura et al 2006). Some possible mechanisms can be suggested for the protective actions of water-soluble low-molecular-weight β-glucan isolated from *A. pullulans* 1A1 strain against restraint stress. The first mechanism may be that water-soluble low-molecular-weight β-glucan inhibits the elevation in blood corticosterone level induced by restraint stress by abrogating the IL-6 reduction caused by restraint stress. The second mechanism may be that water-soluble low-molecular-weight β-glucan attenuates the reduction of NK activity by abrogating IL-12 reduction caused by restraint stress. In this study, the adrenal weight was not significantly different among untreated mice (normal), food- and water-deprived mice, restraint-stressed mice (control) and water-soluble low-molecular-weight β-glucan-treated mice since the adrenal weight could not be measured exactly, being too small (data not shown). Further work is needed to investigate the effects of water-soluble low-molecular-weight β-glucan on histopathplogy of adrenals caused by restraint stress. Water-soluble low-molecular-weight β-glucan may be an effective dietary supplement for the prevention of stress.

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