

## Serum levels of $\beta$ -1,3-1,6 glucan-specific antibodies and immune biomarkers in normal individuals

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### Abstract

In this clinical study, we examined the serum levels of  $\beta$ -1,3-1,6 glucan (BG)-specific antibodies (IgG, IgA2 and IgM) and the peripheral blood (PB) levels of immune biomarkers, including the serum total immunoglobulin (IgG, IgA and IgM), interleukin-6 (IL-6), IL-12 and soluble-form CD44 (sCD44), in 15 normal individuals, using an originally developed enzyme-linked immunoassay (EIA). We found that the sera of even normal healthy individuals consistently contain BG-specific IgG, IgA2 and IgM, and that the titers of these antibodies vary widely among individuals. However, the serum titers of BG-specific IgM were strongly and significantly correlated with the serum total IgM titers ( $r = 0.914$ ,  $P < 0.001$ ). Furthermore, the serum titers of BG-specific IgG were significantly correlated with the serum levels of sCD44 ( $r = 0.611$ ,  $P = 0.015$ ), and the serum titers of BG-specific IgA2 were significantly correlated with the serum levels of IL-6 ( $r = 0.588$ ,  $P = 0.021$ ). In addition, the serum titers of BG-specific IgG, IgA2 and IgM showed a tendency to be correlated with the serum levels of IL-12 ( $r = 0.449$ ,  $P = 0.081$ ;  $r = 0.429$ ,  $P = 0.092$ ;  $r = 0.473$ ,  $P = 0.070$ , respectively). Taken together, these findings show that normal human sera consistently and naturally contain BG-specific IgG, IgA2 and IgM. Since the titers of these BG-specific antibodies are correlated with the serum levels of some immune biomarkers, it may be surmised that they are closely associated with various immunological responses in the living body to maintain a normal health condition.

**Key words :**  $\beta$ -1,3-1,6 glucan-specific antibodies, biomarker, IL-6, IL-12, sCD44

### Introduction

$\beta$ -glucans are mainly found in the extracts of some species of mushrooms and in microbes, such as black yeast, and possess some unique immunological activities<sup>1,2</sup>.  $\beta$ -glucans have been shown to exert cytotoxic activity against cancer cells<sup>3</sup> accompanied by activating the production of interleukin-2 (IL-2), IL-6, IL-12, interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>4</sup>. These findings indicate that  $\beta$ -glucans enhance the immune system underlying the activation

of lymphocytes, monocytes, macrophages, granulocytes, and natural killer (NK) cells<sup>5</sup>. In general, the anti-cancer actions of  $\beta$ -glucans are not attributable to their direct actions on cancer cells, as is the case with chemical anti-cancer drugs, but depends on the immunological enhancement of the host, e.g., by acting as a biological response modifier (BRM)<sup>3</sup>. On the other hand, several studies have provided evidence to suggest that Dectin-1 is the  $\beta$ -glucan receptor<sup>6</sup>.

We established and succeeded in purifying  $\beta$ -1,3-1,6 glucan produced by the black yeast *Aureobasidium*

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*pullulans* (*A. pullulans*) strain AFO-202 using the latest culture technology, and examined the immunological actions of this  $\beta$ -1,3-1,6 glucan *in vitro* using U937, a human monocyte-like cell line, and human peripheral blood mononuclear cells (PBMCs)<sup>1)</sup>.

Recently, it was reported that human and animal sera consistently contain  $\beta$ -glucan-specific antibodies<sup>7,8)</sup>. However, the immunological roles of these antibodies in the living body, in particular, the relationship between the  $\beta$ -glucan-specific antibodies and immune biomarkers such as various cytokines and soluble-form molecules (CD antigens) have not yet been examined.

In this clinical study, we examined the serum levels of  $\beta$ -1,3-1,6 glucan (BG)-specific antibodies (IgG, IgA2 and IgM) and the peripheral blood (PB) levels of immune biomarkers, including those of serum total immunoglobulin (IgG, IgA and IgM), interleukin-6 (IL-6), IL-12 and soluble-form CD44 (sCD44), in 15 normal individuals, using an originally developed enzyme-linked immunoassay (EIA) in our laboratories.

## Materials and Methods

### Reagents

A  $\beta$ -1,3-1,6 glucan was synthesized by Sophy Co. (Kochi-city, Kochi) from *Aureobasidium pullulans* (*A. pullulans*) strain AFO-202 using the latest biological culture and preparation techniques, and is currently available commercially as a health food supplement. In this study, the purified  $\beta$ -1,3-1,6 glucan described above was kindly provided to us for this study by Sophy Co. Enzyme-linked immunoassay (EIA) kits for measuring the blood levels of interleukin-6 (IL-6), IL-12 (p35+p40) and soluble-form CD44 (sCD44) were purchased from Diaclone laboratories Co. (USA). Horseradish peroxidase (HRPOD)-conjugated rabbit anti-human IgG, HRPOD-conjugated rabbit anti-human IgM and HRPOD-conjugated mouse anti-human IgA2 monoclonal antibody (mAb) were purchased from MBL Co. (Nagoya).

### Ethics statement

The study protocol was approved by the institutional

review boards (IRB) of Kyushu University of Health and Welfare and Chikamori Hospital; the IRB numbers were 15-048 and 161, respectively. Informed consent was obtained from all the donors prior to their participation in this study.

### Processing of the blood and serum samples

Blood samples of 15 normal individuals (5 males, age  $38.2 \pm 9.5$  yr; 10 females, age  $28.7 \pm 5.2$  yr) were obtained at Chikamori Hospital (Kochi-city, Kochi). The serum samples were prepared using the standard method and the sera were stored at  $-80^{\circ}\text{C}$  until use.

### Measurement of $\beta$ -1,3-1,6 glucan-specific antibodies in the sera

EIA plates (Sumitomo Co., Tokyo) were coated with  $1 \mu\text{g}/\text{mL}$  of purified  $\beta$ -1,3-1,6 glucan (BG) in carbonate-bicarbonate buffer ( $0.010 \text{ M NaCO}_3$ ,  $0.035 \text{ M NaHCO}_3$ , pH9.6) for 24 hr at  $4^{\circ}\text{C}$ . The wells were washed four times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST) and blocked with PBST containing 2% BSA (BSA-PBST) for 60 min at room temperature. Thereafter, the wells were washed three times with PBST. The sera (x1,000 dilution for IgG, x50 dilution for IgA2 and x500 dilution for IgM with 0.1% BSA-PBST;  $50 \mu\text{L}$ ) were then added to each well, followed by incubation of the plate for 60 min at room temperature with shaking. Then, the wells were washed five times with PBST, followed by addition of HRPOD-conjugated rabbit anti-human IgG, HRPOD-conjugated mouse anti-human IgA2 mAb or HRPOD-conjugated rabbit anti-human IgM (x5,000 dilution with 0.1% BSA-PBST, respectively;  $50 \mu\text{L}$ ) to each well and incubation of the plate for 60 min at room temperature with shaking. The wells were then washed 10 times with PBST, followed by addition of the substrate-chromogen (TMB; Cosmo Bio Co.;  $50 \mu\text{L}$ ) to each well and incubation of the plate for 20 min at room temperature with gentle shaking. The reaction was stopped by the addition of  $0.5 \text{ M-HCl}$  ( $50 \mu\text{L}$ ), and the optical density (O.D.) was read at 450 nm using a multichannel EIA-microplate reader (TOSHO Co.). The experiment was repeated five times. The specificity

and precision were confirmed by a blocking test using purified rabbit anti- $\beta$ -1,3-1,6 glucan-specific IgG established in our laboratories as described previously<sup>8</sup>.

### Measurement of IL-6, IL-12 and sCD44 in the sera

The measurement of IL-6, IL-12 and sCD44 in the serum samples was performed using an IL-6 detection EIA kit, IL-12 detection kit and sCD44 detection kit, respectively. Each of the measurements was repeated three times.

### Measurement of serum total IgG, IgA and IgM

Measurement of serum total IgG, IgA and IgM in the samples was outsourced to a Special Reference Laboratory (SRL). Each experiment was repeated three times.

### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation (SD). Statistical analysis of the correlations of the serum titers of the BG-specific antibodies (IgG, IgA2 and IgM) with the serum total immunoglobulin (IgG, IgA and IgM), IL-6, IL-12 and sCD44 levels was performed by the Pearson correlation test. Differences at  $P < 0.05$  were considered to indicate statistical significance.

## Results and Discussion

First, we examined the serum titers of BG-specific IgG, IgA2 and IgM in 15 normal individuals using an originally developed enzyme-linked immunoassay (EIA). As shown in Figure 1, we consistently found that BG-specific IgG, IgA2 and IgM in the sera of the normal individuals with the titers varying widely among individuals. BG-specific IgG ( $\delta$  OD:  $0.81 \pm 0.51$ ) and BG-specific IgM ( $\delta$  OD:  $0.67 \pm 0.50$ ) occurred in high titers, while BG-specific IgA2 ( $\delta$  OD:  $0.09 \pm 0.07$ ) occurred in low titers (Fig. 1) in the serum samples.

Next, we examined the correlations between the serum titers of BG-specific IgG, IgA2 and IgM and the

peripheral blood (PB) levels of immune biomarkers, including those of serum total immunoglobulin (IgG, IgA and IgM), IL-6, IL-12 and sCD44. Figure 2 shows that the serum titers of BG-specific IgM were strongly and significantly correlated with the serum total IgM titers ( $r = 0.914$ ,  $P < 0.001$ ). Furthermore, the serum titers of BG-specific IgG were significantly correlated with the serum levels of sCD44 ( $r = 0.611$ ,  $P = 0.015$ ) (Fig. 3), and the serum titers of BG-specific IgA2 were significantly correlated with the serum levels of IL-6 ( $r = 0.588$ ,  $P = 0.021$ ) (Fig. 4). In addition, the serum titers of BG-specific IgG, IgA2 and IgM showed a tendency to be correlated with the serum levels of IL-12 ( $r = 0.449$ ,  $P = 0.081$ ;  $r = 0.429$ ,  $P = 0.092$ ;  $r = 0.473$ ,  $P = 0.070$ , respectively) (Fig. 5).

It is common knowledge that IgM is the first antibody to appear as part of the immunological response to initial exposure to an antigen. In this study, since the serum titers of BG-specific IgM were strongly and significantly correlated with the serum total IgM titers, it may be surmised that BG-specific IgM may also play an important role in some immunobiological responses to maintain the health condition.

IL-6 is a cytokine produced by T-lymphocytes, B-lymphocytes, monocytes, fibroblasts, endothelial cells, etc., and plays some important roles in both natural and acquired immune responses; in particular, IL-6 regulates the growth and maturation of B-lymphocytes to produce antibodies<sup>9</sup>. Since the serum titers of BG-specific IgA2 were significantly correlated with the serum levels of IL-6, it is probable that BG-specific IgA2 influences IgA antibody production under IL-6 action in the mucosal and gut immune systems.

CD44 (molecular mass approx. 80-kDa) is expressed on lymphocytes, macrophages, granulocytes, fibroblasts, endothelial cells, natural killer (NK) cells, etc. In particular, CD44 plays an important role in stimulating NK cell and lymphokine-activated killer cell (LAK) functions underlying IFN- $\gamma$  production, activation cell surface antigen expression mediated by protein tyrosine kinase (PTK) phosphorylation and intracellular  $Ca^{2+}$  flux<sup>10, 11</sup>. On the other hand, there are three types of CD44 (CD44H, CD44E and sCD44) with differing biological functions. The sCD44 level in the

serum has been reported to be increased in association with immunological activities. Since the serum titers of BG-specific IgG were significantly correlated with the serum levels of sCD44, it is probable that BG-specific IgG is related to NK cell and LAK cell functions.

IL-12 is a cytokine produced by the dendritic cells (DCs), macrophages and neutrophils in response to antigenic stimulation. IL-12 plays an important role in the activities of NK cells and cytotoxic T-lymphocytes (CTLs), and directly mediates enhancement of the cytotoxic activity of NK cells and CTLs accompanied by IFN- $\gamma$  production<sup>12)</sup>. Since the serum titers of BG-specific IgG, IgA2 and IgM showed a tendency to be correlated with the serum levels of IL-12, it may be assumed that BG-specific antibodies also indirectly regulate NK cell and CTL functions in immune system.

We examined the serum titers of BG-specific antibodies (IgG, IgA2 and IgM) in normal individuals. Our finding of the existence of correlations between the serum titers of BG-specific antibodies and the serum levels of various immune biomarkers strongly suggest the possible involvement of BG-specific antibodies in the regulation of the immune system. The detailed mechanism(s) underlying the immunological roles of the BG-specific antibodies in the sera of normal individuals remains unclear at the present time.

It is possible that the intestinal bacteria flora influence the production of these antibodies. On the basis of the relationships between the serum titers of BG-specific antibodies and the serum levels of some immune biomarkers, it may be considered that when we consume BG as a health food supplement, BG-specific antibodies may more effectively regulate the immune system in the body as a biological response modifier (BRM). Further analyses are needed to examine the detailed production mechanism(s) of BG-specific antibodies at the cellular and molecular levels and the immunological roles of these antibodies in the sera of normal individuals.

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## Disclosure

None of the authors has any conflict of interest to disclose.

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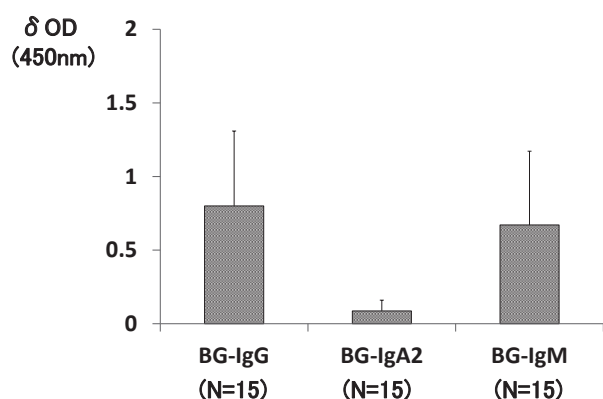


Figure 1. Measurement of BG-specific IgG, IgA2 and IgM in the sera of 15 normal individuals. The measurements were performed using an originally developed EIA system (See Materials and Methods for details). Each measurement was repeated four times.

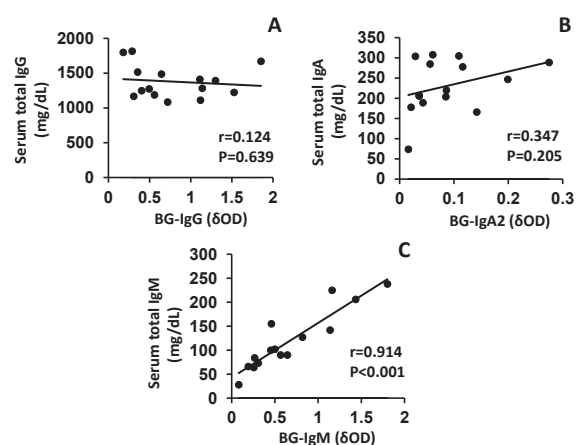


Figure 2. Analysis of the correlations of the serum titers of BG-specific antibodies (IgG, IgA2 and IgM) and the serum total immunoglobulin (IgG, IgA and IgM) titers in normal individuals (n = 15). A: Correlation between the serum titers of BG-specific IgG and serum total IgG titers ( $r = 0.124$ ,  $P = 0.639$ ) in all normal individuals. B: Correlation between the serum titers of BG-specific IgA2 and serum total IgA titers ( $r = 0.347$ ,  $P = 0.205$ ) in all normal individuals. C: Correlation between the serum titers of BG-specific IgM and the serum total IgM titers ( $r = 0.914$ ,  $P < 0.001$ ) in all normal individuals.

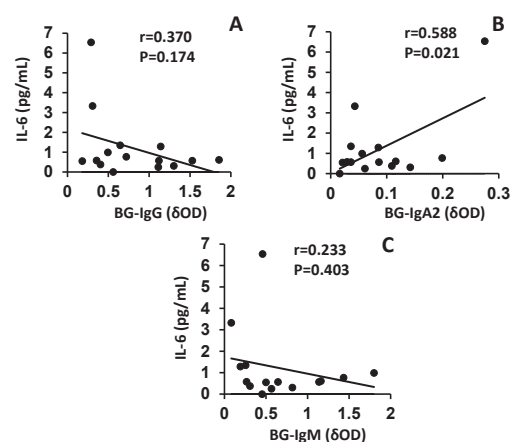


Figure 3. Analysis of the correlations of the serum titers of BG-specific antibodies (IgG, IgA2 and IgM) and the serum levels of IL-6 in normal individuals (n = 15). A: Correlation between the serum titers of BG-specific IgG and the serum levels of IL-6 ( $r = 0.370$ ,  $P = 0.174$ ) in all normal individuals. B: Correlation between the serum titers of BG-specific IgA2 and the serum levels of IL-6 ( $r = 0.588$ ,  $P = 0.021$ ) in all normal individuals. C: Correlation between the serum titers of BG-specific IgM and the serum levels of IL-6 ( $r = 0.233$ ,  $P = 0.403$ ) in all normal individuals.

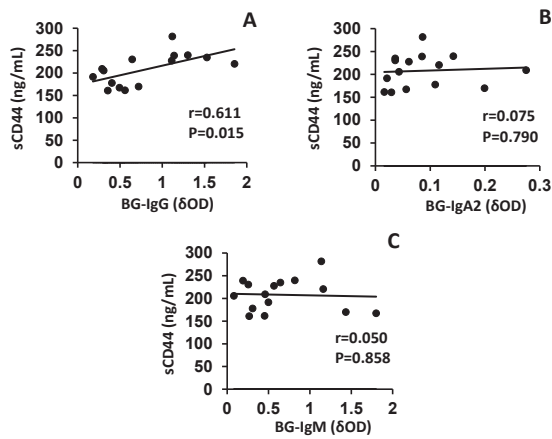


Figure 4. Analysis of the correlations of the serum titers of BG-specific antibodies (IgG, IgA2 and IgM) and the serum levels of sCD44 in normal individuals (n = 15). A: Correlation between the serum titers of BG-specific IgG and the serum levels of sCD44 ( $r = 0.611$ ,  $P = 0.015$ ) in all normal individuals. B: Correlation between the serum titers of BG-specific IgA2 and the serum levels of sCD44 ( $r = 0.075$ ,  $P = 0.790$ ) in all normal individuals. C: Correlation between the serum titers of BG-specific IgM and the serum levels of sCD44 ( $r = 0.050$ ,  $P = 0.858$ ) in all normal individuals.

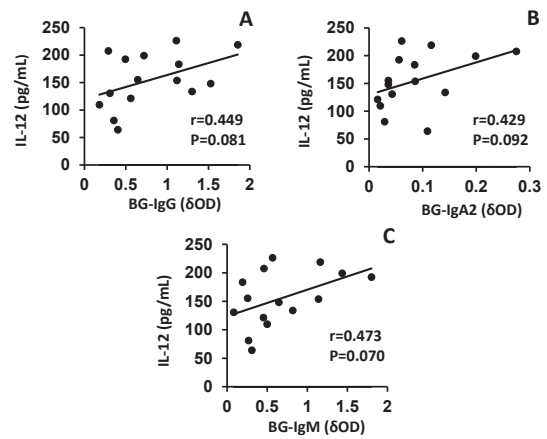


Figure 5. Analysis of the correlations of the serum titers of BG-specific antibodies (IgG, IgA2 and IgM) and the serum levels of IL-12 in normal individuals (n = 15). A: Correlation between the serum titers of BG-specific IgG and the serum levels of IL-12 ( $r = 0.449$ ,  $P = 0.081$ ) in all normal individuals. B: Correlation between the serum titers of BG-specific IgA2 and the serum levels of IL-12 ( $r = 0.429$ ,  $P = 0.092$ ) in all normal individuals. C: Correlation between the serum titers of BG-specific IgM and the serum levels of IL-12 ( $r = 0.473$ ,  $P = 0.070$ ) in all normal individuals.

## 健常人における $\beta$ -1,3-1,6 glucan特異抗体と 免疫バイオマーカーの血清レベル

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### 要旨

健常人(15名)における $\beta$ -1,3-1,6 glucan(BG)特異抗体と免疫バイオマーカーの血清レベルを自主開発した酵素抗体法(enzyme immunoassay: EIA)を用いて検討した。その結果、健常人血清中にはBG特異IgG、IgA2およびIgMが恒常的に存在することが分かった。次に、BG特異抗体と免疫バイオマーカーとの相関性を検討したところ、BG特異IgMは血清総IgMと強い相関( $r=0.914$ ,  $P<0.001$ )を示した。また、BG特異IgGは可溶性CD44(sCD44)と有意な相関( $r=0.611$ ,  $P=0.015$ )を示し、BG特異IgA2はIL-6と有意な相関( $r=0.588$ ,  $P=0.021$ )を示した。さらに、BG特異IgG、IgA2およびIgMとIL-12との相関は有意傾向( $r=0.449$ ,  $P=0.081$ ;  $r=0.429$ ,  $P=0.092$ ;  $r=0.473$ ,  $P=0.070$ )を各々示した。以上の結果は、BG特異IgG、IgA2およびIgMが生体恒常性維持のための免疫応答に密接に関与することを示唆している。

キーワード: BG特異抗体、バイオマーカー、インターロイキン6、インターロイキン12、可溶性CD44