We recently determined the structure of a unique type of 1,3-β-D-glucan obtained from *Aureobasidium pullulans* (AP-FBG) and found that it reacted with the antibodies in human sera. The reactivity of AP-FBG to the antibodies was stronger than that of 1,3-β-D-glucan obtained *Grifola frondosa* (GRN) but weaker than that of 1,3-β-D-glucan from *Candida albicans* (CSBG). Here, we demonstrated that AP-FBG reacted to IgG antibodies, especially those of the subclasses IgG2, IgG1, and IgG3, in human sera. Moreover, the results of competitive enzyme-linked immunosorbent assays (ELISAs) using various glucan competitors showed that these IgGs recognized branched chains at position 6. This is the first study to report that the branched chains at position 6 of β-D-glucan strongly contribute to its recognition by antibodies in human sera. This high reactivity of AP-FBG to human IgG could be advantageous for the use of this glucan in medicine, e.g., as an immunostimulatory agent.

**Key words:** *Aureobasidium pullulans*; β-D-glucan; antibody; human sera; structure

β-Glucan is a well-known biological response modifier (BRM) widely distributed in nature. Various β-glucans have been isolated from different sources, e.g., fungi, plants, and seaweeds. The physicochemical properties of β-glucans differ depending on their primary structure, including linkage type; degree of branching (DB); degree of polymerization (DP); conformation (e.g., triple helix, single helix, and random coil structure), and molecular weight. Recent reports have highlighted the significant role played by β-glucans in the treatment of cancer and infectious diseases in both modern medicine and traditional oriental medicine. β-Glucans also play an important role as dietary substances because they lower the plasma cholesterol level, enhance the hematopoietic response, and possess antitumor and immunomodulating properties. For instance, lentinan and sonifilan (SPG) obtained from *Lentinus edodes* and *Schizophyllum commune*, respectively, have been clinically used for cancer therapy in Japan.

Recent studies have proposed that the pivotal mechanisms underlying the immunostimulatory effects of β-glucans stem from the ability of β-glucans to induce innate immune responses via specific receptors that recognize them. Host molecules that serve as β-glucan receptors have recently been reported, e.g., complement receptor type 3 (CR3) and lactosylceramide (LacCer). More recently, dectin-1 has also been described as a 1,3-β-glucan receptor. It is important to determine the relationship between the structure and receptor-binding ability of β-glucans.

Furthermore, although β-glucans have long been considered to exhibit weak antigenicity, we reported that not only innate immune responses but also acquired immune responses, such as the production of antibodies against β-glucans, are involved in the immunostimulatory effects of β-glucans. Very recently, we found that a unique type of β-D-glucan obtained from the polymorphic fungus *Aureobasidium pullulans* (this β-D-glucan is termed as AP-FBG) reacted to the antibodies in human sera; this β-D-glucan has 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). The reactivity of AP-FBG was stronger than that of β-glucan obtained from *Grifola frondosa* (GRN), which comprises a 1,3-β-D-glucan backbone with single 1,6-β-D-glucopyranosyl-branching units at every 3rd residue, while it was weaker than that of β-glucan from *Candida albicans* (CSBG), which comprises a 1,3-β-D-glucan backbone with long 1,6-β-D-glucopyranosyl-branching units. The difference between these β-D-glucans that react strongly and weakly with human antibodies is not completely understood. It is important

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**Abbreviations:** AP-FBG, *Aureobasidium pullulans*-fermented β-D-glucan; BRM, biological response modifier; CR3, complement receptor type 3; CSBG, 1,3-β-D-glucan from *Candida albicans*; DB, degree of branching; DPX, dextran T500; DP, degree of polymerization; ELISAs, enzyme-linked immunosorbent assays; GRN, 1,3-β-D-glucan obtained *Grifola frondosa*; LacCer, lactosylceramide; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; SPG, sonifilan; TMB, tetramethylbenzidine
to clearly understand this difference, not only to promote the clinical application of β-D-glucans in immunotherapy, but also to determine the mechanisms underlying the recognition of β-D-glucans by host immune systems and the biological effects of these BRMs.

Therefore, in this study, we postulated that the difference in the reactivity of β-D-glucans to antibodies in human sera depends on the presence of branching chains at position 6 of the β-D-glucan molecule. This is because the reactivities of human sera to β-D-glucans differ depending on the frequency and length of the branched chains at position 6. Here, we report the reactivity and specificity of AP-FBG to human sera and discuss the relationship between its reactivity and primary structure.

Materials and Methods

Materials. D$_2$O (deuteration degree min, 99.96%) and Me$_2$SO-d$_6$ (99.96%) were purchased from Merck (Darmstadt, Germany). CSBG, AP-FBG, and GRN were prepared as described previously. Curdlan and pustulan were purchased from Sigma (St. Louis, USA). Peroxidase-conjugated anti-human IgG1, IgG2, IgG3, and IgG4 were purchased from Bayer (Leverkusen, Germany). Peroxidase-conjugated anti-human IgG was purchased from Sigma (St. Louis, USA). Peroxidase-conjugated anti-human IgG1, IgG2, IgG3, and IgG4 were from Invitrogen (USA).

Use of ELISA for detecting anti-β-glucan antibody. A 96-well Nunc plate was coated with β-glucan preparations in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plate was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and blocked with 1% bovine serum albumin (BSA; PBST) at 37 °C for 60 min. The plate was then washed with PBST, treated with peroxidase-conjugated anti-human Ig secondary antibody in PBST, and developed using a tetramethylbenzidine (TMB) substrate system. Color development was terminated using 1N phosphoric acid, and the optical density was measured at 450 nm.

Competitive ELISA used to determine the structure required for anti-β-glucan antibody binding. The specificity of the anti-β-glucan antibody was examined using competitive ELISAs. Competitive ELISAs were essentially conducted as described above except for the following modifications: Polyglobin N diluted with blocking buffer was mixed with known quantities of various soluble glucan competitors dissolved in blocking buffer. Before they were added to the wells of the microtiter plates, the competitor solutions were diluted with equal volumes of polyglobin N and preincubated for 1 h at 37 °C.

NMR spectroscopy. Exchangeable protons were removed by suspending the β-glucans in D$_2$O and lyophilizing this mixture. This exchange process was repeated 3 times. All the spectra were recorded in a mixed solvent of Me$_2$SO-d$_6$/D$_2$O (6:1) (15 mg/ml) at 70 °C by using a Bruker Avance 500 spectrometer equipped with a TXI-xyz three gradient probe by using the method described by Kim et al. We used the internal dimethylsulfoxide (Me$_2$SO) signal (δ$_H$ = 2.53 ppm) for H as a reference and expressed the chemical shift in terms of parts per million (ppm). The Bruker standard pulse sequence was used to perform the 1D-1H experiment, and the spectral width was 5,000 Hz in 64 K complex data points. A relaxation delay of ST1 was used in order to accurately achieve signal integration. Prior to Fourier transformation, zero-filling was used 4 times, and the noise was reduced by using the TRAP function.

Results and Discussion

Reactivity of AP-FBG to human γ-globulin preparation

Since we had previously found antibodies against β-D-glucan in human sera, we first examined the reactivity of AP-FBG to polyglobin N. As shown in Fig. 1A, we found that AP-FBG exhibited high reactivity to antibodies in the human sera; this reactivity was stronger than that of GRN but weaker than that of CSBG. Thus, the reactivities differed among β-D-glucans.

Next, we examined the reactivity of AP-FBG to each of the IgG subclasses. This is because it is well known that the activated capacities of IgG-mediated effector function through interaction with FcyRs and activation of the complement system are differ with IgG subclass. As shown in Fig. 1B, the β-D-glucan AP-FBG was largely reactive to the IgG2 subclass. In addition, it was also reactive to the IgG1 and IgG3 subclasses, although the extent of reactivity was substantially less than that to the IgG2 subclass; the IgG4 subclass was not detected in the human sera. In general, antigen-binding IgGs such as IgG1, IgG2, and IgG3 not only lead to the activation of the host immune system (immune cell
activation) via suitable FcγRs but also lead to the activation of the complement system. Together, these facts imply that the anti-AP-FBG IgGs antibodies in human sera recognize AP-FBG and can subsequently lead to activation of the host immune system.

Structural specificity of anti-AP-FBG IgG in human sera

We found that AP-FBG, a unique variety of 1,3-β-D-glucan with highly branched chains at position 6, reacted with antibodies in human sera. Its reactivity was found to be stronger than that of GRN but weaker than that of CSBG (Fig. 1A). The exact mechanism underlying the difference in reactivity has not yet been elucidated. Therefore, we focused our attention on the differences in the primary structure of three varieties of β-D-glucans, since the characteristic differences among the three varieties were in the frequency presence and length of the branched chains at position 6. Next, by using competitive ELISAs, we examined the specificity of anti-AP-FBG IgGs by using various glucans as competitors. Figure 2 shows the 1D-1H NMR spectra of the various glucans used in this study. Briefly, the down-fielded signals derived from 1,6-β-glucopyranosyl residue (δH = 4.29 ppm) of CSBG and pustulan indicated the existence of a long 1,6-β-glucopyranosyl chain. On the other hand, up-fielded signals derived from 1,6-β-glucosyl residue (δH = 4.25 ppm) of AP-FBG and GRN indicated the existence of a 1,6-β-monoglucopyranosyl chain. In addition, the difference between AP-FBG and GRN are in the frequencies of the 1,6-β-monoglucopyranosyl chain according to their signal intensities. This structural information is summarized in Table 1. From the results of the competitive ELISA experiments, it was apparent that 1,6-β-D-glucans such as pustulan inhibit the binding of anti-AP-FBG IgGs to AP-FBG to a greater extent than the 1,3-β-D-glucans such as curdlan do (Fig. 3). In addition, the difference of inhibitory effect between AP-FBG and GRN might be reflected in their frequencies, that is thickness condition, of the 6-branched side chain units. Besides, the inhibitory effect of AP-FBG was stronger than CSBG. This result may indicate that the binding amounts of anti-CSBG antibodies are higher, because the long 6-branched side chain units within CSBG could be attributed to its binding amount, but binding affinity (avidity) is lower than anti-AP-FBG antibodies, because the density of the 6-branched side chain units could be attributed to its avidity. These facts strongly suggest that the reactivity of β-D-glucans to IgG in human sera depends on the branched chains at position 6. It is well known that the 1,6-β-D-glucopyranosyl side chains extend from the outer side of the 1,3-β-D-glucopyranosyl backbone.22,23) This may explain the strong influence of the 1,6-β-D-glucopyranosyl side chains on the reactivity of β-D-glucans to IgG in human sera.

Table 1. Primary Structure of Polysaccharides Analyzed in This Study

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Source</th>
<th>Primary structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-FBG</td>
<td>Aureobasidium pullulans</td>
<td>1,6-β-monoglucopyranosyl branched 1,3-β-glucan</td>
<td>17</td>
</tr>
<tr>
<td>GRN</td>
<td>Grifora frondosa</td>
<td>1,6-β-monoglucopyranosyl branched 1,3-β-glucan</td>
<td>18, 19</td>
</tr>
<tr>
<td>Curdlan</td>
<td>Alcaligenes faecalis</td>
<td>Linear 1,3-β-glucan</td>
<td>24</td>
</tr>
<tr>
<td>CSBG</td>
<td>Candida albicans</td>
<td>1,6-β-long glucopyranosyl branched 1,3-β-glucan</td>
<td>20</td>
</tr>
<tr>
<td>Pustulan</td>
<td>Umbilicaria papillosa</td>
<td>Linear 1,6-β-glucan</td>
<td>25</td>
</tr>
<tr>
<td>Dextran (DEX)</td>
<td>Leuconostoc dextraniun</td>
<td>1,3-α-glucopyranosyl branched 1,6-α-glucan</td>
<td>26</td>
</tr>
</tbody>
</table>
Involvement of Branched Units in Reactivity of β-β-Glucan to Antibody

Conclusion

In the present study, we demonstrated that a unique type of A. pullulans β-β-glucan that comprises a 1,3-β-β-glucan backbone and single 1,6-β-β-glucopyranosyl side chains at every 2nd or 3rd residue reacted with IgG antibodies, especially those of the IgG2, IgG1, and IgG3 subclasses, in human sera. Moreover, we found that these IgGs recognized the β-β-glucan molecule on the basis of the branched chains at position 6. These data imply that the branching chains at position 6 of β-β-glucan might be responsible for recognition via the acquired immune systems, such as recognition by antibodies, in humans. Thus, these recognitions of antibodies to highly 6-branched β-glucans might contribute to activate immune cell activations such as FcγR-mediated effects by complexes of antibodies and β-glucans. This characteristic of AP-FBG could be an advantage for its use in medicine, e.g., as an immunostimulatory agent.

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References