Activation of Complement and *Limulus* Coagulation Systems by an Alkali-Soluble Glucan Isolated from *Omphalia lapidescens* and Its Less-Branched Derivatives. (Studies on Fungal Polysaccharide. XXXIX[1])

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Activation of the alternative pathway of complement (APC) and the *Limulus* factor G cascade by an alkali-soluble glucan, OL-2, isolated from a crude drug "leiwan" (*Omphalia lapidescens*) and its Smith-type degradation products were examined using human whole complement and *Limulus* lysate. OL-2 is a (1→3)-β-D-glucan having two branches at every third main chain glucosyl unit at each C-6 position (degree of branching (DB): 2/3), and DB of the derivatives used in this study were OL-2-I (1/3), OL-2-II (1/2), OL-2-III (1/24) and OR-OL-2c (no branch: 0/1). Activation of APC by these glucans was dependent on incubation time and concentration. Under optimal conditions, OL-2-I, OL-2-II and OL-2-III showed stronger activation of APC than did the original OL-2, and OR-OL-2c showed relatively weak activity. The structure–activity relationship of the activation of the factor G cascade was similar to that of APC. Considering the fact that the less branched derivatives of OL-2 also showed significantly stronger antitumor activity than OL-2, too much branching would suppress the recognition of (1→3)-β-D-glucan by the host–self defense mechanisms.

Keywords leiwan (*Omphalia lapidescens*); 6-O-branched (1→3)-β-D-glucan; Smith-type degradation product; branching degree; antitumor activity; complement alternative pathway; *Limulus* coagulation system

Introduction

Some of the (1→3)-β-D-glucans, lentinan (LNT) and schizophyllan (SPG), have been used clinically as immunopotentiators for cancer therapy in Japan.[2,3] We have studied antitumor activities and structure–function relationships of (1→3)-β-D-glucans.[1,4-7] OL-2 is obtained from *Omphalia lapidescens*[1] and is a gel-forming (1→6)-branched (1→3)-β-D-glucan having two branches at every third main chain unit.[1,8] We have previously reported that the degree of β-linked branches at position 6 is remarkably related to the antitumor activity. The antitumor mechanisms of these glucans have been examined in detail using several murine systems and it is suggested to be based on the modulation of the host immune systems.[4-7]

We have also demonstrated that parent OL-2 did not show antitumor activity, but controlled Smith-type degradation products which had fewer branches than OL-2 showed significantly stronger antitumor activity.[1] On the other hand, OL-2 showed a strong hematopoietic response, similarly to other antitumor glucans (unpublished results). These facts indicate that the characterization of OL-2 may be used to clarify the biological and immunopharmacological activities of (1→3)-β-D-glucan. Thus systematic investigations of OL-2 would be fruitful.

The complement system is important for many aspects of immune regulation and self defense mechanisms.[9-11] First of all, the complement system is an important barrier against infections by fungi, bacteria, viruses and so on. The complement system is also important for tumor cell killing. Recent studies suggested the contribution of complement components to the regulation of the cytokine network. Activation of the complement system proceeds *via* the classical (CPC) and the alternative (APC) pathways. It is suggested that APC participates in the initiation and augmentation of those immune responses accompanied by inflammatory responses by phagocytic cells. The importance of molecular weight and charge for the stability of C3b has recently been reported using model compounds.[12-15] It is interesting to establish the structure–function relationship of complement activation by (1→3)-β-D-glucans in comparison with its antitumor activity.

The amoebocytes of the horseshoe crab (*Limulus*) cause coagulation of haemolymph in the presence of minute amounts of endotoxins (lipopolysaccharides, LPS) or (1→3)-β-D-glucans.[16] These pathways are mediated by factor C and factor G and are believed to be important for the host defence mechanism of *Limulus* amoebocytes.[17-20] Each of these pathways involves several proteases that are activated sequentially. Some of these proteases are common to both pathways, and the cascades are similar to those of blood coagulation and the complement systems in mammals.

In this paper, we examined the activation of APC and the coagulation of *Limulus* lysates by OL-2 and its derivatives which have different degree of branching (DB). We also discuss the structure–activity relationships between antitumor activity and the cascades of the host defence mechanism.

Materials and Methods

Materials OL-2, OL-2-I, OL-2-II, OL-2-III and OR-OL-2c were prepared as described.[1,8] SPG was provided by Kaken Chemical Co. Human whole complement was purchased from Diamedix Corp. It was reconstituted by dissolving it in 5 ml of distilled water.

Measurement of APC[21] Human whole complement (25 μl) and VEM [veronal buffered saline (VBS)–ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–$MgCl_2$, 5 μl] were mixed in a 96-well plate for ELISA (enzyme-linked immunosorbent assay), and the mixture was incubated at 37°C for 10 min. Subsequently, 50 μl of various concentrations of glucans were added and the mixture was incubated at 37°C for an additional 60 min. After the incubation, 50 μl of rabbit red blood cells (RRBC, 7.5×10^8 cells/ml) were added to this mixture, and the plate was incubated at 25 °C. The turbidity of RRBC was measured by a microplate reader at 620 nm every 15 min for 90 min. For each reaction mixture, the incubation time required to lyse half of the RRBC ($T_{1/2}$) was calculated. The results were shown as an index in comparison with control, $T_{1/2}$/control:

$$T_{1/2}/\text{control} = T_{1/2} \text{ of sample}/T_{1/2} \text{ of control}$$

***Limulus* Test** The activation of factor G by glucans was measured by a colorimetric method, using an endotoxin quantitative kit (Toxicolor LS-1, Seikagaku Kogyo Co.) which contained factors C and G (LS-1 No. 310128). Disposable plastics for tissue culture and clinical work were used, and all glassware was sterilized for 2 h at 250 °C. All operations were performed under aseptic conditions. Reactions were performed in flat-bottomed 96-well plates (endotoxin and β-glucan free; Seikagaku Kogyo Co.) as follows. Each sample (25 μl; 10 pg—10 μg/ml) dissolved in 0.5 M NaOH was diluted with 0.01 M NaOH (prepared with distilled water for injection), and placed in a culture plate. The reagent (LS-1, 25 μl) was added to each well. The plate was incubated for 40 min at 37 °C, then 0.8 M acetic acid (100 μl) was added to each well to stop the reaction, and the absorbance at 405 nm was measured using the microplate reader MTP-32 (Corona Electric). Re-LPS (Sigma, L-9764), prepared from *Salmonella minnesota* Re595, was used as the reference endotoxin. The results are expressed as the absorbance relative to that of 100 ng/ml of Re-LPS, which was given an index of 10.

Results

Activation of APC by (1→3)-β-D-Glucans Having Various DB In the first experiment, OL-2 was incubated with human whole complement for 30, 45, 60 and 90 min, then the activation of APC was measured. The activity was dependent on incubation time (Fig. 1), and dose (Fig. 2). Optimum volumes of human complement were also examined using 10, 25 and 50 μl, and the results are shown in Fig. 3. All the data shown in this section suggested that the optimum conditions for examination of APC was after 60 min of incubation time and using 25 μl of human whole complement.

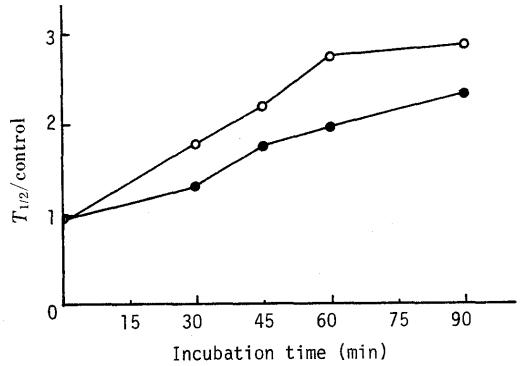

Fig. 1. Time Course of the Activation of APC by OL-2

Human whole complement and OL-2 at doses of 1 and 2 mg/ml were mixed and incubated at 37 °C for 0, 30, 45, 60, and 90 min. RRBC (7.5×10^8 cells/ml) were added to this mixture and the turbidity of RRBC was measured using a microplate reader at 620 nm every 15 min for 90 min. The results are shown as the index: $T_{1/2}$/control. ○, OL-2 2.0 mg/ml; ●, OL-2 1.0 mg/ml.

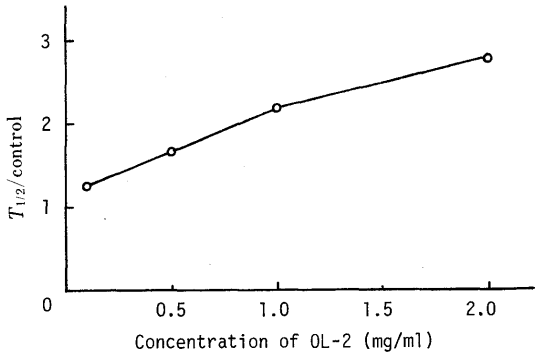

Fig. 2. Dose–Response of OL-2 on the Activation of APC

Human whole complement and OL-2 at doses of 0.1, 0.5, 1.0 and 2.0 mg/ml were mixed and incubated at 37 °C for 60 min. Activation of APC was measured as in Fig. 1. ○, OL-2.

To examine the effect of the side chains of glucans on APC activation, the activities of OR-OL-2c (no branch), OL-2-I [one (1→6)-β-D-glucosyl branch at every third main chain unit (1/3)], OL-2-II (1/2), OL-2-III (1/24) and OL-2 (2/3) were compared. All β-D-glucans were used at a concentration of 0.1—2 mg/ml. All (1→6)-branched (1→3)-β-D-glucans activated APC, but OL-2 was the weakest among OL-2 groups, suggesting some negative contribution of its side chain to the activity (Fig. 4). Moreover, OR-OL-2c, with no side chain, did not activate APC.

We previously confirmed that OL-2 (2/3) did not show antitumor activity, and that OL-2-I (1/3) and OL-2-II (1/2) showed strong antitumor activity. Also, OL-2-III at (1/24) still showed significant activity, but OR-OL-2c (no branch) did not show any activity. We have also previously described that DB of 6-O-branched (1→3)-β-D-glucan was remarkably related to its antitumor activity.[5] The intensity of activation of APC by these (1→3)-β-D-glucans was correlated with antitumor activity except for OL-2-III. These results suggested that, in this particular case, the intensity of antitumor activity was dependent on APC activation. Another important factor for this structure–activity relationship is the solubility of OL-2.

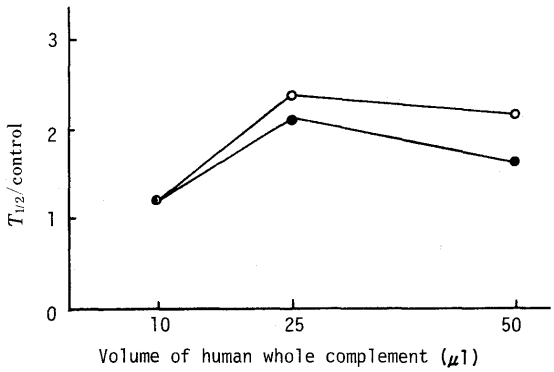

Fig. 3. Volume of Human Whole Complement on the Activation of APC by OL-2

Human whole complement and OL-2 at doses of 1.0 and 2.0 mg/ml were mixed and incubated at 37 °C for 60 min. Activation of APC was measured as in Fig. 1. ○, OL-2 2.0 mg/ml; ●, OL-2 1.0 mg/ml.

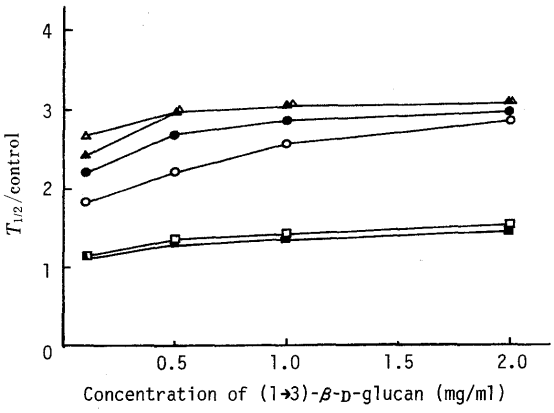

Fig. 4. Dose–Response of Various (1→3)-β-D-Glucans on the Activation of APC

Human whole complement and various (1→3)-β-D-glucans at doses of 0.1, 0.5, 1.0 and 2.0 mg/ml were mixed and incubated at 37 °C for 60 min. Activation of APC was measured as in Fig. 1. ○, OL-2; ●, OL-2-I; △, OL-2-II; ▲, OL-2-III; □, OR-OL-2c; ■, SPG.

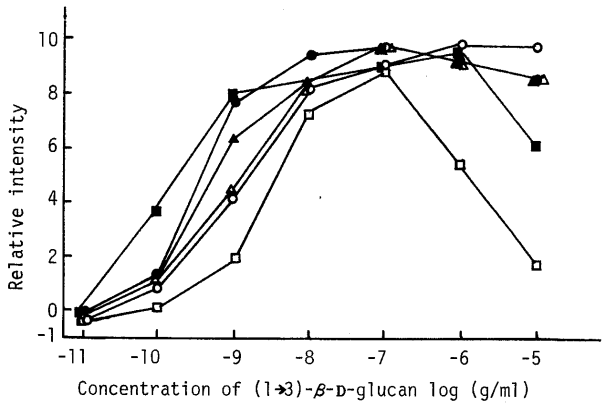

Fig. 5. Dose–Response Curves of LS-1 to Various (1→3)-β-D-Glucans
○, OL-2; ●, OL-2-I; △, OL-2-II; ▲, OL-2-III; □, OR-OL-2c; ■, SPG.

OL-2 is only slightly soluble in water. Since the particulate materials, such as zymozan, activate APC strongly, the activity of OL-2 must be related to its insolubility.

Activation of *Limulus* Coagulation Systems by (1→3)-β-D-Glucans Having Various DB Toxicolor LS-1 is a diagnostic kit used to measure the content of LPS and (1→3)-β-D-glucans. The specificity of LS-1 for (1→3)-β-D-linkage has already been established. However, the structure–activity relationship in various kinds of (1→3)-β-D-glucans has not been sufficiently examined.[22]

The activity of LS-1 towards OL-2 and its derivatives was compared by preparing serial dilutions (10 pg—10 μg/ml) of each glucan in 0.01 M NaOH (Fig. 5). The concentrations for optimum reactions were dependent on the structure of glucans and were OL-2 10 μg/ml; OL-2-I 10 ng/ml; OL-2-II 100 ng/ml; OL-2-III 100 ng/ml; and OR-OL-2c 100 ng/ml. OL-2-I gave the strongest response and OL-2 gave the weakest. Thus, the reactivity of LS-1 to (1→3)-β-D-glucans decreased with an increase in DB. In addition, OR-OL-2c showed a different dose–response curve from the other derivatives and showed the weakest responses. OL-2 and OR-OL-2c showed no antitumor activity. Thus, in this particular case, the structure–activity relationship for the reaction with the *Limulus* lysate also correlates with antitumor activity.

Discussion

The complement system is important for the initial immune responses. Activation of APC by (1→3)-β-D-glucans is thought to contribute to its antitumor activity. However, the mechanisms of APC activation by (1→3)-β-D-glucans and structure–function relationships of (1→3)-β-D-glucans have not yet been clarified. The available evidence suggests the importance of the fate of the C3 component, including its binding ability, and the conversion of C3 to C3b and C3bi in relation to factors H and I.[9,10,23,24] As C3b binds nonspecifically to the hydroxyl or amino groups of a surface, it is thought that the extent of the amplification system or inactivation processes are important for the activity. Carreno *et al.* reported that the interaction between surface-bound C3b and factor H was enhanced by a nonactivator of APC.[23] In addition, the hydrophobicity of glucans in relation to gel productivity may be important for these processes. Furthermore, the side chain moiety of (1→3)-β-D-glucan must also significantly contribute to the activation of APC. The data shown in this paper strongly support the negative contribution of side chains to the activation of APC.

In this paper, we found that the reactivity of *Limulus* amoebocyte lysate to (1→3)-β-D-glucans was also dependent on DB. The activity of LS-1 in relation to (1→3)-β-D-glucans decreased with an increase in DB. We have also demonstrated that curdlan, (1→3)-β-D-glucan without a side chain, showed strong reactivity.[22] However, OR-OL-2c, whose structure is similar to that of curdlan, had low reactivity. The low reactivity of this derivative suggested significant structural changes during chemical modifications: for example, the absorption maximum of Congo red shifted largely to a longer wavelength in the presence of OL-2; however, it did not shift in the presence of OR-OL-2c.[8] Reduction of DB by chemical modifications would change the conformation from a gel to a sol.

The complement system shows some similarities to the blood coagulation system. For example, S-protein (vitronectin) inhibits the formation of C5b-9 and the binding of antithrombin III to thrombin, and C1q inhibits the activation of factor XII. Activation of the *Limulus* coagulation system by (1→3)-β-D-glucans and a similar structure–activity relationship between APC and *Limulus* coagulation would strongly suggest a similar general function of both pathways in host/self defense mechanisms.

The ultrastructure of (1→3)-β-D-glucan is thought to be critically important for its immunopharmacological activities. Studies on the structure–activity relationships for varieties of biological activities revealed the presence of more than one structure–activity relationship dependent on the activity. Representative physicochemical parameters which affect the biological activities are: solubility in water (sol, gel, insoluble), molecular weight, and side chain.[5,21,22,25] In addition, single helix or triple helix conformation would also be important.[22] The derivatives used in this paper can be divided into three groups, namely: i) parent OL-2, which is too branched to show significant antitumor activity; ii) OL-2-I, OL-2-II and OL-2-III, which contained the appropriate amount of branching to show antitumor activity and retained gel productivity; and iii) OR-OL-2c, which is of low molecular weight showing no gel productivity.[8] The structure–activity relationships for APC as well as *Limulus* were well correlated to the above groupings. There is no doubt that the low reactivity of OR-OL-2c is due to the low molecular weight showing no gel productivity. The structure–activity relationship among OL-2, OL-2-I, OL-2-II, and OL-2-III must be dependent on DB, but included some obscurity, because those activities were similar to each other. It is quite interesting, especially in APC, to note whether a slight change in activity affects the immunopharmacological and antitumor activities of OL-2.

As described above, the activation of complement induces a variety of reactions related to host–defense systems. There are many reports that some of the bioactive peptides, such as C3a and C5a, produced from these reactions would activate host–defense systems. However, the structure–activity relationship of complement activation and antitumor activity is thought to be not completely related, and is not completely demonstrated by the earliest

reactions in the host to activate its self defense system. In conclusion, it would be necessary to investigate the glucans by biological, biochemical, as well as physical approaches to fully understand the biological activities of the glucans at the molecular level.

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