Comparison of the Blood Clearance of Triple- and Single-Helical Schizophyllan in Mice

Noriko N. MIURA,^a Naohito OHNO,^a Yoshiyuki ADACHI,^a Jun AKETAGAWA,^b
Hiroshi TAMURA,^b Shigenori TANAKA,^b and Toshiro YADOMAE^{*,a}

Laboratory of Immunopharmacology of Microbial Products, Tokyo College of Pharmacy,^a Horinouchi, Hachioji, Tokyo 192-03, Japan and Tokyo Research Institute, Seikagaku Corporation,^b Higashiyamato, Tokyo 207, Japan.

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(1→3)- β -D-Glucans exhibit a variety of biological and immunopharmacological activities, and the degree of these activities depends on the nature of the individual glucans e.g. molecular weight, degree of branching and conformation. Based on the generally accepted evidence that the conformation of Sonifilan (SPG) used clinically is a triple helix, we prepared alkali-denatured SPG (SPG-OH) as a single helix conformer. In this report, we measured the concentration of β -glucan administered to mice by using a β -glucan-specific reagent prepared from limulus amoebocyte lysate (Gluspecy [G test], Seikagaku Corporation, Tokyo) and discuss the blood clearance of SPG and SPG-OH following intraperitoneal (i.p.) or intravenous (i.v.) administration. Comparing the clearance of SPG-OH from the blood with that of SPG, SPG-OH was removed faster than SPG following both i.p. and i.v. administration. This strongly suggests that the clearance of β -glucans is dependent on their conformation.

Keywords limulus test; (1→3)- β -D-glucan; sonifilan; factor G; clearance rate; conformation

(1→3)- β -D-Glucans are present in variety of organisms, including fungi, yeast, algae, bacteria, and higher plants, and compared with lipopolysaccharides (LPS), their structures also differ significantly depending on the molecular weight, degree of branching and ultrastructure. In addition to this structural variability, (1→3)- β -D-glucans exhibit a variety of biological activities.¹⁾ The mechanism of (1→3)- β -D-glucan-mediated antitumor activity has been shown to be closely related to the host defense. Thus, the structure-activity relationship of (1→3)- β -D-glucans and their action on the host defense mechanisms are worth investigating. Interestingly, the structure required to exhibit each type of activity was different.²⁾

The amoebocyte lysate from horseshoe crab (limulus) coagulates in the presence of gram-negative bacterial endotoxins (LPS) or (1→3)- β -D-glucans. Initiation of LPS and (1→3)- β -D-glucan-triggered reactions is mediated by specific zymogens called factor C and factor G, respectively.³⁾ Measurement of the amidolytic activity of clotting enzyme with chromogenic substrate, instead of the gelation assay, improved the sensitivity and accuracy of the reaction.⁴⁾ Recently, a new limulus reagent, not containing factor C (G-test), was developed and this has been used to determine β -glucan concentrations in various solutions.

It is very important to establish the diagnosis of mycosis clinically. Mycosis, especially candidiasis, is a frequent opportunistic infection in neutropenic patients. The method used for diagnosis of candidiasis, which is superior to conventional blood culture, still requires several days.^{5–11)} Recently, a G-test was applied clinically to diagnose mycosis, because of its very high sensitivity to (1→3)- β -D-glucans which are a major component of the fungal cell wall.¹²⁾ To understand the molecular mechanism of the biological activities of glucans *in vivo*, their fate and tissue distribution play an important role as far as their pathophysiology and immunopharmacology are concerned. In a previous paper, we investigated the tissue distribution of an antitumor glucan, SSG, obtained from

Sclerotinia sclerotiorum, using the metabolically radio-labeled compound and found that most of the SSG was transported to liver and spleen *via* the blood from the peritoneal cavity over a period of several days.¹³⁾ It is probable that the molecular weight, degree of branching, and ultrastructure of the (1→3)- β -D-glucans might affect their transport from organ to blood and their clearance from the blood. To understand the fate of glucans, it is important to define the clearance rate of a variety of chemically-defined compounds. To demonstrate the conformation-dependence of the clearance of β -glucans from the blood, as the first step in our investigation, we used Sonifilan (SPG) and alkali-denatured SPG because of their well characterized solution and solid conformations.^{14,15)}

MATERIALS AND METHODS

Materials SPG (Sonifilan prepared from schizophyllan^{15,16)}) was kindly provided by Kaken pharmaceutical Co., Ltd. (Tokyo). Distilled water was purchased from Otuka Co., Ltd. (Tokyo) and sodium hydroxide was purchased from Wako Pure Chemical Industries, Ltd. Alkali-denatured SPG (SPG-OH) was prepared by treating SPG (25 μ l) with 225 μ l 0.5N NaOH and neutralization.

Animals Specific-pathogen-free male ICR mice were purchased from Japan SLC (Shizuoka) and used at 5–8 weeks of age.

Preparation of Plasma for Limulus Test SPG or SPG-OH was diluted with physiological saline and aliquots were administered to mice. After this, whole blood was collected several times from the tail vein using heparinized capillaries and stored at 4°C before carrying out the G-test.

Limulus Test (G-Test) The activation of factor G by glucans was measured by colorimetry using a glucan-specific chromogenic reagent (Gluspecy [G test], Seikagaku Corporation, Tokyo) which does not contain factor C. Plasma was treated with 0.5N NaOH and diluted with

0.01 N NaOH. Reactions were performed in flat-bottomed 96-well Toxipet Plates (Seikagaku Corp. Tokyo) as follows. Samples (25 μ l) were placed in the wells and the reagent (Gluspey 25 μ l) was added to each. The plate was incubated for 40 min at 37 °C, and, during incubation, the absorbance at 405 nm was measured using a microplate reader (Wellreader SK601, Seikagaku Corp.) at 5 min intervals. Disposable plastics for the tissue culture or the clinic were used, and all glassware was sterilized at 260 °C for 3 h. All operations were performed under aseptic conditions. The β -glucan concentration in blood was calculated from the relative reactivity to SPG. Curve fitting was performed by a program, Cricket Graph ver. 1.3 (Cricket Software, Malvern, PA).

RESULTS

Effect of Blood Components on the Reactivity of the G-Test Even if only one glucan is used, its conformation significantly influences the reactivity of the G-test.¹⁷⁾ Thus, it is necessary to normalize the conformation of the glucan to show the highest reactivity. We used alkaline treatment to normalize the conformation, because we have already demonstrated that such treatment changes the conformation of the glucan to a random coil and, following neutralization, the single helix conformation is adopted.^{14,16)} Figure 1 shows the dose response curve of SPG to the G-test after sodium hydroxide treatment. Under these conditions, more than 10 ng/ml of SPG reacted.

To determine the blood concentration of the glucan, in addition to the conformation change, it is necessary to eliminate the effects of blood components on the limulus reaction. It is known that the reactivity of the limulus test is affected by substances in the blood, such as thrombin, plasmin, trypsin, antithrombin III, antitrypsin, and plasmin inhibitor.¹⁸⁾ Alkaline treatment, as described above, was used for the blood components. As shown in Fig. 1, the reactivity of SPG was unchanged in the presence of plasma. Similar results were obtained in the presence of either whole blood or serum (data not shown). Because

Fig. 1. Effect of Plasma on the Reactivity of SPG in the G-Test

Each 10 mg/ml of SPG (25 μ l) was mixed with 9 volumes of distilled saline (○) or plasma (●) and treated with aqueous NaOH (final concn. of 0.5 N). The resulting mixture was serially diluted with 0.01 N NaOH and used for the G-test. The reaction time of the G-test was 15 min.

the dose-response of SPG in the presence or absence of blood components was indistinguishable, we decided to use sodium hydroxide treatment to determine the glucan concentration.

Dose-Response of Clearance Rate of β -Glucan in Plasma by i.v. or i.p. Administration It was our intention to examine the factors which influence the clearance of β -glucan from plasma. As a first experiment, several doses of SPG were administered intravenously (i.v.) or intraperitoneally (i.p.) and blood samples were collected at various times. In the case of i.v. administration, doses of 1, 10, and 100 μ g/mouse were studied. As shown in Fig. 2A, blood glucan concentrations following the 1, 10 and 100 μ g administrations could be measured after serial dilution of the plasma samples. In the case of the 100 μ g dose, the maximum concentration was ca. 70 μ g/ml immediately after administration and the concentration

Fig. 2. Dose-Response of β -Glucan Clearance from Plasma Following i.p. or i.v. Administration of SPG

Several doses of SPG (1–100 μ g/mouse for i.v., and 10–1000 μ g/mouse for i.p.) were administered intravenously (i.v.) (A) or intraperitoneally (i.p.) (B) to mice ($n=2$), and blood samples were collected at various times. —○—, i.v. -1, i.p. -10; —●—, i.v. -10, i.p. -100; —□—, i.v. -100, i.p. -1000. Plasma was obtained and the β -glucan concentration measured by the G-test. Before carrying out the test, each sample was treated with 0.5 N NaOH.

TABLE I. Pharmacokinetic Parameters of Intravenously Administered SPG in Mice^{a)}

Experiments shown in	Dose (μ g)	K (h^{-1})	$t_{1/2}$ (h)	V_d (ml)	CL_{tot} (ml/h)
Fig. 2	100	0.14	4.91	1.97	0.23
	10	0.10	6.73	2.75	0.28
Fig. 3	200	0.118	5.87	3.67	0.43
	200(OH ⁻) ^{b)}	0.308	2.25	6.5	2.0

a) Each value was estimated from curve fitting (Exponential) of the data shown in Fig. 2 or Fig. 3. b) Alkali treated SPG.

then gradually fell. In the case of the 10 and 1 μg doses, the maximum concentrations were *ca.* 5 $\mu\text{g}/\text{ml}$ and *ca.* 300 ng/ml , respectively, and these fell below the limit of detection (10 ng/ml) within a day. To compare the clearance of SPG in these mice, several pharmacokinetic parameters were estimated and the data are summarized in Table I. These values were calculated using the one-compartment model. Interestingly, the elimination rate constant (K), half-life ($t_{1/2}$), total body clearance (CL_{tot}), and distribution volume (V_d) in the 10 and 100 $\mu\text{g}/\text{mouse}$ groups were similar. However, the 1 $\mu\text{g}/\text{mouse}$ group had significantly different values (data not shown). Considering that the concentration after the lowest dose are close to the detection limit (10 ng/ml), there was great variability in these results. From these observations, we tentatively conclude that the clearance of SPG is independent of the administered dose.

In the case of i.p. administration, doses of 10, 100, 1000 $\mu\text{g}/\text{mouse}$ were studied. As shown in Fig. 2B, blood glucan concentrations following all three doses could be measured. In the case of the 1000 μg dose, the maximum concentration observed was *ca.* 70 $\mu\text{g}/\text{ml}$ at 4 h. However, as the clearance of compounds from the blood given by i.p. administration involves the first-order absorption rate constant from the peritoneal cavity, in addition to the clearance constant, more complicated calculations are required. We need more experiments to decide whether it is practical to use pharmacokinetic parameters to estimate the clearance of SPG, and so we simply compared the profiles of the blood concentrations in Fig. 2. We tentatively concluded that the clearance rates of the 1000,

100 and 10 μg i.p. doses were similar.

Comparison of the Clearance of Native and Alkali-Denatured SPG from Blood Vessels in Mice (1 $\rightarrow$ 3)- β -D-Glucans have been obtained from various sources using various preparation and purification methods. As described above, the ratio of triple and single helices in various glucans varied with the structure, sample preparation and molecular weight. It is generally accepted that sodium hydroxide treatment increases the ratio of the single helix conformer while treatment at 150 $^{\circ}\text{C}$ increased that of the triple helix.^{14,19)} The relationship between conformation and biological activity is interesting and to date, in addition to the limulus reactivity, several biological activities have been found to be influenced by conformation, such as complement activation, plasma clotting and antitumor activity. We investigated the blood clearance of native and alkali-denatured SPG in mice. In order to examine the clearance of β -glucans with different conformers, we used single (SPG-OH) and triple helix (helical) conformers (SPG) of SPG. SPG or SPG-OH, 200 or 1000 μg , were administered i.v. or i.p. As shown in Fig. 3A and Table I, the clearance of SPG-OH from blood was significantly faster than that of SPG. Estimated from the value following i.v. administration in Table I, the clearance of SPG-OH was *ca.* 3 times faster than that of SPG. This was also true in the case of i.p. administration (Fig. 3B).

Interestingly, SPG concentrations could be measured even one week after i.p. administration in this experiment while SPG-OH were at or below the limit of detection. As discussed later, these observations are important when using the G-test to diagnose mycosis.

DISCUSSION

In this paper, we measured the blood clearance of β -glucan using SPG and alkali-denatured SPG (SPG-OH), and found that the clearance of the single helix conformer (SPG-OH) was faster than that of the triple helix conformer (SPG). The fact that the clearance of SPG found in this study was very close to the value obtained using radiolabeled SPG,²⁰⁾ strongly suggests that the G test can be used to measure β -glucan concentrations in blood. The difference in clearance between SPG and SPG-OH may be due to several underlying mechanisms, such as the glucan receptor, protein-binding and the gel productivity.

Czop *et al.*²¹⁾ suggested the presence of β -glucan receptor(s) on the mononuclear phagocytes. Basically, although the β -glucan receptor(s) has been identified functionally as the phagocytic receptor, its contribution to the immunopharmacological activity has not been characterized. We have also shown that hydrogen peroxide synthesis of murine peritoneal macrophages triggered by zymosan particles is inhibited by soluble glucans.²²⁾ Interestingly, the clearance of zymosan particles was significantly faster than that of soluble glucans. In addition, some of the soluble glucans act as an antagonist of the phagocytosis of particulate glucan; however, it is not yet known if the glucan receptor is the major receptor for soluble glucans. We have recently reported, using metabolically radiolabeled soluble β -glucan, that the

Fig. 3. Comparison of the Clearance Rates of Native and Alkali-Denatured SPG from Blood Vessels of Mice

SPG (—○—) or alkali-treated SPG (SPG-OH, —●—) were administered i.v. (200 μg each) (A) or i.p. (1 mg each) (B) to mice ($n=2$). Blood samples were collected from the mice at various times, and the β -glucan concentration measured in plasma by the G-test. Before carrying out the test, each sample was treated with 0.5 N NaOH.

major fraction of glucan was found in liver and spleen, and was located in the intercellular space of the organs instead of inside the cell.¹³⁾ This suggests that the glucan receptor might not contribute to the clearance of soluble glucan.

Although we can not yet discuss the specific receptor for soluble glucan, protein-binding to the glucan might contribute, at least in part, to the clearance, because the soluble glucan could activate the complement and coagulation systems. We previously showed that the protein-binding is higher in the case of the single helix conformer.¹⁾ The data shown in this study that the single helix conformer is cleared faster, also correlates well with the protein-binding.

β -Glucans including SPG have capacity to form a "gel" under physiological conditions. The major physicochemical process involved in producing the gel is an interchain interaction. This interaction can produce huge macromolecules the size of which depends on the physical conditions such as concentration, temperature and pH.^{2,23)} In the case of Sonifilan, which is used clinically, the average molecular weight under physiological conditions is ca. 450 kDa. The molecular weight of the alkali-denatured SPG would gradually change during keeping because of multiple chain interactions, and might include a larger population than native SPG. If the average molecular weight of the denatured SPG was larger than native SPG, it would also affect the clearance.

It is generally accepted that the conformation of the gel producing β -glucan depends on various circumstances, such as its preparation, molecular weight, solvent and primary structure. The conformation is especially sensitive to alkaline treatment and, once treated with alkali, produces an unstable single helix part, which gradually changes to stable single and triple helix conformations.¹⁹⁾ It would be interesting to elucidate the conformation of SPG and SPG-OH in blood. Preliminary investigation shows that none of the SPG in blood reacted while the SPG-OH in blood reacted in the G-test without alkaline pretreatment. This data suggests that the conformation of both SPG and SPG-OH are relatively stable even in blood. However, we can not discuss this quantitatively because reactivity in the G-test is strongly affected by blood components when appropriate pretreatment is not carried out.

Under clinical circumstances, the G-test has been investigated for the diagnosis of fungal infection. Higher concentrations of the soluble glucan in blood suggest the possibility of fungal infection. Fungal β -glucan was originally described as a water-insoluble cell wall constituent, but recent research strongly suggests that a significant amount of soluble β -glucan, reacting with the G-test, is produced by fungi.⁴⁾ We have shown that a part of the cell wall glucan is solubilized during ingestion by phagocytes.²⁴⁾ These facts suggest that a soluble form of fungal glucan really exists. The data in this paper show that blood concentrations of SPG (200 μ g dose) can be detected over a week, while SPG-OH fell below the limit of detection. Intraperitoneal administration of SPG or SPG-OH further delays blood clearance. These facts, unfortunately, suggest that the blood concentration of

glucan released from the infected fungi might be below the limit of detection after a certain period of delay from recovery.

In this paper we used SPG and alkali-denatured SPG as model compounds and found that the conformation affects the glucan clearance. Other factors, such as sol conformer, molecular weight and degree of branching, may also affect the clearance. In addition, we used two-months-old ICR male mice raised under the specific-pathogen-free conditions and this may also affect glucan clearance.

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