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Solubilization of Limulus Test Reactive Material(s) from *Candida* Cells by Murine Phagocytes

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Solubilization of limulus test reactive materials from *Candida* was examined in the presence or absence of phagocytic cells. Solubilized limulus test reactive materials (LTRM) were detected in culture supernatant, and hot water and sodium hydroxide extracts of the acetone dried cells of *Candida parapsilosis*. Suspensions of *Candida* cells also reacted with limulus test, and LTRM were released from the acetone dried cells by serum treatment. After treatment of the acetone dried cells with polymorphonuclear leucocytes (PMN) or macrophages (Mϕ), a significant amount of LTRM was solubilized. Significant amounts of LTRM were also released by PMN during treatment of live and growing *C. parapsilosis*. The reactivity of LTRM was completely inhibited by the addition of excess amount of purified (1→3)-β-D-glucan, suggesting LTRM from *Candida* cells as described above would contain (1→3)-β-D-glucan. These results suggested that LTRM during fungal infection would come from the extracellular water soluble polysaccharide fraction as well as the insoluble cell wall fraction solubilized by the action of phagocytes.

Keywords limulus test; *Candida*; (1→3)-β-D-glucan; PMN; macrophage

Introduction

During the last few decades, the incidence of life-threatening systemic infection caused by *Candida* spp. and other fungi has increased remarkably.[1-3] These infections occur worldwide and now represent a major threat to many hospitalized patients. Most of the increase is due to the superinfections arising in patients compromised by severe underlying diseases, *e.g.*, leukemia, acquired immunodeficiency syndrome, and by immunosuppressive drugs for various kinds of diseases. Additionally, the introduction of sophisticated cardiac surgery, the use of indwelling prostheses and shunts, transplantation, and the direct access of organisms through indwelling vascular catheters and needles increase the infection by eumycetes.

Several approaches have been used to identify the fungal infection. The limulus test is used for this purpose because of the release of soluble factor G reactive materials (LTRM) at the early phase of the infection; nevertheless, the mechanism of release and/or production of LTRM from fungi has not yet been clearly demonstrated.[4]

The amoebocyte lysate of the horseshoe crab (limulus) causes coagulation in the presence of minute amounts of endotoxin (lipopolysaccharide; LPS) through the factor C mediated pathway (C pathway) or of (1→3)-β-D-glucan through the factor G mediated pathway (G pathway).[5,6b] These properties have been used to determine the concentration of LPS in medicine and biological fluids. As described above, a similar method has recently been applied to determine the concentration of (1→3)-β-D-glucans in biological fluid, since (1→3)-β-D-glucan concentration was increased in patients with fungal infection or treatment with biological responses modifier, or hemodialysis.[4] In the previous study, using structurally defined glucans, we demonstrated that the reactivity of the G pathway was strongly dependent on the molecular weight, degree of branching, and conformation of the glucans.[7,8] It is noteworthy that, even in (1→3)-β-D-glucans, there are certain structural units which do not react with limulus test. In the case of clinical isolates, it has been shown that the reactivity of factor G is strongly correlated with fungal infection. The fine structure of LTRM, however, has not been demonstrated. Although LTRM have already been determined semi-quantitatively by utilizing the factor G pathway, the method still entails some quantitative difficulties, such as; 1) how LTRM are solubilized during infection, 2) what the exact structure of LTRM is during infection, and 3) what the best standard material is to monitor clinical materials.

Host defense mechanisms for candidiasis, especially at an early phase of the infection, depend on the phagocytic cells.[9] Oxygen dependent as well as oxygen independent mechanisms work together to induce cytostatic, killing, as well as lytic actions against fungal cells. (1→3)-β-D-Glucan is one of the major constituents of the cell wall of fugi,[10] and LTRM come from the cell wall which has been solubilized for some reason. No detailed explanation has been made, however, of how LTRM are released during infection. In this paper, we will demonstrate the source of LTRM released from live as well as acetone dried *Candida* cells during various situations including treatment with phagocytic cells.

Experimental

Animals Specific-pathogen-free male mice of ICR were purchased from Japan SLC (Shizuoka) and used at 5—7 weeks of age. The animals were bred under specific-pathogen-free conditions.

SPG SPG (Sonifiran®️ prepared from schizophyllan) was kindly provided by Kaken Pharmaceutical Co., Ltd. (Tokyo).

Macrophages (Mϕ) Cells were collected from the peritoneal cavity of normal mice by washing twice with 5 ml of Hank's balanced salt solution (HBSS, Nissui Seiyaku Co., Ltd., Tokyo) containing heparin (5 U/ml). The cells were washed and resuspended in RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 5 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (Hepes), penicillin (100 U/ml) and streptomycin (100 μg/ml). The total cell number was counted with a hemocytometer, and cells were differentiated by using a Diff-Quik Stain Kit (Kokusai Shiyaku, Hyogo). Aliquots of the cell suspension containing an adequate number of macrophages were plated in a flat-bottomed 96-well tissue culture plate (Sumitomo Bakelite Co., Ltd., Tokyo). After incubation for 2 h at 37 °C in a CO_2 incubator, nonadherent cells were removed by washing twice with RPMI 1640 medium. The resultant cells were used as macrophages.

Polymorphonuclear Leucocyte (PMN) Cells were collected from the peritoneal cavity of mice, and administered 8% casein intraperitoneally (i.p.) 6 h before the assay, by washing twice with 5 ml of HBSS. The lavage fluid was combined and centrifuged at 300 × *g* for 5 min to pellet the cells. The cells were washed and resuspended in RPMI-1640 medium, and the total cell number was counted with the hemocytometer, then adjusted to

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an appropriate concentration.

Fungi Stock cultures of *C. albicans* (IFO 1296, 1397, 1974, 10108) and *C. parapsilosis* (IFO 0585, 0640, 0708, 1068) were obtained from the Institute for Fermentation, Osaka, maintained on Sabouraud agar (Difco, U.S.A.) at 25 °C and transferred at once every three months. To prepare yeast cells grown in a stationary phase, *Candida* cells were inoculated into Sabouraud broth, and incubated 1 to 2 d at 37 °C with continuous rocking. Immediately before use, cells were washed and suspended with RPMI-1640 medium.

Candidacidal Assay Candidacidal activity was assayed by the method of Sasada and Johnston[11] with minor modifications.[12] *Candida* cells in RPMI-1640 medium were added to the macrophage monolayer or PMN in 96-well tissue culture plates, and incubated for from 3 h to 1 d at 37 °C. After appropriate dilution, the cell suspension was placed on Sabouraud agar, and the number of *Candida* colonies was counted after incubation for 48 h at 28 °C.

Limulus Test The activation of factor G by glucans was measured by a colorimetric method, using an endotoxin quantitative kit (Toxicolor LS-1, Seikagaku Corporation, Tokyo) which contains factors C and G (LS-1 No. 310128). Disposable plastic for the tissue culture or the clinic was used, and all glassware was sterilized by dry-heating for 2 h at 260 °C. All operations were performed under aseptic conditions. Reactions were performed in flat-bottomed 96-well microtiter plates free of endotoxin and β-glucan (Seikagaku Corporation, Tokyo) as follows. Each sample (25 μl), diluted with pyrogen free distilled water or 0.5 N or 0.01 N NaOH, was placed in a culture plate, and the reagent (LS-1, 25 μl) was added to each well. The plate was incubated for 40 min at 37 °C on a Hotplate CT-961 (Seikagaku Corporation, Tokyo). Then, 0.8 M of acetic acid (100 μl) was added to each well to stop the reaction, and the absorbance at 405 nm and reference at 630 nm was measured using a 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 as an index of 10. Representative protocol to prepare test samples was as follows. PMN (1×10^7/ml) and *Candida* cells (4×10^7/ml) were plated and incubated for 0, 3 and 24 h at 37 °C in a humidified CO_2 incubator. The cell suspension was centrifuged at 1000 × *g* for 5 min, then supernatant and pellet were collected, and maintained at 4 °C. After appropriate serial dilution, these were used for the limulus test.

Results

Preparation of LTRM from the Cells of *Candida* by Physical and Chemical Treatments Prior to determining the release of LTRM from *Candida* cells by the treatment with phagocytic cells, reactivity for the limulus test of various subfractions of *Candida* cells was examined. Figure 1a shows the reactivity of the limulus amebocyte lysates to the culture supernatant obtained from overnight culture of *C. parapsilosis* IFO 0640 in RPMI 1640 medium, which is one of the most commonly used media for cells and tissue culture, in a humidified 5% CO_2 incubator. After the cultivation, *C. parapsilosis* had grown to produce visually observable colonies. As shown in Fig. 1, RPMI 1640 medium did not contain any LTRM before the cultivation, however, strong reaction was observed after the cultivation. In comparison with NaOH treated SPG, which is a well-known (1→3), (1→6)-β-D-glucan, the concentration of LTRM in the culture supernatant is approximately 0.78 μg/ml.

Cell wall (1→3)-β-D-glucan is thought to be water insoluble and can be extracted by extensive alkaline treatment.[13] Before preparing various extracts, the reactivities of *Candida* cell suspensions to the limulus lysate were measured. As shown in Fig. 1b, acetone dried cells of *C. parapsilosis* and *C. albicans* reacted to the lysate almost equally. To prepare various extracts, we selected acetone dried *C. parapsilosis* because of its ease of handling. The cells of *C. parapsilosis* IFO 0640 were extracted with cold

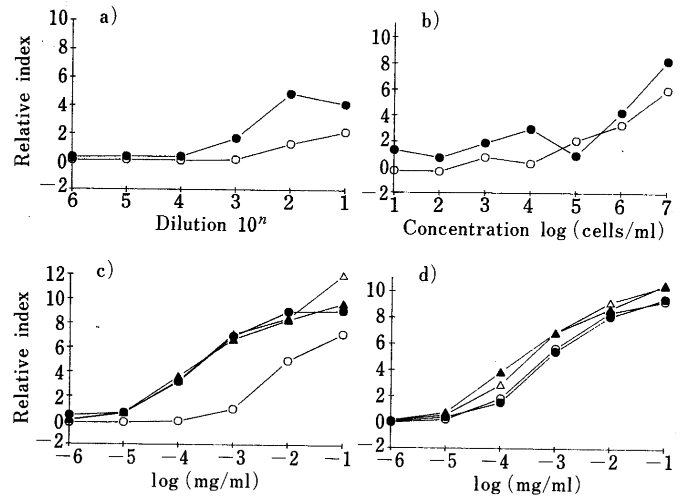

Fig. 1. Reactivity of Limulus Amebocyte Lysate to Cells and Preparations of *Candida*

a) Culture supernatant obtained from 0 h (—○—) and overnight culture (—●—) of *C. parapsilosis* IFO 0640 in RPMI 1640. b) Acetone dried *Candida* whole cells. *C. parapsilosis* 0640 (—○—), and *C. albicans* 1269 (—●—) suspended in RPMI 1640. c) Various extracts of *C. parapsilosis* 0640 prepared as follows: Acetone dried *Candida* cells were suspended in cold water (4 °C for 3 h, —○—), hot water (autoclaved for 1.5 h, —●—), 0.5 N NaOH (room temp. for 24 h, —△—), or 0.5 N NaOH in a sonic bath (room temp. for 24 h, —▲—). After each treatment extract and residual cells were separated by centrifugation. Each extract was used in c). Abscissa indicates the basis of weight of the cells. d) Residual cell preparations of *C. parapsilosis* 0640 were prepared as described for 1-c above. When the limulus test was completed, all samples (a—d) were diluted by 0.5 N NaOH, and subsequently dilution was made by 0.01 N NaOH.

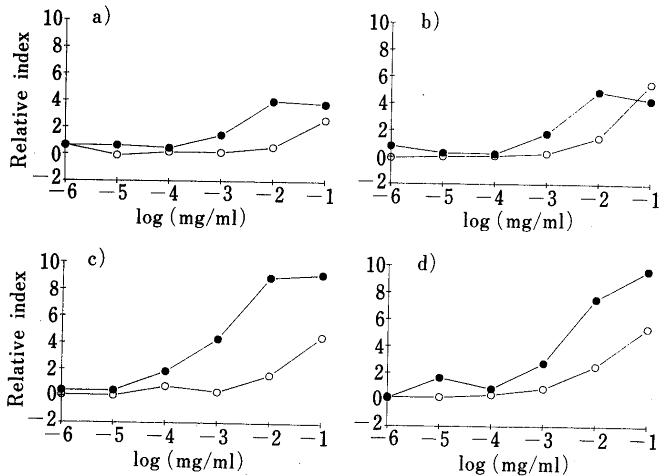

Fig. 2. Solubilization of LTRM from Acetone Dried *C. parapsilosis* 0640 by Host Defense Systems

Acetone dried cells of *C. parapsilosis* were suspended in saline and incubated at 37 °C with a) saline, b) normal mouse serum, c) peritoneal Mϕ (ICR mice), or d) PMN (ICR mice, 6 h after 2 ml of 8% casein injection) for 0 h (—○—) or 24 h (—●—). Abscissa indicates the basis of weight of the cells.

water, hot water at 121 °C, 0.5 N sodium hydroxide at room temperature, and 0.5 N sodium hydroxide in a sonic bath, and the reactivities of these preparations to the limulus lysates were compared. The alkaline extracts are thought to react significantly higher with the lysates than the hot water extract, but, interestingly, as shown in Fig. 1c, all of the extracts except for the cold water extract reacted strongly. It is well known that the major constituents extractable by hot water and sodium hydroxide are mannan and β-glucan, respectively, thus the concentration of β-glucan in the sodium hydroxide extracts would be

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significantly higher than that in the hot water extract. In the previous publication it was demonstrated that yeast mannan showed quite low reactivity to the lysate.[6b,14] It is suggested that, besides the water insoluble cell wall glucan, the water soluble glucan in the cell wall and the glucan easily released from the cell wall would also significantly react with the lysates. In the next experiment, Fig. 1d, reactivities of cell suspensions remaining following the above extractions were found to be similar in all preparations. In comparison with SPG, relative reactivity of the cell suspensions is approximately 4.91 μg glucan/mg dried cell. Interestingly, reactivities of all of the suspensions were quite similar, regardless of the quantities of remaining cell wall β-glucan. These results indicate that solubilized β-glucan has significantly higher reactivity to the lysate than the cell suspension.

Release of LTRM from *Candida* Cells by Serum, PMN, or Mϕ Treatment Prior to examining the release of LTRM from the intact/viable *Candida* cells by the machinery of the self defense mechanism, acetone dried cells were used to demonstrate LTRM release by this machinery. Figure 2a shows the release of LTRM from acetone dried *Candida* cells by incubation with saline as the control experiment for the serum treatment. Similar to the results described above, some part of the LTRM was released by saline treatment. In the serum treatment (Fig. 2b), the serum itself showed some interfering reaction, because samples could not be treated with protein-denaturing reagents; however, significantly higher reaction was observed after 24 h of incubation (●) compared to without incubation (○). It is noteworthy that LTRM were quite easily released into the supernatant fraction in the acetone dried cells. Addition of excess amount of SPG completely inhibited the reactivity of LTRM from *Candida* cells by the serum (Fig. 3a). This confirms that these LTRM are not LPSs or due to any artifact but a kind of (1→3)-β-D-glucan, as excess concentration of soluble (1→3)-β-D-glucan selectively inhibits the factor G pathway of the lysate.[14] These results suggested that, in addition to the spontaneous release of LTRM even under mild reaction conditions (Fig. 1c), the self defense factors in the serum such as lysozyme might act on the *Candida* cells and release LTRM into the serum.

In the next experiment, the acetone dried cells were incubated with PMN or Mϕ for 24 h to determine the release of LTRM during phagocytosis and digestion. As shown in Figs. 2c and 2d, LTRM were significantly increased in both PMN and Mϕ after 24 h incubation. As shown in Fig. 3b, the reaction mediated by solubilized products from *Candida* cells treated with Mϕ was almost completely inhibited by this treatment, confirming the released LTRM during phagocytosis and digestion to be a kind of (1→3)-β-D-glucan. In addition, comparing the data of Figs. 2c and 2d, PMN and Mϕ appear to equally contribute to releasing LTRM from *Candida* cells.

Cytostasis, Killing, and Digestion of *Candida* Cells by Peritoneal Macrophages or Polymorphonuclear Leukocytes Thus, it was demonstrated that PMN and Mϕ equally contribute to release LTRM from *Candida* cells. During the infection by *Candida* cells, and prior to their digestion, phagocytosis and killing of the fungi by these phagocytic cells are very important initial steps. Meanwhile, it is important to know the stages of digesting as well as killing to understand the full mechanism involved in the release of LTRM from the fungi. To demonstrate the candidacidal and candidastatic action of PMN and Mϕ against several strains of *Candida* spp., the following experiments were performed. Preliminary experiments showed that phagocytosis and killing of *Candida* cells by Mϕ was significantly increased by peritoneal exudate macrophages (PEM). Thus, we used Mϕ and casein induced PMN as the source of the cells, and candidacidal activities of these cells to several strains of *C. albicans* and *C. parapsilosis* were measured. The data summarized in Table I suggest that candidacidal activity of PMN was generally stronger than that of Mϕ and was also dependent on the strains. Considering the data shown above that a) PMN and Mϕ almost equally release LTRM from acetone dried *Candida* (Figs. 2c and 2d, and 2b) that PMN killed *Candida* more effectively than Mϕ (Table I), we used PMN in the following experiments.

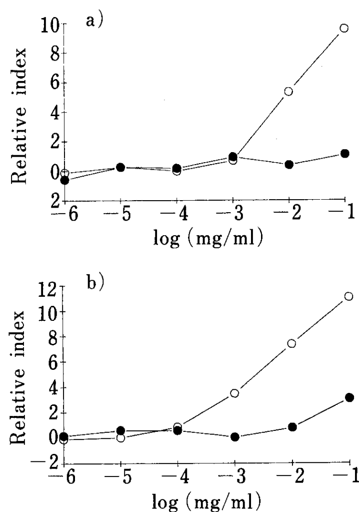

Fig. 3. Inhibition of Limulus Test Reactivity of LTRM from *C. parapsilosis* by Higher Concentration of (1→3)-β-D-Glucan

Acetone dried *Candida* cells were treated with a) normal mouse serum or b) Mϕ for 24 h. Each supernatant fraction were collected and assayed for limulus test in the presence (—●—) or absence (—○—) of 1 mg/ml SPG.

Table I. Candidacidal Activity of PMN and Mϕ[a)]

Strain	0 h	3 h		
		+Saline	+PMN	+Mϕ
C. albicans				
1269	225.8 (100)	252.5 (111.8)	209.5 (92.8)	385.5 (170.7)
1974	163.0 (100)	262.5 (161.0)	62.5 (38.3)	155.5 (95.4)
10108	175.5 (100)	190.5 (108.5)	221.5 (126.2)	233.5 (133.0)
1397	203.3 (100)	477.0 (234.6)	88.0 (43.3)	325.5 (160.1)
C. parapsilosis				
0585	238.3 (100)	301.0 (126.3)	162.5 (68.2)	227.5 (95.5)
0640	205.8 (100)	352.0 (171.0)	123.0 (59.8)	181.5 (88.2)
0708	200.5 (100)	262.0 (130.7)	115.0 (57.4)	175.0 (87.3)
1068	191.0 (100)	256.5 (134.3)	126.5 (66.2)	157.0 (82.2)

a) Each strain of *Candida albicans* and *Candida parapsilosis* shown in this table was grown overnight in Sabouraud medium at 37 °C. PMN and Mϕ were obtained from the peritoneal cavity of ICR mice injected i.p. with 8% casein (2 ml) 6 h earlier and with 1 KE of OK-432 3 d earlier. *Candida* cells (2×10^6/ml, 50 μl) were mixed with PMN (2×10^6/ml, 50 μl) or Mϕ (2×10^6/ml 50 μl) and incubated at 37 °C for 3 h in humidified CO_2 incubator. Each mixture was diluted and placed on Sabouraud agar. The number of survivors was counted after incubation at 28 °C for 48 h.

Release of LTRM from the Intact *Candida* Cells by

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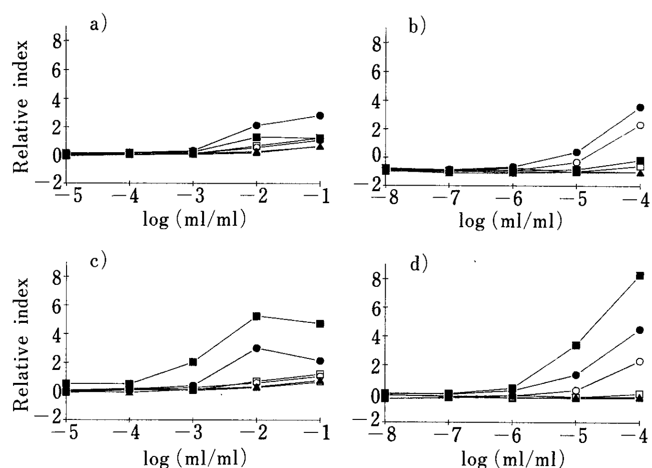

Fig. 4. Solubilization of LTRM from Viable *C. parapsilosis* by PMN

Candida cells prepared by overnight culture (37 °C) in Sabouraud medium were mixed with PMN and incubated at 37 °C in humidified CO_2 incubator for 3 h (a, b) or 24 h (c, d). After incubation, each reaction mixture was centrifuged. Reactivity of the limulus lysate to both supernatants (a, c) and the residues (b, d) was measured. (a) —○—, PMN+*Candida* 0 h; —●—, PMN+*Candida* 3 h; —△—, PMN 0 h; —▲—, PMN 3 h; —□—, *Candida* 0 h; —■—, *Candida* 3 h, (b) —○—, PMN+*Candida* 0 h; —●—, PMN+*Candida* 3 h; —△—, PMN 0 h; —▲—, PMN 3 h; —□—, *Candida* 0 h; —■—, *Candida* 3 h; (c) —○—, PMN+*Candida* 0 h, —●—, PMN+*Candida* 24 h, —△—, PMN 0 h; —▲—, PMN 24 h; —□—, *Candida* 0 h; —■—, *Candida* 24 h; (d) —○—, PMN+*Candida* 0 h; —●—, PMN+*Candida* 24 h; —△—, PMN 0 hr; —▲—, PMN 24 h; —□—, *Candida* 0 h; —■—, *Candida* 24 h.

Digestion with PMN To demonstrate the release of LTRM from the intact *Candida* cells, 1×10^5 cells of *C. parapsilosis* IFO 0640 were mixed with 1×10^5 cells of casein induced PMN in a volume of 100 μl and incubated at 37 °C for 0, 3, of 24 h. Figure 4 shows the reactivities of the supernatants (4a) and precipitates (4b) after 3 h incubation, and those after 24 h incubation (4c, 4d). Significantly higher reactivities of fractions [PMN+*Candida*] for 3 h (●) in 4a, and 24 h (●) in 4c compared to the corresponding 0 h (○) strongly indicated the progress of a series of reactions: phagocytosis, killing, digestion of *Candida* cells and the release of LTRM in the reaction mixture.

Especially, release of LTRM during phagocytosis was clearly demonstrated in the 3 h incubation, because spontaneous release of LTRM from *Candida* was negligible at 3 h. In contrast, the spontaneous release of LTRM was significant at 24 h because of the growth of *Candida* during incubation.

As shown in Fig. 4b, 4d reactivities of the precipitates of these fractions were only slightly increased during incubation. It is noteworthy that *Candida* itself grew significantly over 24 h (■) and revealed stronger reactivity to the lysates than all of the fractions shown in Fig. 4. These results also confirm that PMN killed a part of the *Candida* cells and inhibited the production of LTRM from the killed cells during the incubation period.

Discussion

In this paper we have demonstrated the origin of the limulus test reactive materials (LTRM) from *Candida*, especially using *Candida parapsilosis*. LTRM have been released by two different mechanisms in this particular case. One is the spontaneous release or production in the culture medium by the *Candida* cells during growth; the other is the solubilization of cell wall by phagocytes. These conclusions were also confirmed by adding a higher concentration of (1→3)-β-D-glucan in the reaction mixture (Fig. 3a, 3b). The *Candida* cell is constructed by various kinds of polysaccharides such as mannan, chitin, and β-glucan. During the cell growth and digestion by phagocytes, all of these constituents have a chance to degrade and be solubilized. However, the specificity of the limulus amebocyte lysates which has been demonstrated by previous investigations strongly suggests that the reaction pattern obtained in this paper is closely related to the release of (1→3)-β-D-glucan from *Candida*.[7] It is quite interesting that significant quantities of (1→3)-β-D-glucan were produced or spontaneously released during growth of *Candida* cells, and solubilized under relatively mild cold water and hot water extractions. The exact quantities of these (1→3)-β-D-glucans could not be estimated for reasons discussed later; however, a majority of the cell wall β-glucan would still have remained during these treatments because cell wall glucans are usually extracted under much more drastic conditions, and the reactivities of the cell suspensions after the extractions used in this paper (Fig. 1) were little changed. The data presented in this paper were obtained from only one strain. It is important to trace using other strains.

Solubilization of (1→3)-β-D-glucan by phagocytic cells is quite interesting. Hydrolytic enzymes for (1→3)-β-D-glucosyl linkages have not been discovered in mammals, thus, non-specific oxidative degradation by reactive oxygen, nitrogen, and halogens must contribute to these degradations. Indeed, oxidative products of the antitumor glucan have been identified from the peritoneal cavity of mice.[15] However, in general, as PMN has significantly higher capacity to oxidatively degrade invading substances, the data indicating that PMN and Mϕ contribute equally to the release of LTRM (Fig. 2) suggest the presence of a certain specific control mechanism on LTRM release. On the contrary, in the case of antitumor glucans, the glucans injected into mice have remained in the organs of the reticuloendothelial system for more than a month with little physicochemical change.[16] Considering these facts, it would be quite difficult to fully solubilize the water-insoluble (1→3)-β-D-glucan to the water-soluble form within a day by the host/self-defense systems, and it is probable that only a part of the water-insoluble (1→3)-β-D-glucan would be solubilized during phagocytosis.

During the course of study on the mechanisms of antitumor activity of (1→3)-β-D-glucans, interaction of the glucan with Mϕ and the resulting activation of the cell is critical.[17,18] At a very early period during the glucan and Mϕ interaction, binding and phagocytosis (pinocytosis) of the glucan molecules by Mϕ are believed to be important, and available experimental data directly demonstrate the incorporation of the glucan molecules in the Mϕ.[19] Content of gel-forming, water-soluble, antitumor glucan in the Mϕ *in vitro* increases for at least 4 d (unpublished results).

In addition, the glucan particles, which include zymosan and probably *Candida* cells, can be phagocytosed by PMN and Mϕ within an hour. In contrast to these facts, the data shown in this paper demonstrate the release of (1→3)-β-D-glucan from PMN and Mϕ within a day. These facts clearly indicate that phagocytes not only endocytose β-glucans but also exocytose them under a certain control mechanism or a certain equilibrium. There is another possibility that the glucans remaining inside of the PMN and Mϕ are a mixture

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of intermediates before being exocytosed as the end-product(s), and thus not the same structure as that of glucans already exocytosed by the phagocytic cells.

Structure–activity relationship of (1→3)-β-D-glucans for the factor G activation has not been fully investigated. However, at least it is true that not all of the glucans can react with the limulus amebocyte lysate. In addition, low reactivity of the (1→3)-β-D-glucans having no gel productivity, such as laminaran, has not been clearly explained. Biochemical and physicochemical examinations of the polysaccharide components of *Candida* species used in this paper have not yet been made, but β-glucans constituting the cell wall of *Candida* cells are a mixture of various degrees of branching and architectures. (1→3)-β-D-Glucans having gel productivity which include a majority of the antitumor glucans do not show detectable signals on conventional aqueous nuclear magnetic resonance (NMR) spectroscopy[20] under neutral physiological conditions. However, many kinds of yeast cells contained β-glucans showing detectable signals on conventional aqueous NMR spectrum[21] under the conditions discussed above. This physical property is strongly consistent with the "sol" property of the molecule except for the presence of a "gel" producing microenvironment, which suggests the presence of β-glucan having "sol" property in the cells of *Candida*. Additionally, LTRM was demonstrated to be water soluble and easily extractable by hot water extraction (Fig. 1). Available evidence suggests that factor G would react with "sol" conformer of (1→3)-β-D-glucans in addition to "gel", especially with the single helix conformer. Further systematic investigation is important to confirm this structure–activity relationship.

In trial runs the limulus test has already been clinically applied to monitor fungal infections. As described, the reactivity of the limulus amebocyte lysates is known to be dependent on molecular weight, branching, as well as conformation of (1→3)-β-D-glucans. In clinically isolated *Candida* glucans, it is assumable that the primary structure would not be a simple "repeating unit" type, thus the primary structure would also affect the reactivity of the lysate. Standardized material should be readied to quatitatively monitor the concentration of glucans in clinical materials.

The increasing incidence of fungal infection recently calls for development of new monitoring approaches like immunochemical detection using mannan or cell wall proteins and molecular biological detection using polymerase chain reaction. Within a decade, each monitoring method could establish its own field and demonstrate its potential. The applicability of the limulus amebocyte lysates as one of these approaches must be demonstrated in greater detail.

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