12 : 1062.
10) 佐地　勉，小澤安文，竹内大二，ほか．川崎病におけるウリナスタチン療法．小児科 1999 ; 40 : 1049.
11) 加藤裕久，馬場　清，原田研介，ほか．川崎病冠動脈後遺症のカテーテル治療に関する指針．心臓 2002 ; 34 : 578.
12) 増田弘毅，直江史郎，田中　昇．川崎病(MCLS)における冠状動脈の病理学的検討－特に冠状動脈炎と動脈瘤の形態発生の関連について－．脈管学 1981 ; 21 : 899.
13) Naoe S, Takahashi K, Masuda H, et al. Kawasaki disease with particular emphasis on arterial lesions. Acta Pathol Jpn 1991 ; 41 : 785.
14) 大原関利章，高橋　啓，若山　恵，ほか．マウス冠状動脈炎部の拡張ならびに瘤形成機序に関する検討．日小循誌 2000 ; 16 : 860.
15) Takahashi K, Oharaseki T, Naoe S, et al. Neutrophilic involvement in the damage to coronary arteries in acute stage of Kawasaki Disease. Periatr Int 2005 ; 47 : 305.
16) Suzuki A, Miyagawa TS, Komatsu K, et al. Active remodeling of the coronary arterial lesions in the late phase of Kawasaki disease : immunohistochemical study. Circulation 2000 ; 101 : 2935.
17) 高橋　啓．瘤を確認できなかった冠状動脈の狭窄性病変への進展の可能性について．Prog Med 2002 ; 22 : 1676.
18) Takahashi K, Oharaseki T, Naoe S. Pathological study of postcoronary arteritis in adolescents and young adults : with reference to the relationship between sequelae of Kawasaki disease and atherosclerosis. Pediatr Cardiol 2001 ; 22 : 138.
19) 佐地　勉，薗部友良，上村　茂，ほか．川崎病急性期治療のガイドライン．日小循誌 2004 ; 20 : 54.

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Candida albicans Derived Fungal PAMPS, CAWS, Water Soluble Mannoprotein–β-Glucan Complex Shows Similar Immunotoxicological Activity with Bacterial Endotoxin from *Escherichia coli* O9

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***Candida albicans* water soluble fraction (CAWS), water soluble fraction of *Candida albicans* mainly composed of mannoprotein–β-glucan complex, has various biological effects, such as anaphylactoid shock and coronary arteritis. These toxicological effects fit CAWS as one of PAMPs, pathogen-associated molecular patterns. Acute anaphylactoid reaction is known to be induced by lipopolysaccharide from *Escherichia coli* O9 (O9 LPS), which possesses the mannose homopolysaccharide as the O-antigen region. In the present study, we compared immunotoxicological and immunochemical similarity between CAWS and O9 LPS. CAWS strongly reacted with *Candida* serum factors, and the reactivity was found to be partially competed with O9 LPS. CAWS induced lethal toxicity was inhibited by pretreatment of mice with i.v. injection of CAWS. The lethality was found to be inhibited by i.v. injection of O9 LPS. *Vice versa*, O9 LPS induced acute lethal toxicity was also inhibited by pretreatment of mice with CAWS. These results suggested that CAWS, fungal PAMPs, and O9 LPS from Gram-negative bacteria share, at least in part, immunochemical and immunotoxicological activities.**

Key words *Candida albicans*; mannan; lipopolysaccharide; O-antigen; anaphylactoid shock

Shock is a serious clinical problem because of high mortality. There are many mechanisms inducing shock and major mechanisms related to immune dysfunction are anaphylaxis, septic shock, and endotoxin shock. Shock is usually difficulty to treat, because it is a systemic response involving not only changes in immunological parameters but inducing dysfunction of circulation, coagulation and fibrinolysis.[1] There have been many studies establishing and analyzing animal models of shock. We have also long been analyzing septic and endotoxic shock from various points of view such as, the detoxification of endotoxin by lysozyme, modulation of antibiotic action by lysozyme,[2,3] development of a monoclonal anti-CD14 antibody and its application,[4,5] and establishing an endogenous septic shock model using non-steroidal anti-inflammatory drugs.[6,7] In the course of studying early diagnosis of deep seated fungal infection we have found a limulus factor G activating activity in a water soluble polysaccharide fraction rich in mannan, named *Candida albicans* water soluble fraction (CAWS), released from *Candida albicans*, and it strongly caused acute anaphylaxis-like shock in mice.[8]

The pathogenic yeast *Candida albicans*, a commensal of the human digestive tract and vaginal mucosa, is now one of the most common microbes of bloodstream infections in immunocompromised or intensive-care patients.[9] The invasive mycoses including candidiasis, aspergillosis and cryptococcosis are increasingly becoming associated with immunosuppressive therapies and immunodeficiency associated with infections of the human immunodeficiency virus. The associated crude mortality is extensively high (38 to 75%), despite appropriate treatment with anti-fungal drugs.[10—13] Sepsis, which is caused by a Gram-negative bacterial infection, has also increased for the reasons described above and the associated mortality is markedly high.[14]

We previously obtained a water-soluble fraction (CAWS), prepared from the culture supernatants of *Candida albicans* grown in a completely synthetic medium. CAWS is mainly composed of a complex of mannoprotein and β-1,3-, β-1,6-glucans.[15,16] CAWS exhibits various potential biological activities, such as cytokine synthesis by leukocytes, platelet aggregation, lethal toxicity like anaphylactoid shock, induction of coronary arteritis in various strains of mice, and so on.[17—20] However, the detailed mechanisms of these biological activities are unclear.

It is reported that the intravenous injection of lipopolysaccharide from *Escherichia coli* O9, which possesses the mannose homopolysaccharide (MHP) as the O-antigen region, induced anaphylactoid shock within 15—60 min, but not lipopolysaccharide (LPS) from *Escherichia coli* O111, which possesses a heterosaccharide (not containing mannose).[21,22] The characteristics of this shock differ from those of the well-known endotoxic shock with respect to time until shock occurs and can be evoked in LPS-hyposensitive mice, like C3H/HeJ strain,[23] with a defect in Toll-like receptor 4 (TLR4). It is suggested by this induction of anaphylactoid shock that the structure of the O-antigen region MHP of LPS has played an important role. Namely, MHP-carrying LPS activated the complement system *via* the lectin pathway in tissues and induced the degradation of platelets. This degradation might cause the release of their contents (*e.g.* serotonin *etc.*) resulting in the induction of anaphylactoid shock.[24—28]

The lethal toxicity of CAWS[8] and O9 LPS[29—31] is similar with respect to the following.[1] Lethal toxicity occurs 15—60 min after intravenous injection.[2] The complement C5-deficient mice, DBA/2 and AKR/N,[32] exhibit markedly low sensitivity. Activation of the complement system is strongly posturated in the lethality.[3] Since LPS-hyposensitive mice, C3H/HeJ, exhibit high sensitivity, the involvement of TLR4 was excluded.[4] Since mast cell-deficient mice, W/Wv exhibit high sensitivity, the involvement of mast cells was also excluded.

On the basis of the background described above, we postu-

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lated that a mannan moiety of CAWS is responsible for the lethal toxicity including anaphylactoid shock in mice. In the present study, we compared CAWS with O9 LPS in terms of immunochemical characteristics and biological activity.

MATERIALS AND METHODS

Animals, Microbes, and Materials *Candida albicans* IFO1385 was obtained from the Institute for Fermentation, Osaka, Japan. *Escherichia coli* ATCC23505 was obtained from American Type Culture Collection. All mice were purchased from Japan SLC. Yeast-mannan from *Saccharomyces cerevisiae* was purchased from Nacalai. *Candida* Check was from Mitsubishi Kagaku Iatron. Anti-core LPS-MAb WN1 222-5 was purchased from Hycult Biotechnology b.v. D-mannose was obtained from Wako. LPS extracted by the phenol water method from *E. coli* O111 : B4 was purchased from Sigma. Chemically synthesized muramyl dipeptide was also from Sigma. C-limiting medium contains (per liter): sucrose 10 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 2 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $ZnSO_4 \cdot 7H_2O$ 1 mg, $CuSO_4 \cdot 5H_2O$ 1 mg, $FeSO_4 \cdot 7\ H_2O$ 0.01 g and biotin 25 μg (final pH, 5.2).[33] LB medium contains (per liter): Bacto tryptone peptone 10 g, Yeast-extract 5 g and NaCl 10 g (final pH, 7.0). Phosphate-buffered saline (PBS) contains 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl and 1.47 mM KH_2PO_4. NaCl/Pi contains 15 mM Na_2HPO_4 and 150 mM NaCl (final pH, 7.2). TBS contains 137 mM NaCl, 2.68 mM KCl and 25 mM Tris (final pH, 8.0).

Preparation of LPS *Escherichia coli* O9 LPS was extracted from *E. coli* ATCC 23505 (O9 : K9B : H12) by the phenol water method.[34] The acetone-dried cells (10 g) cultured in LB medium were suspended in 175 ml of distilled water (preheated at 65 °C), and then 175 ml of 90% liquid phenol (preheated at 65 °C) was added under vigorous stirring and the mixture was stirred for 20 min at 65 °C. This suspension was then allowed to cool to 4 °C. After centrifugation, the upper aqueous layer was carefully collected. The residual layer was re-extracted by the methods described above. The combined aqueous phase was reheated to 65 °C, an equal volume of 90% liquid phenol was added, and the mixture was incubated for 20 min under vigorous stirring. This water extract was dialyzed against distilled water. The dialyzed solution was concentrated at 40 °C *in vacuo* to a volume of about 50 ml. After centrifugation for the removal of traces of insoluble material, the water solution was lyophilized. The lyophilized crude extract was dissolved in water to give a 3% solution, which was centrifuged for 8 h at 105000 ***g***. The sediment was suspended in water, and the suspension was recentrifuged 3 times at 105000 ***g*** for 3 h each. The final sediment was taken up in a minimum amount of water and lyophilized (yield 1.23%).

Preparation of CAWS CAWS was prepared from *Candida albicans* IFO1385 in accordance with conventional methods.[15] The procedure used was as follows: 5 l of medium (C-limiting medium) was added to a fermenter and cultured for 2 d at 27 °C with air supplied at a rate of 5 l/min and rotation at 400 rpm. Following the culture, an equal volume of ethanol was added and after the mixture was left to stand overnight, the precipitate was collected. The precipitate was suspended in 250 ml of distilled water, and collected water soluble fraction. Ethanol was added to the soluble fraction and the mixture was allowed to stand overnight. The precipitate was collected and dried with acetone to obtain CAWS (yield 400 mg/5 l).

ELISAs of the Reactivity of CAWS The reactivity of CAWS to serum factors from *Candida* Check which consists of rabbit polyclonal antibodies against *Candida* cell wall mannan was detected by ELISA. The reactivity of anti-core LPS-MAb, which binds to the distal part of the inner core region, was detected by ELISA similar way. A solution of CAWS in 50 mM carbonate buffer (pH 9.6) was coated onto Nunc immunoplates, which were then incubated at 4 °C overnight. The plates were washed extensively with 0.05% Tween 20 containing PBS (PBST); unbound sites were blocked by the addition of 1% BSA containing PBST (BPBST) to wells for 40 min at 37 °C and then the wells were washed 6 times with PBST. *Candida* serum factors or anti-core LPS-MAb serially diluted with BPBST were added, and incubated for 60 min at 37 °C. After 6 washes with PBST, the wells were treated with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG, respectively, and the 3,3′,5,5′-tetramethylbenzidine microwell peroxidase substrate system (TMB; KPL Inc.). After 45 min, the reaction was stopped with 1 N H_3PO_4, and then the optical density of each well was read at 450 nm on an automatic microplate reader.

ELISAs of the Reactivity of O9 LPS The reactivity of O9 LPS to serum factors from *Candida* Check and anti-core LPS-MAb was detected by ELISA. A solution of O9 LPS in NaCl/Pi (pH 7.2) was coated onto Nunc immunoplates, which were then incubated at 4 °C overnight. The plates were washed with PBS; unbound sites were blocked by the addition of 2% BSA-NaCl/Pi to wells for 40 min at 37 °C and then the wells were washed twice with NaCl/Pi. *Candida* serum factors or anti-core LPS-MAb serial diluted with NaCl/Pi were added, and the plates were incubated for 60 min at 37 °C. After 3 washes with NaCl/Pi, the wells were treated with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG, respectively, and TMB. After 45 min, the reaction was stopped with 1 N H_3PO_4, and then the optical density of each well was read at 450 nm on an automatic microplate reader.

Competitive ELISAs The cross-reactivity between CAWS and O9 LPS was detected by competitive ELISAs. Competitive ELISAs were conducted essentially as described above, except that the following procedures were used. *Candida* serum factors 11, 13b and 13 diluted with blocking buffer were mixed with known quantities of various soluble competitors, CAWS, O9 LPS, O111 LPS, Yeast-mannan and D-mannose, dissolved in blocking buffer. Then, the competitor solutions were diluted with equal volumes of serum factors and were preincubated for 1 h at 37 °C prior to the addition of 0.1-ml aliquots to the wells of microtiter plates.

Western Blotting CAWS and O9 LPS were separated by 6.0 and 12.5%, respectively, polyacrylamide gel electrophoresis. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer onto a nitrocellulose membrane at a constant current of 100 mA for 1 h, unbound sites were blocked by the addition of 3% BSA containing-0.05% Tween 20 containing TBS (BTBST) to the membrane for 1 h at room temperature (RT).

After a wash with TBST, the membrane was incubated with *Candida* serum factors or anti-core LPS-MAb, respectively, overnight at RT, and then after another wash with TBST, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG, respectively, for 1 h at RT. The membrane was visualized using ECL+ (Amersham Biosciences).

Scoring of the Rapid Anaphylactoid Shock Induced by CAWS or O9 LPS The incidence and the severity of the rapid anaphylactoid shock were assessed within 1 h of the injection of CAWS or O9 LPS in muramyl dipeptide (MDP)-primed or non-primed ICR mice. These values and the subsequent mortality (in the first hour after injection) were recorded. The scoring of the shock was as follows: 0, no symptoms of shock; 1, staggering; 2, crawling and prostration; 3, prostration and weak convulsions; 4, prostration and strong convulsions.

Effect of Various Pre-treatments on the Lethal Toxicity of CAWS or O9 LPS Thirty minutes before the intravenous injection of a lethal dose of CAWS or O9 LPS, various saccharides (CAWS, O9 LPS, O111 LPS, mannan from *S. cerevisiae* and D-mannose) were intravenously pre-injected into ICR male mice for examining antagonistic activity of various saccharides. Mortality was recorded within the first hour after the injection.

RESULTS

The Reactivity of CAWS and O9 LPS to *Candida* Serum Factors In order to identify the mannan moiety of CAWS and O9 LPS showing immunochemical cross-reactivity, we first examined the reactivity of CAWS and O9 LPS to *Candida* serum factors, a kit provided by Mitsubishi Kagaku Iatron consisting of monospecific rabbit polyclonal antibodies against *Candida* cell wall mannan,[35,36] by ELISAs and Western blotting. The serum factors has been a good tool for identifying the viable cells of seven medically important *Candida* species in clinical specimens. The results are shown in Figs. 1 and 2. CAWS strongly reacted with *Candida* serum factors 1, 4, 5, 6, 11, 13b and 13, but not serum factors 8, 9 and 34 (Fig. 1). On the other hand, O9 LPS strongly reacted with serum factors 1, 11, 13b and 13, but not serum factors 4, 5, 6, 8, 9 and 34 (Fig. 2). From these ELISAs and Western blotting, it is strongly suggested that both CAWS and O9 LPS share immunochemical epitopes; factors 1, 11, 13b and 13.

We then precisely examined the cross-reactivity of CAWS and O9 LPS with *Candida* serum factors 11, 13b and 13 using competitive ELISAs. Epitope structures of these antigenic factors are shown in Fig. 3A.[37] The results of the ELISAs are summarized in Table 1. The reactivity of CAWS against serum factors 11, 13b and 13 was inhibited by addition of O9 LPS (IC_{50}=2.1, 37 and 15 μg, respectively) as well as CAWS (IC_{50}=0.20, 0.22 and 0.21 μg, respectively) as a soluble inhibitor. Similarly, the reactivity of O9 LPS against serum factors 11, 13b and 13 was inhibited by addition of CAWS (IC_{50}=9.3, 4.2 and 2.4 μg, respectively) as well as O9 LPS (IC_{50}=0.0030, 0.0055 and 0.0048 μg, respectively). Heterologous competitions required higher concentrations than the homologous competitions. It is of note that these inhibitory effects were not observed on addition of mannan from *Saccharomyces cerevisiae* (Yeast-mannan) or O111 LPS which not containing mannose (Fig. 3B), as a soluble inhibitor.[38,39]

To confirm that the carbohydrate moieties of both CAWS and O9 LPS except the mannan moiety do not show cross-re-

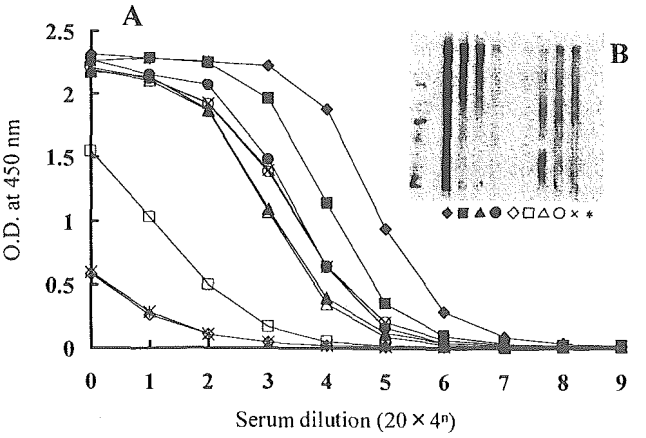

Fig. 1. The Reactivity of CAWS to *Candida* Serum Factors

The reactivity of CAWS to *Candida* serum factors was tested with a kit purchased from Mitsubishi Kagaku Iatron consisting of rabbit polyclonal antibodies against *Candida* cell wall mannan by (A) ELISAs and (B) Western blotting. (A) A solution of CAWS in 50 mM carbonate buffer (pH 9.6) was coated onto Nunc immunoplates. Unbound sites were blocked by the addition of 1% BSA-PBST. Factor sera, (◆) 1, (■) 4, (▲) 5, (●) 6, (◇) 8, (□) 9, (△) 11, (○) 13b, (×) 13 and (*) 34, serially diluted with blocking buffer were added. Binding of Ig to CAWS was detected by peroxidase-conjugated goat anti-rabbit IgG. Then each well was color developed by the addition of TMB solution and the reaction stopped by 1N H_3PO_4. The optical density of each well was read at 450 nm/ref 630 nm on an automatic microplate reader. (B) CAWS was separated by 6.0% polyacrylamide gel electrophoresis. After the separation by SDS-PAGE and transfer onto a nitrocellulose membrane, unbound sites were blocked by the addition of 3% BSA-TBST and then the membrane was treated with *Candida* serum factors, (◆) 1, (■) 4, (▲) 5, (●) 6, (◇) 8, (□) 9, (△) 11, (○) 13b, (×) 13 and (*) 34. Binding of Ig to CAWS was detected by peroxidase-conjugated goat anti-rabbit IgG. After washing, the membrane was visualized by ECL+.

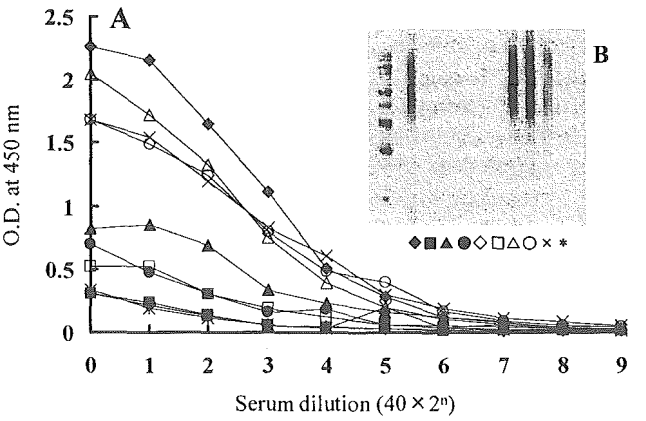

Fig. 2. The Reactivity of O9 LPS to *Candida* Serum Factors

The reactivity of O9 LPS to *Candida* serum factors was tested with a kit purchased from Mitsubishi Kagaku Iatron consisting of rabbit polyclonal antibodies against *Candida* cell wall mannan by (A) ELISAs and (B) Western blotting. (A) A solution of O9 LPS in NaCl/Pi (pH 7.2) was coated onto Nunc immunoplates. Unbound sites were blocked by the addition of 2% BSA-NaCl/Pi. Factor sera, (◆) 1, (■) 4, (▲) 5, (●) 6, (◇) 8, (□) 9, (△) 11, (○) 13b, (×) 13 and (*) 34, serially diluted with NaCl/Pi were added. Binding of Ig to O9 LPS was detected by peroxidase-conjugated goat anti-rabbit IgG. Then each well was color developed by the addition of TMB solution and the reaction stopped by 1N H_3PO_4. Then the optical density of each well was read at 450 nm/ref 630 nm on an automatic microplate reader. (B) O9 LPS was separated by 12.5% polyacrylamide gel electrophoresis. After the separation by SDS-PAGE and transfer onto a nitrocellulose membrane, unbound sites were blocked by the addition of 3% BSA-TBST and then the membrane was treated with *Candida* serum factors, (◆) 1, (■) 4, (▲) 5, (●) 6, (◇) 8, (□) 9, (△) 11, (○) 13b, (×) 13 and (*) 34. Binding of Ig to O9 LPS was detected by peroxidase-conjugated goat anti-rabbit IgG. After washing, the membrane was visualized by ECL+.

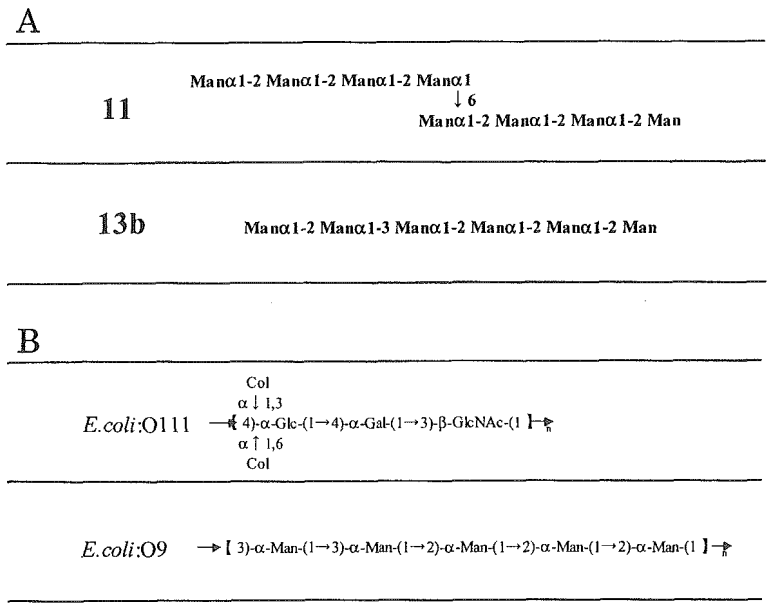

Fig. 3. Chemical Structures of Antigenic Factors, 11, 13b and 13 (A), O-Antigen Repeating Units of LPSs (B)

(A) Chemical structure of antigenic factors used in this study. The chemical structure of 13 was not determined. (B) The structures of O-antigen repeating units of LPSs used in this study. Col; colitose, Glu; glucose, Gal; galactose, GlcNAc; *N*-acetyl glucosamine, Man; mannose.

Table 1. IC_{50} of Inhibition ELISAs of Various Saccharide on CAWS or O9 LPS *Candida* Serum Factors

Serum factor	Inhibitor	50% inhibition dose (μg)	
		CAWS	O9 LPS
11	CAWS	0.20	9.3
	O9 LPS	2.1	0.0030
	O111 LPS	N.I.	N.I.
	Yeast-mannan	N.I.	N.I.
	D-Mannose	N.I.	N.I.
13b	CAWS	0.22	4.2
	O9 LPS	37	0.0055
	O111 LPS	N.I.	N.I.
	Yeast-mannan	N.I.	N.I.
	D-Mannose	N.I.	N.I.
13	CAWS	0.21	2.4
	O9 LPS	15	0.0048
	O111 LPS	N.I.	N.I.
	Yeast-mannan	N.I.	N.I.
	D-Mannose	N.I.	N.I.

The concentration of carbohydrate at which inhibition is 50% of binding to *Candida* serum factors in the absence of carbohydrate (IC_{50}) was obtained by modeling the inhibition curves of the competitive ELISAs. N.I.: not inhibited.

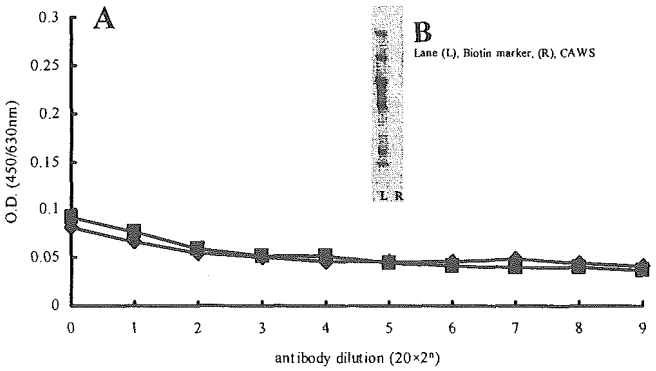

Fig. 4. The Reactivity of CAWS to Anti-core LPS-MAb

The reactivity of CAWS to anti-core LPS-MAb WN1 222-5, which binds to the distal part of the inner core common to LPS from most members of the *Enterobacteriaceae* family, was detected by (A) ELISAs and (B) Western blotting. (A), A solution of CAWS in 50 mM carbonate buffer (pH 9.6), (◆) or vehicle, (■) was coated onto Nunc immunoplates. Unbound sites were blocked by the addition of 1% BSA-PBST. Anti-core LPS-MAb, serially diluted with blocking buffer, was added. Binding of Ig to CAWS was detected by peroxidase-conjugated goat anti-mouse IgG. Then each well was color developed by the addition of TMB solution and the reaction stopped by 1N H_3PO_4. The optical density of each well was read at 450 nm/ref 630 nm on an automatic microplate reader. (B) CAWS was separated by 6% polyacrylamide gel electrophoresis. After the separation by SDS-PAGE and transfer onto a nitrocellulose membrane, unbound sites were blocked by the addition of 3% BSA-TBST and then the membrane was treated with O9-antisera. Binding of Ig to CAWS was detected by peroxidase-conjugated goat anti-mouse IgG. After washing, the membrane was visualized by ECL+.

activity, we examined the reactivity of CAWS and O9 LPS with anti-core LPS-MAb WN1 222-5 which binds to the distal part of the inner core region and recognizes the structural element R_1-α-D-Glc*p*-(1→3)-(L-α-D-Hep*p*-(1→7))-L-α-D-Hep*p* 4P-(1→3)-R_2 (where R_1 represents additional sugars of the outer core and R_2 represents additional sugars of the inner core) common to LPS from most members of the *Enterobacteriaceae* family,[40,41] by ELISA and Western blotting. The results of the ELISAs and Western blotting with anti-core LPS-MAb are shown in Figs. 4 and 5. O9 LPS reacted with anti-core LPS-MAb strongly, but CAWS did not at all (Figs. 4, 5). In the Western blot analysis of O9 LPS to anti-core LPS-MAb, the slow and the fast moving bands were detected those corresponds to longer O-specific chains (more repeating units), and small or no O-side chains (Fig. 5B).

From these ELISAs and Western-blotting, mannan moieties of CAWS and O9 LPS share strong and significant immunochemical similarity.

The Rapid Anaphylactoid Shock Induced by CAWS and O9 LPS We primarily examined the lethal toxicity of CAWS and O9 LPS by intravenous injection in MDP-primed (Table 2) or non-primed (Table 3) ICR mice. As shown in Table 2, CAWS and O9 LPS concurrently induced a severe anaphylactoid shock in MDP-primed ICR mice. At 2.0 mg/kg, O9 LPS killed 2 of 3 mice within 1 h after the injection. At 2.0 mg/kg, CAWS killed 1 of 3 mice, and at 4.0 mg/kg, all

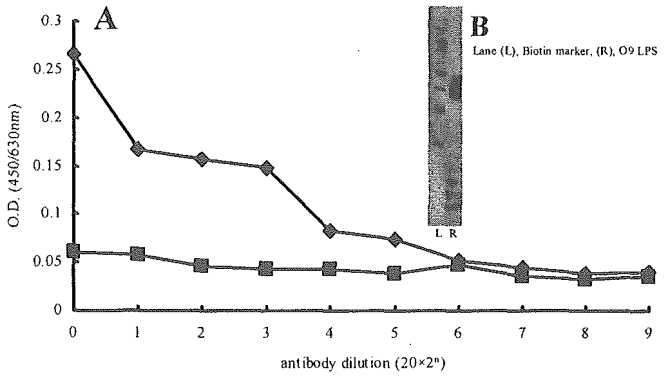

Fig. 5. The Reactivity of O9 LPS to Anti-core LPS-MAb

The reactivity of O9 LPS to anti-core LPS-MAb WN1 222-5, which binds to the distal part of the inner core common to LPS from most members of the *Enterobacteriaceae* family, was detected by (A) ELISAs and (B) Western blotting. (A) A solution of O9 LPS in NaCl/Pi (pH 7.2), (◆) or vehicle, (■) was coated in Nunc immunoplates. Unbound sites were blocked by the addition of 2% BSA-NaCl/Pi. Anti-core LPS-MAb, serially diluted with NaCl/Pi, was added. Binding of Ig to O9 LPS was detected by peroxidase-conjugated goat anti-mouse IgG. Then each well was color developed by the addition of TMB solution and the reaction stopped by 1 N H_3PO_4. Then the optical density of each well was read at 450 nm/ref 630 nm on an automatic microplate reader. (B) O9 LPS was separated by 12.5% polyacrylamide gel electrophoresis. After the separation by SDS-PAGE and transfer onto a nitrocellulose membrane, unbound sites were blocked by the addition of 3% BSA-TBST and then the membrane was treated with O9-antisera. Binding of Ig to O9 LPS was detected by peroxidase-conjugated goat anti-mouse IgG. After washing, the membrane was visualized by ECL+.

Table 2. Ability of CAWS, O9 LPS to Induce Rapid Anaphylactoid Shock in MDP-Primed ICR Mice

	Dose (mg/kg)	Anaphylactoid shock		
		Incidence	Score[a)]	Mortality
CAWS	0	0/3	0	0/3
	1	0/3	0	0/3
	2	2/3	3–4	1/3
	4	3/3	4	3/3
	8	3/3	3–4	2/3
O9 LPS	0	0/3	0	0/3
	1	3/3	2–3	0/3
	2	3/3	3–4	2/3
	4	3/3	3–4	2/3
	8	3/3	3–4	2/3

a) The scoring of the shock was as follows: 0, no symptoms of shock; 1, staggering; 2, crawling and prostration; 3, prostration and weak convulsions; 4, prostration and strong convulsions.

3 mice. However, O111 LPS did not cause rapid death (data not shown). Table 3 summarized the anaphylactoid reaction tested in normal, MDP-non primed mice. Of quite interest, anaphylactoid reaction was only induced by CAWS in this experimental condition. Comparing the data of Tables 2 and 3, MDP-priming enhanced the toxicity of O9 LPS, but the toxicity of CAWS was significantly high without priming and the severity was not enhanced by priming. It is of note that, anaphylactoid reaction was induced by extremely high dose of O9 LPS in MDP-non-primed mice (Table 4). Now it is obvious that priming of mice by MDP is essential to induce anaphylactoid reaction by O9 LPS. Thus, these results indicated that the mechanisms behind the rapid lethal effects of CAWS and O9 LPS were partially the same due to the mannan structures.

Effect of Various Saccharides on Lethal Toxicity We next examined the antagonistic effects of various saccharides (CAWS, O9 LPS, O111 LPS, Yeast-mannan and D-mannose)

Table 3. Ability of CAWS, O9 LPS to Induce Rapid Anaphylactoid Shock in MDP-Non Primed ICR Mice

	Dose (mg/kg)	Anaphylactoid shock		
		Incidence	Score[a)]	Mortality
CAWS	0	0/3	0	0/3
	1	0/3	0	0/3
	2	2/3	2—3	0/3
	4	2/3	4	2/3
	8	3/3	3—4	2/3
O9 LPS	0	0/3	0	0/3
	1	0/3	0	0/3
	2	0/3	0	0/3
	4	0/3	0	0/3
	8	0/3	0	0/3

a) The scoring of the shock was as follows: 0, no symptoms of shock; 1, staggering; 2, crawling and prostration; 3, prostration and weak convulsions; 4, prostration and strong convulsions.

Table 4. Ability of Extremely High Dose of O9 LPS to Induce Rapid Anaphylactoid Shock in MDP-Non Primed ICR Mice[a)]

	Dose (mg/kg)	Anaphylactoid shock		
		Incidence	Score[a)]	Mortality
O9 LPS	0	0/3	2	0/3
	10	2/3	2	0/3
	20	2/3	2—4	1/3
	40	3/3	3—4	1/3
	80	3/3	3—4	2/3

a) The scoring of the shock was as follows: 0, no symptoms of shock; 1, staggering; 2, crawling and prostration; 3, prostration and weak convulsions; 4, prostration and strong convulsions.

Table 5. Effect of Various Pre-treatments on Toxicity of CAWS and O9 LPS in MDP-Non Primed ICR Mice

Pretreatment	Dose (mg/kg)	Mortality	
		CAWS (50 mg/kg)	O9 LPS (50 mg/kg)
Saline		3/3	3/3
O9 LPS	4	0/3	1/3
O111 LPS	4	2/3	3/3
CAWS	1	0/3	1/3
Yeast-mannan	4	3/3	3/3
D-Mannose	20	2/3	3/3

on CAWS or O9 LPS induced anaphylactoid reaction, in order to confirm the crucial role of mannan moiety. The results are summarized in Table 5. Lethal toxicity of CAWS was inhibited by pre-treatment with O9 LPS, but not O111 LPS. Moreover, O9 LPS-induced toxicity was also inhibited by pre-treatment with CAWS (Table 5). The lethal effects of both CAWS and O9 LPS were inhibited by a large dose of Yeast-mannan (data not shown). Interestingly, the late phase endotoxic shock induced by O9 LPS was not inhibited by intravenous pre-treatment with CAWS unlike the inhibitory effect on acute phase shock (data not shown).

DISCUSSION

In this study, we demonstrated that the mannan moieties of CAWS and O9 LPS shared immunochemical as well as im-

munotoxicological similarity. This immunochemical similarity was not observed if the mannan structures were absent. Because anti-core LPS-MAb WN1 222-5 binds to the distal part of the inner core region of LPS, it did not react with CAWS. In addition, CAWS and O9 LPS concurrently induced rapid anaphylactoid shock in mice. The shock was found to be inhibited by pretreatment with CAWS, O9 LPS or a large dose of Yeast-mannan from *S. cerevisiae*, but not O111 LPS (does not include mannose). This result strongly suggested that the toxicity is evoked through the recognition of the mannan moiety by CAWS and O9 LPS. These results implied that CAWS from fungi and LPS (*E. coli* O9) from Gram-negative bacteria, at least in part, share immunochemical and biological activities.

We first investigated whether the mannan moieties of CAWS and O9 LPS cross-react. CAWS and O9 LPS strongly reacted with *Candida* serum factors No. 11, 13b and 13. The reactivity was specific according to the results of the competitive ELISAs. CAWS did not cross-react with any other region of LPS except the mannan moiety, because it did not react with anti-core LPS-MAb WN1 222-5. This result indicated that the mannan moieties of CAWS and O9 LPS cross-reacted. These results suggested that the mannan moieties of CAWS and O9 LPS are responsible for some of these biological activities, especially lethal toxicity like anaphylactoid shock.

We previously reported that CAWS induced rapid shock in mice, however the mechanisms are not clear. On the other hand, Takada *et al.* reported that O9 LPS induced a rapid shock different from the well-known endotoxic shock in mice.[21,22] The mechanism which O9 LPS induced anaphylactoid shock was reported previously: strong complement activation and platelet response (include serotonin release due to platelet degradation) in the lungs and liver *via* the lectin pathway through recognition of MHP as the O-antigen region depends on mannose-binding lectin (MBL). These responses result in acute inflammation accompanied by severe tissue destruction, especially in the lungs, which in turn leads to an anaphylactoid reaction.[24—28]

Additionally, Mikami *et al.* reported that a neutral subfraction of mannan of *S. cerevisiae* (WNM) showed strong lethal toxicity in mice when it was administered intravenously, but the acidic fraction of mannan from the same yeast and chemically phosphorylated WNM was practically ineffective. Synthetic linear α-1, 2-, α-1, 3-mannans lacking branching moieties showed even less toxicity. These results demonstrated that the density of branches is important to the lethal effect.[42,43] But Kind *et al.* reported that the lethal toxicity of some yeast mannans appeared to depend on the α-1, 2-, α-1, 6-linkage in their main chain.[44] In addition, the complement systems may be an important factor in this toxicity. No toxicity was observed in C5-deficient mice and following the consumption of whole hemolytic complement after the addition of mannans to mouse serum *in vitro*.

In our study, CAWS and O9 LPS concurrently induced a rapid anaphylactoid shock following intravenous injection in mice. The toxicity could be modulated by pretreatment with various saccharides. These toxic effects were found to be inhibited by intravenous injection of CAWS, O9 LPS and a large dose of Yeast-mannan, but not O111 LPS. These inhibitory effects may due to antagonistic effects against some of mannose receptors, such as mannose-binding lectin and mannose receptors. Interestingly, this inhibitory pretreatment was not effective against late-phase lethality caused by O9 LPS dependent on Lipid A, so-called "endotoxic shock". Both agents were less toxic to C5-deficient mice, DBA/2 and AKR/N, whereas LPS-hyposensitive mice, C3H/HeJ, mast cell-deficient mice, W/Wv exhibited high sensitivity.

Interestingly, the lethal effects of CAWS and O9 LPS are in some respects different. The toxicity of O9 LPS was markedly enhanced by MDP-primed, but that of CAWS was not efficient. The cause of these differences is not clear. Others have reported a synergistic effect of MDP with LPS or other bacterial components. Flak *et al.* reported synergistic interactions between muramyl peptide and LPS in the induction of inflammatory processes, such as induction of interleukin (IL)-1α, inducible nitric-oxide synthase, nitric oxide production, and inhibition of DNA synthesis within hamster trachea epithelial cells.[45] Yang *et al.* reported a synergistic effect of MDP with LPS or lipoteichoic acid to induce production of the inflammatory cytokine IL-8 in human monocytic cells in culture.[46] Wang *et al.* reported that co-administration of peptideglycan (PGN) or MDP with LPS caused significantly increased concentrations of TNF-α and IL-6 in cultures of whole human blood, whereas the release of IL-10 was not influenced.[47] Margreet *et al.* reported that although MDP alone produced a minimal amount of TNF-α, it caused significant expression of TNF-α mRNA. The majority of the TNF-α mRNA induced by MDP alone is not translated into protein.[48] These findings indicated that the apparent synergistic effect of MDP on TNF-α production induced by either LPS or PGN is due to removal of a block in translation of the mRNA expressed in response to MDP.

These findings on lethal toxicity strongly suggested that the mechanism of CAWS-induced anaphylactoid shock is partially similar to that of O9 LPS-induced shock. Namely, the toxicity of CAWS might be evoked through the recognition of the mannan moiety, especially α-mannan by some mannan-binding protein or receptors. On the other hand, the toxicity of O9 LPS might be evoked through a synergistic effect involving the recognition of the O-antigen region *via* some mannan-binding protein or receptors and the lipid moiety. Thus, no MDP-primed effect was observed for CAWS, unlike O9 LPS.

Septic shock is induced by both bacteria and fungi. *Candida*-induced septic shock is as much a clinical problem as bacterial septic shock. The mechanism of shock is not simple, and includes various immunological as well as inflammatory parameters. The mechanism of septic shock induced by *Candida* is little understood and almost no information is available. In the present study, we have found that a *Candida* component, CAWS, shows similar properties to bacterial O9 LPS. We also found that the mechanisms of CAWS and O9 LPS-induced shock partly differ, especially in the need for a priming agent, such as MDP. The data obtained in this study clearly demonstrated that sepsis induced by both bacteria and *Candida* share partly the same underlying molecular mechanism and should be quite helpful for understanding sepsis induced by bacteria as well as *Candida*.

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REFERENCES

1) Matuschak G. M., Lechner A. J., *Crit. Care Med.*, **25**, 110—120 (2003).
2) Takada K., Ohno N., Yadomae T., *Infect. Immun.*, **62**, 1171—1175 (1994).
3) Ohno N., Morrison D. C., *Eur. J. Biochem.*, **186**, 629—636 (1989).
4) Adachi Y., Satokawa C., Saeki M., Ohno N., Tamura H., Tanaka S., Yadomae T., *J. Endotoxin Res.*, **5**, 139—146 (1999).
5) Tasaka S., Ishizaka A., Yamada W., Shimizu M., Koh H., Hasegawa N., Adachi Y., Yamaguchi K., *Am. J. Respir. Cell Mol. Biol.*, **29**, 252—258 (2003).
6) Yoshioka S., Ohno N., Miura T., Adachi Y., Yadomae T., *FEMS Immunol. Med. Microbiol.*, **21**, 171—179 (1998).
7) Nameda S., Saito M., Miura N. N., Adachi Y., Ohno N., *Biol. Pharm. Bull.*, **28**, 1254—1258 (2005).
8) Ohno N., *Microbiol. Immunol.*, **47**, 479—490 (2003).
9) Rangel-Frausto M. S., Wiblin T., Blumberg H. M., Saiman L., Patterson J., Rinaldi M., Pfaller M., Edwards J. E., Jr., Jarvis W., Dawson J., Wenzel R. P., *Clin. Infect. Dis.*, **29**, 253—258 (1999).
10) Beck-Sague C., Jarvis W. R., *J. Infect. Dis.*, **167**, 1247—1251 (1993).
11) Voss A., Kluytmans J. A., Koeleman J. G., Spanjaard L., Vandenbroucke-Grauls C. M., Verbrugh H. A., Vos M. C., Weersink A. Y., Hoogkamp-Korstanje J. A., Meis J. F., *Eur. J. Clin. Microbiol. Infect. Dis.*, **15**, 909—912 (1996).
12) McNeil M. M., Nash S. L., Hajjeh R. A., Phelan M. A., Conn L. A., Plikaytis B. D., Warnock D. W., *Clin. Infect. Dis.*, **33**, 641—647 (2001).
13) Pfaller M. A., Diekema D. J., *J. Clin. Microbiol.*, **40**, 3551—3557 (2002).
14) Martin M. A., Silverman H. J., *Clin. Infect. Dis.*, **14**, 1213—1228 (1992).
15) Uchiyama M., Ohno N., Miura N. N., Adachi Y., Aizawa M. W., Tamura H., Tanaka S., Yadomae T., *FEMS Immunol. Med. Microbiol.*, **24**, 411—420 (1999).
16) Kurihara K., Miura N. N., Uchiyama M., Ohno N., Adachi Y., Aizawa M., Tamura H., Tanaka S., Yadomae T., *FEMS Immunol. Med. Microbiol.*, **29**, 69—76 (2000).
17) Kurihara K., Shingo Y., Miura N. N., Horie S., Usui Y., Adachi Y., Yadomae T., Ohno N., *Biol. Pharm. Bull.*, **26**, 233—240 (2003).
18) Ohno N., *Jpn. J. Infect. Dis.*, **57**, 9—10 (2004).
19) Nagi-Miura N., Shingo Y., Adachi Y., Ishida-Okawara A., Oharaseki T., Takahashi K., Naoe S., Suzuki K., Ohno N., *Immunopharmacol. Immunotoxicol.*, **26**, 527—543 (2004).
20) Miura N. N., Harada T., Shinohara H., Kurihara K., Adachi Y., Okawara I. A., Oharaseki T., Takahashi K., Naoe S., Suzuki K., Ohno N., *Atherosclerosis*, in press.
21) Takada H., Galanos C., *Infect. Immun.*, **55**, 409—413 (1987).
22) Takada H., Hirai H., Fujiwara T., Koga T., Ogawa T., Hamada S., *J. Infect. Dis.*, **162**, 428—434 (1990).
23) Qureshi S. T., Lariviere L., Leveque G., Clermont S., Moore K. J., Gros P., Malo D., *J. Exp. Med.*, **189**, 605—609 (1999).
24) Shibazaki M., Nakamura M., Endo Y., *Infect. Immun.*, **64**, 5290—5294 (1996).
25) Endo Y., Shibazaki M., Nakamura M., Takada H., *J. Infect. Dis.*, **175**, 1404—1412 (1997).
26) Yokochi T., Inoue Y., Kimura Y., Kato N., *J. Immunol.*, **144**, 3106—3110 (1990).
27) Yokochi T., Fukada M., Kawai M., Zhang Y. H., Jiang G. Z., Takahashi K., *Infect. Immun.*, **60**, 4953—4956 (1992).
28) Jiang G. Z., Sugiyama T., Kato Y., Koide N., Yokochi T., *Infect. Immun.*, **63**, 2537—2540 (1995).
29) Zhao L., Ohtaki Y., Yamaguchi K., Matsushita M., Fujita T., Yokochi T., Takada H., Endo Y., *Blood*, **100**, 3233—3239 (2002).
30) Endo Y., Yokochi T., Matsushita M., Fujita T., Takada H., *J. Endotoxin Res.*, **7**, 451—455 (2001).
31) Swierzko A. S., Cedzynski M., Kirikae T., Nakano M., Klink M., Kirikae F., Ziolkowski A., Vinogradov E. V., Kawakami M., *Eur. J. Immunol.*, **33**, 2842—2852 (2003).
32) Ooi Y. M., Colten H. R., *Nature* (London), **282**, 207—208 (1979).
33) Shepherd M. G., Sullivan P. A., *J. Gen. Microbiol.*, **93**, 361—370 (1976).
34) Westphal O., Jann K., *Methods Carbohydr. Chem.*, **5**, 83—91 (1965).
35) Tsuchiya T., Fukazawa Y., Kawakita S., *Mycopathol. Mycol. Appl.*, **10**, 191—206 (1959).
36) Fukazawa Y., Shinoda T., Tsuchiya T., *J. Bacteriol.*, **95**, 754—763 (1968).
37) Suzuki S., Suzuki M., *Fungal Cells in Biodefense Mechanism*, **1997**, 1—15 (1997).
38) Prehm P., Jann B., Jann K., *Eur. J. Biochem.*, **67**, 53—56 (1976).
39) Parolis L. A., Parolis H., Dutton G. G., *Carbohydr. Res.*, **155**, 272—276 (1986).
40) Di Padova F. E., Brade H., Barclay G. R., Poxton I. R., Liehl E., Schuetze E., Kocher H. P., Ramsay G., Schreier M. H., McClelland D. B., Rietschel E. T., *Infect. Immun.*, **61**, 3863—3872 (1993).
41) Muller-Loennies S., Brade L., MacKenzie C. R., Di Padova F. E., Brade H., *J. Biol. Chem.*, **278**, 25618—25627 (2003).
42) Nagase T., Mikami T., Suzuki S., Schuerch C., Suzuki M., *Microbiol. Immunol.*, **28**, 997—1007 (1984).
43) Mikami T., Fukushi K., Ishitani M., Ishitani K., Suzuki S., Suzuki M., *Lipids*, **26**, 1404—1407 (1991).
44) Kind L. S., Kaushal P. K., Drury P., *Infect. Immun.*, **5**, 180—182 (1972).
45) Flak T. A., Heiss L. N., Engle J. T., Goldman W. E., *Infect. Immun.*, **68**, 1235—1242 (2000).
46) Yang S., Tamai R., Akashi S., Takeuchi O., Akira S., Sugawara S., Takada H., *Infect. Immun.*, **69**, 2045—2053 (2001).
47) Wang J. E., Jorgensen P. F., Ellingsen E. A., Almiof M., Thiemermann C., Foster S. J., Aasen A. O., Solberg R., *Shock*, **16**, 178—182 (2001).
48) Wolfert M. A., Murray T. F., Boons G. J., Moore J. N., *J. Biol. Chem.*, **277**, 39179—39186 (2002).

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Role of anti-β-glucan antibody in host defense against fungi

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Abstract

We have recently detected an anti-β-glucan antibody in normal human and normal mouse sera. The anti-β-glucan antibody showed reactivity to pathogenic fungal *Aspergillus* and *Candida* cell wall glucan. Anti-β-glucan antibody could bind whole *Candida* cells. It also enhanced the candidacidal activity of macrophages in vitro. The anti-β-glucan antibody titer of DBA/2 mice intravenously administered either *Candida* or *Aspergillus* solubilized cell wall β-glucan decreased remarkably dependent on dose. Moreover, in deep mycosis patients, the anti-β-glucan antibody titer decreased, and this change correlated with clinical symptoms and other parameters such as C-reactive protein. It was suggested that the anti-β-glucan antibody formed an antigen–antibody complex and participated in the immune response as a molecule recognizing pathogenic fungi.

Keywords: β-Glucan; Anti-β-glucan antibody; *Candida* solubilized cell wall β-glucan; *Aspergillus* solubilized cell wall β-glucan; Fungal infection

1. Introduction

The incidence of deep mycosis has been increasing with improvements in chemotherapy for malignant diseases and the popularization of marrow transplant and organ transplant medical care [1]. Moreover, changes in the usage of immunosuppressive, antibacterial and antiviral drugs are affecting the incidence of the disease [2]. *Candida* spp. and *Aspergillus* spp. are major fungi and account for most of the fungi causing deep mycosis. Recently, the number of case of aspergillosis in particular has been increasing. Also, strains resistant to antifungal agents are emerging [3,4]. Many researchers have examined the identification and mechanism of fungal pathogenic factors and reported many candidates for *Candida* and *Aspergillus* spp. [5–8]. However, a fungal pathogenic factor equivalent to bacterial lipopolysaccharide (LPS), a well-known pathogen, has not been reported.

Approximately, 80% of the fungal cell wall is composed of polysaccharides, the main constituents of which are β-glucan (BG), mannan, galactomannan and chitin [9–11]. Moreover, the cell wall also contains protein and a small amount of lipid. β-(1 → 3)-glucan forms a rigid skeleton and imparts physical strength to the fungal cell wall (excluding Zymomycetes) and is basically insoluble in water and alkali solutions and can not be solubilized easily. The limulus G test which utilized β-(1 → 3)-glucan-sensitive factor G of the horseshoe crab coagulation enzyme is useful for the screening of general fungal infections [12]. It is reported that patients with deep mycosis such as candidasis, aspergillosis, trichosporosis and carinii pneumonia but

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not mucormycosis or cryptococcosis show a positive reaction correlated with clinical symptoms and pathological changes [13–15]. Also, it was reported that β-(1 → 3)-glucan showed various biological activities triggering the activation of complement and the production of inflammatory mediators such as leukotriene, TNF-α and so on [16,17]. It is possible that β-(1 → 3)-glucan has some influence on, and can be a parameter which reflects, the immune response and inflammatory reactions of the host. It is strongly suggested that a variety of these biological activities depend on its solubility in water, molecular mass, degree of branching and conformation [18,19].

Many pattern recognition receptors involved in the innate immune response to fungi, such as the Toll-like receptor and mannose receptor, have been identified [20,21]. Also, dectin-1, complement receptor 3 and lactosylceramide were cited as candidates for a β-D-glucan receptor [22–24]. They may be important for phagocytosis and other biological activities. We also demonstrated the binding specificity and recognition site of dectin-1 using standard glucan, and showed that dectin-1 recognized the-(1 → 3)-D-glucosyl linkage but not α-glucans [25]. On the other hand, there are few reports of an antibody to β-glucan. It is generally thought that β-glucan was the inner layer component of the cell wall and has poor immunogenicity. In *Candida* spp., cell surface mannan is considered the dominant antigen. We attempted to establish a monoclonal antibody to 6-branched β-1 → 3-D-glucans from mushroom in mice and rats [26]. But this was quite difficult to achieve. However, recently, anti-β-glucan (anti-BG) antibody was detected in sera from human volunteers, and the titer of this antibody varied in each individual [27]. Also, the sera of DBA/1 and DBA/2 mice contained a higher anti-BG antibody titer than those of other strains [28].

Many researchers have examined the *Candida*–host relationship and anti-*Candida* protective immune mechanism using a murine systemic, lethal *Candida* infection model. The importance of innate immunity (i.e., macrophages, neutrophils) and cellular immunity (T-helper type 1 cells) to defend against fungi is widely acknowledged [29–31]. Little is known about the effects of antibody on the host response to fungal infection, and few antigens have been characterized for their ability to elicit the production of protective antibodies. However, antibody has been shown to benefit host defense against pathogens by promoting phagocytosis, enhancing the presentation of antigens and co-stimulatory molecules, and modifying cytokine production and so on. The role of antibodies in fungal infections is controversial. Anti-BG antibody may be able to interact with the fungal cell wall or extracellular glucan, and modify the host defense system. In this study, we examined the antigen–antibody interaction of anti-BG antibody in vivo and vitro, and the role of this antibody in fungal infections.

2. Materials and methods

2.1. Materials

All strains of *Aspergillus niger* and *Candida albicans* were purchased from the Institute for Fermentation (Osaka, Japan) and maintained on Sabouraud agar (Difco, USA) at 25 °C and transferred once every three months. Sodium hypochlorite solution and sodium hydroxide were obtained from Wako Pure Chemical Industries, Ltd. Distilled water (DIW) was from Otsuka Co., Ltd. (Tokyo, Japan). Lipopolysaccharides (LPS) from *Escherichia coli* O111:B4 were purchased from Sigma (USA). Grifolan (GRN) was prepared by fermentation of the mycelium of *Grifola frondosa* as described in a previous report [32]. Sonifilan (SPG) was purchased from Kaken Pharm. Co. (Tokyo, Japan).

2.2. Animals

Male DBA/2 mice between 7 and 10 weeks of age were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained under specific pathogen-free conditions.

2.3. Media

A C-limiting medium originally described by Shepherd and Sullivan [33] was used to grow all strains of yeast unless otherwise stated. The medium contained (per liter): sucrose, 10 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 2 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $ZnSO_4 \cdot 7H_2O$, 1 mg; $CuSO_4 \cdot 5H_2O$, 1 mg; $FeSO_4 \cdot 7H_2O$, 0.01 g; biotin, 25 μg; final pH, 5.2. Five liters of medium was placed in the glass jar of a microferm fermentor (New Branswick Scientific Co., Inc., USA) and cultured at 27 °C with 5 l min^{-1} of aeration and stirring at 400 rpm.

2.4. Preparation of Candida and Aspergillus cell wall glucans

The acetone-dried cells of *Candida albicans* IFO1385 or *Aspergillus niger* IFO 6342 (2 g) were suspended in 200 ml of 0.1 M NaOH with NaClO for 1 d at 4 °C. After the reaction was completed, the reaction mixture was centrifuged to collect the insoluble fraction. The insoluble fraction was then dried by washing with ethanol and acetone (NaClO-treated *Candida*, OX-CA or *Aspergillus*, OX-Asp). OX-CA suspended in dimethylsulfoxide (DMSO) was ultrasonically disrupted to obtain the supernatant (*Candida* solubilized cell wall β-glucan, CSBG).

OX-Asp suspended in 8 M urea was autoclaved (120 °C, 20 min) and the resulting solutions were centrifuged (12 000 rpm, 20 min) and divided into supernatant

(*Aspergillus* solubilized cell wall β-glucan, ASBG) and precipitate. Each fraction was dried with EtOH and acetone.

2.5. Preparation of sera

Sera obtained with fully informed consent from patients ($N = 5$, male = 3, female = 2) with fungal infections and normal volunteers ($N = 23$), and stocked in the Hachioji Medical center of Tokyo Medical University were used.

The optimal dose of the glucan preparation was administered to DBA/2 mice by intravenous injection. An aliquot of blood was collected from the tail vein at appropriate intervals using heparinized capillaries. After centrifugation, samples were stored at −20 °C before carrying out an ELISA for anti-BG antibody.

2.6. ELISA for anti-BG antibody

A 96-well Nunc plate was coated with the glucan preparation (25 μg ml^{-1}) in 0.1 M carbonate buffer (pH 9.6) by incubation at 4 °C overnight. The plate was washed with phosphate-buffered saline buffer (PBS) containing 0.05% Tween 20 (Wako Pure Chemical Co. Japan) (PBST) and blocked with 0.5% bovine serum albumin (BSA, Sigma) (PBST) at 37 °C for 60 min. After a wash, the plate was incubated with human or murine serum at 37 °C for 60 min. The plate was washed with PBST and then treated with antibody for peroxidase-conjugated anti-human IgG + M + A (Sigma) or anti-mouse IgG + M (sigma) in PBST containing 0.1% BSA (BPBST) and developed with a TMB substrate system (KPL Inc., MD). Color development was stopped with 1 M phosphoric acid and the optical density was measured at 450 nm. Immune plates (Nunc 442404, F96 Maxisorp) were used for all ELISA experiments in this study.

2.7. Affinity purification of anti-BG antibody

Aliquots of the human γ-globulin preparation (polyglobin N: BAYER, Swiss), or PBS as control were mixed with particles of OX-CA pretreated with 0.1% BSA containing PBST (BPBST) and incubated at 4 °C overnight with occasional shaking. After OX-CA was washed with saline, the resulting particles were washed with a 0.2 M glycine–HCl buffer solution to release the anti-BG antibody. The eluted antibody was immediately neutralized with 0.2 M Tris–HCl buffer and frozen at −80 °C prior to use.

2.8. Immunohistochemical analysis of Candida cell wall

Candida albicans acetone-dried and living cells were fixed with cold methanol, attached to microscope slides (Nunc) and then denatured with cold methanol and acetone. The slides were incubated with purified anti-BG antibodies diluted in a solution of 1% casein in PBS. Cells were then treated with FITC-conjugated anti-human Ig G (MBL, Nagoya, Japan) and observed by fluorescence microscopy. Confocal images were gathered using the Bio-Rad laser scanning confocal microscope (Bio-Rad, Hermel Hampstead, UK).

2.9. Assay for candidacidal activity of THP-1

THP-1 cells (Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) were treated with phorbol 12-myristate 13-acetate to induce maturation of the monocytes to a macrophage-like, adherent phenotype.Cell suspensions of *Candida albicans* IFO 1385 in FCS-RPMI were added to the THP-1 monolayer (5×10^5 cells well^{-1}; 24-well culture plate) with optimal effector/target ratios; 50:1), and incubated at 37 °C for 3 h. After incubation, non-phagocytosed *Candida* cells (supernatants) were collected and placed on YPG agar. The colonies of *Candida* were enumerated after incubation at 27 °C for 24 h. The candida-killing activity was calculated using the following formula: Candida-killing activity (%) = (total number of colonies without THP-1 − number of colonies from THP-1 supernatant)/total number of colonies without THP-1 × 100.

3. Results

3.1. Reactivity of anti-BG antibody to pathogenic fungi, Candida and Aspergillus

We have prepared *Candida* solubilized cell wall β-glucan (CSBG) by the NaClO–DMSO method [34]. We recently used this method to prepare *Aspergillus* solubilized cell wall β-glucan (ASBG) with NaClO, followed by urea-autoclave treatment [35]. We examined the reactivity of a human normal immune globulin preparation from pooled human sera, to CSBG and ASBG, pathogenic fungal cell wall glucan (Fig. 1). It showed higher reactivity to CSBG and ASBG, on the other hand, only a low response to mushroom glucan such as Grifolan (GRN) from *Grifola fronfosa* [32] and Sonifilan (SPG) from *Schizophyllum commune* [36]. The reactivity of the sera varied among volunteers, but a similar tendency was observed (Fig. 2). Next, specificity of anti-BG antibody was examined by mixing soluble polysaccharides as a competitor to ELISA using CSBG-coated plate (Fig. 3). The binding to CSBG was inhibited by adding soluble ASBG as a competitor. These results suggested that

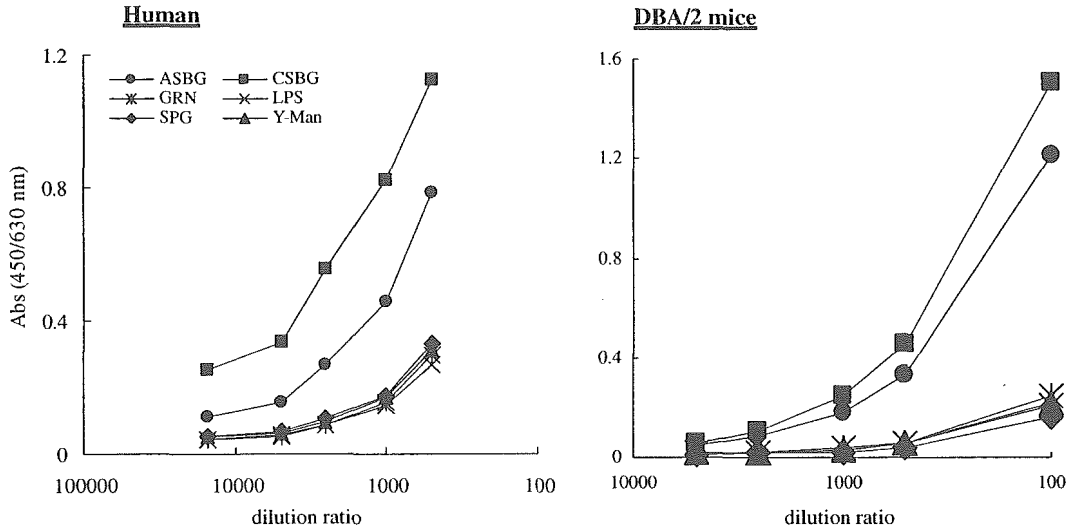


Fig. 1. Comparison of reactivity of human and murine sera to various antigen-coated plates. An ELISA plate was coated with various antigen (25 μg ml^{-1} in carbonate buffer) and blocked by BSA before use. Human immunoglobulin preparation (polyglobin N) or DBA/2 mice sera were diluted and the plate-bound Ig was determined by peroxidase-conjugated anti-human IgG + M + A or anti-mouse IgG + M antibody.

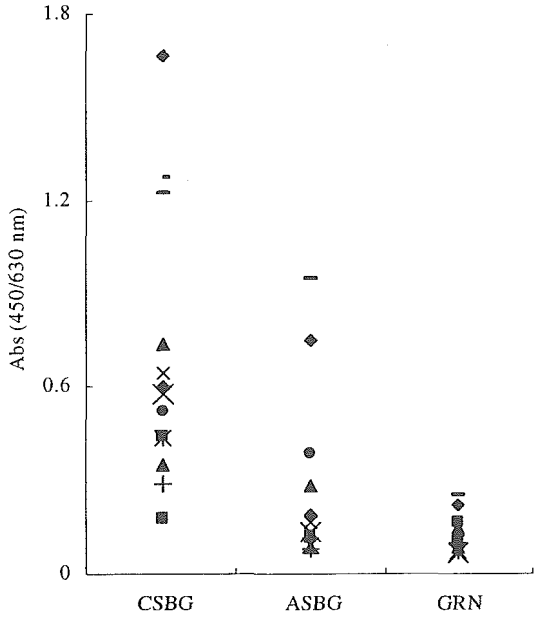


Fig. 2. Reactivity of human sera to CSBG, ASBG and GRN-coated plates. An ELISA plate was coated with CSBG, ASBG or GRN (25 μg ml^{-1} in carbonate buffer) and blocked by BSA before use. Human sera were serially diluted and the plate-bound Ig was determined by peroxidase conjugated anti-human IgG + M + A. Data from 12 volunteers are shown.

CSBG and ASBG contained a common antigenic epitope and anti-BG antibody was more reactive to glucan derived from pathogenic fungal cell wall rather than mushroom. Also, anti-BG antibody in DBA/2 mouse sera showed a more specific reaction to pathogenic fungal cell wall glucan, CSBG and ASBG (Figs. 1 and 3).

3.2. *Effect of the anti-BG antibody on* Candida *cells*

We examined whether or not anti-BG antibody could act on *Candida* cells using confocal microscopy. The binding of anti-BG antibody to living and acetone-dried cells was observed, although it was weaker than that to OX-CA, *Candida* -purified particle glucan (Fig. 4(a)). No binding to polybeads as a control was observed. Moreover, a quantitative examination of this binding was also performed by mixing whole cell or OX-CA as soluble antigen in an ELISA using CSBG-coated plates (Fig. 4(b)). It was found that whole *Candida* cells inhibited by about fifty-fold the cell concentration compared to OX-CA. These results suggested that anti-BG antibody binds certain sites on *Candida* cells.

Next, we examined the effect of anti-BG antibody on the candidacidal activity of human macrophages derived from THP-1 (Table 1). Anti-BG antibody enhanced the candidacidal activity. Therefore, it was suggested that anti-BG antibody could modify the host defense against *Candida* through opsonization.

3.3. *Binding of anti-BG antibody to* Candida *cell wall BG in vivo*

We examined the glucan-anti-BG antibody interaction in vivo and the behavior of anti-BG antibody titer in blood upon administration of glucan to DBA/2 mice. The anti-BG antibody titer of mice intravenous administered CSBG decreased remarkably in a dose-dependent manner compared with the titer before administration and showed a maximal decrease at 2 h (Fig. 5a). Also, on the administration of OX-CA, a particulate *Candida* cell wall glucan that has a primary

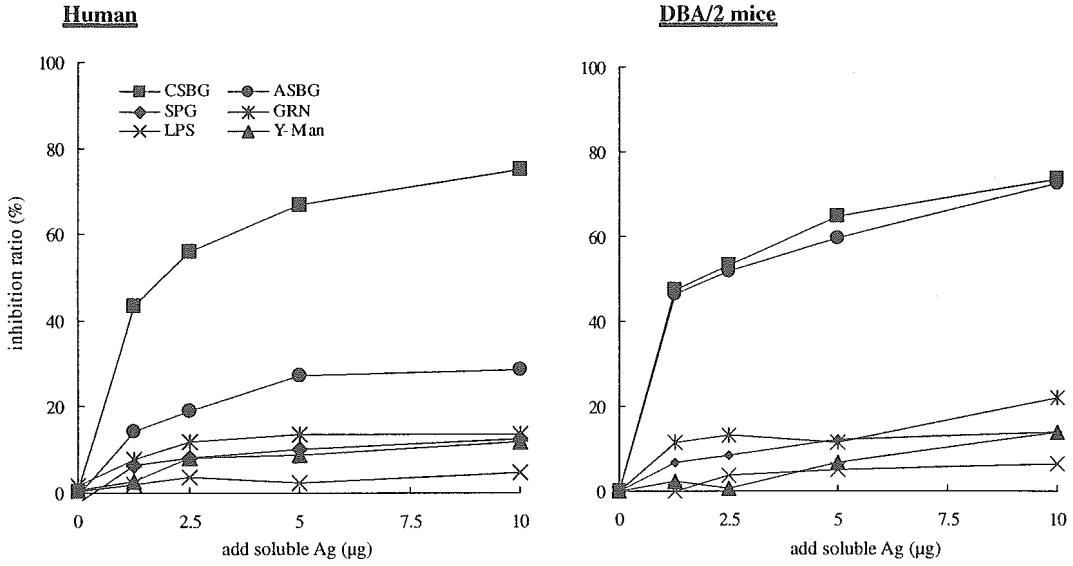


Fig. 3. Specificity of sera against plate-bound CSBG. ELISA plate was coated with CSBG. Human immunoglobulin preparation (polyglobin N) or DBA/2 mice sera were mixed with serially diluted polysaccharides and then applied to the ELISA plate. The plate-bound antibody was determined by peroxidase-conjugated anti-human IgG + M + A.

structure like CSBG, a decrease in the titer was observed (Fig. 5(b)). These results suggested that anti-BG antibody formed a complex with *Candida* cell wall glucan and was eliminated from the blood.

3.4. The reduction of the anti-BG antibody in response to Aspergillus *cell wall BG*

Next, to confirm the specificity of the decrease, the change in the anti-BG antibody titer following the intravenous administration of other glucans was examined (Fig. 6). On the administration of ASBG, the anti-BG antibody titer decreased significantly as in the case of CSBG administration (Fig. 6(a)). However, the anti-BG antibody titer of mice administered with SPG, which showed low reactivity to anti-BG antibody, decreased little and behaved like that in the saline-administered group (Fig. 6(b)). These results agreed with the results obtained in vitro as mentioned above.

Next, we examined the anti-LPS antibody titer of mice administered CSBG. The titer changed insignificantly like in the saline-administered group (Fig. 7). Therefore, these results suggested that anti-BG antibody interacted with pathogenic fungal cell wall glucan specifically in vivo and was eliminated from the blood as an antigen–antibody complex.

3.5. The behavior of anti-BG antibody in the mycosis patients

Anti-BG antibody interacted with pathogenic fungal cell wall glucan in vivo. Then, we measured anti-BG antibody titer in mycosis patients (aspergillosis: $N = 2$, carinii pneumonia: $N = 3$), whose sera is β-(1 → 3)-glucan positive (Fig. 8). Mycosis patients showed a significantly low titer (414 ± 355 U ml^{-1}) compared with normal volunteers (2371 ± 1107 U/ml^{-1}).

A case study of anti-BG titer during fungal infection (Fig. 9) was monitored in a patient. This clinical case involves 67-year-old female suffering from anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV), rapidly progressive glomerulonephritis (RPGN), and interstitial pneumonia, who was hospitalized in June. MPO-ANCA, one of the diagnostic markers of AAV decreased below the cutoff value (10 mg ml^{-1}) on steroid pulse treatment as immunosuppressive therapy. Following the decrease, the symptoms of AAV and dyspnea resulting from interstitial pneumonia abated. On the other hand, the creatinine value as a nephritic functional index increased from 8.8 to 2.2 mg dl^{-1}. Then, the patient was diagnosed with RPGN, and subjected to dialysis. After a third steroid pulse, her white blood cell count was strikingly decreased to about 1000. Therefore, it was thought that this patient was immunocompromised. Blood culture indicated no fungal infection. However, a serological analysis with β-(1 → 3)-glucan and galactomannan, suggested that a fungal infection was conceivable. Then, the administration of an anti-fungal agent was started on July 4. Subsequently, dyspnea developed exponentially and she died. At necropsy, the diagnosis of aspergillosis was confirmed. Her serum β-(1 → 3)-glucan concentration was higher (>300 pg ml^{-1}) than the cutoff value (20 pg ml^{-1}). Galactomannan, which is used as a serological marker for aspergillosis was also positive

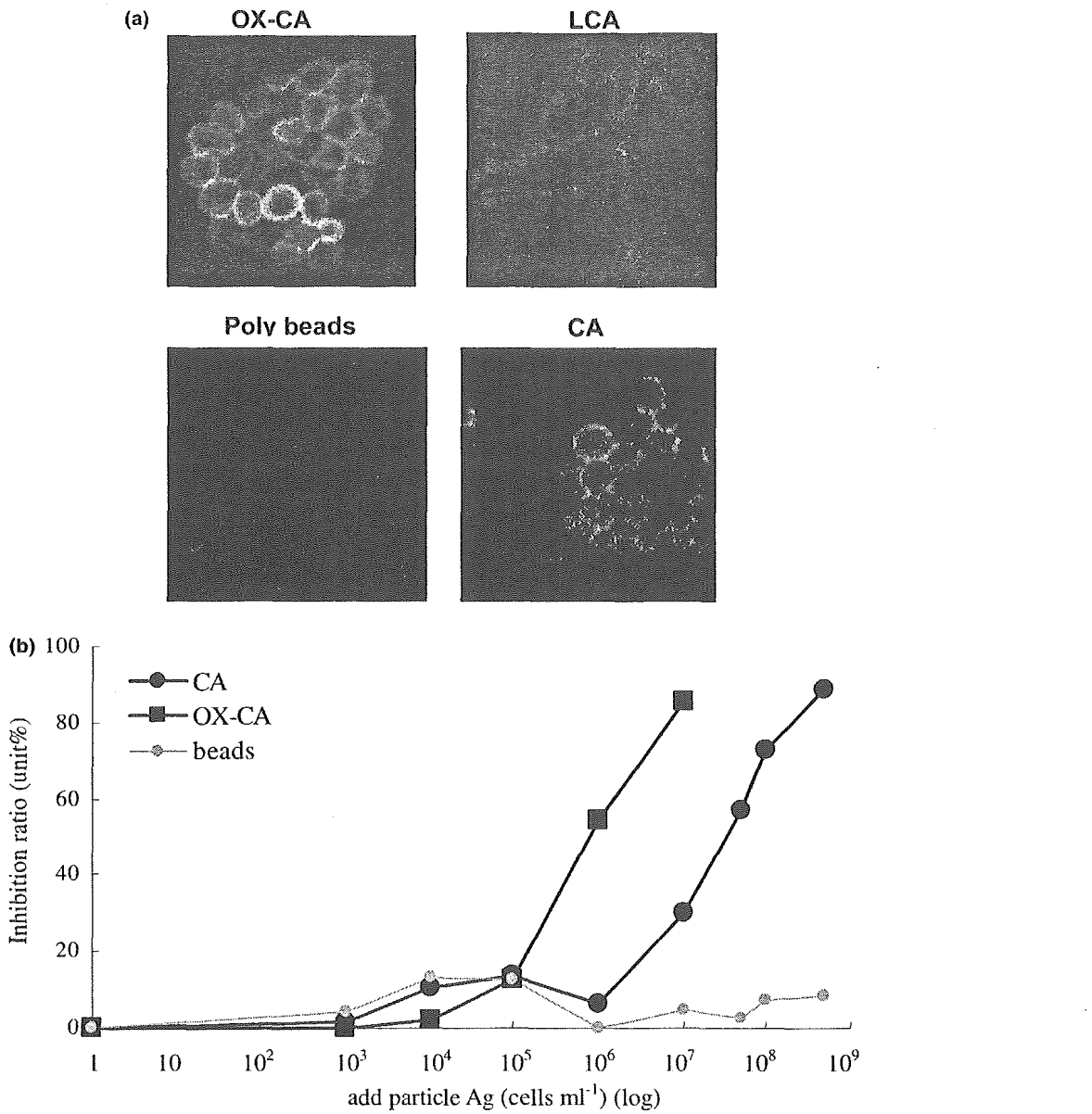


Fig. 4. Reactivity of anti-BG antibody to *Candida* dried cells and OX-CA. (a) Methanol-fixed OX-CA, *Candida* acetone dried (CA) and living (LCA) cells were stained with purified anti-BG antibody and determined with fluorescein isothiocyanate (FITC) anti-human IgG. We used polybeads as negative controls. (b) ELISA plate was coated with CSBG. Ig fraction was mixed with serially diluted CA, OX-CA and polybeads. Then, each preparation applied to the ELISA plate. The plate-bound Ig was determined with peroxidase-conjugated anti-human IgG + M + A. Enzyme activity was measured by the addition of TMB substrate.

Table 1
Effect of anti-BG antibody on candidacidal activity of macrophage

	Candidacidal activity (%)[b]
Cell	72.9 ± 1.4
Cell + control	76.8 ± 0.1
Cell + anti-BG Ab[a]	92.8 ± 1.4[c]

[a] OX-CA purified anti-BG antibody as described in Section 2.
[b] Results are expressed as arithmetic means ± SD of two separate experiments performed in duplicate.
[c] $p < 0.01$ (vs. cell), $p < 0.05$ (vs. cell + control).

and once, after becoming negative, again, it showed positive. This parameter reflects clinical symptoms such as dyspnea and C-reactive protein (CRP). The anti-BG antibody titer fluctuated at low levels (about 1000 units) and decreased to 500 units. The anti-BG antibody titer showed a reverse correlation to the clinical symptoms and galactomannan. It was suggested that anti-BG antibody decreased according to the progression of fungal infection in mycosis patients.

4. Discussion

In deep mycosis, specifically aspergillosis, candidasis has become a clinical problem. β-(1 → 3)-Glucan is one of the main components of the fungal cell wall

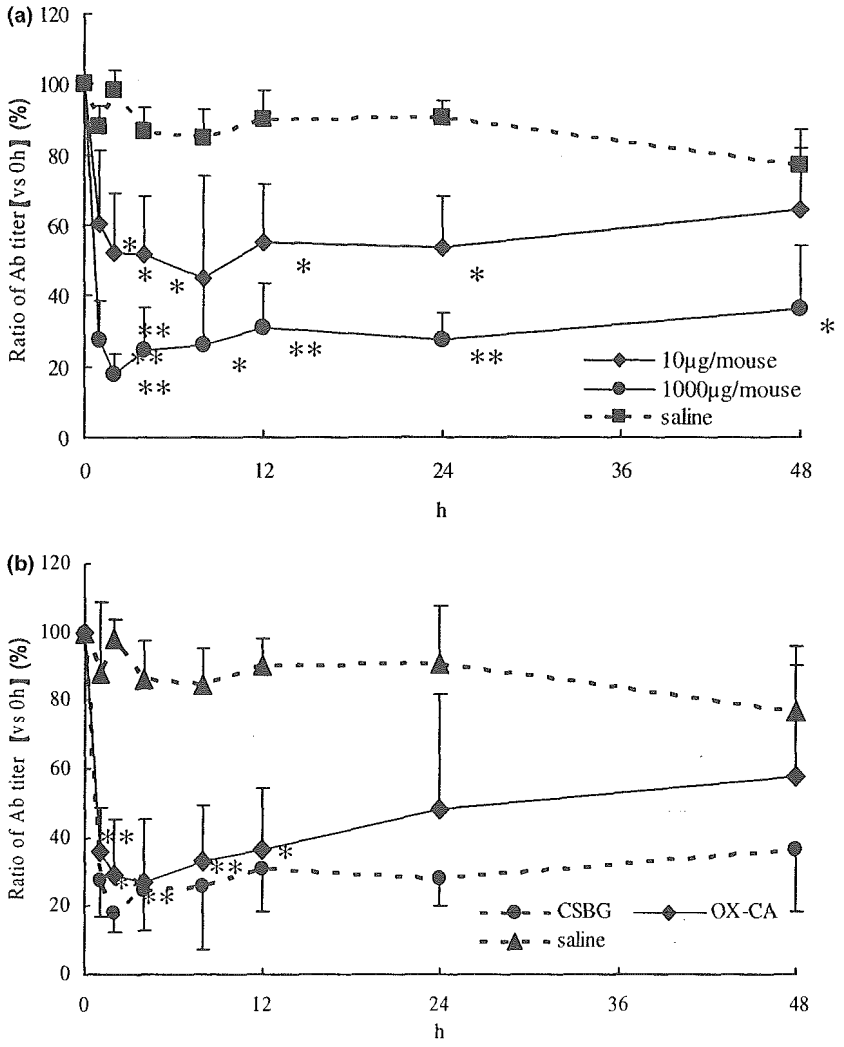


Fig. 5. Kinetics of anti-BG antibody titer from plasma following i.v. administration of *Candida* cell wall glucan. (a) CSBG (1000, 10 μg mouse^{-1}) or (b) CSBG, OX-CA (1000 μg mouse^{-1}) was intravenously administered to DBA/2 mice (three per group). An aliquot of blood was collected from the tail vein at appropriate intervals using heparinized capillaries. Subsequently, the plasma was collected, and anti-BG antibody titer was measured as described in Section 2. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. saline.

and released into a patient's blood during fungal infection. Recently, anti-BG antibody was detected in normal human sera. In this study, we examined the role of anti-BG antibody in host defense against fungi.

We first confirmed the reactivity of anti-BG antibody in sera from humans and DBA/2 mice, using glucan-coated ELISA plate. Anti-BG antibody in sera from humans and DBA/2 mice showed greater reactivity to pathogenic fungal cell wall glucan, CSBG and ASBG than to mushroom glucan. These results supported the previous concept that immunogenicity of glucan from mushroom and yeast were different, and the antigen-specific responses to mushroom glucan are weak. However, in the *Aspergillus* cell wall glucan, the β-(1 → 6)-glucan long chain present in CSBG is absent [35,37]. Hence, the existence of an antigenic epitope that is peculiar to pathogenic fungi is suggested. Next, We examined the behavior of anti-BG antibody titer in blood on administration of glucan to DBA/2 mice. On the administration of pathogenic fungal cell wall glucan, CSBG and ASBG, a remarkable decrease in the antibody titer was specifically observed, which suggested the formation of an antigen antibody complex and prompt clearance from the blood. Anti-BG antibody also showed great reactivity to the pathogenic fungal cell wall glucan in vivo. Furthermore, in deep mycosis patients, whose sera was positive for β-(1 → 3)-glucan, the anti-BG antibody titer decreased and this decrease correlated with the clinical symptoms and other parameters such as CRP. It was suggested that anti-BG antibody could play a role for β-glucan recognition molecule, and induce clearance of pathogenic fungi and biological activity by collaboration with other recognition molecule such as β-glucan receptor or complement in human.

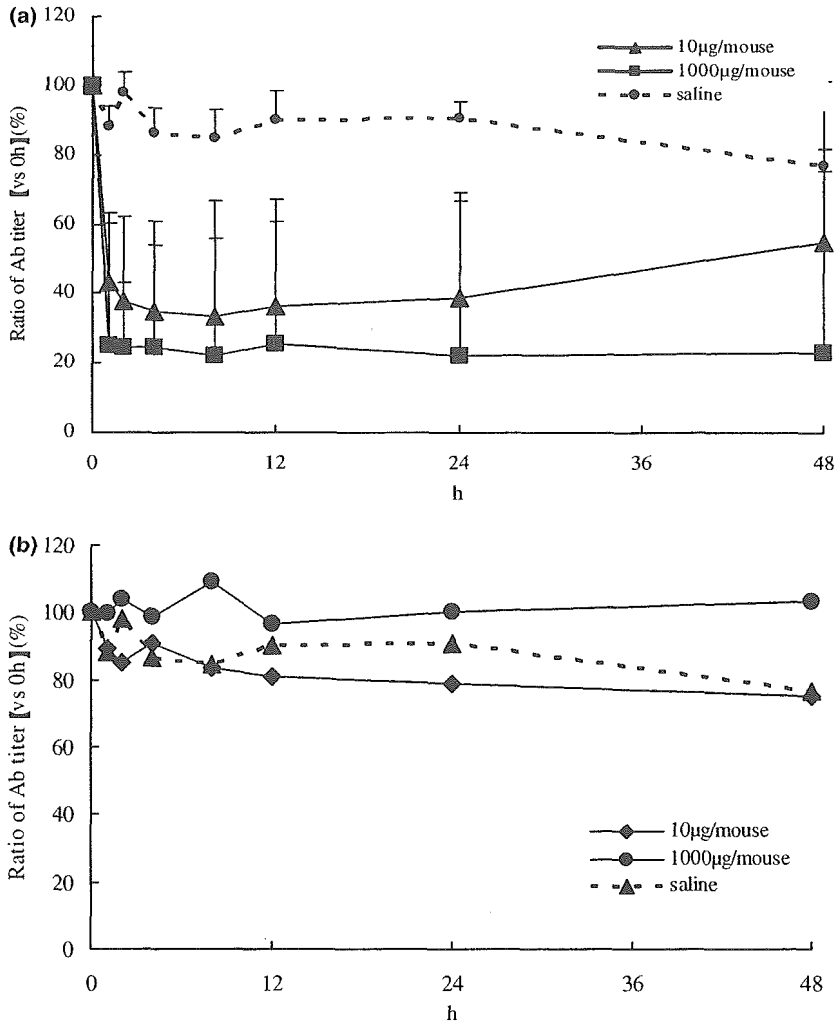


Fig. 6. Kinetics of anti-BG antibody titer in plasma following i.v. administration of SPG and ASBG. (a) ASBG or (b) SPG (1000, 10 μg mouse^{-1}) was intravenously administered to DBA/2 mice (three per group). An aliquot of blood was collected from the tail vein at appropriate intervals using heparinized capillaries. Subsequently, the plasma was collected, and anti-BG antibody titer was measured as described in Section 2.

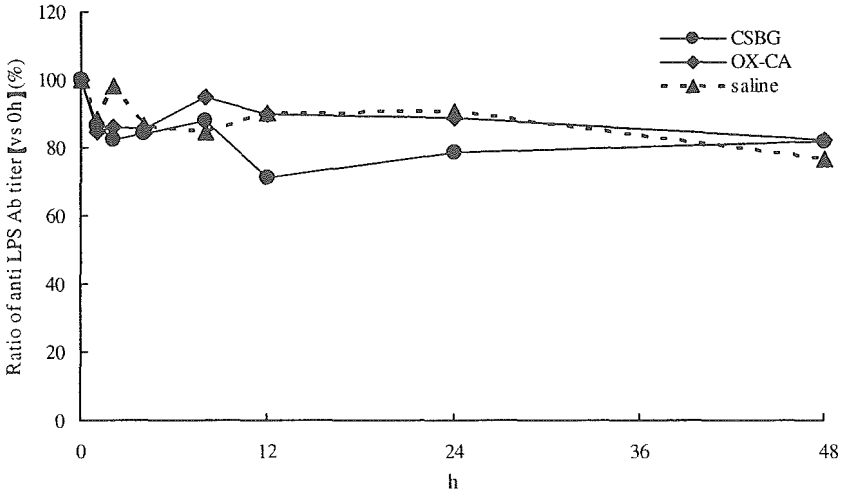


Fig. 7. Kinetics of anti-LPS antibody titer in plasma following i.v. administration of *Candida* cell wall glucan. CSBG or OX-CA (1000 μg mouse^{-1}) was intravenously administered to DBA/2 mice. An aliquot of blood was collected from the tail vein at appropriate intervals using heparinized capillaries. Subsequently, the plasma was collected, and anti-LPS antibody titer was measured with LPS (O111)-coated plates (25 μg ml^{-1}).

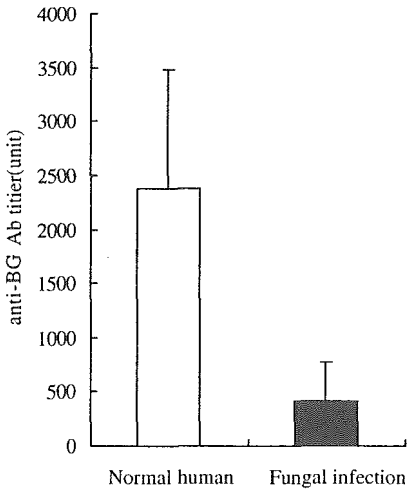
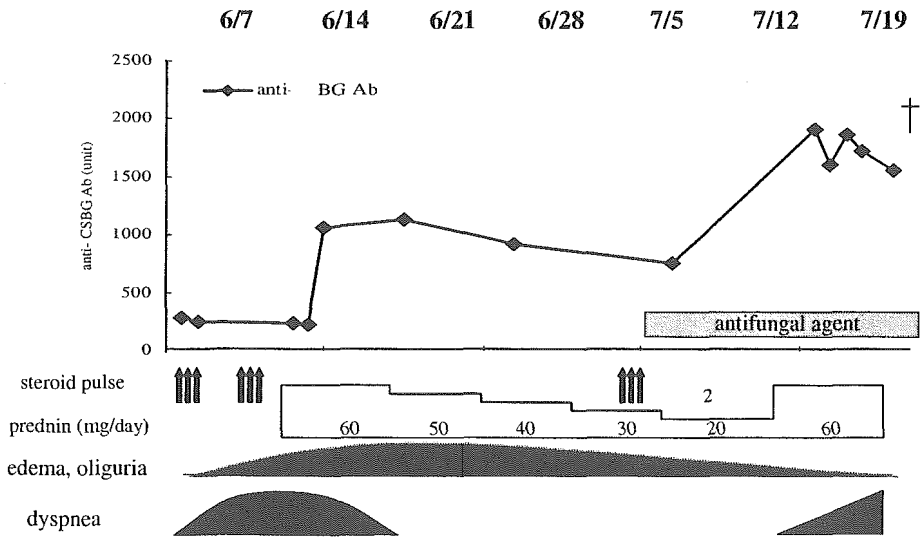


Fig. 8. Comparison of anti-BG antibody titer in patients with fungal infection and healthy volunteers. An ELISA plate was coated with CSBG (25 μg ml^{-1}). The plate-bound immunoglobulin was determined using peroxidase-conjugated anti-human IgG + M + A antibody.

The recognition system to β-D-glucan, which plays a role in the induction of biological activity, has been identified. Also in those, the limulus G test which utilized β-(1 → 3)-glucan-sensitive factor G of the horseshoe crab coagulation enzyme is useful for the sensitive screening of general fungal infections. Because 1,3-β-D-glucans widely distributed in microbes especially in fungi, limulus G test indicates a nonspecific response in patients using β-glucan preparation and undergoing dialysis with a cellulose membrane dialyzer [38]. Whereas, we showed that the anti-BG antibody titer specifically decreased on interaction with the pathogenic fungal cell wall as antigen β-glucan in vivo. It was possible that anti-BG antibody could be parameter correlated with clinical symptoms and pathological change from view of not pathogenic fungi as antigen but host.

Nonspecific cellular immunity and T-cell mediated immunity are generally believed to provide the main defense mechanism for protection against fungi. However, it is reported that the antibody plays a major role in protection against fungal infection [39,40]. Also, it was reported that anti-*Candida* antibody enhanced phagocytosis and killing activity, and activated the complement system [41,42]. On the other hand, the existence of anti-non-protective antibodies that inhibit phagocyte functions and are deleterious was reported [43], and only few antigens have been characterized for anti-fungal antibodies.

In this study, we showed that the anti-BG antibody could bind *Candida* whole cells. Also, this antibody could enhance the candidacidal activity of macrophages in vitro. These results suggested that the anti-BG antibody recognized and protected against pathogenic fungi. Bromuro et al. [44], recently reported that the sera of mice immunized with *C. albicans* cells treated with dithiothreitol and protease which is β-glucan-rich, was more effective against a lethal fungal challenge than that of



β- glucan (pg mL^{-1})	300↑	300↑	300↑	300↑	300↑	57↑	
Aspergilus antigen	1.4(+)	1.4(+)	0.1(-)	5.0(+)	1.7(+)	1.4(+)	
MPO-ANCA (U mL^{-1})	45	68	<10				20
WBC/CRP (mg dL^{-1})	21400/16.7		21300/0.8	23280/0.8	6630/7.1	1200/16.1	900/20.1
creatinin (mg mL^{-1})	2.2			8.8	6.5	5.1	0.7

Fig. 9. Clinical course of anti-BG antibody titer. A 67-year-old female with anti-neutrophil cytoplasmic antibody-associated vasculitis was diagnosed as having invasive aspergillosis. Serum was collected, and anti-CSBG or -BG antibody titer was measured as described in Section 2.

mice immunized with whole cells. It was also reported that β-glucan immunostimulation increased survival and reduced pathology for post-operative *Candida* sepsis infection [45]. These reports support that anti-BG antibody is a protective antibody. Therefore, anti-BG antibody plays a major role in host defense against pathogenic fungi. It is generally recognized that mycosis is an opportunistic infection. Anti-BG antibody titer in serum from deep mycosis patients showed low titer. By immunosuppressive therapy, the decrease of anti-BG antibody titer may be a risk factor for mycosis.

In this study, we showed that anti-BG antibody, which showed high reactivity to CSBG and ASBG, formed an antigen-antibody complex and participated in immune response to pathogenic fungi. Anti-BG antibody is expected to be useful as a response index of pathogenic fungal cell wall β-glucan.

References

[1] Clark, T.A. and Hajjeh, R.A. (2002) Recent trends in the epidemiology of invasive mycoses. Curr. Opin. Infect. Dis. 15, 569–574.

[2] Singh, N. (2003) Impact of current transplantation practices on the changing epidemiology of infections in transplant recipients. Lancet Infect. Dis. 3, 156–161.

[3] Masia, C.M. and Gutierrez, R.F. (2002) Antifungal drug resistance to azoles and polyenes. Lancet Infect. Dis. 2, 550–563.

[4] Helmerhorst, E.J., Reijnders, I.M., van't Hof, W., Simoons-Smit, I., Veerman, E.C. and Amerongen, A.V. (1999) Amphotericin B- and fluconazole-resistant *Candida* spp., *Aspergillus fumigatus*, and other newly emerging pathogenic fungi are susceptible to basic antifungal peptides. Antimicrob. Agents Chemother. 43, 702–704.

[5] Ghannoum, M.A. (2000) Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev. 13, 122–143.

[6] Douglas, L.J. (1988) *Candida* proteinases and candidosis. Crit. Rev. Biotechnol. 8, 121–129.

[7] Tomee, J.F. and Kauffman, H.F. (2000) Putative virulence factors of *Aspergillus fumigatus*. Clin. Exp. Allergy 30, 476–484.

[8] Moss, M.O. (1989) Mycotoxins of *Aspergillus* and other filamentous fungi. Soc. Appl. Bacteriol. Symp., 1869S–1881S.

[9] Chaffin, W.L., Lopez-Ribot, J.L., Casanova, M., Gozalbo, D. and Martinez, J.P. (1998) Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol. Mol. Biol. Rev. 62, 130–180.

[10] Lipke, P.N. and Ovalle, R. (1998) Cell wall architecture in yeast: new structure and new challenges. J. Bacteriol. 180, 3735–3740.

[11] Hearn, V.M. and Sietsma, J.H. (1994) Chemical and immunological analysis of the *Aspergillus fumigatus* cell wall. Microbiology 140, 789–795.

[12] Obayashi, T., Yoshida, M., Mori, T., Goto, H., Yasuoka, A., Iwasaki, H., Teshima, H., Kohno, S., Horiuchi, A. and Ito, A., et al. (1995) Plasma (1 → 3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. Lancet 345, 17–20.

[13] Yoshida, M., Obayashi, T., Iwama, A., Ito, M., Tsunoda, S., Suzuki, T., Muroi, K., Ohta, M., Sakamoto, S. and Miura, Y. (1997) Detection of plasma (1 → 3)-beta-D-glucan in patients with *Fusarium*, *Trichosporon*, *Saccharomyces* and *Acremonium fungaemias*. J. Med. Vet. Mycol. 35, 371–374.

[14] Miyazaki, T., Kohno, S., Mitsutake, K., Maesaki, S., Tanaka, K., Ishikawa, N. and Hara, K. (1995) Plasma (1 → 3)-beta-D-glucan and fungal antigenemiain patients with candidemia, aspergillosis, and cryptococcosis. J. Clin. Microbiol. 33, 3115–3118.

[15] Yuasa, K., Goto, H., Iguchi, M., Okamura, T. and Ieki, R. (1996) Evaluation of the diagnostic value of the measurement of (1 → 3)-beta-D-glucan in patients with pulmonary aspergillosis. Respiration 63, 78–83.

[16] Czop, J.K. and Austen, K.F. (1985) Generation of leukotrienes by human monocytes upon stimulation of their beta-glucan receptor during phagocytosis. Proc. Natl. Acad. Sci. USA 82, 2751–2755.

[17] Hoffman, O.A., Standing, J.E. and Limper, A.H. (1993) *Pneumocystis carinii* stimulates tumor necrosis factor-alpha release from alveolar macrophages through a beta-glucan-mediated mechanism. J. Immunol. 150, 3932–3940.

[18] Yadomae, T. (2000) Structure and biological activities of fungal beta-1,3-glucans. Yakugaku Zasshi 120, 413–431.

[19] Ohno, N. and Yadomae, T. (1996) Structure–activity relationship of immunomodulating (1 → 3)-β-D-glucans. Recent Res. Dev. Chem. Pharm. Sci. 1, 23–33.

[20] Gordon, S. (2002) Pattern recognition receptors: doubling up for the innate immune response. Cell 111, 927–930.

[21] Roeder, A., Kirschning, C.J., Rupec, R.A., Schaller, M. and Korting, H.C. (2004) Toll-like receptors and innate antifungal responses. Trends Microbiol. 12, 44–49.

[22] Ross, G.D., Cain, J.A., Myones, B.L., Newman, S.L. and Lachmann, P.J. (1987) Specificity of membrane complement receptor type three (CR3) for beta-glucans. Complement 4, 61–74.

[23] Zimmerman, J.W., Lindermuth, J., Fish, P.A., Palace, G.P., Stevenson, T.T. and DeMong, D.E. (1998) A novel carbohydrate-glycosphingolipid interaction between a beta-(1–3)-glucan immunomodulator, PGG-glucan, and lactosylceramide of human leukocytes. J. Biol. Chem. 273, 22014–22020.

[24] Brown, G.D. and Gordon, S. (2001) Immune recognition. Anew receptor for beta-glucans. Nature 413, 36–37.

[25] Adachi, Y., Ishii, T., Ikeda, Y., Hoshino, A., Tamura, H., Aketagawa, J., Tanaka, S. and Ohno, N. (2004) Characterization of β-glucan recognition site on C-type lectin, dectin 1. Infect. Immun. 72, 4159–4171.

[26] Adachi, Y., Ohno, N. and Yadomae, T. (1994) Preparation and antigen specificity of an anti-(1 → 3)-beta-D-glucan antibody. Biol. Pharm. Bull. 17, 1508–1512.

[27] Masuzawa, S., Yoshida, M., Ishibsahi, K., Saito, N., Akashi, M., Yoshikawa, N., Suzuki, T., Nameda, S., Miura, N.N., Adachi, Y. and Ohno, N. (2003) Solubilized *Candida* cell wall β-glucan, CSBG, is epitope of natural human antibody. Drug Dev. Res. 58, 179–189.

[28] Harada, T., Miura, N.N., Adachi, Y., Nakajima, M., Yadomae, T. and Ohno, N. (2003) Antibody to soluble 1,3/1,6-beta-D-glucan, SCG in sera of naive DBA/2 mice. Biol. Pharm. Bull. 26, 1225–1228.

[29] Bistoni, F., Vecchiarelli, A., Cenci, E., Puccetti, P., Marconi, P. and Cassone, A. (1986) Evidence for macrophage-mediated protection against lethal *Candida albicans* infection. Infect. Immun. 51, 668–674.

[30] Romani, L. (1999) Immunity to *Candida albicans*: Th1, Th2 cells and beyond. Curr. Opin. Microbiol. 2, 363–367.

[31] Romani, L., Mencacci, A., Cenci, E., Mosci, P., Vitellozzi, G., Grohmann, U., Puccetti, P. and Bistoni, F. (1992) Course of primary candidiasis in T cell-depleted mice infected with attenuated variant cells. J. Infect. Dis. 166, 1384–1392.

[32] Ohno, N., Adachi, Y., Suzuki, I., Oikawa, S., Sato, K., Ohsawa, M. and Yadomae, T. (1986) Antitumor activity of a beta-1,3-glucan obtained from liquid cultured mycelium of *Grifola frondosa*. J. Pharmacobiodyn. 9, 861–864.

[33] Shepherd, M.G. and Sullivan, P.A. (1976) The production and growth characteristics of yeast and mycelial forms of *Candida albicans* in continuous culture. J. Gen. Microbiol. 93, 361–370.
[34] Ohno, N., Uchiyama, M., Tsuzuki, A., Tokunaka, K., Miura, N.N., Adachi, Y., Aizawa, M.W., Tamura, H., Tanaka, S. and Yadomae, T. (1999) Solubilization of yeast cell-wall beta-(1 → 3)-D-glucan by sodium hypochlorite oxidation and dimethyl sulfoxide extraction. Carbohydr. Res. 316, 161–172.
[35] Ishibashi, K., Miura, N.N., Adachi, Y., Tamura, H., Tanaka, S. and Ohno, N. (2004) The solubilization and biological activities of *Aspergillus*-(1 → 3)-D-glucan. FEMS Immunol. Med. Microbiol. 42, 155–166.
[36] Sietsma, J.H. and Wessels, J.G. (1977) Chemical analysis of the hyphal wall of *Schizophyllum commune*. Biochim. Biophys. Acta 496, 225–239.
[37] Fontaine, T., Simenel, C., Dubreucq, G.., Adam, O., Delepierre, M., Lemoine, J., Vorgias, C.E., Diaquin, M. and Latgé, J.P. (2000) Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. J. Biol. Chem. 275, 27594–27607.
[38] Ikemura, K., Shiono, S., Yokota, J., Sakamoto, T., Uenishi, M., Hashimoto, K., Shibuya, M., Yoshioka, T. and Sugimoto, T. (1986) Study on limulus amebocyte lysate reactive material derived from dialyzers. Rinsho. Byori. 34, 313–318.
[39] Maiti, P.K., Kumar, A., Kumar, R. and Mohapatra, L.N. (1985) Role of antibodies and effect of BCG vaccination in experimental candidiasis in mice. Mycopathologia 91, 79–85.
[40] Bozza, S., Gaziano, R., Spreca, A., Bacci, A., Montagnoli, C., di Francesco, P. and Romani, L. (2002) Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. J. Immunol. 168, 1362–1371.
[41] van Spriel, A.B., van den Herik-Oudijk, I.E., van Sorge, N.M., Vile, H.A., van Strijp, J.A. and van de Winkel, J.G. (1999) Effective phagocytosis and killing of *Candida albicans* via targeting FcgammaRI (CD64) or FcalphaRI (CD89) on neutrophils. J. Infect. Dis. 179, 661–669.
[42] Wellington, M., Bliss, J.M. and Haidaris, C.G. (2003) Enhanced phagocytosis of *Candida* species mediated by opsonization with a recombinant human antibody single-chain variable fragment. Infect. Immun. 71, 7228–7231.
[43] Bromuro, C., LaValle, R., Sandini, S., Urbani, F., Ausiello, C.M., Morelli, L., Féd'ostiani, C., Romani, L. and Cassone, A. (1998) A 70-kilodalton recombinant heat shock protein of*Candida albicans* is highly immunogenic and enhances systemic murine candidiasis. Infect. Immun. 66, 2154–2162.
[44] Bromuro, C., Torosantucci, A., Chiani, P., Conti, S., Polonelli, L. and Cassone, A. (2002) Interplay between protective and inhibitory antibodies dictates the outcome of experimentally disseminated candidiasis in recipients of a *Candida albicans* vaccine. Infect. Immun. 70, 5462–5470.
[45] Browder, I.W., Williams, D.L., Kitahama, A. and DiLuizo, N.R. (1984) Modification of post-operative *C. albicans* sepsis by glucan immunostimulation. Int. J. Immunopharmacol. 6, 19–26.

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Effect of *Candida albicans* cell wall glucan as adjuvant for induction of autoimmune arthritis in mice

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Abstract

Collagen-induced arthritis (CIA) is an experimental model of rheumatoid arthritis (RA) and has aided research into the pathogenesis of inflammatory joint disease. Typically, Type II collagen (CII) emulsified with Freund's complete adjuvant (FCA) is injected into DBA/1 mice. After a booster injection, the mice develop inflammation of the paws. But the fact that the immunization of CII alone does not induce arthritis suggests that activation of the immune system by an adjuvant is necessary for induction of the arthritis. In the present study, we investigated the ability of β-glucans derived from *Candida albicans* to act as an adjuvant to induce autoimmune arthritis. DBA/1 mice were injected with CII emulsified with FCA or particulate β-glucan, OX-CA, on day 0 and given a booster at day 21. Mice immunized with CII plus OX-CA developed arthritis at around 7–10 days after the booster injection. Similarly, mice administered CII emulsified with FCA developed arthritis with the same time course. The mice immunized with CII and OX-CA had a more severe arthritis than those immunized with CII and FCA. Histological changes and production of anti-CII antibody were observed regardless of the type of injection. In addition, components of *C. albicans* were also tested for their ability to induce arthritis as an adjuvant. The results showed that CSBG, which is a soluble β-glucan, acted as an adjuvant for CIA but CAWS, which is a mannoprotein-β-glucan complex, did not. In conclusion, β-glucan derived from *C. albicans* acted as an adjuvant and the injection with CII resulted in arthritis with the production of anti-CII autoantibody. The results strongly suggested that fungal metabolites such as β-glucans have the capacity to induce and exacerbate autoimmune diseases such as RA.

Keywords: Collagen-induced arthritis; β-Glucan; Adjuvant; Autoimmunity

1. Introduction

Collagen-induced arthritis (CIA) is an experimental model of rheumatoid arthritis (RA), and has helped researchers to analyze the role of cellular and molecular mediators in the pathogenesis of inflammatory joint disease [1,2]. The CIA model is produced by immunization with type II collagen (CII) emulsified with Freund's complete adjuvant (FCA) consisting of mineral oil (Freund's incomplete adjuvant) and heat-killed mycobacterium, followed by a booster injection [3]. With this protocol, the production of anti-CII autoantibody is induced. It has been shown that not only humoral but cellular immune responses to CII are involved in the pathogenesis of CIA [4,5].

Recently, it was reported that treatment with a mixture of four monoclonal anti-CII antibodies and lipopolysaccharide (LPS) induced CIA in various strains of mice [6]. In this model, the monoclonal antibodies (mAbs) and LPS acted synergistically in the induction of arthritis [7]. In the classical CIA model, FCA is essential for the induction of arthritis in mice. IgG2a antibody production, which is essential for activating complement and subsequently inducing arthritis, depends upon the concentration of *Mycobacterium tuberculosis* in FCA [8]. Furthermore, it is reported that the administration of LPS from *Escherichia coli*, *Salmonella*, and *Klebsiella* exacerbates CIA [9]. Additionally, Yoshino et al. reported that

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