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Beta-Mannosyl Linkages Negatively Regulate Anaphylaxis and Vasculitis in Mice, Induced by CAWS, Fungal PAMPs Composed of Mannoprotein–Beta-Glucan Complex Secreted by *Candida albicans*

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Candida albicans water soluble fraction (CAWS) is a water-soluble extracellular mannoprotein–beta-glucan complex obtained from the culture supernatant of *Candida albicans*, which grows in a chemically defined medium. CAWS induced toxic reactions, such as acute anaphylactoid reaction, by intravenous administration and coronary arteritis by intraperitoneal administration. To clarify the structure responsible for these toxic reactions, *C. albicans* was cultured in pH- and temperature-controlled conditions and prepared with CAWS with or without the beta-1,2-linked mannosyl segment (BM). The structure of CAWS was assessed by immunochemical and spectroscopic methodologies, and we found that CAWS prepared under the natural culture conditions contained only small amounts of BM and CAWS prepared at neutral conditions at 27°C contained a significantly higher percentage of BM. Both the acute lethal toxicity and coronary arteritis induction was significantly more severe in the absence of BM. Activation of a complement pathway, the lectin pathway, by CAWS was significantly stronger in the absence of BM. These facts strongly suggest that BM linkages in CAWS negatively modulate acute and chronic toxicity of CAWS, and may be strongly related to the lectin pathway of the complement activation.

Key words *Candida albicans*; arteritis; mannoprotein; beta-1,2-mannan; DBA/2 mice

Candida spp. is a medically important fungus that frequently induces deep mycosis and fungemia, especially in immunocompromised hosts.¹⁾ Pathogenic microbes have various toxic mechanisms that are unique to each species and are essential for survival and evolution. Pathogenic mechanisms of eukaryotic microbes are complicated compared to those of bacteria and viruses. Although there have been extensive basic and clinical studies, the major pathogenic factors for *Candida* spp. are still unknown and precise characterization of each of the constituents would be valuable.

Cardiovascular disease is one of the most life-threatening diseases, and various new compounds and new methods of pharmaceutical care, such as gene therapy and drug delivery systems, have been developed to treat it. There are several lines of evidence suggesting that inflammatory cells, particularly macrophages and neutrophils, regulate endothelial cell function and dysfunction in atherosclerosis, via the release of mediators that display proinflammatory activity. Analysis of such diseases by animal models will give promising feedback to human health.

Murata *et al.*²⁾ reported that Kawasaki-disease-like coronary arteritis was induced in mice by administration of an alkaline extract of *C. albicans* (CADS) isolated from patients with Kawasaki disease (KD). KD, a disease of unknown cause that mainly affects children aged 4 and under, was first reported by Kawasaki in 1967.³⁾ The patient presents with systemic coronary arteritis in nearly 10% of cases. Although the occurrence of such coronary artery disorders has decreased with γ -globulin therapy, the mechanism of occurrence along with the pharmacological mechanism of treat-

ment is unknown.⁴⁾ Murata's murine model has been extensively examined from various points of view, *e.g.* anti-myeloperoxidase antibody production, susceptibility loci, and histopathological features.^{5,6)}

Previously, we prepared a water-soluble polysaccharide fraction of *C. albicans* released into a culture supernatant (*Candida albicans* water-soluble fraction: CAWS) and performed various analyses.^{7–10)} The most important point concerning this system is that we use a completely synthetic medium, named C-limiting medium, to eliminate contaminants from the culture medium, such as endotoxins, peptidoglycans, and nucleic acids. Series of our studies found that CAWS shows various toxic activities, such as cytokine synthesis by leukocytes, platelet aggregation, lethal toxicity, enhancement of side effect of indomethacin, induction of coronary arteritis in mice, and so on. Thus, CAWS may be a pathogen-associated microbial product (PAMPs) of *Candida albicans*.

In a previous study we found that intraperitoneal administration of CAWS to mice induces coronary arteritis similar to that induced by CADS.^{8–10)} It is of note that CAWS-induced arteritis showed significant strain dependency: The incidence of arteritis was 100% in C57BL/6, C3H/HeN and DBA/2 mice, but only 10% in CBA/J mice. The coronary arteritis observed in DBA/2 mice was the most serious, with the majority of mice expiring during the observation period.

The coronary arteritis induced by CAWS was accompanied by hypertrophy of the tunica intima, the rupture of elastic fibers and a diffuse invasion by lymphocytes, histiocytes, fibroblasts, smooth muscle cells and eosinophils of vascular

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endothelial cells and the regions surrounding blood vessels. Based on such characteristics, the coronary arteritis induced by CAWS was presumed to be proliferative granulomatous coronary arteritis, and is clearly different from fibrinoid arteritis. In DBA/2 mice, it was observed to cover nearly the entire periphery of the vessels, and those mice were considered to demonstrate the most virulent form of coronary arteritis.

Structure of yeast cell wall, such as in *Saccharomyces cerevisiae*, *Candida albicans*, and *Schizosaccharomyces pombe*, have been extensively examined and it was found that the overall structure is quite complex and regulated by expression of multiple genes.^{11,12)} According to the results of NMR and biochemical analyses, CAWS was found to be composed of a mannoprotein and β -glucan portion, which are the main components of the *C. albicans* cell wall.⁸⁾ The mannoprotein region of *Candida albicans* is composed of both *N*- and *O*-glycosyl linkages with α - and β -linked mannosyl residues.^{13,14)} Phosphodiester linkages are also included. Mannan synthesis is regulated by several specific glycosyl transferases.^{15–17)} It is also an immunochemical determinant of *Candida* spp.^{18,19)} It is of note that the structure of mannan is modulated by various culture conditions, such as temperature and pH.^{20,21)} The structure of mannan is also known to be modulated by various stresses. The structure of mannan is a key molecule for recognition of *Candida* by host biodefense system and might be strictly regulated by the affinity for specific receptors. Analysis of the disease from the structural point of view is an indispensable study.

In a preliminary experiment, the pH of a culture medium was measured and it was found that shifted from 5.2 to 2.3 at the end. In the present study, we have prepared CAWS using different culture conditions and examined CAWS-induced anaphylactoid reaction and vasculitis. We demonstrated that β -1,2-mannosyl linkages are the negative regulators for both anaphylactoid reaction and vasculitis.

MATERIALS AND METHODS

Mice Male ICR and DBA/2 mice were purchased from Japan SLC. The mice were housed in a specific pathogen-free (SPF) environment and were used in the study at 5 weeks of age. All animal experiments were followed by the guideline of laboratory animal experiments in Tokyo University of Pharmacy and Life Sciences (TUPLS), and each of the experimental protocol was approved by the committee of laboratory animal experiments in TUPLS.

Preparation of CAWS *Candida albicans* strain IFO1385 was purchased from the Institute for Fermentation, Osaka (IFO), stored at 25 °C on Sabauroud's agar (Difco, U.S.A.) and passaged once every three months. CAWS was prepared from *C. albicans* strain IFO1385 in accordance with conventional methods.^{7,9,10)} The procedure used was as follows: 5 l of medium (C-limiting medium) was added to a glass incubator 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 allowed to stand overnight, the precipitate was collected. The precipitate was dissolved in 250 ml of distilled water, ethanol was added and the mixture was allowed to stand overnight. The precipitate was collected and dried with

acetone to obtain CAWS.

Administration Schedule for Induction of Coronary Arteritis (10) CAWS (4 mg/mouse) was administered intraperitoneally for 5 consecutive days to each mouse in week 1. At week 5, the hearts of the animals were fixed with 10% neutral formalin and prepared in paraffin blocks. Tissue sections were stained with hematoxylin–eosin (HE) stain.

ELISAs of *Candida* Typing Sera to CAWS The reactivity of CAWS to serum factors from *Candida* that consists of rabbit polyclonal antibodies against *Candida* cell wall mannan was detected by ELISA. 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 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 and the 3,3',5,5'-tetramethylbenzidine microwell peroxidase substrate system (TMB; KPL inc). After 45 min, the reaction was stopped with 1 N H₃PO₄, and then the optical density of each well was read at 450 nm on an automatic microplate reader.

Measurement of Complement Activation The complement kit for assessment of classical, alternative and MBL pathway activity was developed by the EU consortium and is now commercially available (Wielisa COMPL300 Total Complement Functional Screen kit from Wieslab AB, Lund, Sweden). The kit was operated by the instructions provided in the manual with slight modifications. In brief, strips of wells for classical pathway (CP) evaluation were precoated with IgM, strips for alternative pathway (AP) determination were coated with LPS, and mannose binding lectin (MBL) pathway (MBL-P) strips were coated with mannan. Sera were diluted 1/101 for the CP and MBL-P assay and 1/18 for the AP assay in specific buffers, serial dilutions of CAWS solution was added to the diluted sera and incubated. The resulting sera were added to the strips of the kit and were incubated for 1 h at 37 °C. After washing the strips, alkaline phosphatase-conjugated antihuman C5b-9 was added and incubated at room temperature for 30 min. Additional washing was performed, substrate was added, and the wells were incubated for 30 min. Finally, absorbance values were read at 405 nm. In each assay, standard positive and negative control sera provided in the kit as lyophilised material were reconstituted with distilled water. Complement activity was calculated using the following formula: activity = 100% × (mean A405 (sample) – mean A405 (negative control)) / (mean A405 (standard serum) – mean A405 (negative control)). Samples as well as standard serum and negative control serum were tested in duplicate at a fixed dilution.

Assay for Anaphylactoid Reaction Indicated dose of CAWS solution was i.v. administered to ICR mice. The incidence and the severity of the rapid anaphylactoid shock were assessed within 30 min. For measuring tolerance, low dose CAWS was i.v. administered to ICR mice 1 h prior to induce anaphylactoid reaction by CAWS (400 μ g/mouse).

NMR Spectroscopy ¹H-NMR experiments were performed with a Bruker DPX 400 equipped with XWIN-NMR

software. The spectra were recorded using a solution of each soluble fraction (10 mg/ml) in D₂O at 45 °C with acetone as an internal standard.

RESULTS

Preparation of CAWS by Various Culture Conditions

Figure 1 shows the data of pH monitoring during *Candida* culture in C-limiting medium. The data clearly demonstrated that at pH uncontrolled natural time course, pH was 5.2 at the beginning and was lowered to around 2 at the end, thus the naturally expressed CAWS was produced in this culture condition. In order to prepare CAWS with modulated mannan structure, *C. albicans* IFO1385 was cultured at either 27 °C or 37 °C in C-limiting medium. In addition, pH of the culture medium was controlled at either pH 2.3, 5.2, or 7.0 by automatically added sodium hydroxide solution. The extracellular mannoprotein fraction was prepared by a previously established procedure that was the same as naturally produced CAWS. The names of each fraction were designated as CAWS 27-2.3, CAWS 27-5.2, CAWS 27-7.0, CAWS 37-2.3, CAWS 37-5.2, and CAWS 37-7.0. The name of CAWS prepared at natural pH course was designated as 27-(–) and 37-(–), respectively. In addition, CAWS prepared at 27 °C was designated as CAWS 27s which include 27-2.3, 27-5.2, 27-7.0, and 27-(–), and prepared at 37 °C as CAWS 37s which include 37-2.3, 37-5.2, 37-7.0, and 37-(–). Yield and some parameters of each CAWS are shown in Table 1. The structure of the mannan moiety was characterized by the reactivity to the typing sera and NMR spectral analysis.

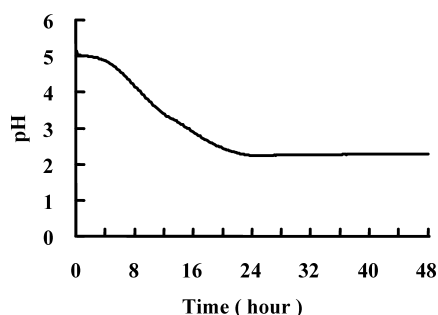


Fig. 1. Monitoring pH of *C. albicans* IFO1385 Culture in C-Limiting Medium at 27 °C

One hundred milliliters of *C. albicans* IFO1385 precultured in liquid to logarithmic phase was added to 4 l of C-limiting medium in a jar fermenter with 4 l air/min and 400 rpm stirring. The pH of the culture medium was measured automatically with a pH meter.

Table 1. Properties of CAWS Prepared by Various Culture Conditions

CAWS	Yield (mg/l)	Cell (g/l)	C (%)	H (%)	N (%)	Factor G	Factor C
27-(–)	147±30	4.3±0.7	30.1±2.7	5.5±0.4	1.6±0.3	—	<2
27-2.3	132±19	4.0±0.2	27.5±4.7	5.0±0.7	1.6±0.1	0.73±0.57	0.25±0.1
27-5.2	247±26	3.5±0.4	28.6±3.5	5.2±0.4	2.8±0.3	ND	0.33±0.45
27-7.0	381±77	3.4±0.1	18.1±5.3	4.0±0.8	2.1±0.6	ND	0.71±0.73
37-(–)	84±33	2.0±0.7	26.5±1.8	4.8±0.4	1.1±0.1	1.24±0.21	0.03±0.02
37-2.3	160±17	3.3±0.1	18.5±9.1	3.7±1.5	1.1±0.2	0.56±0.16	ND
37-5.2	255±87	3.0±0.2	20.3±2.7	4.0±0.7	0.9±0.2	ND	0.1±0.03
37-7.0	263±12	3.2±0.5	24.3±1.1	4.9±0.1	1.5±0.1	0.07±0.01	0.08±0.01

27-(–); cultured at 27 °C without pH control, 27-2.3; cultured at 27 °C and pH was maintained at 2.3, 27-5.2; cultured at 27 °C and pH was maintained at 5.2, 27-7.0; cultured at 27 °C and pH was maintained at 7.0, cell; yield of dried cells. C, H, N; elemental analysis (carbon, hydrogen, nitrogen), factor G; content of 1,3-beta-glucan (ng/ml), factor C; content of endotoxin (ng/ml), ND; not detected.

Figure 2 shows relative reactivity to the typing sera no. 5, 6, and 11. Experiments were done using three doses of CAWS solution adsorbed onto the ELISA plates. The pattern of reactivity of each CAWS preparation to the sera were similar between no. 5 and no. 6. In contrast, reactivity to no. 11 was significantly different. In the cases of no. 5 and no. 6, comparing the dose to show absorbance 1.0, reactivity of CAWS 27-5.2 and CAWS 27-7.0 was significantly increased compared to CAWS 27-(–), and other preparations. In contrast, relative activity of CAWS 27-5.2 and CAWS 27-7.0

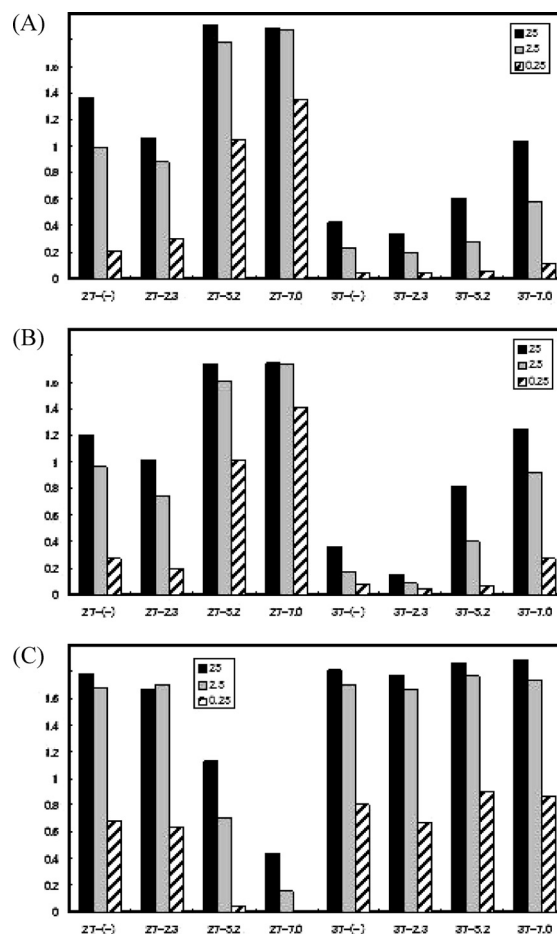


Fig. 2. Reactivity of CAWS Prepared under Various Conditions to *Candida* Typing Sera no. 5, 6, and 11

Each 2.5, 2.5, or 0.25 µg/ml solution of CAWS was adsorbed on ELISA plate and diluted typing sera was added. After appropriate incubation time, plate-bound antibody was measured by peroxidase conjugated anti-rabbit IgG with TMB reagent. (A) Anti no. 5; (B) anti no. 6; (C) anti no. 11.

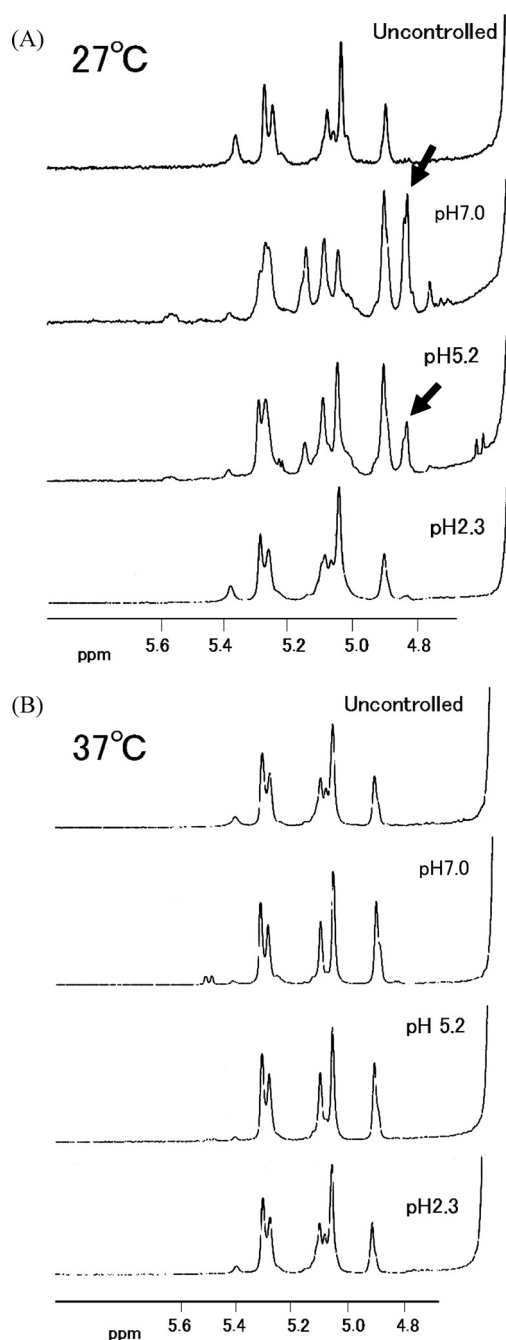


Fig. 3. Proton-NMR Spectra of CAWS in D₂O

(A) CAWS 27s prepared from the 27°C culture. (B) CAWS 37s prepared from the 37°C culture. Arrow indicates signals corresponding to beta-1,2-mannosyl linkages.

was significantly decreased in the case of typing sera no. 11. It is proposed that the specific epitope of no. 5 and no. 6 are for the beta-1,2-linked manno-oligosaccharides, and no. 11 for the alpha-1,2 and 1,6-manno-oligosaccharides.^{20,21)} These data strongly suggested that CAWS 27-5.2 and CAWS 27-7.0 contained significantly higher concentration of beta-1,2-linkages and reduced concentration of alpha-linkages.

Figure 3 shows NMR spectra of CAWS preparations. Each of the spectra contained many signals in the anomeric region (4.8–5.5), and thus, we could not do complete assessment at this time. However, comparing the published spectra of the cell wall mannan moiety of *Candida* spp. by Professor Suzuki *et al.*,^{20,21)} major signals could be assigned. Chemical

Table 2. Anaphylactoid Reaction Induced by CAWS Prepared under Various Conditions

CAWS	3200	800	200	50	12.5
27-(–)			3/3	0/3	
27-2.3			3/3	0/3	
27-5.2	2/3	0/3	0/3		
27-7.0	0/3	0/3	0/3		
37-(–)			2/3	1/3	0/3
37-2.3			3/3	1/3	0/3
37-5.2			2/3	0/3	
37-7.0			2/3	0/3	

Indicated dose (μ g/mouse) of CAWS was i.v. administered to mice ($n=3$). Lethality of each mouse was monitored 30 min later. */*, number of mice, dead/total.

shift around 4.85, shown as arrow in the spectra, would be beta-linked manno-oligosaccharides. The signal around 4.85 was only detected in the spectra of CAWS 27-5.2 and CAWS 27-7.0. Signals around 5.13 also appeared in those spectra would be the residues linked to beta-mannosyl linkages. In contrast, signals around 5.05 which include alpha-mannose residues was reduced in relation to the substitution of beta-mannosyl linkages. It is of note that even in the same pH conditions, CAWS 37-5.2 and CAWS 37-7.0 did not show such signals, and this observation was strongly supported by immunochemical reactivity. NMR spectra of CAWS 37-(–), CAWS 37-2.3, CAWS 37-5.2, and CAWS 37-7.0, shown in Fig. 3b show simple anomeric signals compared to those of CAWS 27s. Shibata *et al.* examined the cell wall mannan structure of *Candida parapsilosis* and *Candida albicans* by use of controlled hydrolysis and NMR spectroscopy.²²⁾ Comparing Proton NMR spectra of *C. parapsilosis* and *C. albicans*, that of *C. parapsilosis* is simple and shows only 5 major signals in the anomeric region of 1D-NMR spectrum. Those signals correspond to alpha mannosyl linkages. At least in 1D-NMR spectrum, CAWS 37s resembles that of *C. parapsilosis*, also supporting that beta-1,2-linkages are missing in CAWS 37s. Regulation of beta-1,2-mannosyl linkages by culture condition is also reported in *Candida guilliermondii*.²³⁾ From the data of immunochemistry and NMR spectra, CAWS 27-5.2 and CAWS 27-7.0 contained significantly higher concentrations of beta-1,2-linked manno-oligosaccharides.

Acute Toxicity of CAWS Prepared by Various Culture Conditions

We have previously shown that CAWS induced acute anaphylactoid reaction, similar to *E. coli* O9 LPS and yeast mannan.^{24,25)} We compared acute anaphylactoid reaction of various CAWS preparations. As shown in Table 2, various concentrations of CAWS were intravenously administered to ICR mice and survival within 30 min was compared. In this experiment, the majority of CAWS preparations induced almost complete lethal toxicity at doses of 200 μ g/mouse. However, CAWS 27-5.2 required 3200 μ g for lethal toxicity and CAWS 27-7.0 did not show any lethal toxicity in this experimental condition. These facts strongly suggested beta-1,2-linked manno-oligosaccharide would be a negative regulator for acute anaphylactoid reaction. To confirm the possibility, tolerance induction by CAWS preparations was also tested. As shown in Table 3, administration of 16 μ g of CAWS 27-(–) and CAWS 27-2.3 significantly reduced lethality of subsequent high dose CAWS administration. However, in the case of CAWS 27-5.2, a relatively

Table 3. Tolerance Induction in CAWS Induced Anaphylactoid Reaction by Pretreatment with CAWS Prepared under Various Conditions

CAWS	64	16	4	1
27(-)		1/3	3/3	
27-2.3		0/3	2/3	2/3
27-5.2	0/3	2/3	2/3	

Indicated dose of CAWS ($\mu\text{g/ml}$) was i.v. administered to mice ($n=3$). CAWS(-) was again i.v. administered to each mouse, and lethality was monitored 30 min later. */*, number of mice, dead/total.

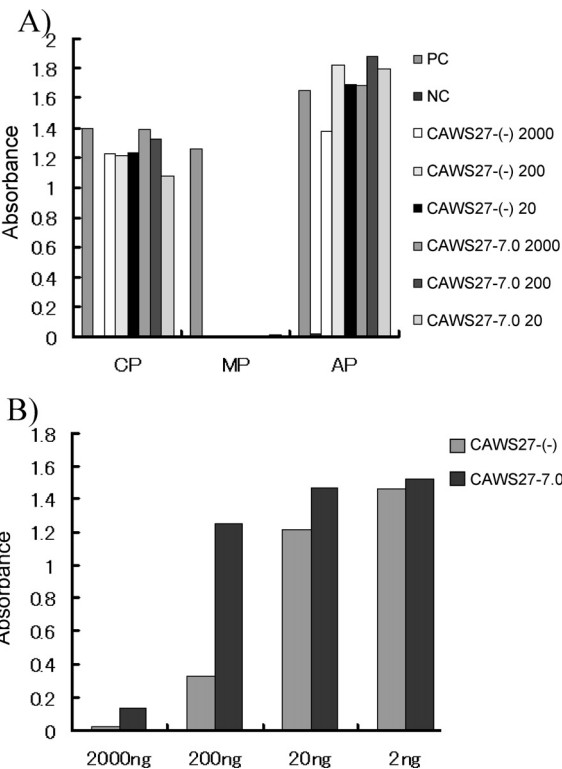


Fig. 4. Complement Activation by CAWS (A) Screening of classical (CP), lectin (MP), and alternative (AP) pathway of complement activation by CAWS. (B) Dose response of CAWS-induced activation of the lectin pathway of complement. CAWS 27(-) and CAWS 27-7.0 were used in this experiment. PC; positive control serum, NC; negative control serum.

higher dose, 64 μg , was required for tolerance induction. This result also strongly suggested that beta-1,2-manno-oligosaccharides negatively modulate acute lethal toxicity of CAWS.

Complement activation and the resulting anaphylactic peptides, C3a and C5a, production are the most commonly postulated mechanism for acute toxicity. In the preliminary experiments, incubation of CAWS with human sera produced high concentration of C5a anaphylatoxic peptide. Because of the difficulty in measuring mouse complement activation, we used a commercially available kit for human complement activation in the present study. Activity of classical, lectin, and alternative pathways were measured using IgM, mannan, and LPS-coated ELISA plate and specific reaction buffer systems. As shown in Fig. 4a, CAWS of 20 to 2000 $\mu\text{g/ml}$ were added, and it was found that CAWS activated complement was almost specifically mediated by the lectin pathway. In this dose range, CAWS 27(-) and CAWS 27-7.0 did not show any differences; however, comparing the activity be-

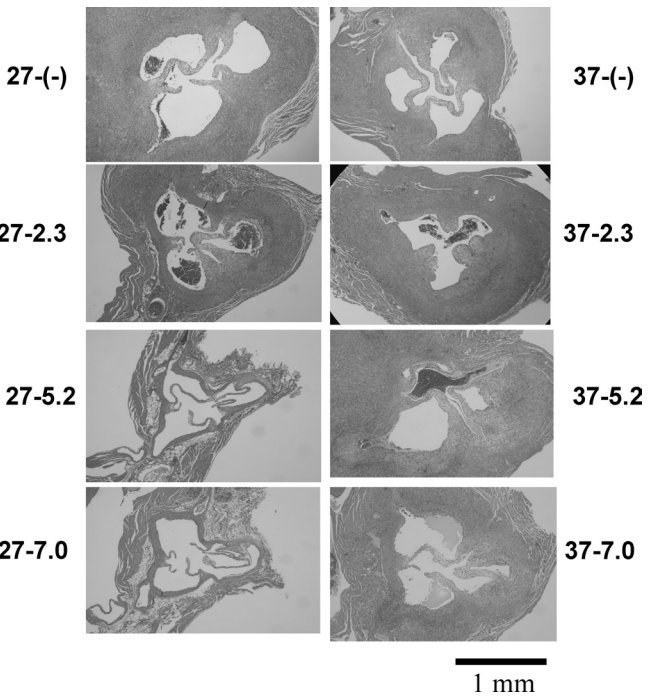


Fig. 5. Histological Analysis of DBA/2 Mice Administered with CAWS CAWS prepared under various conditions (4mg/mouse) was administered i.p. to DBA/2 mice for five consecutive days. Five weeks later, the aorta with coronary of these mice were stained with hematoxylin-eosin.

tween doses of 20 ng and 200 ng shown in Fig 4b, CAWS 27(-) showed almost ten times higher activity than CAWS 27-7.0. These results strongly suggest that beta-1,2-linked manno-oligosaccharides inhibited complement activation by alpha-linked mannose residues.

Arteritis Induction by CAWS Prepared by Various Culture Conditions Intraperitoneal injection of CAWS induced coronary arteritis in mice.⁸⁻¹⁰ Severity is significantly dependent on strains of mice and DBA/2 shows the most severe reaction, resulting in death. Coronary arteritis induction by CAWS prepared as above was compared. CAWS was administered i.p. to each mouse at 4 mg/mouse for 5 consecutive days and sections of coronary artery were prepared at 4 weeks after the final CAWS administration. Figures 5 to 8 show hematoxylin eosin staining of aorta, aortic valve, and the coronary artery in CAWS-administered mice. CAWS prepared by the standard protocol induced strong inflammation in this area. CAWS 27-2.3, CAWS 37(-), CAWS 37-2.3, CAWS 37-5.2, and CAWS 37-7.0 show similar inflammation. However, CAWS 27-5.2 and CAWS 27-7.0, both of which contained higher content of beta-1,2-manno-oligosaccharides, show significantly weaker inflammation. The histology of the aorta, aortic valve, and coronary artery using higher magnification (Figs. 7, 8) shows similar phenotypes.

Coronary arteritis was also examined by survival. As shown in Fig. 9, almost all of the mice that had CAWS administered died except for CAWS 27-5.2, in which only one mouse out of five died. Figure 10 shows the dose response of survival and corresponding histology of DBA/2 mice administered CAWS 27(-). Arteritis was induced even with 250 $\mu\text{g/mouse}$ administration and 2/5 mice died in this condition. Considering the data of survival and histology of CAWS 27(-) and CAWS 27-5.2, the relative activity of

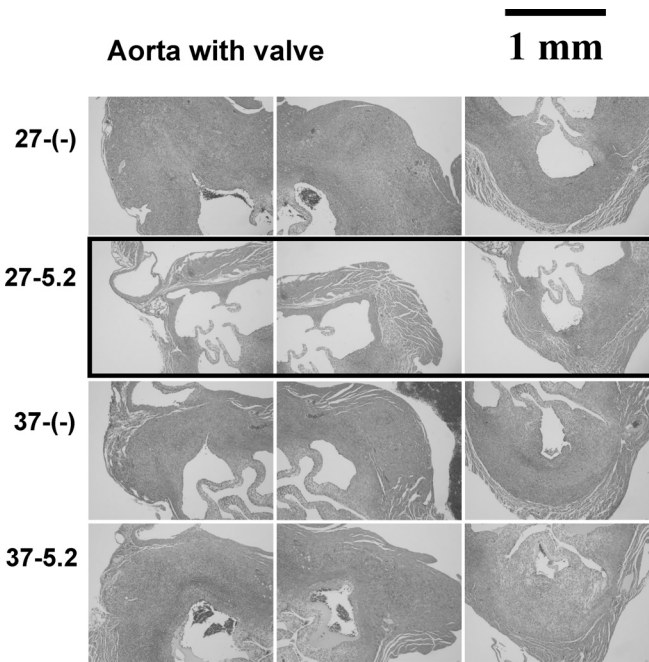


Fig. 6. Comparison of Histology of Aorta in DBA/2 Mice Administered Native and pH-Controlled CAWS

CAWS 27-(-), CAWS 27-5.2, CAWS 37-(-), CAWS 37-5.2 (4 mg/mouse) were administered i.p. to DBA/2 mice for five consecutive days. Five weeks later, the aorta with coronary of these mice were stained with hematoxylin-eosin.

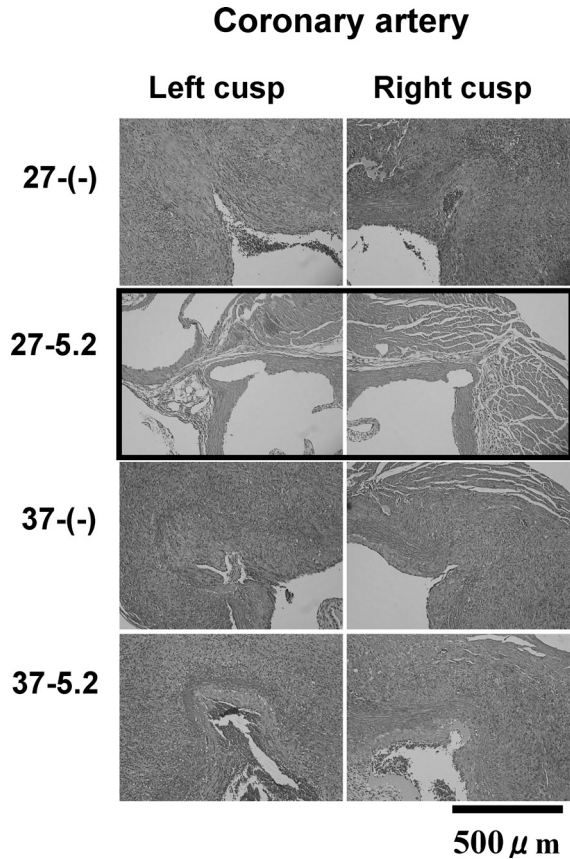


Fig. 7. Comparison of Histology of Cusp Region of Coronary Artery in DBA/2 Mice Administered Native and pH-Controlled CAWS

CAWS 27-(-), CAWS 27-5.2, CAWS 37-(-), CAWS 37-5.2 (4 mg/mouse) were administered i.p. to DBA/2 mice for five consecutive days. Five weeks later, the aorta with coronary of these mice were stained with hematoxylin-eosin.

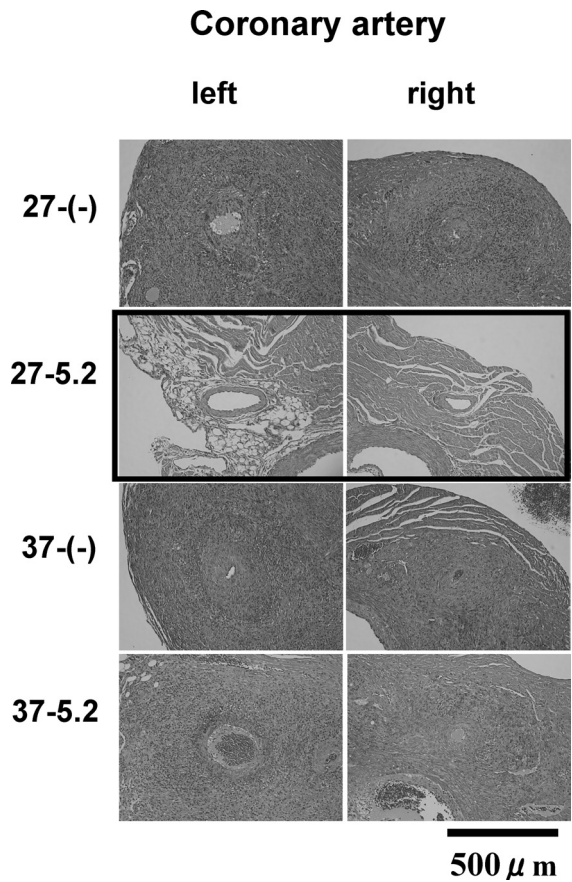


Fig. 8. Comparison of Histology of Coronary Artery in DBA/2 Mice Administered Native and pH-Controlled CAWS

CAWS 27-(-), CAWS 27-5.2, CAWS 37-(-), CAWS 37-5.2 (4 mg/mouse) were administered i.p. to DBA/2 mice for five consecutive days. Five weeks later, the artery with coronary of these mice were stained with hematoxylin-eosin.

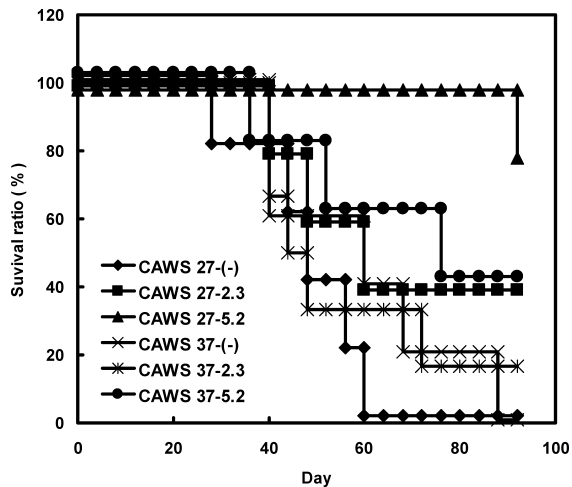


Fig. 9. Survival Time of CAWS-Administered Mice

CAWS 27s and CAWS 37s (4 mg/mouse) was administered i.p. to DBA/2 mice for five consecutive days in the 1st week. Survival was observed for 12 weeks.

CAWS 27-(-) was at least 20 times higher than that of CAWS 27-5.2. From these findings, CAWS with beta-1,2-linked mannooligosaccharides showed significantly weaker inflammatory reaction in this experimental model.

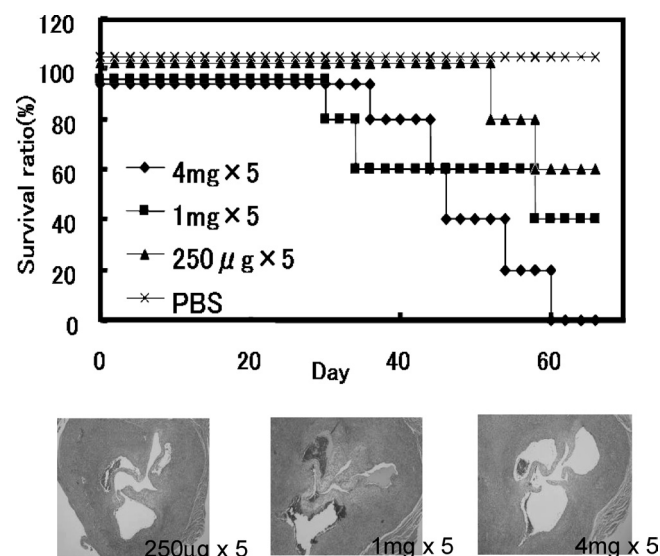


Fig. 10. Dose Response of Survival Time of CAWS-Administered Mice

CAWS 27(-) (0.25, 1, or 4 mg/mouse) was administered i.p. to DBA/2 mice for five consecutive days in the 1st week. Survival was observed for 9 weeks. Hematoxylin-eosin stain of the aorta with coronary were also shown.

DISCUSSION

Candida albicans is a clinically important fungus and is known to cause disseminated candidiasis and candidemia in immunocompromised hosts. Patients with deep mycoses, such as that induced by *C. albicans* and *A. fumigatus*, have been demonstrated to release β -glucans into the blood. However, these are present in extremely small amounts, and the overall structure of the factor G activating substance present in the blood is unknown.²⁶⁾ The factor G activating substance has the potential to exhibit various biological activities, but the details of this are also unknown. In order to clarify these matters, we first cultured *C. albicans* in a completely synthetic medium in order to obtain water-soluble limulus factor G activating substance that is released from the cells, and obtained a water-soluble polysaccharide fraction released into the culture supernatant (CAWS), which is thought to be similar to the β -1,3-D-glucan actually present in patient blood.⁸⁾ Murata *et al.* conducted an analysis on children with KD and found that *C. albicans* extract (CADS) isolated from the stool specimens of the patients induced coronary angiitis in mice that resembled KD.^{5,6)} During the course of joint research, we found that administration of CAWS according to this protocol induced a similar coronary angiitis in mice.⁸⁻¹⁰⁾

In the present study, the relationship between culture conditions of *Candida* and induction of some of the pathologic parameters, such as acute shock, arteritis, and complement activation, was examined, and the condition producing beta-1,2-linkage significantly reduced all three parameters measured in the present study. It is of note that in the standard culture medium, C-limiting medium, the pH was acidified during culture. Thus, in the present study we have prepared CAWS in significantly regulated conditions and we analyzed both activity and structure. We have confirmed that CAWS prepared under the condition of neutral at 27 °C culture significantly reduced activity and is strongly related to the presence of beta-1,2-mannosyl linkages. These facts clearly

demonstrated that beta-1,2-linkages are negative regulators for pathologic parameters, such as acute shock, arteritis, and complement activation. At this time, the role of beta-1,2-linkages on the anaphylactoid reaction and arteritis induction could not be fully clarified. As shown in Table 3, pretreatment of mice with CAWS 27-5.2 induced tolerance in the CAWS-induced anaphylactoid reaction, but this was less than that observed with CAWS 27(-) and CAWS 27-2.3. In addition, small numbers of beta-1,2-linkages are present in CAWS 27(-), which is comparable arteritis to CAWS 27-2.3. These facts strongly suggest that beta-1,2-linkages cover the active site, the alpha mannan moiety, of CAWS. Beta-1,2-linkage itself might not show receptor-mediated, specific and negative regulatory reactions.

Acute anaphylactoid reaction is known to be induced by lipopolysaccharide from *E. coli* O9 (O9 LPS), which possesses the mannose homopolysaccharide as the O-antigen region.²⁵⁾ Very recently, we compared immunotoxicological and immunochemical similarity between CAWS and O9 LPS.²⁴⁾ CAWS strongly reacted with *Candida* serum factors, and the reactivity was found to be partially competed with O9 LPS. CAWS-induced anaphylactoid reaction was inhibited by pretreatment of mice with i.p. injection of CAWS. The lethality was found to be inhibited by i.v. injection of O9 LPS. *Vice versa*, O9 LPS-induced acute anaphylactoid reaction in muramyl-dipeptide primed mice was also inhibited by pretreatment of mice with CAWS. These results suggested that CAWS and O9 LPS from gram-negative bacteria share, at least in part, immunochemical and immunotoxicological activities.

Mannose binding lectin (MBL) is a key molecule for innate immune response.²⁷⁾ Neth *et al.* analyzed the concentration of MBL in various inbred strains and found that all of the strains tested contained MBL.²⁸⁾ The MBL-A and MBL-C levels in 10 laboratory mice strains were 4 to 12 μ g/ml, and 16 to 118 μ g/ml, respectively. Higher concentration of MBL was detected in patients of rheumatic heart disease.²⁹⁾ Point mutation in MBL is also related to severe atherosclerosis.³⁰⁾ In the present study, we have shown contribution of the complement lectin pathway for the initiation of CAWS-induced anaphylactoid reaction and arteritis. We did not measure concentration of each complement components, and concentration of each component in inbred strains might be related to the phenotype of anaphylactoid reaction and arteritis.

The anaphylatoxin receptor is a family of G-protein coupled receptors (G-PCR).³¹⁾ It is well known that signaling of G-PCR induced cross talk and cross desensitization.³²⁾ Cross desensitization of C5a receptor was reported with receptors of fMLP, IL-8, LTB4 and so on. Exaggerated inflammatory phenotype of bleomycin-induced lung fibrosis was shown in C5 knockout mice. DBA/2 is a strain deficient in C5. Severity of arteritis might be related to cross desensitization and may follow anti-inflammatory action of C5a receptor mediated signaling.

In the series of studies, we have analyzed CAWS-induced arteritis from various points of view. The most striking result was the significant strain dependency of arteritis as well as acute anaphylactoid reaction. For example, C3H/HeN is the most sensitive strain for anaphylactoid reaction but arteritis is moderate. DBA/2 is completely resistant to anaphylactoid reaction but shows the most severe arteritis. CBA/J is the

most resistant strain for arteritis but induced anaphylactoid reaction (unpublished results). B6 is sensitive to both anaphylactoid reaction and arteritis. These facts strongly suggest that the genes responsible for anaphylactoid reaction and arteritis would be at least in part different, and thus regulated by multiple genetic factors. However, from the point of early signaling events, activation of the lectin pathway of the complement by alpha-mannan moiety of CAWS might be key for both of the activities. Several reports indicated that *Candida albicans* could bind to MBL.^{27,28)}

We have shown that CAWS-induced arteritis and anaphylactoid reaction is dependent on strains of mice. In the present study, we demonstrated the essential structure to induce arteritis and anaphylactoid reaction. Strain specificity of both reactions was independent. We also analyzed the kinetics of arteritis induction and determined that it is separated into at least three phases, *i.e.*, early, middle, and late.¹⁰⁾ In the early phase, a variety of biochemical events is induced. In the middle phase, remodeling of arteries is induced. In the late phase, arteritis and coronary arteritis stressed heart function, resulting in cardiomegaly. Undoubtedly, multiple genes are involved in arteritis and cardiomegaly. This model proved very useful for analyzing whole steps of cardiac diseases.

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REFERENCES

- Shoham S., Levitz S. M., *Br. J. Haematol.*, **129**, 569—582 (2005).
- Murata H., Iijima H., Naoe S., Atobe T., Uchiyama T., Arakawa S., *Jpn. J. Exp. Med.*, **57**, 305—313 (1987).
- Kawasaki T., Kosaki F., Okawa S., Shigematsu I., Yanagawa H., *Pediatrics*, **54**, 271—276 (1974).
- Furusko K., Kamiya T., Nakano H., Kiyosawa N., Shinomiya K., Hayashidera T., Tamura T., Hirose O., Manabe Y., Yokoyama T., *Lancet*, **2**, 1055—1058 (1984).
- Oharaseki T., Kameoka Y., Kura F., Persad A. S., Suzuki K., Naoe S., *Microbiol. Immunol.*, **49**, 181—189 (2005).
- Takahashi K., Oharaseki T., Wakayama M., Yokouchi Y., Naoe S., Murata H., *Inflamm. Res.*, **53**, 72—77 (2004).
- Kurihara K., Shingo Y., Miura N. N., Horie S., Usui Y., Adachi Y., Yadomae T., Ohno N., *Biol. Pharm. Bull.*, **26**, 233—240 (2003).
- Ohno N., *Microbiol. Immunol.*, **47**, 479—490 (2003).
- 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).
- Nagi-Miura N., Harada T., Shinohara H., Kurihara K., Adachi Y., Ishida-Okawara A., Oharaseki T., Takahashi K., Naoe S., Suzuki K., Ohno N., *Atherosclerosis*, **186**, 310—320 (2006).
- Poulain D., Jouault T., *Curr. Opin. Microbiol.*, **7**, 342—349 (2004).
- Masuoka J., *Clin. Microbiol. Rev.*, **17**, 281—310 (2004).
- Bates S., Hughes H. B., Munro C. A., Thomas W. P., Maccallum D. M., Bertram G., Atrih A., Ferguson M. A., Brown A. J., Odds F. C., Gow N. A., *J. Biol. Chem.*, **281**, 90—98 (2006).
- Arroyo-Flores B. L., Calvo-Mendez C., Flores-Carreón A., López-Romero E., *FEMS Immunol. Med. Microbiol.*, **45**, 429—434 (2005).
- Rouabhi M., Schaller M., Corbucci C., Vecchiarelli A., Prill S. K., Giasson L., Ernst J. F., *Infect. Immun.*, **73**, 4571—4580 (2005).
- Munro C. A., Bates S., Buurman E. T., Hughes H. B., Maccallum D. M., Bertram G., Atrih A., Ferguson M. A., Bain J. M., Brand A., Hamilton S., Westwater C., Thomson L. M., Brown A. J., Odds F. C., Gow N. A., *J. Biol. Chem.*, **280**, 1051—1060 (2005).
- Suzuki A., Takata Y., Oshie A., Tezuka A., Shibata N., Kobayashi H., Okawa Y., Suzuki S., *FEBS Lett.*, **373**, 275—279 (1995).
- Jones J. M., *Clin. Microbiol. Rev.*, **3**, 32—45 (1990).
- Fukazawa Y., Shinoda T., Tsuchiya T., *J. Bacteriol.*, **95**, 754—763 (1968).
- Kobayashi H., Takahashi S., Shibata N., Miyauchi M., Ishida M., Sato J., Maeda K., Suzuki S., *Infect. Immun.*, **62**, 968—973 (1994).
- Okawa Y., Goto K., Nemoto S., Akashi M., Sugawara C., Hanzawa M., Kawamata M., Takahata T., Shibata N., Kobayashi H., Suzuki S., *Clin. Diagn. Lab. Immunol.*, **3**, 331—336 (1996).
- Shibata N., Ikuta K., Imai T., Satoh Y., Satoh R., Suzuki A., Kojima C., Kobayashi H., Hisamichi K., Suzuki S., *J. Biol. Chem.*, **270**, 1113—1122 (1995).
- Okawa Y., Oikawa S., Suzuki S., *Biol. Pharm. Bull.*, **29**, 388—391 (2006).
- Tada R., Nagi-Miura N., Adachi Y., Ohno N., *Biol. Pharm. Bull.*, **29**, 240—246 (2006).
- Zhao L., Ohtaki Y., Yamaguchi K., Matsushita M., Fujita T., Yokochi T., Takada H., Endo Y., *Blood*, **100**, 3233—3239 (2002).
- Miyazaki T., Kohn S., Mitsutake K., Maesaki S., Tanaka K., Ishikawa N., Hara K., *J. Clin. Microbiol.*, **33**, 3115—3118 (1995).
- Ip W. K., Lau Y. L., *J. Infect. Dis.*, **190**, 632—640 (2004).
- Neth O., Jack D. L., Dodds A. W., Holzel H., Klein N. J., Turner M. W., *Infect. Immun.*, **68**, 688—693 (2000).
- Schafrański M. D., Stier A., Nishihara R., Messias-Reason I. J., *Clin. Exp. Immunol.*, **138**, 521—525 (2004).
- Hegele R. A., Ban M. R., Anderson C. M., Spence J. D., *J. Invest. Med.*, **48**, 198—202 (2000).
- Richardson R. M., Ali H., Tomhave E. D., Haribabu B., Snyderman R., *J. Biol. Chem.*, **270**, 27829—27833 (1995).
- Addis-Lieser E., Kohl J., Chiaramonte M. G., *J. Immunol.*, **175**, 1894—1902 (2005).