present in those with atherosclerosis are known to express MPO [48]. It was reported that GM-CSF treatment resulted in a significant amount of MPO in activated macrophages. These findings strongly suggested that GM-CSF activates both neutrophils and macrophages to aggravate inflammatory responses.

Activity of GM-CSF is mediated by the specific GM-CSF receptor. Metcalf et al. precisely examined the clearance of GM-CSF from the bloodstream and found that its uptake by the specific receptor is very high, thus only a limited percentage of GM-CSF could be detected in the blood in a very short period [49]. In the present study, splenomegaly characterized by increased numbers of neutrophils and macrophages was induced in both strains of mice, but was severer in DBA/2. A major cytokine responsible for splenomegaly would be GM-CSF and in the experiments in vitro, GM-CSF was only detected in DBA/2. Considering Metcalf's observation, this does not mean that C3H/HeN mice did not produce GM-CSF at all, rather, the quantity is not great enough to register in culture supernatant. As shown in Fig. 3, splenic cell numbers increased in both strains in response to CAWS and were maintained for more than 5 weeks. The splenomegaly would be due, at least in part to GM-CSF acting as a growth factor for both strains. It is also suggested that DBA/2 might be produced higher concentration of GM-CSF, and thus excessive GM-CSF has an additional function for example in the differentiation, maturation and activation of leukocytes, and enhanced production of IFN-γ, [50,51] resulting in augmented cytokine production.

As shown in Figs. 4 and 6, the production of IFN-γ and release of MPO by splenocytes was most intense in the mice just after the administration of CAWS. This observation would also be related to the production of GM-CSF. From the results of the experiment in vitro shown in Fig. 8, IFN-γ production was significantly enhanced in the presence of GM-CSF. Harada et al reported that IFN-γ synthesis is induced by cell–cell contact between the adherent and non-adherent populations [35,36]. In addition, this reaction did not occur in nude mice, strongly suggesting a crucial role for the mature T cell populations. In a normal host, the T lymphocyte represents the major cellular source of IFN-γ. All $CD8^+$ T cell populations and certain subsets of $CD4^+$ T cells can produce the protein in response to antigenic stimulation mediated by antigen presenting cells [50]. Frucht et al. proposed the "jump start model" for production of IFN-γ by various types of antigen presenting cells such as resting peritoneal, peritoneal exudates, alveolar, bone-marrow-derived and splenic macrophages, and both $CD8^+$ and $CD8^-$ DCs [51]. Even though the type of cell synthesizing IFN-γ in this study was not identified and should soon be clarified, IFN-γ is a key cytokine for the maturation and activation of a variety of lymphocytes, and is closely involved in the induction of arteritis.

Previously, we have shown that CBA/j mice were resistant to CAWS-induced vasculitis and the resistance is strongly related to high levels of IL-10 [33]. In the present study, we showed that GM-CSF is the factor exacerbating the arteritis. IL-10 is known to be immunosuppressive. Hashimoto et al reported that IL-10 suppressed macrophage survival promoted by GM-CSF [52]. Some other reports have shown that IL-10 competes with GM-CSF [53–55]. In pulmonary alveolar proteinosis, IL-10 inhibited the synthesis of GM-CSF [56]. All these findings strongly support the underlying mechanism of CAWS-induced arteritis.

The identification of candidate genes involved in disease susceptibility is important for understanding diseases at a molecular level. It is also important for medical treatment and public health, such as for predicting the effects of medicines and side effects, and controlling the intake of foods for preventing diabetes, hyperlipidemia and cancer. The identification of such genes in experimental animals gives indispensable information for understanding human diseases. The major histocompatilibity complex is one of the most important genes for immune responses and several autoimmune diseases are known to be associated with a specific MHC. It is well recognized that collagen-induced rheumatoid arthritis (CIA) is specifically induced in DBA/1 mice which carry H2-q [57,58]. In the case of CAWS-induced coronary arteritis, we have already found it to be induced in a variety of inbred strains, such as A/J (H2-a), C57Bl (H2-b), BALB/c (H2-d), DBA/2 (H2-d), C3H (H2-k) and DBA/1 (H2-q), with the vasculitis in DBA/2 being lethal (unpublished results). These findings strongly suggested that MHC is not the major gene for determining susceptibility to CAWS-induced vasculitis. The pathogenesis of heart failure is well known to be complex, polygenic and multifactorial, thus the identification of candidate genes is difficult. Suzuki et al. analyzed genes for murine dilated cardiomyopathy, by establishing transgenic mice that develop severe dilated cardiomyopathy due to the cardiac-specific overexpression of calsequestrin [59]. A reciprocal backcross strategy was employed using two inbred strains, DBA/2 and C57Bl/6, showing distinct differences in survival and cardiac function and identified two loci significantly linked to survival on chromosomes 2 and 3, and one locus significantly linked to cardiac function on chromosome 3.

Matsumori and co-workers have developed a murine model of viral myocarditis induced by the encephalomyocarditis (EMC) virus in which severe myocarditis, congestive heart failure and dilated cardiomyopathy occur with high incidence [60–62]. When various inbred strains were tested, myocardial lesions developed in BALB/c (48.7%), C3H/He (61.8%) and DBA/2 (66.1%). In C3H/He and DBA/2 mice, dilatation and hypertrophy of the heart accompanying myocardial lesions persisted up to the 8th month after the virus' inoculation. In contrast, no pathologic findings were noted in A/J and C57BL/6 mice. Compared with CAWS-induced vasculitis, the susceptibility to this viral infection-triggered model has a narrow strain specificity. Considering these findings, CAWS-induced vasculitis might also be regulated by a polygene.

In the present study, we have identified GM-CSF as the principal cytokine responsible for exacerbating coronary

arteritis. The arteritis induced by CAWS in DBA/2 mice is lethal, but not only local inflammatory reactions but also systemic inflammation would be important. We have already described the biological activities of CAWS including, activation of complement, down-regulation of thrombomoduline synthesis, activation of neutrophils and macrophages, and so on [30,33]. The severity of arteritis might be regulated by each of these factors and related modulators. Research is in progress to clarify the molecular mechanism.

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Neutrophil activation and induced by *C. albicans* water-soluble mannoprotein-β-glucan complex (CAWS)

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Abstract

We have established a mouse model which shows the symptoms of coronary arteritis after consecutive injections of CAWS, which is released from *Candida albicans*. In this study, we examined neutrophil activation in the initial period after CAWS injection intraperitoneally. During 10 min to 16 h after the injection, blood profiles and neutrophil functions were determined. At the same time, levels of inflammatory cytokines and chemokines in plasma were measured. Furthermore, level of ICAM-1 as a marker of lesion endothelial cell. Counts of the peripheral leukocytes increased immediately after CAWS injection, especially neutrophil. In vitro sensitivity of neutrophils to stimuli was enhanced. Moreover, proinflammatory cytokines (IL-1β, IL-12 and IL-6) in plasma initially followed by an increase in IL-10, G-CSF, MIP-2 and soluble ICAM-1. Locally, ICAM-1 message in arterial walls was significantly increased 16 h after CAWS injection. A decrease in C3 levels was observed in plasma, suggesting complement activation and consumption. In summary, neutrophil activation occurred after CAWS injection, followed by complement activation, and production of proinflammatory cytokines chemokines and G-CSF which may be involved in development of coronary arteritis.

Keywords: Complement activation; Inflammatory cytokines; Endothelium injury

Introduction

Recently, clinical relevance of fungal diseases has increased, mainly because of an increasing population of immunocompromised individuals including those infected with HIV, transplant recipients and patients with cancer (Zupanic-Krmek and Nemet, 2004). Neutrophils play a key role during *Candida albicans* infection (Urban et al., 2006; Fradin et al., 2005). *C. albicans* exposed to erythrocytes, mononuclear cells, plasma or blood lacking neutrophils physiologically active and rapidly switch to filamentous growth. In contrast, the presence of neutrophils arrested *C. albicans* growth (Urban et al., 2006; Fradin et al., 2005). Recently, immune responses to some components of *C. albicans* such as mannan (Muller and Melchinger, 2004) or β-glucan (Gantner et al., 2005) have been reported.

Abbreviations: CAWS, *C. albicans* water-soluble mannoprotein-β-glucan complex; fMLP, fMet-Leu-Phe.

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Animal model is useful to clarify mechanism of infection process, genetic background differences and to strategy for therapy (Nagi-Miura et al., 2004; Aratani et al., 2002; Spellberg et al., 2005).

Ome microorganisms or microorganism-derived products are specific diseases. For example, certain *Campylobacter jejuni* strains trigger Guillain–Barre syndrome while other strains trigger the Fisher syndrome in the Japanese population (Takahashi et al., 2005). Esper et al. have reported that New Haven coronavirus (HCoV-NH) infection is associated with Kawasaki disease (Esper et al., 2005). Recently, Iwai et al. have identified oral microorganisms in the lesions of Buerger disease, suggesting a possible etiologic link between Buerger disease and chronic infections such as oral bacterial infections (Iwai et al., 2005). We have established a mouse model which shows the symptoms of coronary arteritis following consecutive injections into mice of a water-soluble polysaccharide (CAWS) (Nagi-Miura et al., 2004; Ohno, 2003). In this study, we examined neutrophil activation and subsequent events after a single injection of CAWS (Fig. 1).

Materials and methods

Reagents

ELISA kits for mouse IL-1β, IL-6, IL-10, IL-12 p70, IFN-γ, TNF-α were purchased from BD Biosciences (CA, USA), IL-18 was from Medical and Biological Laboratories (Tokyo, Japan), soluble ICAM-1 and MIP-2 were from R&D Systems (MN, USA), G-CSF and GM-CSF were from AN'ALYZA (MN, US). fMet-Leu-Phe (fMLP) was purchased from Peptide Institute (Osaka, Japan), 3,3, 5,5-tetrametylbendizine (TMB), cytochalasin B (CB), cytochrome *c*, RPMI 1640 medium, aprotinin and PMSF were purchased from Sigma Chemical Co. (MO, USA). Casein was purchased from Calbiochem. Co. (Darmstadt, Germany). TaqMan Universal PCR master mix was purchased from PE Biosystems (NJ, USA). Monoclonal antibody and peroxidase-conjugated IgG fraction to mouse C3 were purchased from HyCult biotechnology (Uden, The Netherlands) and MP Biochemicals, Inc. (CA, USA), respectively.

Mice

C57BL/6N male mice were purchased from Charles River Japan, kept under SPF conditions, and used according to a guideline for animal care of the National Institute of Infectious Diseases. The mice were used in the experiment at 6 weeks of age.

Preparation and administration of CAWS

CAWS was prepared from *C. albicans* strain IFO1385 in accordance with conventional methods (Nagi-Miura et al., 2004). CAWS was dissolved in PBS autoclaving, 0.2 ml of the (20 mg/ml) was intraperitoneally injected into a mouse. For vitro assay, neutrophils isolated from 8% casein-induced peritoneal exudated cells were co-cultured with CAWS in RPMI medium containing 0.3 mM PMSF and 0.4 μg/ml aprotinin.

Histological observations of coronary arteritis

Coronary artery segments were fixed in 10% buffered (pH7.2) formaldehyde, paraffin-embedded, and sections stained with hematoxylin and eosin.

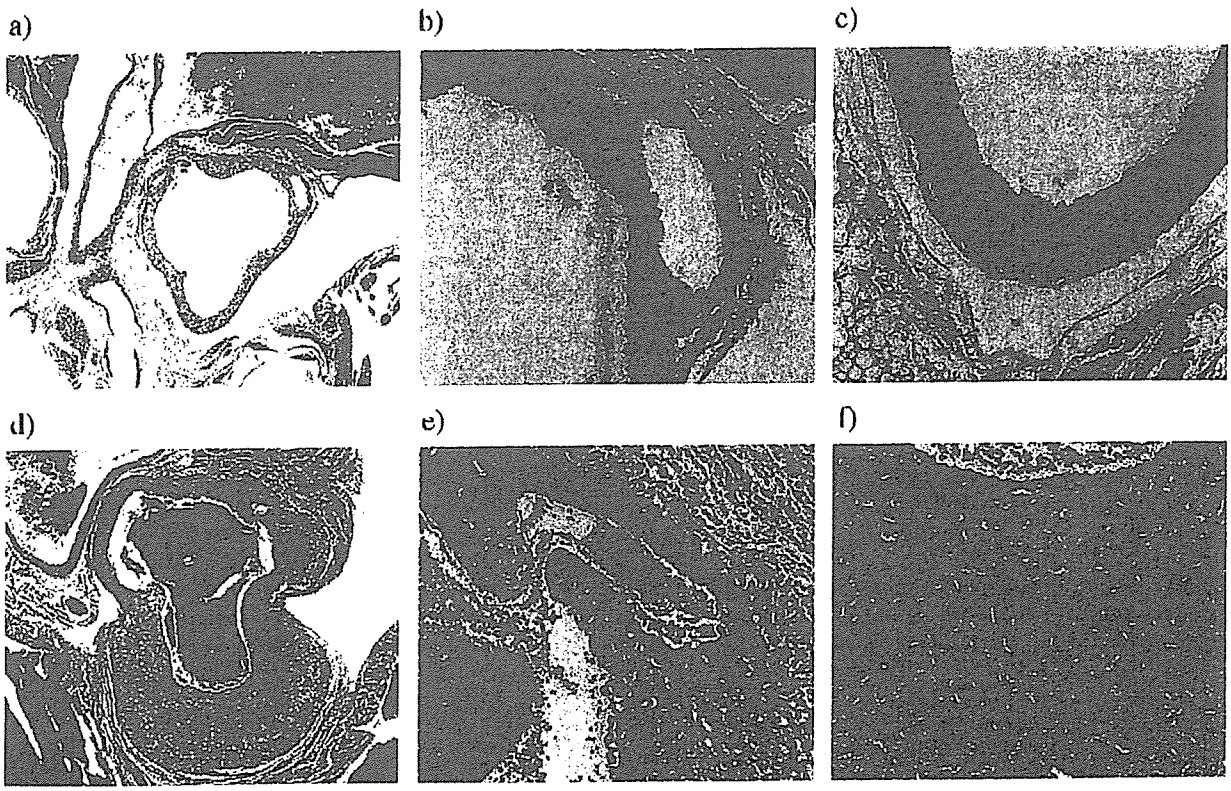

Fig. 1. Histological analysis of coronary arteritis administration of a single dose of CAWS. Normal (a) aortic root including coronary artery ×40, (b) coronary artery ×200 and (c) aorta, ×200. Histology 4 weeks after CAWS injection a single dose of 4 mg/mouse vasculitis at the coronary artery and aortic root, ×40, (e) coronary arteritis, ×200, (f) aortitis, ×200.

Analysis of leukocytes profile

Peripheral blood was collected from heart and EDTA-2K was added. Leukocyte profiles were analyzed by use of automation instruments (Mitsubishi BCL Corp., Tokyo, Japan).

Flow cytometric analysis

Peripheral blood was collected from heart and EDTA-2K was added. After lysis for 5 min at 4 °C, subsequent centrifugation (1000×*g* at 4 °C, cells) was suspended in HBSS. Bone marrow cells were prepared from a shinbone. The cells were incubated with mAb against surface markers (BD Pharmingen, CA, USA). Gr-1 (RB6-8C5), Mac-1 (M1/70) were used for flow cytometric analysis, and then analyzed with FACSCalibur using CellQuest software (BD Biosciences, CA, USA).

Neutrophil function assay

Heparinized blood was collected from heart, and neutrophil was prepared. MPO release and superoxide generation were measured as described previously (Ishida-Okawara et al., 1991).

Measurement of proinflammatory cytokines and chemokine in plasma

Levels of proinflammatory cytokines IL-1β, IL-6, IL-10, IL-12, IL-18, TNF-α, INF-γ, MIP-2, soluble ICAM-1, G-CSF, GM-CSF in plasma using individual ELISA.

Preparation of peritoneal exudate neutrophils and measurement of cytokine production level by co-cultured neutrophil with CAWS

Normal C57BL/6N mice were intraperitoneally injected with 4 ml of 8% casein in PBS and exudate cells containing resident macrophage or casein-induced neutrophils were recovered 8 h lavage with 5 ml of PBS. The exudate cells were put onto M-SMF (Japan Immunoresearch Laboratories Co., Ltd., Takasaki, Japan), centrifuged 1200×*g* for 20 min at room temperature. Neutrophils were suspended (5×10^6 cells/ml) in RPMI-1640 medium containing 0.3 mM PMSF and 0.4 μg/ml aprotinin and co-cultured with 1 mg/ml CAWS for 0.5 to 4 h. At the end of culture, culture supernatant was prepared by centrifugation and levels of cytokines IL-1β, IL-6, IL-10 determined using by ELISA kits.

Measurement of ICAM-1 mRNA expressed in aortic wall after CAWS injection

The thoracic aortas of mice were was isolated and frozen immediately for detection of ICAM-1 mRNA. The total aortic RNA for each mouse was isolated using ISOGEN (Nippon ene, Tokyo Japan). One microgram of RNA was reverse transcribed with ReverTra Ace (Toyobo, Osaka, Japan) to obtain cDNA. Real-time PCR was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA, US) according to the manufacture's protocol. Primers and TaqMan probes specific for ICAM-1 was obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems). For endogenous control, the level of GAPDH in each sample was measured using TaqMan Rodent GAPDH Control Reagents VIC (Applied Biosystems). Data analyses were performed on ABI PRISM 7000 SDS software version 1.0 (Applied Biosystems).

Detection of mouse complement 3 (C3) by ELISA and Western blotting

Peripheral blood was collected from heart and EDTA-2K was added. One μg/ml of C3 monoclonal antibody was coated to ELISA plate for overnight. After washing, blocking was done by 50% FCS for 1 h. Sample plasma (×100) was added to the plate for 1 h, after washing peroxidase labeled-2nd antibody (×1000) was added to the plate. After washing, *o*-phenylenediamine chloride was added to the plate for 3 min. Finally, reaction was stopped 2 N hydrogen sulphate and measured at 490 nm by auto reader (Nippon Bunko, Tokyo, Japan). Western blotting was performed by using mouse C3 monoclonal antibody and peroxidase-labeled anti-Rat IgG as 2nd antibody.

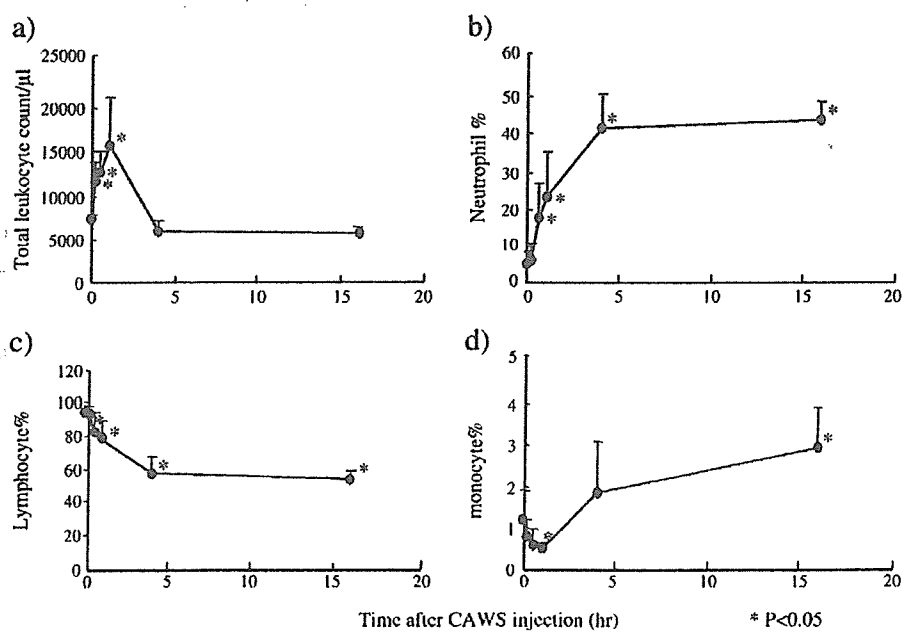

Fig. 2. Change of blood profile after CAWS injection. Peripheral blood was collected from heart EDTA-2K. Blood profile was analyzed by Mitsubishi Kagaku BCL Inc. (a) total leukocyte number, ratio of (b) neutrophil, (c) lymphocyte and (d) monocyte, respectively. $N=6$ in each group.

t1.1 Table 1
t1.2 Change of Gr-1$^+$ cells ratio after CAWS injection

t1.3		Time after CAWS injection (hr)					
t1.4		0	0.17	0.5	1	4	16
t1.5	Bone marrow	19.47±5.35	15.85±1.89	9.65±1.14 *	10.42±1.26 *	7.39±1.97 *	11.73±6.58
t1.6	Peripheral blood	5.24±6.92	5.30±0.36	10.35±2.00 *	9.78±3.59	8.06±6.69	6.12±5.34

t1.7 * $P<0.05$.

Statistical analysis

Results expressed as the mean±SD. Statistical analysis was using Mann–Whitney *U*-test. The probability value of <0.05 considered significant.

Results

Observation of coronary arteritis administration of a single dose of CAWS

We have severe coronary arteritis by daily injection of CAWS (4 mg/mouse/day) for 5 days. In order to compare initial events and development of coronary arteritis, the current studies employed a one shot injection of CAWS at a dose of 4 mg/mouse. Four weeks after CAWS injection with this protocol, arteritis was evident in both coronary and aorta.

Profiles of peripheral leukocytes

After CAWS injection, total leukocyte counts were increased and later returned to baseline levels 4 h (Fig. 2a). The ratio of lymphocytes decreased after CAWS injection, while ratio of neutrophils promptly increased (Figs. 2b, c), the ratio of monocyte increased (Fig. 2d).

Profile of leukocyte by flow cytometric analysis

To confirm the leukocyte counts, flow cytometric analysis of peripheral and bone marrow cells after CAWS injection was done (Table 1). Bone marrow cell numbers the tibia were significantly increased 4 h after CAWS injection (data not shown). The ratio of Gr-1 cells in bone marrow significantly decreased at 0.5, 1, 4 h after CAWS injection, while the ratio of the cells in peripheral blood increased suggesting that neutrophils in bone marrow promptly migrate to peripheral blood CAWS injection.

CAWS effect on neutrophil activation

To the functional response of neutrophils after CAWS injection, MPO release and superoxide generation were

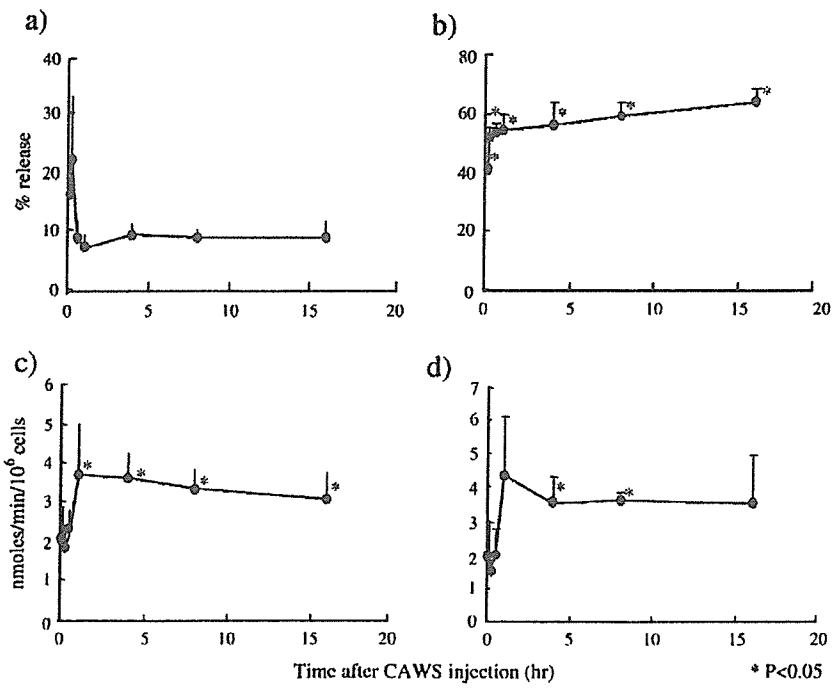

Fig. 3. Neutrophil activation after CAWS injection. (a, b) MPO release from neutrophils after CAWS injection. Neutrophils (10^6 cells/ml) were stimulated in the (a) absence, or (b) presence of (10^{-5} M) and (5 μg/ml). (c, d) Superoxide generation after CAWS injection. Neutrophils (10^6 cells/ml) were stimulated with (c) (10^{-5} M) and (5 μg/ml) or (d) PMA (1 μg/ml). $N=6$ in each group.

examined (Fig. 3). fMLP-induced MPO release from neutrophils was enhanced after CAWS injection (Fig. 3b), while spontaneous MPO release was not changed (Fig. 3a). In addition, both fMLP and PMA-induced superoxide generation was enhanced following in vivo CAWS injection (Figs. 3c, d).

CAWS effects on proinflammatory cytokine production

Since activation of neutrophils was observed, after in vivo injection of CAWS, proinflammatory cytokines levels in plasma were measured (Fig. 4). IL-12 p70 production was increased IL-1β (b), IL-10 (c), 1L-6 (d) significantly increased CAWS injection. Levels of MIP-2 and G-CSF in plasma increased after CAWS injection and 16 h after CAWS injection (Figs. 4e, f). On the other hand, IL-18, TNF-α, INF-γ, GM-CSF were not detected up to 16 h after CAWS injection (data not shown). Since IL-1β, IL-10, IL-6 was significantly increased, production of these cytokines by casein-induced neutrophils was also measured (Fig. 5). IL-6 production was enhanced by exposure of neutrophils to CAWS (Fig. 5c), while IL-11L-10 was nearly the same in presence or absence of CAWS (Figs. 5a, b).

CAWS effect of ICAM-1 expression and soluble ICAM release

Sine ICAM-1 is a marker of activation of endothelial cells, we ICAM-1 gradually increased in plasma after CAWS injection (Fig. 6a). In addition, ICAM-1 the thoracic aortic wall was also significantly increased 16 h after CAWS injection (Fig. 6b).

CAWS C3 activation

Activation of C3 was examined (Fig. 7). C3 decreased time dependently after CAWS injection and gradually (Fig. 7a). This was confirmed by Western blotting analysis (Fig. 7b). These results suggest that neutrophil activation triggered through complement activation by a single injection.

Discussion

In study, we focused on neutrophil activation related to development of coronary arteritis. Single injection of CAWS at a dose of 4 mg/mouse induced coronary arteritis 4 weeks featuring neutrophil accumulation in the coronary arterial wall. This observation was similar to the

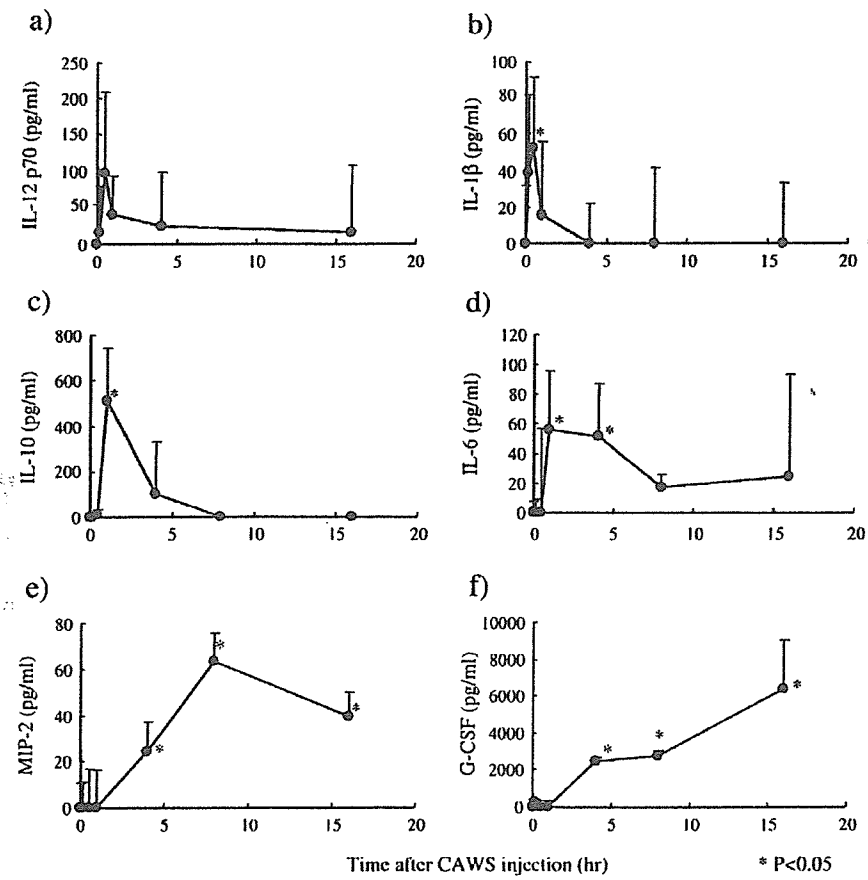

Fig. 4. Cytokine after CAWS injection. Heparinized blood was obtained from heart after CAWS injection. Plasma was separated and proinflammatory cytokines level was measured by ELISA kit. IL-12 p70, (b) IL-1β, (c) IL-10, (d) IL-6, (e) MIP-1, (f) G-CSF, respectively. $N=6$ in each group.

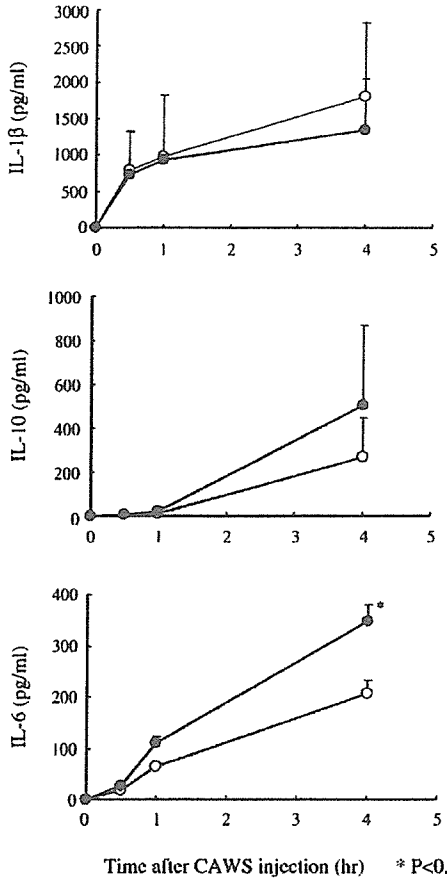

Fig. 5. Cytokine production by neutrophil. Casein-induced neutrophil (5×10^6 cells/ml) suspended in RPMI-1640 containing 0.3 mM PMSF and 1.4 μg/ml aprotinin and co-cultured with 1 mg/ml CAWS for 0.5 to 4 h. culture supernatant was prepared by centrifugation and level of cytokine. (a) IL-1β, (b) IL-10 and (c) IL-6, respectively. O not treated by CAWS ● 1 mg/ml of CAWS. N=3–6 in each group.

development of coronary arteritis daily injection of CAWS (4 mg/mouse/day) 5 days (Nagi-Miura et al., 2004; Ohno, 2003).

Blood neutrophils and subsequently monocyte increased, while lymphocyte counts decreased. In addition, the ratio of Gr-1 cells in bone marrow decreased, suggesting migration of neutrophils from bone marrow into peripheral blood after CAWS injection. G-CSF after CAWS injection, fMLP-induced MPO release from neutrophils was enhanced as was PMA and fMLP-induced superoxide generation in vivo injection. These results suggest that number of blood neutrophils their activation early after CAWS injection.

Levels of IL-1β, IL-6, and 1L-10 significantly increased in plasma after CAWS injection. These cytokines are also blood leukocytes live *C. albicans* (Netea et al., 2002; Gasparoto et al., 2004). The of cytokines seems to be different depend on *Candida* strain, component and virulence (Villar et al., 2005). With regard to casein-induced neutrophils, IL-6 was significantly enhanced by co-culture with CAWS, but IL-1β and 1L-10 production virtually unchanged in presence of CAWS. These results suggest IL-6 production especially enhanced by CAWS. MIP-2 and G-CSF in plasma were maintained for 16 h, neutrophil levels in blood MIP-2 and G-CSF in blood.

Because of increase and activation of peripheral neutrophils, complement activation products may well be candidate because C5a is a chemoattractant for neutrophils (Guo and Ward, 2005). Ohno et al. have already demonstrated activation of the lectin complement pathway by CAWS (unpublished). Mullick A. et al. have confirmed dysregulated cytokine response during *C. albicans* infection (Mullick et al., 2004).

Soluble ICAM-1, which is a marker of activated endothelial cells (Iiyama et al., 1999), in blood increased after CAWS injection. In addition, ICAM-1 message was significantly increased in the thoracic aortic wall 16 h after CAWS injection. Both systemic and local increases in ICAM-1 could be involved to subsequent endothelial cell lesion development (Di Lorenzo et al., 2004).

In summary, increased numbers and activation of peripheral blood neutrophils are the initial events after CAWS injection, perhaps followed by macrophage activation and adaptive immune responses. Neutrophil a primary role in biodefense

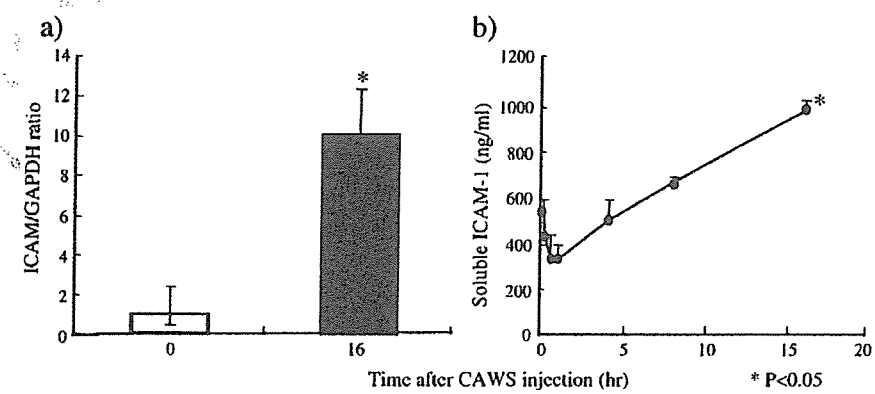

Fig. 6. Soluble ICAM-1 in plasma and ICAM-1 mRNA in aorta after CAWS injection. (a) Heparinized blood was obtained from heart after CAWS injection. Plasma was separated and ICAM-1level was measured by ELISA kit. (b) Total RNA was extracted and mRNA isolated. cDNA was prepared from 1 μg of mRNA. Real-time PCR was performed and analyzed. GAPDH was used as an internal control. White bar shows not treated by CAWS, black bar shows treated by 4 mg/mouse of CAWS, respectively. N=6 in each group.

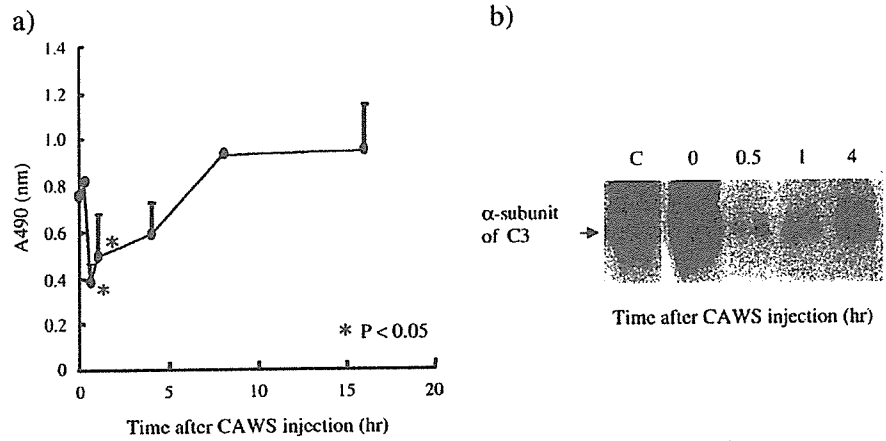

Fig. 7. Mouse complement 3 (C3) was assessed in plasma by sandwich ELISA and Western blotting. 10 mM EDTA treated blood was prepared from heart after CAWS injection. (a) C3 protein was detected by sandwich ELISA using monoclonal and peroxidase-labeled polyclonal antibody, (b) α chain of C3 was detected by monoclonal antibody to mouse C3.

against *C. albicans* or its products. Arteritis induced by CAWS might be.

Uncited reference

Collins et al., 2000

Acknowledgments

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Highlighted paper selected by Editor-in-chief

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 treatment 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 alfa- and beta-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 beta-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 H_3PO_4, 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 μg/mouse).

NMR Spectroscopy H1-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 D_2O 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.

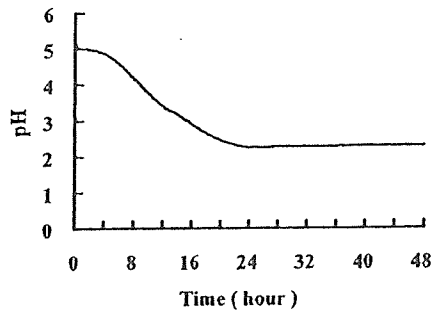

Fig. 1. Monitoring pH of *C. albicans* IFO1385 Culture in C-Limiting Medium at 27 °C

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.

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

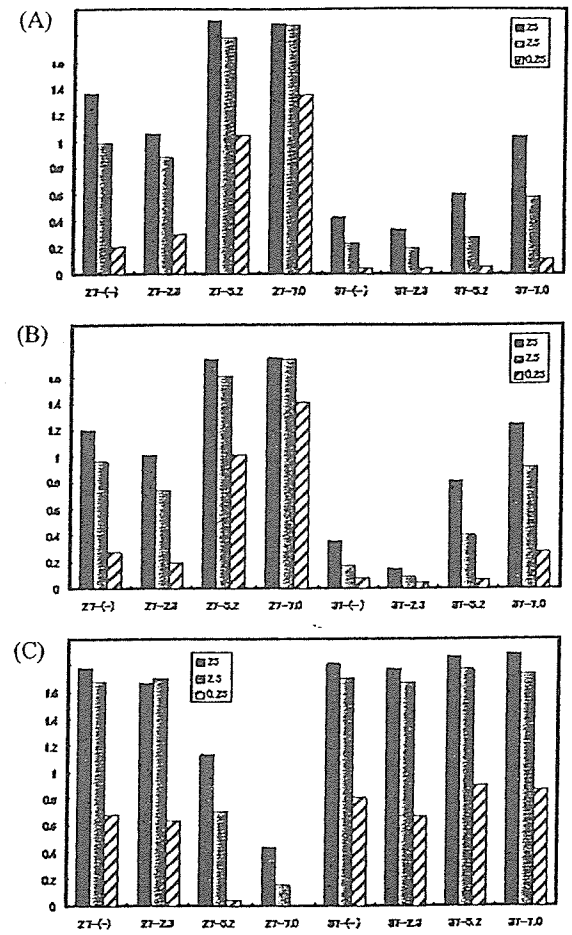

Fig. 2. Reactivity of CAWS Prepared under Various Conditions to *Candida* Typing Sera no. 5, 6, and 11

Each 25, 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.

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 analysys (carbon, hydrogen, nitrogen), factor G; content of 1,3-beta-glucan (ng/ml), factor C; content of endotoxin (ng/ml), ND; not detected.

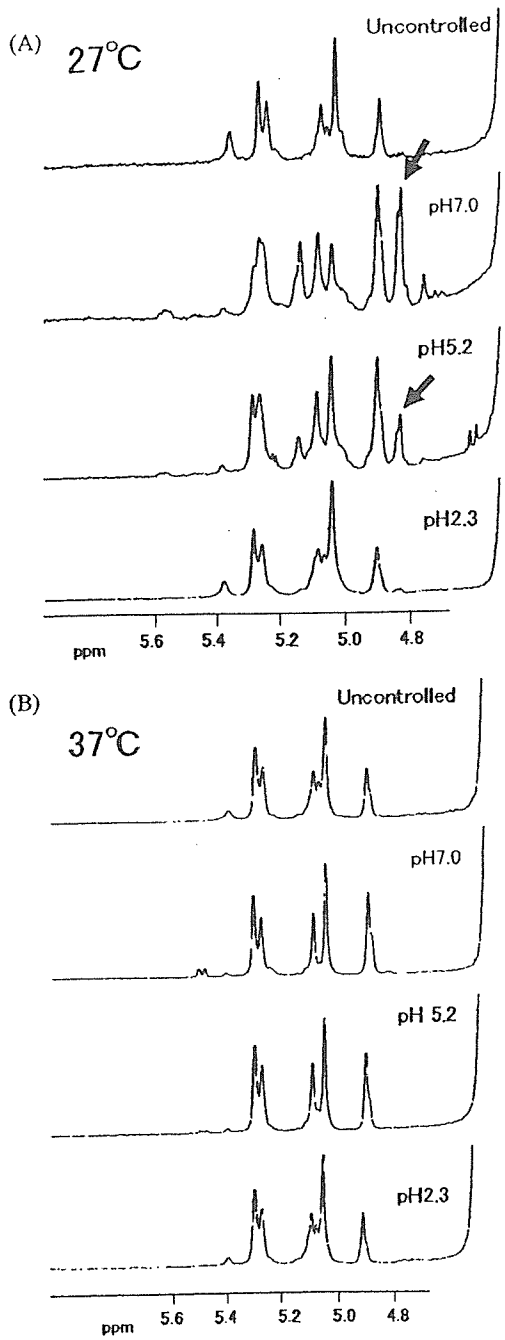

Fig. 3. Proton-NMR Spectra of CAWS in D_2O

(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 mannooligosaccharides, and no. 11 for the alpha-1,2 and 1,6-mannooligosaccharides.[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 alfa-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 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.

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 (μg/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.

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 μg/mouse. However, CAWS 27-5.2 required 3200 μg 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 μg 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 (μg/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.

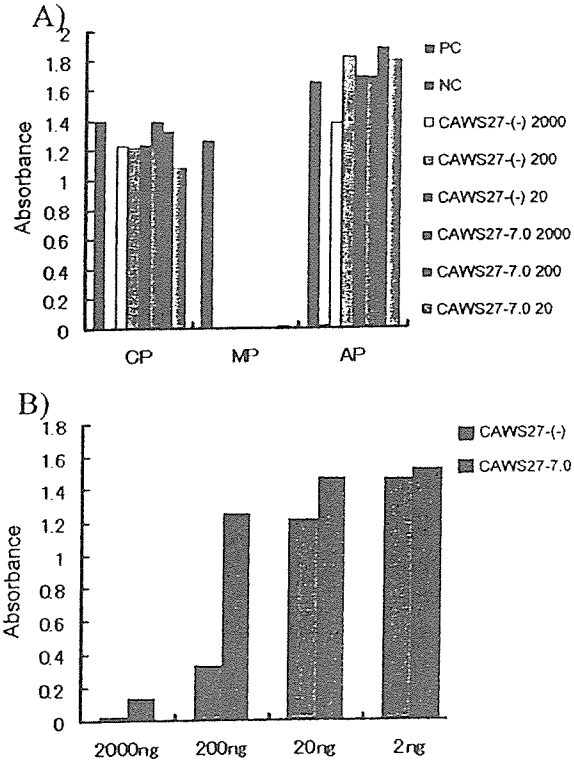

Fig. 4. Complement Activation by CAWS

(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-mannooligosaccharides 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 μg/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 between 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 mannooligosaccharides inhibited complement activation by alfa-linked mannose residues.

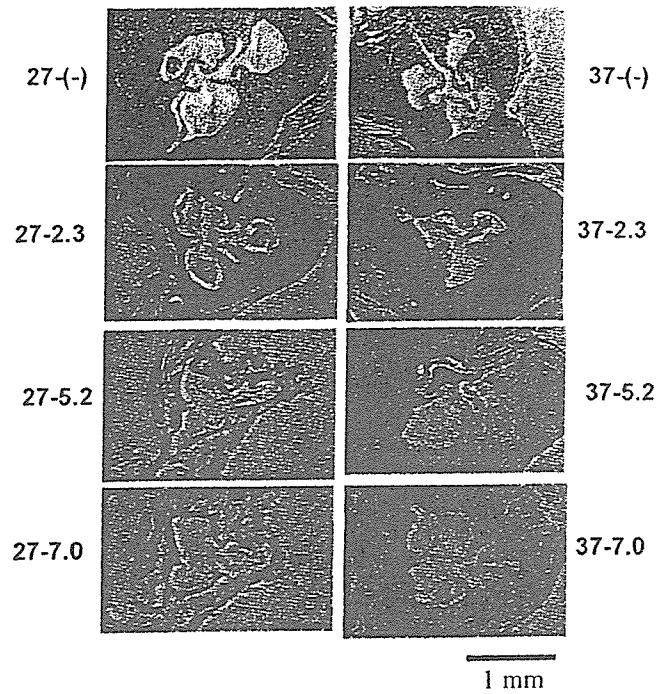

Fig. 5. Histological Analysis of DBA/2 Mice Administered with CAWS

CAWS prepared under various conditions (4 mg/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.

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 μg/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

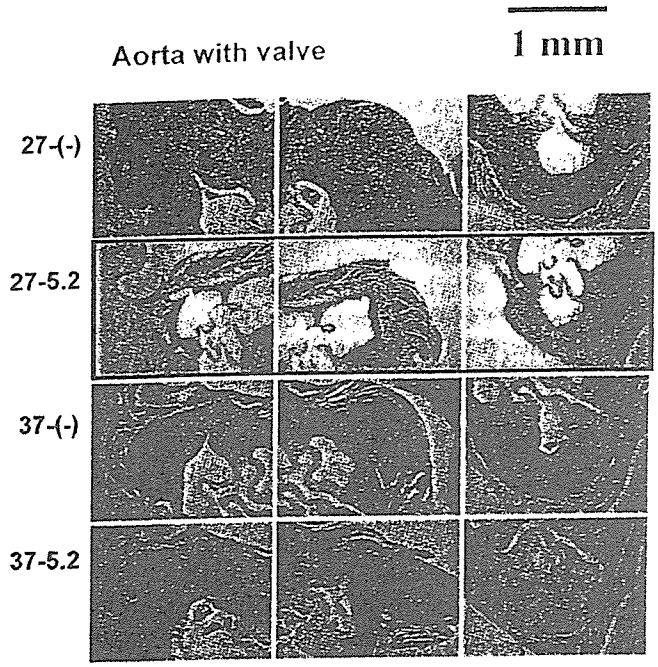

Fig. 6. Comparison of Histology of Aorta in DBA/2 Mice Administered Native and pH-Controlled CAWS

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.

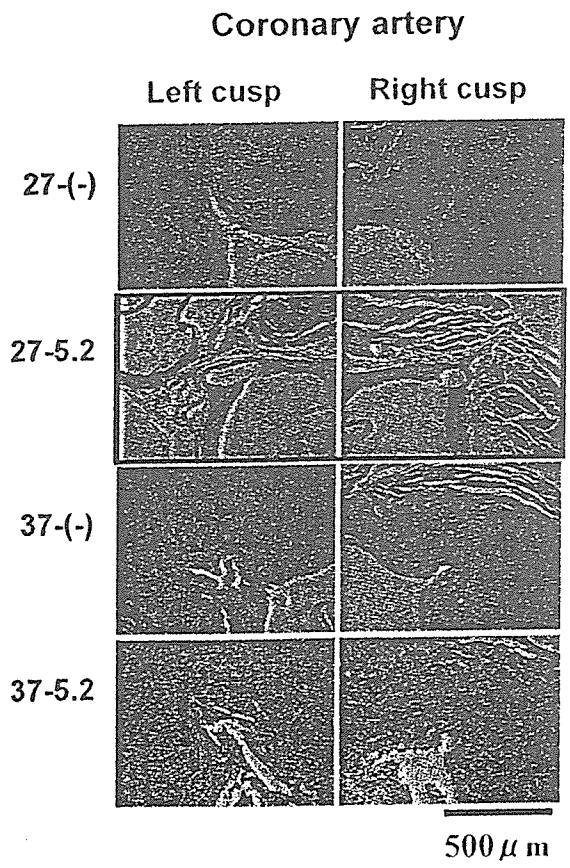

Fig. 7. Comparison of Histology of Cusp Region of Coronary Artery in DBA/2 Mice Administered Native and pH-Controlled CAWS

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

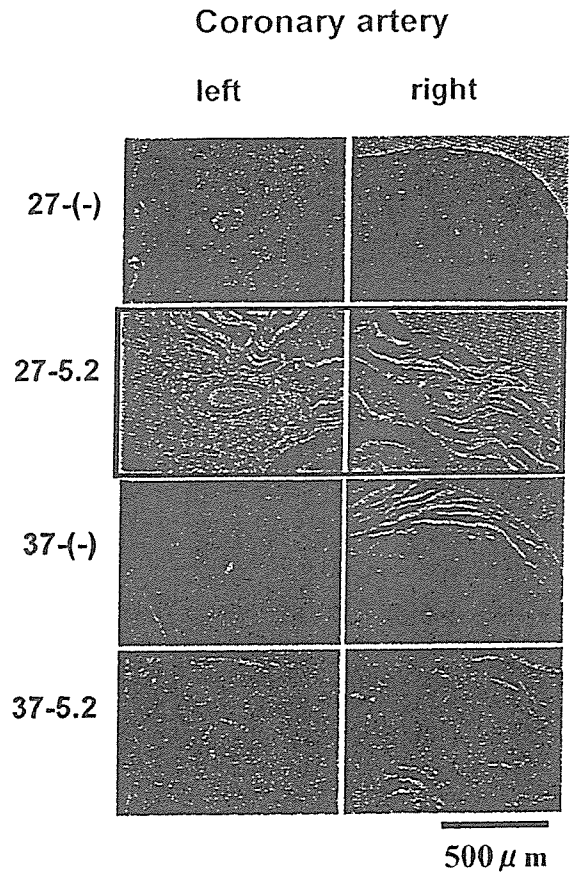

Fig. 8. Comparison of Histology of Coronary Artery in DBA/2 Mice Administered Native and pH-Controlled CAWS

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.

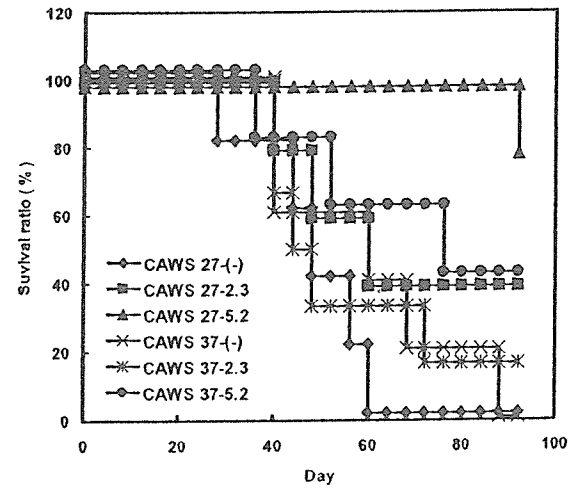

Fig. 9. Survival Time of CAWS-Administered Mice

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.

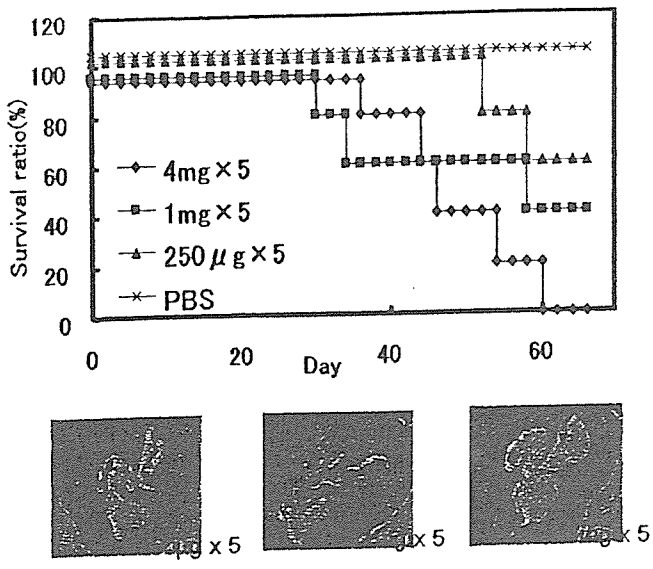

Fig. 10. Dose Response of Survival Time of CAWS-Administered Mice

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 μg/ml, and 16 to 118 μg/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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Contribution of the myeloperoxidase-dependent oxidative system to host defence against *Cryptococcus neoformans*

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The *in vivo* contribution of reactive oxygen species produced by neutrophils against *Cryptococcus* infection is not widely recognized. Myeloperoxidase (MPO) is a neutrophil-specific enzyme that catalyses the production of hypohalous acids such as HOCl from H_2O_2. This study investigated the role of MPO in immunological defence against *Cryptococcus neoformans* in an MPO-deficient ($MPO^{-/-}$) mouse model. The survival of $MPO^{-/-}$ mice infected either intranasally or intravenously with *C. neoformans* was lower than that of identically challenged wild-type mice. The $MPO^{-/-}$ mice that received intranasal injection of *C. neoformans* had significantly larger lung fungal burdens than wild-type mice. On day 7, $MPO^{-/-}$ mice had a significantly higher lung concentration of interleukin (IL)-4 and lower concentrations of IL-2, IL-12p70 and interferon (IFN)-γ than wild-type mice, suggesting a weak Th1 response in the $MPO^{-/-}$ mice to *C. neoformans*. Pathologically, the $MPO^{-/-}$ mice with intranasal infection showed more severe pneumonia than wild-type mice, which was associated with an increase in the levels of IL-1α/β in the lungs. In addition, in $MPO^{-/-}$ mice, the pulmonary infection disseminated to the brain with occasional meningitis. The keratinocyte-derived cytokine (KC) level in the brain of infected $MPO^{-/-}$ mice was higher than that of control mice. Both intranasal and intravenous infections resulted in a higher number of fungi in the spleen of $MPO^{-/-}$ mice compared to wild-type, suggesting decreased resistance to *C. neoformans* not only in the lungs but also in the spleen in the absence of MPO. Taken together, these data suggest a major role of MPO in the response to cryptococcal infection.

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INTRODUCTION

Cryptococcus neoformans is an encapsulated fungus that causes life-threatening infections, particularly in patients with impaired cell-mediated immunity (Chuck & Sande, 1989). The lung is the initial site of *C. neoformans* infection and elicits an influx of macrophages, lymphocytes and neutrophils. Although the infection is typically restricted to the lung, *C. neoformans* disseminates to the central nervous system in the immunosuppressed patient and occasionally in the normal host (Murphy, 1996), and leads to a potentially life-threatening meningoencephalitis (Kozel, 1993). Successful eradication of *C. neoformans* is largely mediated by T cells (Buchanan & Doyle, 2000), and is dependent on protective Th1 cell-derived cytokines (Kawakami *et al.*, 1996; Yuan *et al.*, 1997).

In addition to cell-mediated immunity, neutrophils have been implicated in protection against *C. neoformans* (Graybill *et al.*, 1997; Miller & Mitchell, 1991; Retini *et al.*, 1996; Vecchiarelli *et al.*, 1998). Whereas normal human neutrophils kill *C. neoformans*, those isolated from patients with chronic granulomatous disease lack fungicidal activity (Diamond *et al.*, 1980). Since chronic granulomatous disease is associated with a defect in the phagocyte NADPH oxidase, this suggests that normal neutrophil anti-cryptococcal activity depends on an intact NADPH oxidase. However, the contribution of neutrophil-derived reactive oxygen species (ROS) to host defence against *C. neoformans* has not been fully characterized.

Abbreviations: H&E, haematoxylin and eosin; IFN, interferon; IL, interleukin; KC, keratinocyte-derived cytokine; MPO, myeloperoxidase.

Myeloperoxidase (MPO) (Hansson *et al.*, 2006; Klebanoff, 2005) is found mainly in neutrophils and to a lesser degree in monocytes. In the presence of hydrogen peroxide (H_2O_2), MPO oxidizes chloride to produce the potent microbicidal agent hypochlorous acid. MPO also catalyses the oxidation of bromide, iodide and the pseudohalide ion thiocyanate. MPO deficiency (Lehrer & Cline, 1969; Nauseef, 1998) is the most common inherited neutrophil defect, with an estimated incidence of 1 in 2000–4000 in Europe and the USA (Parry *et al.*, 1981), and of 1 in 55 000 in Japan (Nunoi *et al.*, 2003). Phagocytes deficient in MPO express a mild to moderate defect in bacterial killing but a marked defect in fungicidal activity *in vitro* (Diamond *et al.*, 1980; Parry *et al.*, 1981). Using MPO-deficient ($MPO^{-/-}$) mice, we have previously demonstrated that the MPO-dependent oxidative system is important for *in vivo* host defence against a variety of micro-organisms (Aratani *et al.*, 1999, 2000, 2002a, b). The aim of the present study was to define the contribution of the MPO-dependent antimicrobial system to the *in vivo* host defence against *C. neoformans*.

METHODS

Animals. Animal experimentation was carried out in accordance with the guidelines of Kihara Institute for Biological Research, Yokohama City University. All mice used were 10- to 12-week-old females. C57BL/6 mice were purchased from Japan SLC (Hamamatsu). Homozygous mutant mice for MPO (Aratani *et al.*, 1999) were backcrossed more than ten times with C57BL/6 to ensure similar genetic backgrounds. Before infection, all animals were housed under specific-pathogen-free conditions.

Experimental infection with *C. neoformans*. *C. neoformans* (ATCC 24067) was cultured on agar slants containing 2·1 % YM broth for 4 days at 27 °C. Fungi were enumerated with a haemocytometer, and the viable number determined in c.f.u. For pulmonary infection, wild-type C57BL/6 and $MPO^{-/-}$ mice were intranasally challenged with 0·04 ml fungal suspension. Thirty minutes later, the lungs of two wild-type and two $MPO^{-/-}$ mice were removed aseptically and homogenized in 1 ml sterile saline to determine the initial number of organisms. At various time points after the challenge, selected organs (lungs, brain, spleen) were harvested aseptically and homogenized in sterile saline in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche). Appropriate dilutions of the homogenates were plated in duplicate onto trypticase soy agar plates (Eiken Chemical). After 2 days incubation at 37 °C, the number of viable organisms was determined in c.f.u. Data were recorded as the mean log(c.f.u.) per organ. The remaining homogenates of the lungs and brain were centrifuged at 19 000 *g* for 10 min to remove cell debris and filtered with 0·45 μm pore-size filters (MILLEX-HV; Millipore). The final supernatants were frozen at −20 °C until assayed for cytokines. For intravenous infection, mice were warmed under a heat lamp to vasodilate the tail vein, and fungi were injected in a 0·1 ml volume through a 27-gauge needle. The spleen was removed and homogenized in 1 ml sterile saline and the aliquots were diluted and plated onto the agar plates.

Quantification of cytokine and chemokine levels. The lung and brain supernatants were analysed using the Bio-Plex system and a Luminex 100TM analyser (Bio-Rad) according to the manufacturer's instructions. Results were expressed as the mean ± SD. The detection limit of the assay was 1·95 pg ml^{-1} for all cytokines and chemokines, as stated by the manufacturer.

Preparation of sections and slides. Five wild-type and five $MPO^{-/-}$ mice were sacrificed for histopathologic examination at the same time points as those for c.f.u. measurement. The whole lungs and brain were removed and fixed in a buffered 4 % paraformaldehyde solution, dehydrated in ethanol, and embedded in paraffin for sectioning. The sections of these organs were processed for haematoxylin and eosin (H&E) and Grocott staining using standard protocols. Five well-separated cross sections of each organ were obtained and all available fields were observed by light microscopy.

Statistical analysis. Survival curves were analysed by the Kaplan–Meier log-rank test. Differences in the number of c.f.u. were examined by the Mann–Whitney *U* test. $P<0·05$ was considered significant.

RESULTS AND DISCUSSION

To examine the contribution of MPO to immunological defence against pulmonary infection with *C. neoformans*, wild-type and $MPO^{-/-}$ mice were infected intranasally or intravenously with $3·6\times10^6$ or $8·7\times10^5$ viable yeast, respectively. After 6 months intranasal infection, there was a 94 % survival rate among infected wild-type mice (Fig. 1a), and all of the intravenously infected wild-type mice remained viable after 3 months (Fig. 1b). Thus, the dose of *C. neoformans* induced minimal mortality in C57BL/6 control mice. In contrast, there was rapid death in $MPO^{-/-}$ mice infected with *C. neoformans*, with a 100 % death rate before 1 month and 4 months of intravenous and intranasal infection, respectively (Fig. 1a, b). The difference in survival rates between wild-type and $MPO^{-/-}$ mice was highly significant ($P<0·001$).

We reasoned that the most likely explanation for the increased mortality in $MPO^{-/-}$ mice following intranasal inoculation of *C. neoformans* was an impaired clearance of the initial inoculum from the airway. To assess this possibility, we compared the lung fungal burden in $MPO^{-/-}$ mice to that of wild-type controls (Fig. 2). Nearly half of the inoculum, $1·7\times10^6$ fungi, was recovered from the lung of wild-type and $MPO^{-/-}$ mice 30 min after infection. The number of *C. neoformans* in the lungs increased simultaneously for the first 7 days in both groups and peaked on day 19, with a slightly but significantly higher number of c.f.u. in the $MPO^{-/-}$ mice. In the wild-type mice, a gradual elimination of the fungi occurred between days 19 and 34, but the $MPO^{-/-}$ mice failed to eliminate *C. neoformans* from the lung. Consequently, by day 60, $MPO^{-/-}$ mice contained nearly 200-fold more fungi than the infected control group ($P<0·01$).

Minimal inflammation was observed histopathologically on day 7, irrespective of genotype, and similar degrees of inflammation were observed on day 19 in both groups (Fig. 3). The lungs of infected wild-type mice showed only localized areas of leukocyte infiltration at days 34 and 60, whereas $MPO^{-/-}$ mice at day 34 exhibited extensive areas of inflammatory infiltration, and all of the airway spaces were filled with the inflammatory cells at day 60. Thus, the lung c.f.u. and the development of pulmonary inflammation correlated with the increased mortality in $MPO^{-/-}$ mice.