Effect of CAWS, a Mannoprotein-β-glucan Complex of *Candida albicans*, on Leukocyte, Endothelial Cell, and Platelet Functions *in Vitro*

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Candida albicans **is a medically important fungus which induces a disseminated candidasis and candidemia in immunocompromised hosts, and releases a polysaccharide fraction into the blood. We recently found that *C. albicans* released a water-soluble polysaccharide fraction (CAWS) into synthetic medium and demonstrated that CAWS was mainly composed of a complex of mannan and β-glucan. In the murine system, CAWS showed a lethality resembling anaphylactic shock when administered i.v., and induced coronary arteritis similar to Kawasaki Disease (KD) when given i.p. In the present study, we examined the biological activity of CAWS in the cell culture and found the following: i) CAWS slightly induced production of IFN-γ and IL-6 by splenocytes at lower dose (*ca.* 10 μg/ml), but at a higher dose strongly inhibited the proliferation of splenocytes induced by a B cell mitogen, lipopolysaccharide (LPS) and a T cell mitogen, concanavalin A. ii) The viability of these splenocytes monitored by propidium iodide staining was significantly reduced. iii) The addition of CAWS to a culture of monophage RAW264.7 cells significantly reduced cellular growth rate dose dependently. iv) The LPS-mediated synthesis of cytokines by RAW264.7 cells was significantly inhibited by CAWS. v) CAWS induced an aggregation of platelets in human platelet-rich plasma, and vi) CAWS inhibited the production of thrombomodulin by human umbilical endothelial cells and acted synergistically with TNF-α. Thus, CAWS strongly inhibited the cellular functions of leukocytes *in vitro*, partly through direct cytotoxicity. The enhanced production in injured cells of the vascular endothelium would be related to the local inflammatory response in the coronary artery.**

Key words *Candida albicans*; mannan; cytotoxicity; apoptosis; immunosuppression

Invasive deep-mycoses have been becoming increasingly common due to the growing number of immunocompromised hosts caused by progressed chemotherapy or immuno-suppressants, such as steroids. These infections occur in severely ill patients with hematological malignancies, leukemia, and acquired immune deficiency syndrome (AIDS), and those undergoing myelosuppressive cytotoxic chemotherapy.[1)] From the standpoint of diagnosis, the symptoms of infection by bacteria and fungi are similar because of systemic inflammation; in addition, isolation of the causative microbe is time consuming. Misinterpretation resulting in the wrong choice of antibacterial or antifungal chemotherapy can be fatal. Metabolites of microbes are often used for early detection. *Candida albicans* is a medically important fungus which induces a disseminated candidiasis and candidemia in immunocompromised hosts,[2)] and releases a polysaccharide fraction into the blood. The cell wall of *C. albicans* is mainly composed of polysaccharides. The major polysaccharides of cell wall were β-1,3 and β-1,6-branched glucan (β-glucan), chitin, and mannan covalently associated with proteins (mannoprotein).[3—8)]

In a series of studies, we used a chemically defined liquid-medium to culture *Candida* spp. and obtained a water-soluble high molecular weight fraction, termed CAWS.[9)] We have reported that CAWS is primarily composed of a complex of mannoprotein and β-glucan. CAWS activated limulus factor G, a well-established test for the diagnosis of invasive deep mycosis.[10,11)] Thus, CAWS would be a candidate molecule of the limulus factor G activating substance in a patient's sera. In addition to the patho-physiological interest in the invasive deep-mycoses, CAWS showed lethal toxicity on intravenous administration and induced coronary arteritis on intraperitoneal administration in mice (manuscript in preparation). From the histochemical point of view, the coronary arteritis is similar to Kawasaki Disease (KD). Thus the study of CAWS induced biological activities would be important not only for analyzing the clinical symptoms of candidiasis but also for clarifying the etiology of KD. In the present study, we examined the effect of CAWS on leukocyte function *in vitro*. The effect on endothelial cells and platelets was also examined.

MATERIALS AND METHODS

Materials *C. albicans* IFO 1385 was purchased from the Institute for Fermentation, Osaka, Japan, maintained on Sabouraud agar (Difco, Detroit, MI, U.S.A.) at 25 °C and transferred once every three months. Distilled water and physiological saline were from Otsuka Co., Ltd. (Tokyo, Japan). Lipopolysaccharide (from *Escherichia coli* 0111: B4) was from SIGMA-ALDRICH (St. Louis, MO, U.S.A.). Concanavalin A was from Pharmacia (Uppsala, Sweden). Human fibrinogen (grade L; AB KABI, Stockholm, Sweden) was further purified using DEAE-cellulose as reported by Lawrie *et al.*[12)] Ethylenediaminetetraacetic acid-disodium salt (EDTA) and acetylsalicylic acid (ASA) were obtained from Wako Pure Chemical Industries (Tokyo). Quinacrine dihydrochloride, fibronectin (Fn), and human immunoglobulin G (IgG) were from Sigma (St. Louis, MO, U.S.A.). Collagen was from Niko Bioscience (Horm: Bovine Achilles's tendon, Tokyo). Trypsin was from Seikagaku Kogyo (from beef pancreas; specific activity, 2500 U/mg; Tokyo). Other chemicals

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were of reagent grade.

Mice Six to 12 week-old male DBA/2 and C3H/HeN mice were obtained from Japan SLC Ltd., Shizuoka, Japan. All mice were maintained under specific pathogen free (SPF) conditions before use.

Media The C-limiting medium originally described by Shepherd and Sullivan[13] was used to grow *C. albicans*. The medium contained (per liter): sucrose 10 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 2 g, $CaCl_2 \cdot 2H_2O$ 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $ZnSO_4 \cdot 7H_2O$ 1 mg, $CuSO_4 \cdot 5H_2O$ 1 mg, $FeSO_4 \cdot 7H_2O$ 0.01 g, and biotin 25 μg (final pH, 5.2). Five liters of medium was placed in the glass jar of a microferm fermenter (New Brunswick, NJ, U.S.A.), and cultured at 27 °C with aeration at 5 l/min and stirring at 400 rpm.

Preparation of Water-Soluble Fraction (CAWS) The water-soluble fraction (CAWS) was prepared as previously described.[9] Briefly, an equal volume of ethanol was added to the culture and the precipitate was collected, which included cells and secreted macromolecules. The precipitate was then suspended in an aliquot of distilled water and the solubilized part was collected and dried in acetone. A chemical characterization of CAWS was done previously.[9] Briefly, carbohydrate and protein content was 78±6.6 and 15±6.3%, respectively. The M (mannan)/G (glucan) ratio was 6.3±1.3 in the case of *C. albicans* IFO 1385. CAWS was diluted with physiological saline and autoclaved.

Preparation of Splenocytes Splenocytes were collected from each strain of mice. Erythrocytes in splenocytes were disrupted with ACK-lysing buffer (8.29 g/l NH_4C1, 1 g/l $KHCO_2$, 37.2 mg/l EDTA·2Na). Total cell numbers were counted with a hemocytometer. Splenocytes were maintained in RPMI1640 medium (Sigma, MO, U.S.A.) supplemented with 5 μg/ml gentamycin sulfate (Wako, Osaka, Japan) containing 10% heat inactivated fetal calf serum (FCS; IWAKI, Chiba, Japan).

Effect of CAWS on the Growth and Cytokine Synthesis of Cell Lines Mouse monophage-like RAW264.7 (RIKEN Cell Bank, Tsukuba) cells were cultured in RPMI1640 medium with gentamycin sulfate containing 10% FCS. Cells were suspended at a density of 5×10^4 and 1×10^5 cells/ml and left for 2 h to adhere to the culture plate. They were then cultured with CAWS or sonifilan (SPG) (Kaken Pharmaceutical, Tokyo) (25—500 μg/ml) for 24 h in a 5% CO_2 incubator at 37 °C. At the end of the incubation, cell viability was evaluated with a cell counting kit, WST-1 (Wako, Osaka) or CellTiter-Glo™ luminescent cell viability assay (Promega, Tokyo) following the distributor's instructions. Absorbance at 450 nm was measured with a microplate reader MTP-32 (CORONA ELECTRIC, Tokyo) and luminescence at 380—630 nm was measured using MicroLumat Plus (EG&G BERTHOLD, Germany). For the measurement of cytokine synthesis, cells were cultured with CAWS, SPG or mannan (from yeast) (Nacalai Tesque, Kyoto) (10—200 μg/ml) and LPS (50 ng/ml) for 24 h. After the incubation, the culture supernatant was collected and cytokines were measured.

Alkaline Phosphatase Activity Splenocytes prepared as above were cultured with reagents (CAWS and LPS) for 72 h at a density of 1×10^6 cells/ml in a 5% CO_2 incubator at 37 °C. The cell suspensions were collected and freeze-thawed. The cell lysates were measured for alkaline phosphatase (ALP) activity to estimate the effect of CAWS on the mitogenic activity of LPS.[14] ALP activity was measured by adding 100 μl of *p*-nitrophenylphosphate 2Na (Wako) dissolved in 10% diethanolamine–HCl (pH 9.5) to 25 μl of cell lysate. The reaction mixture was incubated at 37 °C for 60 min and the optical density at 405 nm was measured.

ELISA Splenocytes prepared as outlined above were cultured with reagents (CAWS and concanavalin A (ConA)) for 72 h at a density of 1×10^6 cells/ml in 5% CO_2 at 37 °C. The interferon (IFN)-γ concentration in the culture supernatant was measured by enzyme linked immunosorbent assay (ELISA) to estimate the effect of CAWS on the mitogenic activity of ConA. A 96-well plate (Sumitomo Bakelite Co., Tokyo) was coated with rat anti-mouse IFN-γ monoclonal antibody (mAb) (Pharmingen, CA, U.S.A.) in 0.1 M $NaHCO_3$ (pH 8.2) at 4 °C overnight. The plate was washed with PBS containing 0.05% Tween 20 (Wako, Osaka) (PBST) and blocked with 0.5% bovine serum albumin (Sigma, MO, U.S.A.) (BPBST) at 37 °C for 40 min. After washing, the plate was incubated with recombinant mouse IFN-γ (Pharmingen) or 50 μl of sample at 37 °C for 40 min. The plate was washed with PBST, blocked with BPBST at room temperature for 10 min, and treated with biotinylated rat anti-IFN-γ mAb (Pharmingen). Then, the plate was treated with peroxidase-conjugated streptavidin (ZyMED Laboratories Inc., CA, U.S.A.) and developed with a tetramethylbenzidine (TMB) substrate system (KPL Inc., MD, U.S.A.). Color development was stopped with 1 M phosphoric acid and the optical density at 450 nm was measured.

Interleukin (IL)-6 concentrations in the culture supernatant of RAW264.7 cells prepared as above were measured by ELISA using antibodies and recombinant provided by Pharmingen.

PI Staining Splenocytes, prepared as above, were cultured with CAWS for 24 h at a density of 1×10^6 cells/ml in 5% CO_2 at 37 °C. The cell suspensions were then collected and washed with staining buffer (2% FCS 0.01% NaN_3 in phosphate-buffered saline (PBS)). Rat anti-mouse CD4, rat anti-mouse CD8α, or rat anti-mouse CD45R/B220 mAb (Pharmingen) was added to the tubes and left at 4 °C for 30 min. Next, 1 μl of propidium iodide (PI; 2 mg/ml; Molecular Probes, INC.) was added and the mixture incubated at 4 °C for 5 min. The cell suspensions were then washed with staining buffer twice, and measured with a fluorescence activated cell sorter (FACS calibur, Becton Dickinson).

Preparation of Platelets Blood collected into 0.1 vol. 3.8% (w/v) sodium citrate from a healthy volunteer was used as the source of platelets. Only plastic tubes and pipettes were used. Citrated blood was gently mixed, then centrifuged at 150×***g*** for 10 min at room temperature. Platelet-rich plasma (PRP) was used for the platelet aggregation assay.

Platelet Aggregation Platelet aggregation was evaluated as previously reported.[15] Briefly, a tetra-channel aggregometer (NKK Hema Tracer 1 model Pat-4A PAT-4M, Tokyo) was used to measure the aggregation. In each experiment, PRP or HEPES buffer was used to adjust the instrument to 100% transmission to represent 100% aggregation. A mixture of 200 μl of PRP was used to represent 0% transmission (zero aggregation). In the actual experiments, 200 μl of PRP was added to a cuvette, which was placed in the aggregometer and stirred at 1000 rpm, while being allowed to warm to 37 °C for at least 10 min prior to the aggregation testing;

11 μl of the test solution was then added and aggregation was monitored for over 15 min. Collagen was added to a final concentration of 10 μg/ml.

Culture of Endothelial Cells Human umbilical vein endothelial cells (HUVEC) were harvested according to the method of Jaffe *et al.*[16] The cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratory, Irvine, UK) supplemented with 20% heat-inactivated fetal calf serum (FCS; Filtron, Brooklin, Australia), 72 U/ml penicillin, and 0.05 mg/ml streptomycin under an atmosphere of 95% air and 5% CO_2. After reaching confluence, the cells were harvested by incubation with 0.05% trypsin (Difco Laboratories, Detroit, MI, U.S.A.) in Dulbecco's (PBS; 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 140 mmol/l NaCl, 2.7 mmol/l KCl, pH 7.4) for 5 min at 37 °C. The cells were collected by centrifugation at 1500 rpm for 5 min at room temperature, and washed twice with DMEM. The collected cells were suspended in 10 ml of DMEM and cell numbers were adjusted to 1×10^5 cells/ml. Aliquots of the suspension (200 μl) were placed in gelatin-coated 96-well microplates (Falcon; Becton Dickinson, Lincoln Park, NJ, U.S.A.) for thrombomodulin (TM) assays and 1 ml aliquots of the suspension were placed in gelatin-coated 24-well plates (Falcon; Becton Dickinson) for the assay of tissue factor (TF) activity. The cells were again grown to confluence in the same medium containing 20% FCS under an atmosphere of 95% air and 5% CO_2. The second passaged cells were used for experiments at 1 to 3 d after confluence. The cells were exposed to various concentrations of CAWS and/or tumor necrosis factor (TNF)-α at 37 °C for one day.

Measurement of TM Cultured endothelial cells (96-well microplates, 2×10^4 cells/well) incubated with CAWS and/or TNF-α, and the control cells were washed quickly with DMEM, and the cell monolayers were extracted with 200 μl of 50 mmol/l Tris–HCl containing 0.15 mol/l NaCl, 0.5% Triton X-100, and 1 mmol/l benzamidine hydrochloride (pH 7.5) for 30 min at 4 °C. The TM antigen levels in the cell extracts were measured by enzyme immunoassay using monoclonal IgGs (TMmAb 20, 2, 11) as previously described.[17,18] Purified human placental TM was used as a standard.

Measurement of TF Activity After incubation with CAWS and/or TNF-α, the cells (24-well plates, 1×10^5 cells/well) were washed three times with Dulbecco's PBS and were scrape-harvested into 300 μl of Dulbecco's PBS. The scraped cells were frozen and thawed three times. The cell lysate was assayed in a one-stage clotting assay for TF activity as described by Bevilacqua *et al.*[19] Briefly, 100 μl of cell lysate was incubated with 100 μl of 25 mmol/l $CaCl_2$ at 37 °C for 2 min in prewarmed plastic tubes in a Ball coagulation timer (Amelung KCIA, Medizinische Laborgeräte, Germany). Clotting was initiated by the addition of 100 μl of prewarmed citrated normal human plasma. TF cofactor activity was quantitated by reference to standard curves (log–log plot) constructed with rabbit brain thromboplastin (Sigma) and 1 U of activity was defined as a clotting time of 20 s in a standard assay with normal human plasma. The coagulant activity reflects TF activity because no procoagulant activity was detected in endothelial cells when factor VII- or X-deficient plasma was used instead of normal plasma.

Statistics Results are expressed as the mean±standard deviation (S.D.). The significance of differences between means was determined by Student's *t*-test.

RESULTS

Effect of CAWS on the Proliferation of Splenocytes *in Vitro* As preliminary experiments, the immunostimulatory effect of CAWS was screened by measuring the production of several cytokines (IFN-γ, TNF-α, IL-lα, macrophage inflammatory protein (MIP)-2 and IL-6) by DBA/2 or C3H/HeN splenocytes *in vitro*. IFN-γ and IL-6 were produced at low levels (Fig. 1) but the others were not significantly changed (data not shown). Then we examined the antilymphoproliferative effect of CAWS on splenocytes stimulated with a B cell mitogen, lipopolysaccharide (LPS) and a T cell mitogen, Con A. The activity was measured as alkaline phosphatase (ALP) activity or IFN-γ production. As shown in Fig. 2, ALP and IFN-γ production by LPS and Con A was dose-dependently inhibited by CAWS. Of interest, the dose of CAWS required to inhibit the Con A response was significantly lower than that of LPS, probably due to competition of CAWS with the Con A binding site of lymphocytes.

Viability of Splenocytes Stimulated with CAWS *in Vitro* CAWS showed strong toxicity *in vivo* on intravenous administration. However, no toxicity was observed on intraperitoneal administration.[20] We then examined the toxicity of CAWS in cell cultures. The toxicity was monitored by staining with propidium iodide (PI) and analyzed by FACS. Cells were also stained with markers of lymphocyte populations; CD4, CD8, or CD45R/B220 antibodies. As shown in Fig. 3, the incorporation of PI increased dose-dependently in all populations of CD4+ T cells, CD8+ T cells and CD45R/B220+ B cells. At a concentration of 100 μg/ml of CAWS, almost all of the cell populations were stained by PI. Similar cytotoxicity was observed in splenocytes of DBA/2

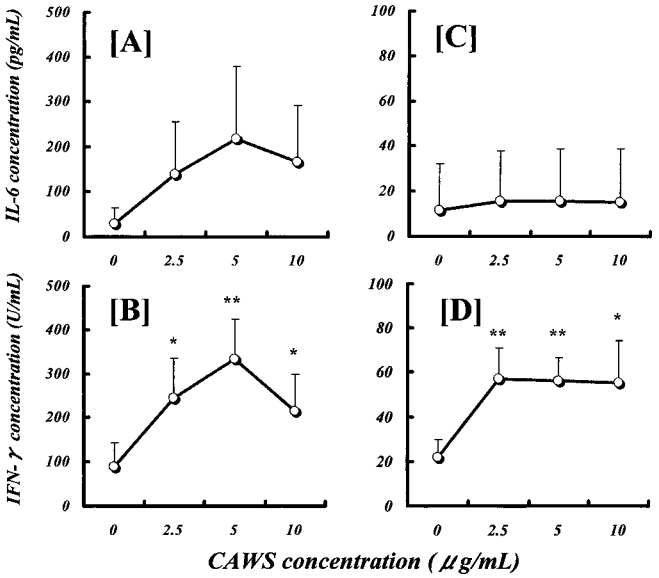

Fig. 1. Production of Cytokines by Splenocytes Stimulated with CAWS *in Vitro*

Splenocytes (1×10^7) of C3H/HeN or DBA/2 mice were stimulated with the indicated dose of CAWS *in vitro*. After 48 h, culture supernatants were collected and levels of IFN-γ and IL-6 measured as described in Materials and Methods. The results represent the mean±S.D. $*p<0.05$ and $**p<0.005$ compared with control [A, B]: DBA/2. [C, D]: C3H/HeN.

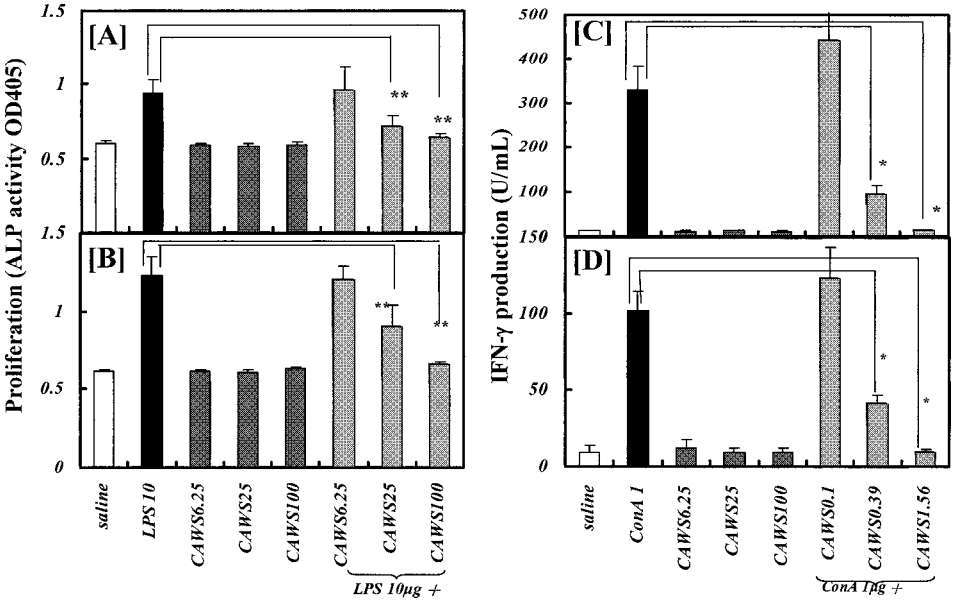

Fig. 2. ALP Activity and IFN-γ Production in Culture Supernatant of Splenocytes Stimulated with LPS or ConA and CAWS *in Vitro*

[A, B] Splenocytes (1×10^6) of C3H/HeN or DBA/2 mice were stimulated with LPS and CAWS *in vitro*. After 72 h, the splenocytes were collected and assayed for alkaline phosphatase activity as described in Materials and Methods. [C, D] Splenocytes (1×10^6) of C3H/HeN or DBA/2 mice were stimulated with ConA and CAWS *in vitro*. After 72 h, the splenocytes were collected and the IFN-γ concentration was measured by ELISA as described in Materials and Methods. The results represent the mean±S.D. $*p<0.05$ and $**p<0.005$ compared with control (LPS; 10 μg/ml or Con A; 1 μg/ml). [A, C]: DBA/2. [B, D]: C3H/HeN.

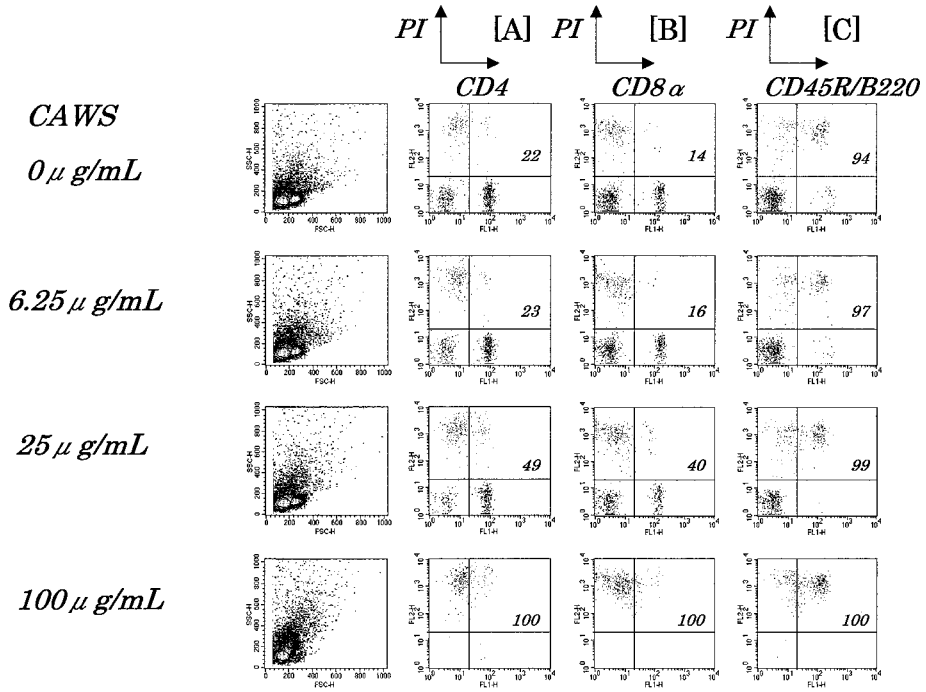

Fig. 3. PI Staining of C3H/HeN Splenocytes Stimulated with CAWS *in Vitro*

Splenocytes (1×10^6) were stimulated with CAWS *in vitro*. After 24 h, they were collected and stained with FITC-labeled anti-CD4 (A), CD8a (B) or CD45R/B220 (C) mAb and PI as described in Materials and Methods. These cells were analyzed by FACS. Numbers represent PI-positive cells in surface antigen-positive cells/total surface antigen-positive cells ×100. The data represent one set of results of similar experiments.

mice (data not shown). These results indicated that CAWS was lethal to splenocytes.

Inhibition of Growth and Cytokine Synthesis of RAW 264.7 by CAWS *in Vitro* To analyze the cytotoxic effect more precisely, the effect of CAWS on macrophage growth and function was measured using a monophage cell line, RAW 264.7. To measure the growth, RAW was cultured at concentrations of 0.5 and 1×10^5 cells/ml in the presence or absence of CAWS and the color development of WST-1, a commercially available cell counting kit, was examined. As shown in Fig. 4A, at higher than 100 μg/ml, CAWS significantly inhibited the growth of RAW cells. Similarly, as shown in Fig. 4B, growth inhibition was shown by chemiluminescent assay. Inhibition ratio of the two methods differed,

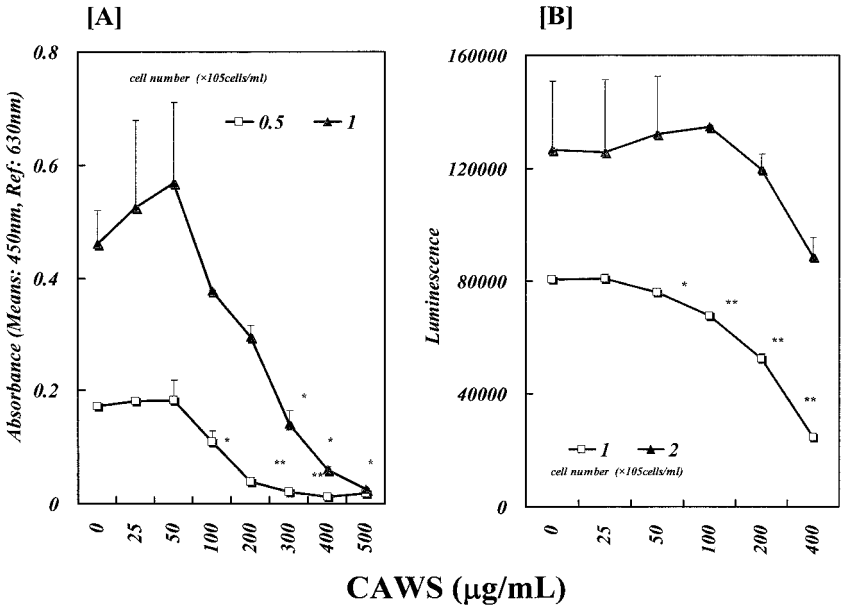

Fig. 4. Cell Viability of RAW264.7 Cells Stimulated with CAWS *in Vitro* Measured with WST-1 or Cell Titer-Gro™

[A] RAW 264.7 cells (100 μl each, 0.5 and 1×10^5) were stimulated with CAWS *in vitro*. After 24 h, WST-1 reagent (10 μl) was added and further incubated for 4 h. Absorbance was measured at 450 nm. [B] RAW264.7 (1 and 2×10^5) cells were stimulated with CAWS *in vitro*. After 24 h, reagent was added and cell viability was measured. The results represent the mean±S.D. $*p<0.05$ and $**p<0.005$ compared with control (saline; 0 μg/ml).

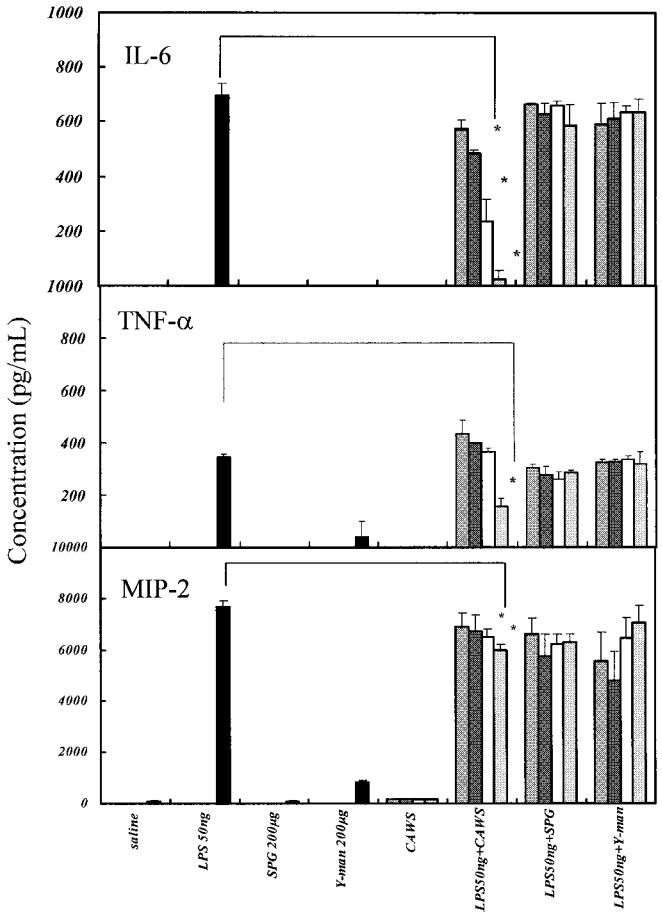

Fig. 5. Inhibition of LPS-Induced Synthesis of Cytokine in RAW 264.7 by CAWS

RAW 264.7 cells (5×10^4) were simulated with CAWS, SPG or yeast-mannan and LPS *in vitro*. After 24 h, the culture supernatant was collected and IL-6, MIP-2 and TNF-α concentrations were measured by ELISA as described in Materials and Methods. The results represent the mean±S.D. $*p<0.05$ and $**p<0.005$ compared with control (LPS; 50 ng/ml). CAWS, SPG, and Y-man; 25, 50, 100, 200 μg/ml, left to right in this order).

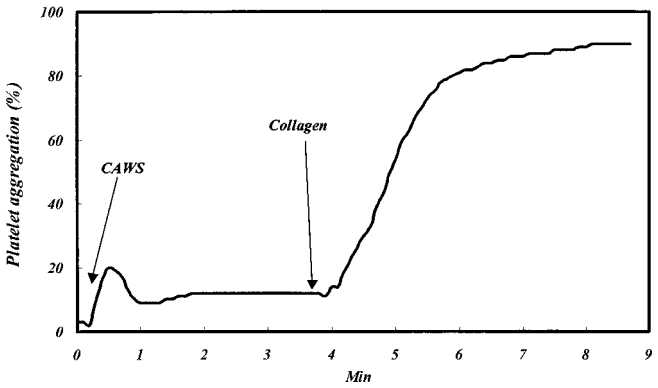

Fig. 6. Effect of CAWS on Platelet Aggregation

PRP (200 μl) placed in the cuvette of an aggregometer was mixed with a solution of CAWS (10 mg/ml, 11 μl) and the aggregation was monitored, then collagen (100 μg/ml, 11 μl) was added as a positive control and monitored further. Experimental details are given in Materials and Methods.

perhaps due to the principle of the methods. As a control experiment, sonifilan, a β-1,3-glucan showing immunomodulating activity was added and measured similarly, however, cytotoxic activity could not be detected as high as 500 μg/ml in WST-1 nor could chemiluminescence.

To assess function, RAW was stimulated with LPS in the presence or absence of CAWS, and levels of the cytokines, IL-6, MIP-2, and TNF-α were measured. As shown in Fig. 5, the synthesis of all three cytokines induced by LPS was inhibited by CAWS concentration-dependently. The inhibitory effect on MIP-2 was not remarkable however. Comparing the inhibition of growth and cytokine-synthesis, the latter was induced by a lower concentration of CAWS. This strongly suggested that CAWS inhibited both the growth and function of macrophages.

Effect of CAWS on Platelet Aggregation To obtain information about blood coagulation, which is strongly related to the lethal shock induced by bacterial infection, the effects

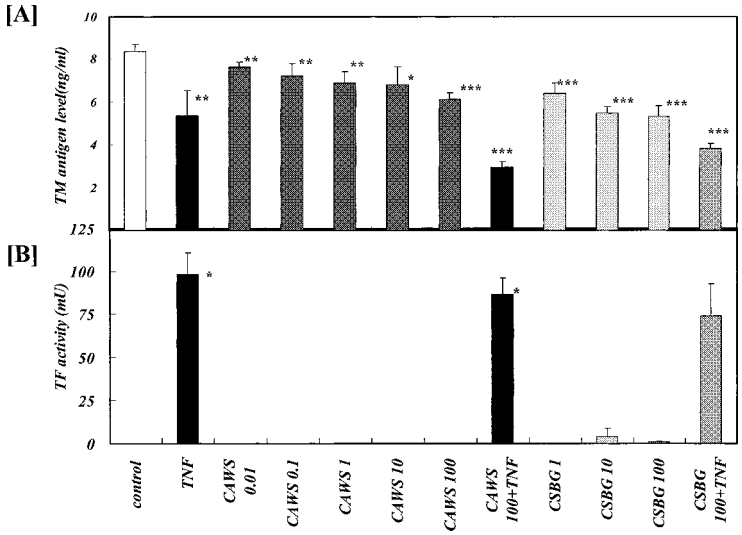

Fig. 7. Modulation of Thrombomodulin Expression or Tissue Factor Activity on Cultured Endothelial Cells by CAWS

[A] Cultured endothelial cells were incubated with the indicated concentration of CAWS, CSBG, and/or TNF-α for one day and then lysed. The concentration of thrombomodulin in the resulting lysate was measured by ELISA as described in Materials and Methods. [B] Cultured endothelial cells were incubated with the indicated concentration of CAWS, CSBG, and/or TNF-α for 6 h and lysed. The concentration of tissue factor in the resulting lysate was measured based on the coagulation of citrated normal human plasma as described. The results represent the mean±S.D. $*p<0.05$, $**p<0.01$ and $***p<0.001$ compared with control.

of CAWS on platelets were examined. Figure 6 shows representative data on CAWS-induced platelet aggregation. Compared with the strong activator, collagen, CAWS induced a weak but significant platelet aggregation, and the pretreatment of platelets with CAWS did not inhibit the collagen-induced aggregation. The effect of CAWS on the platelet aggregation was concentration-dependent (max. 1 mg/ml) but under these experimental conditions, no strong aggregation was observed. No further analysis on platelets was made.

Effect of CAWS on Endothelial Cell Functions The effect of CAWS on blood coagulation was also examined in cell cultures of human umbilical vein endothelial cells, and the expression of TM, an essential cofactor for the anti-coagulant function on the surface of endothelial cells,[21] and TF, a major physiological trigger for the generation of thrombin in the blood coagulation pathway,[21] were examined. As shown in Fig. 7, CAWS reduced the production of TM dose-dependently, but did not enhance the production of TF. TNF-α is a strong inhibitor of TM synthesis and activator of TF. The synergistic effect of TNF-α and CAWS was also examined. In TM production, CAWS was synergistic with TNF-α, but the effect was not observed in TF production.

As control experiments, another polysaccharide fraction of Candida, CSBG, was also examined. CSBG inhibited the synthesis of TM and acted synergistically with TNF-α. These facts suggested that CAWS had little effect on TF activity but inhibited TM production and controlled the blood coagulation system.

DISCUSSION

CAWS is composed of a complex of mannoprotein and β-glucan.[9] The ratio of the former to the latter is about 5 : 1, thus the former is the major component. CAWS shows various types of activity to which both components contribute. For example, CAWS reacts with limulus factor G, specific for 1,3-β-D-glucan.[9] The half-clearance time of CAWS in DBA/2 mice measured by the limulus factor G test was 19 min by i.v. administration, and would be mediated by the mannose receptor. CAWS reacts with human natural antibody against β-glucan, specific for 1,6-β-D-glucan segments (manuscript in preparation). In the murine system, CAWS showed a lethality similar to anaphylactic shock after i.v. administration.[20] The lethality was partially inhibited by pretreatment with yeast mannan and salbutamol. Thus, the lethality would be mediated by the mannan moiety of CAWS. Salbutamol is a relatively selective β_2-adrenoreceptor stimulant with rapid, potent bronchodilator activity and only minor inotropic or chronotropic effects.[23] Thus the CAWS-mediated toxicity might relate to the production of anaphylactic substances.

A potassium hydroxide extract of *C. albicans* (CADS) isolated from a patient with KD has been known to induce a coronary arteritis in mice that resembles KD.[24] The etiology of KD is yet unknown. Recently, we also found that i.p. administration of CAWS induced coronary arteritis (manuscript in preparation), with an incidence higher than that of CADS-induced arteritis. The complex nature of CAWS, a mannoprotein-β-glucan complex, might be related to the higher inductivity.

In the present study, we examined the biological activity of CAWS in cell cultures and found that: i) CAWS inhibited the proliferation of splenocytes induced by a B cell mitogen, LPS and a T cell mitogen, Con A dose-dependently; ii) the viability of these splenocytes monitored by propidium iodide staining was significantly reduced; iii) the addition of CAWS to the culture of monophage RAW264.7 significantly reduced the growth rate of the cells dose-dependently; iv) the LPS-mediated synthesis of cytokines by RAW264.7 was significantly inhibited by CAWS; v) CAWS induced platelet aggregation in human platelet-rich plasma; and vi) CAWS inhibited the production of TM by HUVEC and its activity was synergistic with TNF-α.

The most characteristic feature of CAWS found in this study was the direct cytotoxic effect on leukocytes, and thus the inhibition of cytokine synthesis. The cytotoxic effect also

appeared in other types of cells such as fibroblast and mastocytoma cells (data not shown). LPS is known to be lethal both *in vivo* and *in vitro*.[25] LPS induced toxicity largely dependent on the autocrine and paracrine mechanisms of cytokine synthesis, especially that of TNF-α.[25] Indeed, TNF-α shows toxicity similar to LPS.[25] In contrast, we did not find higher concentrations of cytokines in the cell cultures with CAWS. However, in the human system, CAWS induced production of TNF-α by peripheral blood monocytes (manuscript in preparation). Yet unidentified factors might be produced during cell culture with CAWS. Thus, the cytotoxic mechanisms of LPS and CAWS would be different. The mechanisms are now under investigation.

CAWS was found to exhibit cytotoxicity, thus it is assumed to be produced in damaged cells in mice. These damaged cells would be selectively removed by the immune scavenger function.[26] Several scavenger receptors have already been identified and ligands of macrophage scavenger receptors enhanced cytokine synthesis and cell growth, thus resulting in augmented immune/inflammatory reactions *in vivo*.[27] In addition, oxidized low-density lipoprotein induced an influx of calcium in polymorphonuclear leukocytes, and apoptosis in human endothelial cells.[28,29] These observations, in turn, are closely related to the pathophysiology of atherosclerosis. As shown above, i.p. administration of CAWS induced coronary arteritis, which might be related to the scavenger function. In addition, CAWS was found to modulate the coagulation system in at least two ways, platelet aggregation and down regulation of TM synthesis by endothelial cells. TM is an antithrombotic factor of endothelial cells. Both of these activities suggested the enhancement of thrombus formation in the circulatory system, resulting in an increase in the risk of coronary arteritis.

In the patient's blood of an invasive deep-mycosis, concentration of the limulus factor G activating substance reaches nanogram quantity based on the purified 1,3-β-glucan as a substrate. The fungal metabolite might not be a purified 1,3-β-glucan, thus the blood concentration of the metabolite itself reaches microgram quantity. In the previous study, we showed that the blood concentration of CAWS reached 300 μg/ml as 1,3-β-glucan when given 1 mg i.p. Compared to the proposed concentration of the fungal metabolite in a patient's blood, the concentration of CAWS used in this study would be suitable. However, it is of note that some of the *in vitro* experiments used higher concentration of CAWS. Attentiveness is required to gauge the physiological meaning of these data.

In the spleen cell culture, Con A induced lymphocyte proliferation was inhibited by a lower dose of CAWS compared with cytotoxic activity. Approximately 1 : 1 weight ratio of CAWS inhibited activity of Con A, in this study, and would be a conceivable dose. Con A is a mannan binding lectin and thus CAWS might act as a competitive inhibitor of Con A binding site on the T lymphocytes. Various kinds of mannose binding proteins, collectins, ficolins, MBL, surfactant proteins *etc.*, are identified in sera and on the surface of the cells,[30,31] some of which have important roles in natural as well as acquired immunity. CAWS might interfere and/or modulate these interactions.

The endothelium plays a crucial and a dynamic role as a protective interface between blood and the underlying tissues during the hemostatic process, maintaining blood flow in the circulation and prevents life-threatening blood loss.[32] Healthy endothelial cells, unlike extravascular cells, are anticoagulant and antithrombotic; this is due to the regulated secretion of antiplatelet agents, including prostacyclin and nitric oxide. During vasculitis, the endothelium can be damaged by various mechanisms including neutophil activation and subsequent oxidative burst. Following vessel injury including vasculitis, platelet adhesion to the exposed matrix requires von Willebrand factor, another endothelial cell product. Local generation of thrombin causes a series of receptor-mediated endothelial cell functional responses, while the surface of the endothelium is also the site of inactivation of thrombin by antithrombin, and its conversion to a coagulation inhibitor by interaction with TM. Endothelial cells are the source of circulating tissue-type plasminogen activator and its inhibitor, and TF pathway inhibitor. Following vessel wall injury with initial platelet adhesion and aggregation to exposed subendothelial extracellular matrix, the initiation, amplification, and control of hemostasis depend on structurally unrelated membrane-associated receptors for blood coagulation proteases including TF, TM, G-protein-coupled protease-activatable receptors, and protein C receptor, respectively. In addition to their regulatory role in hemostasis, the respective (pro-)enzyme ligands such as Factors VIIa and Xa, thrombin or protein C mediate specific signaling pathways in vascular cells related to migration, proliferation or adhesion. The functional importance of these receptors beyond hemostasis has been manifested by various lethal and pathological phenotypes in knock-out mice.[32] These protease receptors thereby provide important molecular links in the vascular system and serve to integrate hemostasis with endothelial cell functions which are relevant to the (patho-)physiological responses to injury or inflammatory challenges. Recently, it was suggested that inflammation can potentiate blood clotting.[33] Inflammatory mediators like LPS and TNF-α can cause the expression of TF on monocytes and, possibly, endothelium, thereby initiating the coagulation cascade. Activation of the complement system can lead to exposure of membrane surfaces capable of amplifying the initial TF stimulus by facilitating the assembly of the factor VIIIa-factor IXa and the factor Xa-factor Va complexes. Inflammatory mediators, particularly IL-6, can also increase the levels of fibrinogen, an acute-phase reactant. In addition, the inflammatory mediators can elevate the levels of plasminogen activator inhibitor, thus suppressing the fibrinolytic system. During a preliminary investigation, various activities of CAWS were examined by i.p. administration to clarify the underlying mechanisms for pathophysiology, and various mediators related to inflammatory response were released by CAWS: i) CAWS induced infiltration of macrophages and granulocytes into the peritoneal cavity, ii) IFN-γ, IL-6 and IL-10 production by spleen cells was increased, and iii) the myeloperoxidase (MPO) activity of the spleen cells was increased. Considering these facts, the CAWS-induced inflammatory response might modulate regulation of the endothelium resulting in arteritis.

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