一方、加齢により、免疫系が低下するから、感染症にかかりやすくなり、発癌リスクも上昇すると一般的に考えられてきた。しかしながら、最近の研究は、好中球やマクロファージ、樹状細胞、ナチュラルキラー（NK）細胞などの自然免疫をになう細胞機能への加齢による低下は、比較的軽微である一方、T 細胞や B 細胞の関与する獲得免疫に関しては、ナイーブ T は減少、メモリー T は増加、B 細胞についても同様で、加齢とともに細胞集団の構成変化が起こり、新規抗原への対応力が低下している事が明らかにされている[4,5,6]。

加齢と免疫機能の関係については、大半の研究は、いわゆる若年者と老齢者の集団を比較した横断的研究といわれるもので、対象となる老齢者は 60-70 歳であったり、80-100 歳であったり、比較する集団も様々である。時には年齢差は 80 年に及ぶ集団を比較しており、育ってきた環境変化も考えれば、本当に加齢の影響だけを見ているのか疑問である比較もある。私たちは免疫と加齢の影響を論じるなら、一人の方を長期間追跡してその加齢と免疫機能との関連を検証するべきであると考えている（縦断的研究）。そこで、今回はルイ・パストゥール医学研究センターで蓄積されてきた、IFN 産生能検査を含む検診結果を解析し、IFN 産生能と加齢との関係を検証した。

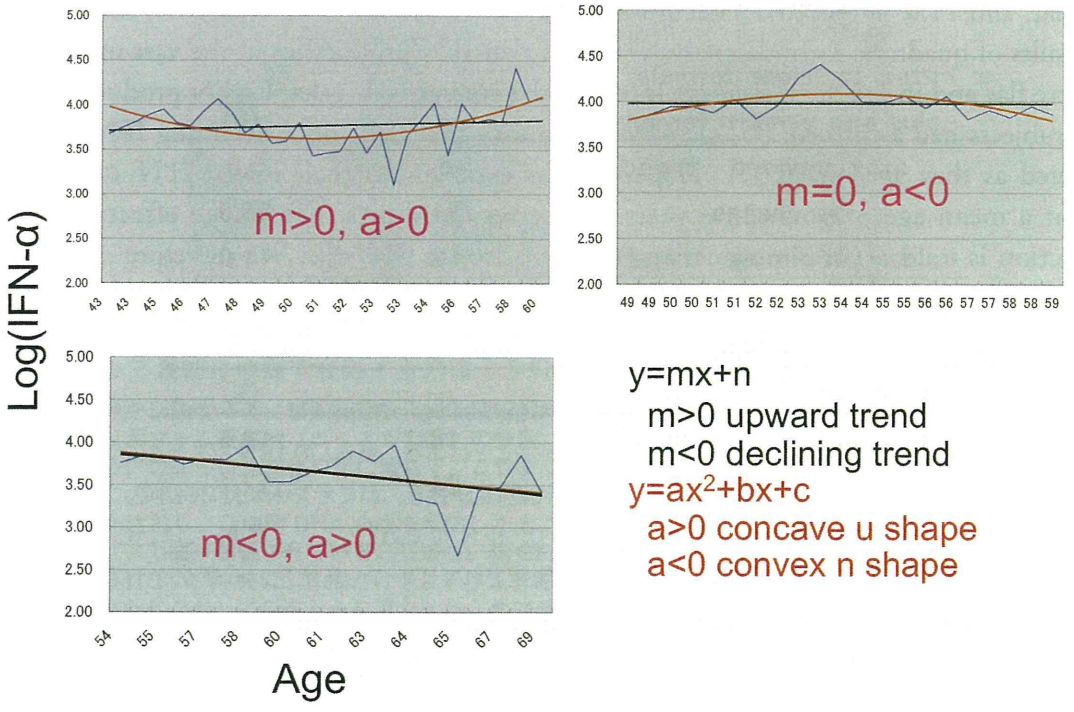
対象

1987 年から 2009 年末までに測定した、総計 2387 名を対象とした。そこから、6 回以上、10 年以上の検査履歴の残っている計 156 名を抽出した。さらに、50 歳以上の測定履歴があり、癌、自己免疫疾患、HCV 感染症履歴のない、ヒトを健常人として抽出した。男性 76 名、女性 33 名が抽出された。

IFN- α 産生能の測定

採血後 8 時間以内に、ヘパリン加採血した血液を、1cc スピッツ遠心管（栄研スピッツ、栄研科学（株））にわけとり、最終濃度 500HA/ml となるようにセンダイウイルスを添加した。37℃のインキュベーターにて 20 時間培養、3000 rpm にて 10 分遠心後上清を回収した。回収した上清は、希釈後、FL 細胞、sindobis virus を用いて、上清中の IFN 活性を測定し、測定値を IFN 産生能値とした。

Fig. 1 Subjects' periodical IFN-α values (y) were plotted vs. age (x) along fitted to two quadratic formulas (① y=mx+n and ② $y=ax^2+bx+c$) expression.



解析

個々人の IFN 産生能値は、対数変換後 y 値、年齢を x として、一次式 $y=mx+b$、二次式 $y=ax^2+bx+c$ にあてはめた。$m>0$ は上昇傾向、$m<0$ は低下傾向となる。また、$a>0$ は凹型、$a<0$ は凸型示す。図 1 に典型例を示す。

結果

1，一次式による解析

一次式に、個々人の結果を当てはめたところ、Table 1 a に示すように、83.5% の方は特に変化無しであり、$p<0.05$ で $m>0$ であった人を上昇傾向（5.5%）、$p<0.05$ で $m<0$ であった人を低下傾向（11.0%）とした。このことは、加齢と共にやや低下するヒトの方が多い傾向があることを意味してはいるが、上昇傾向、低下傾向の間には有意差は認められなかった。

2，二次式による解析

二次式に、個々人の結果を当てはめたところ、Table 1 b に示すように、同じく 77.1% の方は特に変化無しであり、$p<0.05$ で $a<0$ であった人を凹型（16.5%）が、$p<0.05$ で $a>0$ であった人を凸型（6.4%）とした。凹型と凸型では凹型の割合が優位に高かった。また、$-b/(2a)$ は 56.1 ± 2.0 となった。このことは 56 歳が凹型の底辺となることを意味しており、IFN 産生能が一番低下する時期であること、即ちこの時期を境として、上昇するケースが少なからずあるということを意味している。

考察

IFN 産生能の長期にわたる結果を、一次式、二次式に当てはめ、解析した。その結果一次式では、上昇傾向に比べて、やや低下傾向が多いもののその差は優位ではなかった。また、m 値が有意に低下、あるいは上昇とは認められない割合が多かった。このことは、IFN 産生能という人の自然免疫力の一つの指標での加齢の影響は、やや低下傾向が多いものの、あっとしても非常に軽微であることを意味している。

Table 1 Periodical IFN-α trend in healthy subjects #

a: Fitted to linear formula

plug into y=mx+n formula	n	(%)	
m:+(upward trend)	6	5.5	
No change	91	83.5	
m:−(declining trend)	12	11.0	NS

b: Fitting to quadratic expression

plug into $y=ax^2+bx+c$ formula	n	(%)	
a:+(concave u shape)*	18	16.5	p<0.05
No change	84	77.1	
a:−(convex n shape)	7	6.4	

*−b/(2a) : 56.1±2.0(mean±SE)

Healthy subjects were over 50 years old and excluded those with a history of cancer, autoimmune and chronic infectious diseases.

また二次式へのあてはめでは、凹型を示すケースが、凸型を示す割合より優位に多かった。これらの結果は、一度低下したIFN産生能も、加齢につれて、上昇する場合も少なからずあることを示している。また、一番低下する時期は56歳という値が導き出された。このデータベースでは、会社員や自営業の方が多いところから、この年齢は、一番忙しい時期であることを反映している可能性が推察されるが、更なる検討を要する。

また、男女別に解析を行ってみたが、今回の解析範囲では、大きな男女差を認めることは出来なかった。

今回、長期間わたる経時的測定結果から、IFN産生能の加齢との関係について検討した。このようなデータの取り扱いについては、手探りの状態にあり、とりあえず、一次式、二次式への当てはめで解析をした。また、ここでは、10年以上のデータのある50歳以上でのデータのある人を抽出した。

その後、このような一般線型モデルでは満足に解析できない反復測定データや経時観察データに対しては、混合モデルを用いて解析するのが適切であることがわかった[7,8]。現在、混合モデルを用いて、更なる解析を行っている。

謝辞

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引用文献

1. Akahoshi M, Ishihara M, Remus N, Uno K, Miyake K, et al. (2004) Association between IFNA genotype and the risk of sarcoidosis. Hum Genet 114: 503-509.
2. Uno K, Nakano K, Maruo N, Onodera H, Mata H, et al. (1996) Determination of interferon-alpha-producing capacity in whole blood cultures from patients with various diseases and from healthy persons. J Interferon Cytokine Res 16: 911-918.
3. Uno K, Hirosaki M, Kakimi K, Tominaga M, Suginoshita Y, et al. (2007) Impaired IFN-alpha production and the risk of cancer development. J Interferon Cytokine Res 27: 1013-1017.
4. Franceschi C, Bonafe M (2003) Centenarians as a model for healthy aging. Biochem Soc Trans 31: 457-461.
5. Gibson KL, Wu YC, Barnett Y, Duggan O, Vaughan R, et al. (2009) B-cell diversity decreases in old age and is correlated with poor health status. Aging Cell 8: 18-25.
6. Derhovanessian E, Larbi A, Pawelec G (2009) Biomarkers of human immunosenescence: impact of Cytomegalovirus infection. Curr Opin Immunol 21: 440-445.
7. Nayak Savla G, Moore DJ, Roesch SC, Heaton RK, Jeste DV, et al. (2006) An evaluation of longitudinal neurocognitive performance among middle-aged and older schizophrenia patients: use of mixed-model analyses. Schizophr Res 83: 215-223.
8. Fleg JL, Morrell CH, Bos AG, Brant LJ, Talbot LA, et al. (2005) Accelerated longitudinal decline of aerobic capacity in healthy older adults. Circulation 112: 674-682.

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ORIGINAL ARTICLE

Vasculitis and anaphylactoid shock in mice induced by the polysaccharide fraction secreted into culture supernatants by the fungus *Candida metapsilosis*

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ABSTRACT

The biological effects of *Candida metapsilosis* water-soluble fraction (CMWS), prepared using a completely synthesized medium, were examined to determine whether CMWS induces vasculitis similar to that seen in Kawasaki disease, and anaphylactoid shock, in mice. It was found that intraperitoneal injection of CMWS induces coronary arteritis and i.v. injection induces acute anaphylactoid shock in mice, similar to *Candida albicans* water-soluble fraction (CAWS)-induced arteritis and anaphylactoid shock. The mannan structure of the polysaccharide fraction was then analyzed by performing antiserum reactivity tests and nuclear magnetic resonance spectroscopy. The mannan structure was investigated because the present authors have recently found that the mannan moiety within the polysaccharide fraction might be responsible for these pathogenic activities. The structural analysis showed that the mannan structure within CMWS expresses α-mannan residues, but not β-mannan. In addition, the mannan structure of CMWS is quite similar to that of CAWS. The present findings indicate that the polysaccharide fraction from *C. metapsilosis*, which is mainly composed of mannan, contributes to coronary arteritis and acute shock, and that the mannan structure could be responsible for this pathogenicity.

Key words anaphylactoid shock, *Candida metapsilosis*, polysaccharide, vasculitis.

Kawasaki disease is a systemic childhood vasculitis that can result in aneurysms of the coronary arteries (1, 2). The diagnosis of KD is based entirely on clinical features. The diagnosis of classic KD requires that individuals have a fever for more than 5days and either meet at least four of the following five criteria:(i) bilateral conjunctivitis; (ii) erythema of the mouth or pharynx, strawberry tongue, or stomatitis; (iii) polymorphous rash; (iv) erythema or edema of the hands or feet; and (v) nonsuppurative cervical lymphadenopathy; or meet at least three of these criteria and have evidence of coronary artery abnormalities. Incomplete or atypical KD, in which these criteria are not fully met, also occurs and can result in aneurysms of the coronary arteries. Laboratory findings are nonspecific, and there are no diagnostic tests for KD.

The cause of KD remains unknown despite numerous efforts. However, many recent studies have reported that KD may be triggered by responses to an infectious agents such fungi, bacteria, and viruses (3–5). Moreover infection of neonates by invasive *Candida*, such as the pathogenic

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List of Abbreviations: BPBST, BSA containing PBST; *C. albicans*, *Candida albicans*; CAWS, *C. albicans* water-soluble fraction; *C. metapsilosis*, *Candida metapsilosis*; CMWS, *C. metapsilosis* water-soluble fraction; GLC, gas-liquid choromatography; HE, hematoxylin-eosin; HSQC, heteronuclear single quantum coherence; KD, Kawasaki disease; NBRC, National Institute of Technology and Evaluation Biological Resource Center; NMR, nuclear magnetic resonance; PBST, Tween 20 containing phosphate buffered saline; p.p.m., parts per million; TMB, 3,3′,5,5′-tetramethylbenzidine; TOCSY, total correlation spectroscopy.

species *C. albicans*, can cause mycetoma of the right atrium and candidal endocarditis (6). Pathogenic fungi, including *C. albicans*, can also induce septic shock. *Candida*-induced septic shock is as serious a clinical problem as bacterial septic shock.

The pathogenic yeast *C. albicans*, a commensal of the human digestive tract and vaginal mucosa, is now one of the commonest microbes causing bloodstream infections in immunocompromised or intensive-care patients (7, 8).

We have previously found and reported that polysaccharide fractions obtained from culture supernatants, as well as the cell wall of the pathogenic yeast *C. albicans*, dramatically induce coronary arteritis similar to that found in KD, and acute anaphylactoid shock, in mice (9–17). In the course of our studies, we recently found relationships between *C. albicans* culture conditions and the induction of several biological effects of CAWS, including acute anaphylactoid shock, coronary arteritis, and complement activation (10–17). Specifically, culture conditions resulting in a 1,2-β-mannosyl linkage within the mannan moiety of these fractions significantly reduced the biological effects described above (15). This finding was also supported by investigations into the activity and structure of cell wall mannan extracts of *C. albicans*, the structures of which vary with changes in culture conditions such as culture media and growth temperatures (9). Numerous studies have reported that the cell wall mannan of *Candida* species is altered by various culture conditions such as growth temperature (18), pH (19), oxidative stress, and osmotic pressure (20).

However, pathogenic activities in terms of induction of vasculitis and acute anaphylactoid shock of other *Candida* species, such as *C. metapsilosis*, have not been investigated. We thought that polysaccharide fractions from *C. metapsilosis* might induce such activity because it is well known that the cell mannan of *C. metapsilosis* is not expressed as 1,2-β-mannan within its mannan moiety (21). In the present study, we examined whether the secreted polysaccharide fraction from another *Candida* species, *C. metapsilosis*, which is less pathogenic than *C. albicans*, can induce vasculitis similar to that found in KD, and anaphylactoid shock, in mice in the same way as *C. albicans* does. We obtained the secreted polysaccharide fraction from *C. metapsilosis*; assessed its pathogenic activities, such as induction of vasculitis and acute anaphylactoid shock; and analyzed its mannan structure.

MATERIALS AND METHODS

Animals and materials

Male ICR and DBA/2 mice (6weeks old) were purchased from Japan SLC (Hamamatsu, Japan). The mice were housed in a specific pathogen-free environment. All animal experiments followed the guidelines for laboratory animal experiments of the Tokyo University of Pharmacy and Life Sciences, and each experimental protocol was approved by the committee for laboratory animal experiments at this institution. The completely synthetic medium, C-limiting medium (22) 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). The *Candida* Check was from Mitsubishi Kagaku Iatron (Tokyo, Japan).

Yeasts and culture conditions

Candida metapsilosis NBRC 1068 was obtained from the NBRC (Chiba, Japan). CMWS was prepared from *C. metapsilosis* NBRC 1068 in accordance with slightly modified conventional methods (10). The procedure used was as follows: 4L of medium (C-limiting medium) was added to a fermenter and cultured for 2 days at 27°C with air supplied at a rate of 4L/min. Following culture, an equal volume of ethanol was added. After the mixture had been left to stand overnight, the precipitate was collected. The precipitate was suspended in 250 mL of distilled water, and the water-soluble fraction collected. Ethanol was added to the soluble fraction, and the mixture allowed to stand overnight. The precipitate was collected and dried with acetone to obtain crude CMWS. This crude material was dialyzed in distilled water. The water solution was then lyophilized to obtain CMWS.

Sugar analysis

Polysaccharides were completely hydrolyzed in 2.0 M CF_3CO_2H (115°C, 1.5 hr). The sugars were converted to alditol acetates by reduction followed by treatment with acetic anhydride in an equal volume of pyridine (100°C, 1 hr), and then analyzed by GLC using a GC-2014AF instrument (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a 30 m×0.25 mm (0.25 mM) DB-225 capillary column (J&W Scientific, Folsom, CA, USA).

Other chemical analyses

The total carbohydrate concentration was determined by the phenol-sulfuric acid method using a mixture of D-mannose and D-glucose as a standard. Total protein was determined by using the BCA Protein Assay Regent kit (Pierce Biotechnology, Rockford, IL, USA), with BSA as a standard. Endotoxin content was determined by the Toxicolor LS-50M Set (Seikagaku Biobusiness, Tokyo, Japan).

Table 1. Chemical composition of CMWS

	Total carbohydrate (%)	Total protein (%)	Elemental analysis (C:H:N)	Sugar composition (Man:Glc)	Endotoxin content (ng/mg)
CMWS	49.0	9.8	40.44:6.87:2.06	3.9:1.0	N.D.

Glc; glucose, Man; mannose, N.D; not detected.

Administration schedule for induction of coronary arteritis

We used the DBA/2 mouse strain in this experiment because this strain shows the most serious coronary arteritis after treatment with the CAWS that is secreted into the culture supernatant by *Candida albicans* (11). In week1, CMWS (4 mg/mouse) was administered intraperitoneally for 5 consecutive days to each mouse. The hearts of the animals were fixed with 10% neutral formalin and embedded in paraffin blocks. Tissue sections were stained with HE. Preparation of paraffin blocks and HE staining was done by Japan SLC.

Evaluation of rapid anaphylactoid shock

The incidence and severity of rapid anaphylactoid shock was assessed within 1 hr of i.v. injection (0.1 mL/10 g body weight) of CMWS (8 mg/kg) into ICR mice. The subsequent mortality (in the first hour after injection) was recorded.

Enzyme-linked immunosorbent assay of reactivity to *Candida* serum factors

The reactivity of cell wall extracts to serum factors from *Candida* Check, which consists of rabbit polyclonal antibodies against *Candida* cell wall mannan (23–25), was assessed by ELISA. A solution of cell wall extracts in 50 mM carbonate buffer (pH 9.6) was coated onto Nunc immunoplates (Roskilde, Denmark), which were then incubated at 4°C overnight. The plates were washed extensively with PBST; unbound sites were blocked by the addition of BPBST to wells for 40 min at 37°C; and then the wells were washed six times with PBST. *Candida* serum factors serially diluted with BPBST were added and incubated for 60 min at 37°C. After six washes with PBST, the wells were treated with peroxidase-conjugated goat anti-rabbit IgG and the TMB microwell peroxidase substrate system (KPL, Gaithersburg, MD, USA). After 45 min, the reaction was stopped with 1 N H_3PO_4. The optical density of each well was then read at 450 nm on an automatic microplate reader. The reactions were evaluated as positive when the maximum optical density was over 1.0 at an 80-fold dilution ratio of *Candida* serum factor because *Candida* serum factors are polyclonal antibodies.

Nuclear magnetic resonance spectroscopy

Exchangeable protons were removed by dissolving cell wall extracts in D_2O, and samples were then lyophilized. This exchange process was repeated three times. All NMR spectra were recorded in D_2O at 308 K using a Bruker Avance 500 spectrometer equipped with a TXI *xyz* 3-gradient probe for ^1H detection. Chemical shifts are reported in p.p.m. relative to acetone-d_6 as an internal standard (δ_H = 2.189 p.p.m., δ_C = 31.45 p.p.m.). Data processing was performed using XWinNMR software. The 1D-^1H experiment was performed using a Bruker standard pulse sequence with 4310Hz in 64 K complex data points. The relaxation delay used to calculate accurate signal integrations was $5T_1$. Before Fourier transformation, four times zero filling was used, and noise was reduced using the Trafication function. 2D sensitivity improvement ^1H, ^{13}C-HSQC without decoupling during acquisition was conducted to measure $^1J_{H1,C1}$ with 512 increments of 2048 data points, with 32 scans per t_1 increment in the Bruker standard pulse sequence. The spectral width was 3501Hz for t_2 and 12 500 Hz for t_1. 2D-TOCSY was conducted with a mixing time for TOCSY spinlock of 30–180 ms using the pulse sequence of Griesinger *et al.* to suppress ROE signals (26). The spectral width was 2200 Hz in each dimension, and 512 increments of 4096 data points with 16 scans per t_1 increment were recorded. All 2D experiments were zero-filled to 2k and 2k in both dimensions before Fourier transformation. A cosine-bell window function was applied in both dimensions.

RESULTS

Chemical composition of the water-soluble polysaccharide fraction from *Candida metapsilosis*

The chemical composition of CMWS NBRC 1068 is summarized in Table 1. The fraction is mainly composed of carbohydrates (49.0%) and proteins (9.8%), but has less carbohydrate content than CAWS. The monosaccharide content of the water-soluble polysaccharide fraction was determined by GLC analysis and found to be composed of mannose and glucose in a molar ratio of 3.9:1.0.

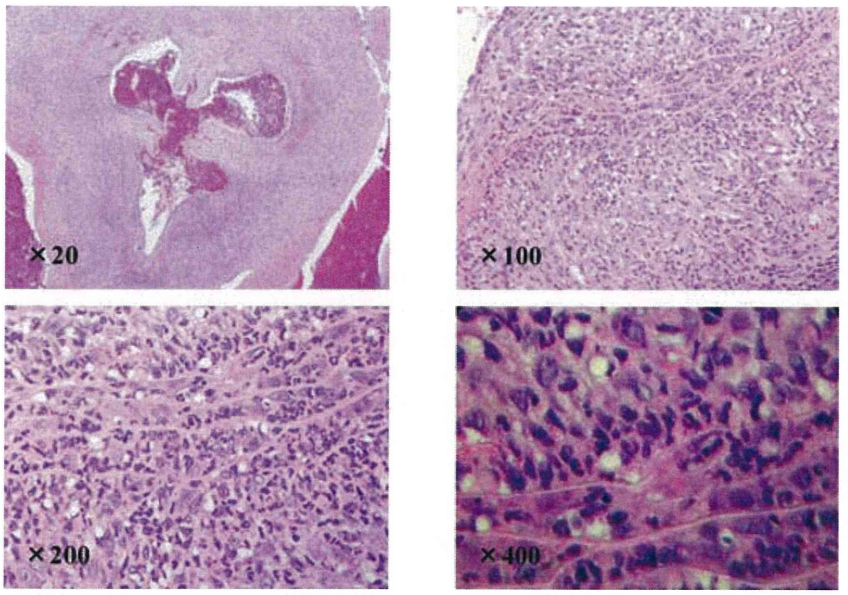

Fig. 1. Histological examination of CMWS-induced coronary arteritis in mice. CMWS (4 mg/mouse) was administered intraperitoneally to DBA/2 mice for 5 consecutive days in the first week. Five weeks later, the hearts of DBA/2 mice were fixed in buffered formalin solution, embedded in paraffin, thin-sectioned, stained with HE, and then observed under a microscope. Magnifications are shown in the figure.

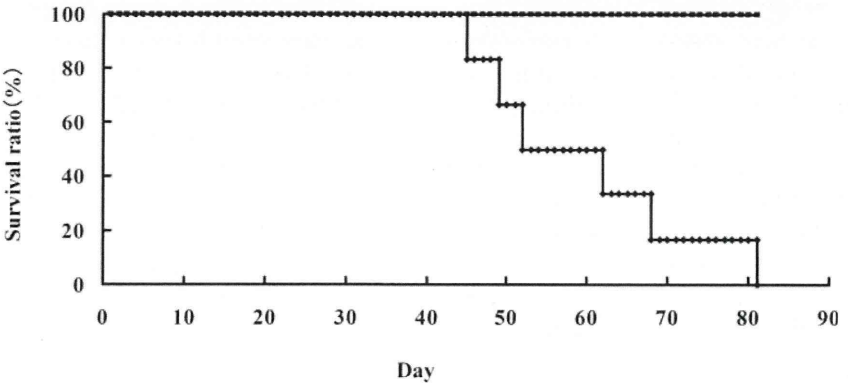

Fig. 2. Survival time of CMWS-administered mice. CMWS (4 mg/mouse) (♦) and negative control (■) were administered intraperitoneally to DBA/2 mice for 5 consecutive days in the first week. Survival was observed for 12weeks.

These analyses reveal that the water-soluble polysaccharide fraction contains the mannoprotein-glucan complex; however, no endotoxin contamination was detected.

Coronary arteritis induced by the *Candida metapsilosis* water-soluble fraction

We first examined the induction of coronary arteritis by CMWS. Figure 1 shows HE staining of the aorta in DBA/2 mice which had been administered CMWS. Histological examination showed that intraperitoneal injection of CMWS induced severe coronary arteritis in DBA/2 mice, which was similar to CAWS-induced arteritis. Coronary arteritis was also examined in terms of the survival rate. As shown in Figure 2, mice given CMWS gradually died. These studies show that not only CAWS, but also CMWS, induces severe coronary arteritis in DBA/2 mice.

Rapid anaphylactoid shock induced by the water-soluble polysaccharide fraction from *Candida metapsilosis*

We next examined another typical biological effect exhibited by CAWS and found that administration of CMWS also resulted in acute anaphylactoid shock in ICR mice (Table 2).

Reactivity of the water-soluble polysaccharide fraction from *Candida metapsilosis* to *Candida* serum factors

Since we had already found that the mannan structure is vital for biological activity, we next examined the structural differences between the mannan residues of *C. metapsilosis and C. albicans*. Figure 3 shows the reactivity of CMWS to *Candida* serum factors, which consist of rabbit polyclonal antibodies against *Candida* cell wall mannan. CMWS strongly reacted with *Candida* serum

Table 2. Ability of CMWS to induce rapid anaphylactoid shock in ICR mice

8 mg/kg	Dead/total
Saline	0/4
CMWS	4/4

factors 1, 11, 13b, and 13, but not serum factors 4, 5, 6, 8, 9, and 34. These results suggest that the mannan within CMWS might be composed only of α-type mannose residues.

Nuclear magnetic resonance analyses of the water-soluble polysaccharide fraction from *Candida metapsilosis*

For further structural characterization, we next analyzed the sample using NMR spectroscopy. Figure 4 shows the 1D-^{1}H NMR spectra of CMWS. The spectrum of CMWS contained many signals in the anomeric region of the mannose residues (δ_H 4.8–5.5p.p.m.). Thus, we could not completely assign the signals using this technique. Therefore, we further examined samples using ^{1}H, ^{13}C-HSQC spectra to detect the number of signals from the mannose residues. Figure 5 shows the overlaid HSQC spectra of CMWS (black) and CAWS (blue). The overlaid HSQC spectra show 10 signals in the anomeric regions of their mannose residues (δ_H 4.8–5.5 p.p.m., δ_C 98–104 p.p.m.) that were arbitrarily labeled numbers 1–10 as described in Table 3. However, we could not completely assign all signals at this time. Therefore, we examined the anomeric conformation of their carbohydrate residues because numerous studies have reported that the anomeric conformation of mannose residues is crucial for their pathogenicity and antigenicity (27, 28). From the observed $^{1}J_{H1,C1}$ obtained from ^{1}H, ^{13}C-HSQC spectra without decoupling during acquisition, all mannose residues were assigned to α-mannose (Table 3). We next examined samples using 2D TOCSY spectra to determine the linkage types of each residue according to the method of Shibata *et al.* (29). The findings are described in Table 3. Notably, no qualitative differences compared to CAWS were identified.

DISCUSSION

In the present study, we clearly revealed that the CMWS, which is composed of a mannoprotein-β-glucan complex, dramatically induces coronary arteritis similar to that of KD, as well as acute anaphylactoid shock, in mice. These pathogenic effects are similar to those induced by CAWS. Moreover, the structure of mannan, which is considered a factor in induction of the above-described pathogenicities, within CMWS was quite similar to that within CAWS. Based on these findings, we concluded that *Candida* mannan, especially α-mannan, might contribute to *Candida* pathogenicity with respect to coronary arteritis and acute shock.

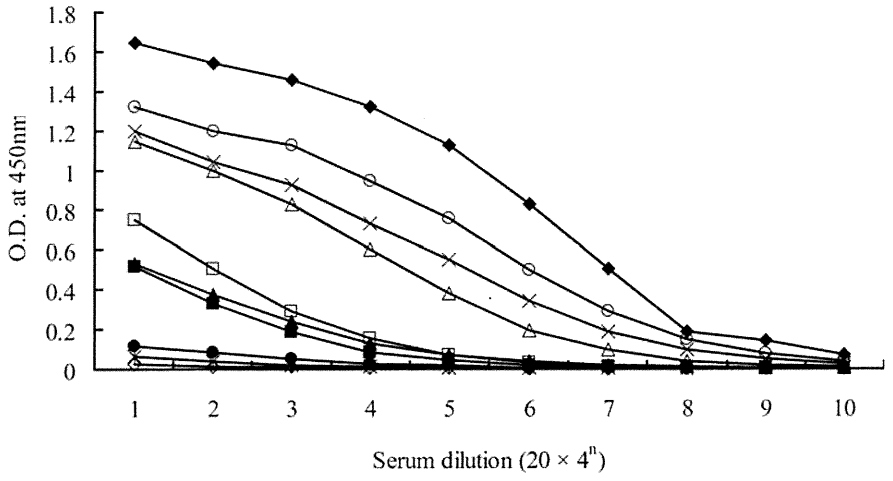

Fig. 3. Reactivity of CMWS to *Candida* serum factors. The reactivity of CMWS to *Candida* serum factors was tested by ELISA using rabbit polyclonal antibodies against *Candida* cell wall mannan. Briefly, a solution of CMWS in 50mM carbonate buffer (pH 9.6) was coated onto Nunc immunoplates. Unbound sites were blocked by the addition of 1% BSA-PBST. Serum factors 1, 4, 5, 6, 8, 9, 11, 13b, 13, and 34, serially diluted with blocking buffer, were added. Binding of immunoglobulin to CMWS was detected by peroxidase-conjugated goat anti-rabbit IgG. Then, each well was color-developed by the addition of TMB solution, and the reaction stopped by 1N H_3PO_4. The optical density of each well was read at 450 nm/ref 630 nm on an automatic microplate reader. ◆, 1; ■, 4; ▲, 5; ●, 6; ◇ 8; □, 9; △, 11; ○ 13b; ×, 13; *, 34.

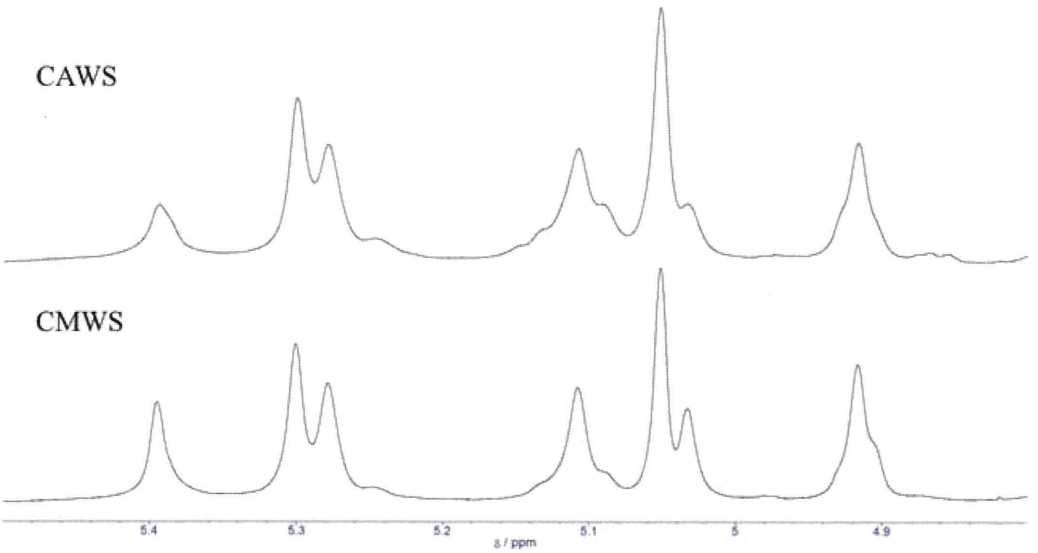

Fig. 4. 1D-^{1}H NMR spectrum of CMWS. 1D-^{1}H NMR spectra of CMWS (lower) and CAWS (upper) were recorded in D_2O at 308 K using a Bruker Avance 500 spectrometer equipped with a TXI *xyz* 3-gradient probe for ^{1}H detection. Chemical shifts are reported in p.p.m. relative to acetone-d_6 as an internal standard (δ_H = 2.189 p.p.m.).

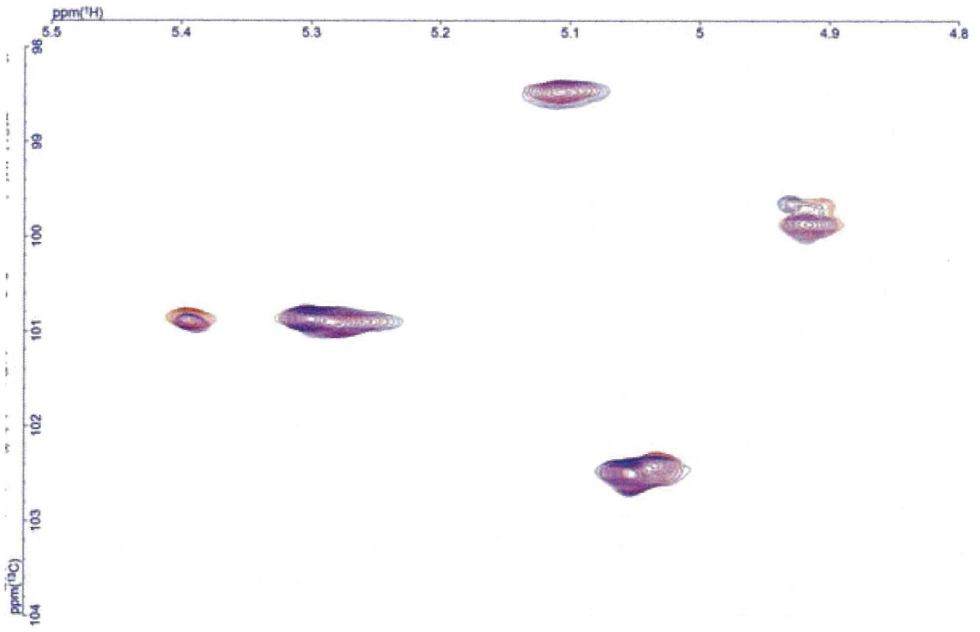

Fig. 5. Overlaid ^{1}H, ^{13}C-HSQC spectrum of CMWS. The overlaid ^{1}H, ^{13}C-HSQC spectra from CMWS (black) and CAWS (blue) were recorded in D_2O at 308 K using a Bruker Avance 500 spectrometer equipped with a TXI *xyz* 3-gradient probe for ^{1}H detection. Chemical shifts are reported in p.p.m. relative to acetone-d_6 as an internal standard (d_H = 2.189 p.p.m., δ_C = 31.45 p.p.m.).

The CMWS used in this study was mainly composed of carbohydrates (mannose and glucose) and protein, with no endotoxin contamination (Table 1). Moreover, CMWS dramatically induced coronary arteritis (Figs 1 and 2) and acute anaphylactoid shock in mice (Table 2) in the same way as CAWS does (10–17). CMWS contains 50% carbohydrates and 10% proteins. Therefore, we attempted to further purify CMWS by dialysis. After dialysis, the carbohydrate content reached 80%, after which we again assessed its biological activity in terms of induction of vasculitis and acute anaphylactoid shock in mice. We found that this purified CMWS also exhibited both pathogenic

Table 3. Anomeric conformation analyses of CMWS

	^{1}H (p.p.m.)	^{13}C (p.p.m.)	$^1J_{H1,C1}$ (Hz)	Conformation	Contains		Residue
					CMWS	CAWS	
1	5.39	100.9	173	α-mannose	•	•	α1→2**Man**α1→3Manα1→2
2	5.30	100.8	172	α-mannose	•	•	Manα1→2**Man**α1→2
3	5.28	100.9	172	α-mannose	•	•	α1→2Manα1→2**Man**α1→2
4	5.13	102.5	173	α-mannose	•	•	**Man**α1→3
5	5.11	98.4	172	α-mannose	•	•	α1→6Manα1→6**Man**α1→6Manα1→6 ↑2 Manα1(→2Manα1)$_n$
6	5.09	98.5	173	α-mannose	•	•	(→6**Man**α1→)$_n$ ↑2 Manα1 (→2Manα1)$_n$
7	5.05	102.9	171	α-mannose	•	•	**Man**α1→2
8	5.04	102.4	172	α-mannose	•	•	α1→3**Man**α1→2
9	4.92	99.9	172	α-mannose	•	•	**Man**α1→6
10	4.87	99.3	171	α-mannose	•	•	Manα1→(6Manα1)$_n$→6**Man**α1→6

effects on mice (data not shown). These results strongly indicate that the mannoprotein-β-glucan complex within CMWS could contribute to the pathogenic effects.

We next examined the mannan structure of CMWS and compared it to that of CAWS, because we have previously found that the mannan moiety might be responsible for these activities (9–15), and many reports have indicated that *Candida* cell wall mannan contributes to its antigenicity and pathogenicity (30). In addition, the structure of mannan from *Candida* differs between species (21, 31–35) and can also be altered by environmental conditions such as growth temperature (18), pH (19), and osmotic pressure (20). As revealed by the reactivity of *Candida* serum factors (Table 3), CMWS reacted to antisera against α-mannan but not β-mannan. Moreover, NMR analysis of CMWS confirmed that CMWS contains only α-mannosyl, and not β-mannosyl, residues. These serum reactivity and NMR data are similar to those of CAWS. These results strongly indicate that α-mannan, but not β-mannan, contributes to these pathogenic effects of CMWS.

Numerous studies on the antigenicity and pathogenicity of fungal cell wall mannans, especially those from *C. albicans* and *Saccharomyces cerevisiae*, have been reported. Kind *et al.* reported that the lethal toxicity and increased vascular permeability of some yeast mannans, including that of *C. albicans*, seem to depend on the 1,2-α-, 1,6-α-linkage in their main chain (30). Garner *et al.* reported that tumor necrosis factor-α is produced *in vivo* in response to mannan derived from *C. albicans* (36). These effects can be regulated by mannan ligands such as anti-mannan antibodies and corticosteroids. On the other hand, numerous studies have shown that 1,2-β-linked mannans, which are only expressed by pathogenic yeasts such as *C. albicans*, are vital for cell adhesion to host cells (27) and cytokine production from various cells (37). This specific glycan does not bind to typical mannan receptors such as the macrophage mannose receptor or mannose-binding lectin. However, some studies have recently reported that galectin-3 is the receptor for 1,2-β-linked mannan (38), and may contribute to some biological effects of mannan (39).

In our studies, CAWS, an extracellular polysaccharide fraction obtained from the culture supernatant of *C. albicans*, has been found to induce coronary arteritis and acute anaphylactoid shock (10–17). These biological effects depend on the pH of the culture process (15). CAWS synthesized in neutral pH conditions that result in the expression of 1,2-β-mannosyl residues produces significantly reduced acute anaphylactoid shock, coronary arteritis, and complement activation. This pattern was most definitely matched by the results of investigations of the activities of mannan from *C. albicans* cell wall (9). Our previous studies have clearly suggested that the β-mannosyl residue attached to nonreducing terminal α-mannosyl branched chains within an acid-stable region is very different in biologically active versus inactive mannan (9, 15). This is probably because β-mannosyl residues mask the nonreducing end of α-mannosyl residues, which leads to nonbinding of lectins, such as mannose-binding lectin, to α-mannan. Very recently, Saijo *et al.* reported that dectin-2 is a crucial receptor for the α-mannan from *C. albicans* and plays an important role in host defense against this fungus. Cytokine production and signal transduction by α-mannan from *C. albicans* are completely abolished in dectin-2$^{-/-}$ mice compared to wild-type mice (28). This implies that the pathogenic effect of CMWS could be

exhibited via dectin-2. However, this possibility needs further examination.

The present study strongly suggests that *C. metapsilosis*, a less pathogenic fungus than *C. albicans*, can cause coronary arteritis, such as that observed during KD, and fungal-induced sepsis in the same way as *C. albicans*. Since CMWS only contains α-mannosyl residue (not expressed as β-mannan), the results of this study support our previous results. However, further studies are needed because the precise mechanism(s) behind these pathogenic activities is not understood. Nevertheless, these findings suggest the possibility of a novel strategy for drug therapy; that is, regulation of the biosynthesis of *Candida* mannan could be a candidate for therapy of coronary arteritis and acute anaphylactoid shock.

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REFERENCES

1. Burns J.C., Glode M.P. (2004) Kawasaki syndrome. *Lancet* **364**: 533–44.
2. Cimaz R., Falcini F. (2003) An update on Kawasaki disease. *Autoimmun Rev* **2**: 258–63.
3. Akiyama T., Yashiro K. (1993) Probable role of *Streptococcus pyrogenes* in Kawasaki disease. *Eur J Pediatr* **152**: 82–92.
4. Esper F., Shapiro E.D., Weibel C., Ferguson D., Landry M.L., Kahn J.S. (2005) Association between a novel human coronavirus and Kawasaki disease. *J Infect Dis* **191**: 499–502.
5. Wang C.L., Wu Y.T., Liu C.A., Kuo H.C., Yang K.D. (2005) Kawasaki disease: infection, immunity and genetics. *Pediatr Infect Dis J* **24**: 998–1004.
6. Levy I., Shalit I., Birk E., Sirota L., Ashkenazi S., German B., Linder N. (2006) *Candida* endocarditis in neonates: report of five cases and review of the literature. *Mycoses* **49**: 43–8.
7. Rangel-Frausto M.S., Wiblin T., Blumberg H.M., Saiman L., Patterson J., Rinaldi M., Pfaller M., Edwards J.E. Jr., Jarvis W., Dawson J., Wenzel R.P. (1999) National epidemiology of mycoses survey (NEMIS): variations in rates of bloodstream infections due to *Candida* species in seven surgical intensive care units and six neonatal intensive care units. *Clin Infect Dis* **29**: 253–8.
8. Beck-Sague C., Jarvis W.R. (1993) Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. National Nosocomial Infections Surveillance System. *J Infect Dis* **167**: 1247–51.
9. Tada R., Nagi-Miura N., Adachi Y., Ohno N. (2008) The influence of culture conditions on vasculitis and anaphylactoid shock induced by fungal pathogen *Candida albicans* cell wall extract in mice. *Microb Pathog* **44**: 379–88.
10. Uchiyama M., Ohno N., Miura N.N., Adachi Y., Aizawa M.W., Tamura H., Tanaka S., Yadomae T. (1999) Chemical and immunochemical characterization of limulus factor G-activating substance of *Candida* spp. *FEMS Immunol Med Microbiol* **24**: 411–20.
11. Nagi-Miura N., Shingo Y., Adachi Y., Ishida-Okawara A., Oharaseki T., Takahashi K., Naoe S., Suzuki K., Ohno N. (2004) Induction of coronary arteritis with administration of CAWS (*Candida albicans* water-soluble fraction) depending on mouse strains. *Immunopharmacol Immunotoxicol* **26**: 527–43.
12. Nagi-Miura N., Harada T., Shinohara H., Kurihara K., Adachi Y., Ishida-Okawara A., Oharaseki T., Takahashi K., Naoe S., Suzuki K., Ohno N. (2006) Lethal and severe coronary arteritis in DBA/2 mice induced by fungal pathogen, CAWS, *Candida albicans* water-soluble fraction. *Atherosclerosis* **186**: 310–20.
13. Kurihara K., Shingo Y., Miura N.N., Naoe S., Usui Y., Adachi Y., Yadomae T., Ohno N. (2003) Effect of CAWS, a mannoprotein-beta-glucan complex of *Candida albicans*, on leukocyte, endothelial cell, and platelet functions *in vitro*. *Biol Pharm Bull* **26**: 233–40.
14. Tada R., Miura N.N., Adachi Y., Ohno N. (2006) *Candida albicans* derived fungal PAMPS, CAWS, water soluble mannoprotein-beta-glucan complex shows similar immunotoxicological activity with bacterial endotoxin from *Escherichia coli* O9. *Biol Pharm Bull* **29**: 240–6.
15. Shinohara H., Miura N.N., Ishibashi K., Adachi Y., Ishida-Okawara A., Oharaseki T., Takahashi K., Naoe S., Suzuki K., Ohno N. (2006) 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*. *Biol Pharm Bull* **29**: 1854–61.
16. Miura N.N, Komai M., Adachi Y., Osada N., Kameoka Y., Suzuki K., Ohno N. (2009) IL-10 is a negative regulatory factor of CAWS-vasculitis in CBA/J mice as assessed by comparison with Bruton's tyrosine kinase-deficient CBA/N mice. *J Immunol* **183**: 3417–24.
17. Ohno N. (2008) A murine model of vasculitis induced by fungal polysaccharide. *Cardiovasc Hematol Agents Med Chem* **6**: 44–52.
18. Okawa Y., Goto K., Nemoto S., Akashi M., Sugawara C., Hanzawa M., Kawamata M., Takahata T., Shibata N., Kobayashi H., Suzuki S. (1996) Antigenicity of cell wall mannans of *Candida albicans* NIH B-792 (serotype B) strain cells cultured at high temperature in yeast extract-containing Sabouraud liquid medium. *Clin Diagn Lab Immunol* **3**: 331–6.
19. Kobayashi H., Takahashi S., Shibata N., Miyauchi M., Ishida M., Sato J., Maeda K., Suzuki S. (1994) Structural modification of cell wall mannans of *Candida albicans* serotype A strains grown in yeast extract-Sabouraud liquid medium under acidic conditions. *Infect Immun* **62**: 968–73.
20. Koyama T., Makita M., Shibata N., Okawa Y. (2009) Influence of oxidative and osmotic stresses on the structure of the cell wall mannan of *Candida albicans* serotype A. *Carbohydr Res* **344**: 2195–200.
21. Shibata N., Ikuta K., Imai T., Satoh Y., Satoh R., Suzuki A., Kojima C., Kobayashi H., Hisamichi K., Suzuki S. (1995) Existence of branched side chains in the cell wall mannan of pathogenic yeast, *Candida albicans*. Structure-antigenicity relationship between the cell wall mannans of *Candida albicans* and *Candida parapsilosis*. *J Biol Chem* **270**: 1113–22.
22. Shepherd M.G., Sullivan P.A. (1976) The production and growth characteristics of yeast and mycelial forms of *Candida albicans* in continuous culture. *J Gen Microbiol* **93**: 361–70.
23. Fukazawa Y., Shinoda T., Tsuchiya T. (1968) Response and specificity of antibodies for *Candida albicans*. *J Bacteriol* **95**: 754–63.

24. Tsuchiya T., Fukazawa Y., Kawakita S. (1959) A method for the rapid identification of the genus *Candida*. *Mycopathol Mycol Appl* **10**: 191–206.
25. Suzuki S., Suzuki M. (1997) Structural investigation of mannans of medically relevant *Candida* species; determination of chemical structures of antigenic factors, 1, 4, 5, 6, 9 and 13b. *Fungal Cells Biodefense Mech* 1–15.
26. Griesinger C., Otting G., Wuthrich K., Ernst R.R. (1988) Clean TOCSY for proton spin system identification in macromolecules. *J Am Chem Soc* **110**: 7870–72.
27. Dalle F., Jouault T., Trinel PA., Esnault J., Mallet J.M., d'Athis P., Poulain D., Bonnin A. (2003) Beta-1,2- and alpha-1,2-linked oligomannosides mediate adherence of *Candida albicans* blastospores to human enterocytes *in vitro*. *Infect Immun* **71**: 7061–8.
28. Saijo S., Ikeda S., Yamabe K., Kakuta S., Ishigame H., Akitsu A., Fujikado N., Kusaka T., Kubo S., Chung S.H., Komatsu R., Miura N., Adachi Y., Ohno N., Shibuya K., Yamamoto N., Kawakami K., Yamasaki S., Saito T., Akira S., Iwakura Y. (2010) Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity* **32**: 681–91.
29. Shibata N., Suzuki A., Kobayashi H., Okawa Y. (2007) Chemical structure of the cell-wall mannan of *Candida albicans* serotype A and its difference in yeast and hyphal forms. *Biochem J* **404**: 365–72.
30. Kind L.S, Kaushal P.K., Drury P. (1972) Fatal anaphylaxis-like reaction induced by yeast mannans in nonsensitized mice. *Infect Immun* **5**: 180–2.
31. Kobayashi H., Mitobe H., Takahashi K., Yamamoto T., Shibata N., Suzuki S. (1992) Structural study of a cell wall mannan-protein complex of the pathogenic yeast *Candida glabrata* IFO 0622 strain. *Arch Biochem Biophys* **294**: 662–9.
32. Kobayashi H., Takaku M., Nishidate Y., Takahashi S., Takikawa M., Shibata N., Suzuki S. (1992) Structure of the D-mannan of the pathogenic yeast, *Candida stellatoidea* ATCC 20408 (type II) strain, in comparison with that of *C. stellatoidea* ATCC 36232 (type I) strain. *Carbohydr Res* **231**: 105–16.
33. Shibata N., Akagi R., Hosoya T., Kawahara K., Suzuki A., Ikuta K., Kobayashi H., Hisamichi K., Okawa Y., Suzuki S. (1996) Existence of novel branched side chains containing beta-1,2 and alpha-1,6 linkages corresponding to antigenic factor 9 in the mannan of *Candida guilliermondii*. *J Biol Chem* **271**: 9259–66.
34. Shibata N., Onozawa M., Tadano N., Hinosawa Y., Suzuki A., Ikuta K., Kobayashi H., Suzuki S., Okawa Y. (1996) Structure and antigenicity of the mannans of *Candida famata* and *Candida saitoana*: comparative study with the mannan of *Candida guilliermondii*. *Arch Biochem Biophys* **336**: 49–58.
35. Shibata N., Senbongi N., Hosoya T., Kawahara K., Akagi R., Suzuki A., Kobayashi H., Suzuki S., Okawa Y. (1997) Demonstration of the presence of alpha-1,6-branched side chains in the mannan of *Candida stellatoidea*. *Eur J Biochem* **246**: 477–85.
36. Garner R.E., Hudson J.A. (1996) Intravenous injection of *Candida*-derived mannan results in elevated tumor necrosis factor alpha levels in serum. *Infect Immun* **64**: 4561–66.
37. Fradin C., Poulain D., Jouault T. (2000) Beta-1,2-linked oligomannosides from *Candida albicans* bind to a 32-kilodalton macrophage membrane protein homologous to the mammalian lectin galectin-3. *Infect Immun* **68**: 4391–8.
38. Jouault T., El Abed-El Behi M., Martinez-Esparza M., Breuilh L., Trinel P.A., Chamaillard M., Trottein F., Poulain D. (2006) Specific recognition of *Candida albicans* by macrophages requires galectin-3 to discriminate *Saccharomyces cerevisiae* and needs association with TLR2 for signaling. *J Immunol* **177**: 4679–87.
39. Kohatsu L., Hsu D.K, Jegalian A.G., Liu F.T., Baum L.G. (2006) Galectin-3 induces death of *Candida* species expressing specific beta-1,2-linked mannans. *J Immunol* **177**: 4718–26.

PEDIATRIC RHEUMATOLOGY

SHORT REPORT Open Access

Mizoribine provides effective treatment of sequential histological change of arteritis and reduction of inflammatory cytokines and chemokines in an animal model of Kawasaki disease

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Abstract

Background: Intravenous immunoglobulin (IVIg) treatment results in an effective response from patients with acute-phase Kawasaki disease (KD), but 16.5% of them remain nonresponsive to IVIg. To address this therapeutic challenge, we tried a new therapeutic drug, mizoribine (MZR), in a mouse model of KD, which we have established using injections of *Candida albicans* water-soluble fractions (CAWS).

Methods: CAWS (4 mg/mouse) were injected intraperitoneally into C57BL/6N mice for 5 consecutive days. MZR or IgG was administered for 5 days. After 4 weeks, the mice were sacrificed and autopsied, the hearts were fixed in 10% neutral formalin, and plasma was taken to measure cytokines and chemokines using the Bio-Plex system. The incidence of panvasculitis in the coronary arteries and aortic root was 100% in the control group. The incidence of panvasculitis in the MZR group decreased to 50%. Moreover, the scope and severity of the inflammation of those sites were significantly reduced in the MZR group as well as the IgG group. On the other hand, increased cytokines and chemokines, such as IL-1α, TNF-α, KC, MIP-1α, GM-CSF, and IL-13, in the nontreatment group were significantly suppressed by treatment with MZR, but the MCP-1 level increased. In addition, IL-1α, TNF-α, IL-10, IL-13, and MIP-1α were suppressed by treatment in the IgG group.

Results: The incidence of panvasculitis in the coronary arteries and aortic root was 100% in the control group. The incidence of panvasculitis in the MZR group decreased to 50%. Moreover, the scope and severity of the inflammation of those sites were significantly reduced in the MZR group as well as the IgG group. On the other hand, increased cytokines and chemokines, such as IL-1α TNF-α, KC, MIP-1α, GM-CSF, and IL-13, in the nontreatment group were significantly suppressed by treatment with MZR, but the MCP-1 level increased. In addition, IL-1α, TNF-α, IL-10, IL-13, and MIP-1α were suppressed by treatment in the IgG group.

Conclusion: MZR treatment suppressed not only the incidence, range, and degree of vasculitis, but also inflammatory cytokines and chemokines in the plasma of the KD vasculitis model mice, suggesting that MZR may be useful for treatment of KD.

Keywords: Kawasaki disease, an animal model, IVIg, coronary arteritis, inflammatory cytokines and chemokines, mizoribine

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Background

Kawasaki disease (KD) is an acute febrile illness that manifests mainly in infancy and early childhood [1]. The most important complication of KD is coronary arteritis, which leads to formation of aneurysms. KD has attracted special interest because it may cause ischemic heart disease in children due to thrombosed coronary aneurysms [2]. Since the etiology and development of KD are thought to be due to the dysfunction of the immune system, intravenous immunoglobulin (IVIg) during the early acute phase has been used with an excellent response in most patients [3]. However, 16.5% of patients did not respond to the first IVIg treatment [4], and some nonresponders to the first IVIg treatment manifested severe coronary arteritis with large aneurysm [5]. Therefore, additional treatments have been tried on the nonresponders to the first treatment with IVIg. To date, a second IVIg treatment [6], plasmapheresis [7-10], pulse steroids [11], cyclophosphamide plus steroids [12], ulinastatin as an elastase inhibitor [13-16], cyclosporin A plus steroids and methotrexate plus steroids [17,18], and anti-tumor necrosis factor-α (infliximab) therapy [19-23] have been tried. Thus, for treatment of patients with KD who do not respond to IVIg, other medicines for immune response and suppression of lymphocyte proliferation have been applied due to immune dysfunction in the patients. One immune modulating medicine, mizoribine (MZR), a drug that inhibits synthesis of purine compounds (GMP), blocks proliferation of lymphocytes and will be useful for application to nonresponders to IVIg treatment. MZR has long been used as therapy for kidney transplantation, lupus nephritis, nephrotic syndrome, and rheumatoid disease with few side-effects [24]. Moreover, it has been reported to have been used for lupus nephritis, nephrotic syndrome, and IgA nephritis in children [25-28], and as a maintenance therapy in anti-neutrophil cytoplasmic autoantibody (ANCA)-associated renal failure, frequently relapsing nephrotic syndrome, and purpura nephritis [29,30]. Therefore, MZR will be a valuable therapeutic strategy for patients with KD who are nonresponsive to IVIg.

Prior to a clinical trial in children with KD, it was necessary to test MZR in a mouse model of KD, which has been established. The model we chose was the mouse model in which coronary arteritis can be induced by administration of *Candida albicans* water-soluble fractions (CAWS) [31]. This model mouse has previously been useful for evaluation of other drug treatments.

Therefore, in the present study, we tested MZR as a immunomodular for treatment of this CAWS-induced coronary arteritis. The evaluation of MZR was performed by histopathological findings and profiles of chemokines and cytokines. Also, this treatment effect was compared with that of IgG.

Methods

Animals

Four-week-old male C57BL/6N mice were purchased from Charles River Japan (Yokohama, Japan). All mice were kept under specific pathogen-free (SPF) conditions, according to the guidelines for animal care of the National Institute of Infectious Diseases in Tokyo (NIID).

Preparation of CAWS

CAWS was prepared from *C. albicans* strain IFO1385 in accordance with the reported method [31]. Briefly, 5 liters of medium (C-limiting medium) was added to a glass incubator, and the culture was maintained for 2 days at 27°C while air was supplied at a rate of 5 liters/min and the mixture was swirled at 400 rpm. Following culture, an equal volume of ethanol was added. After allowing this to stand overnight, the precipitate was collected. After dissolving the precipitate 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 of MZR and IgG to the mice

CAWS (4 mg/mouse/day) in a volume of 0.2 ml was intraperitoneally injected into a C57BL/6N mouse (4-week old male) on each of 5 consecutive days. Subsequently, MZR (a kind gift of Asahikasei Pharma Corporation (Tokyo, Japan)) was administered at a dose of 30 mg/kg/day intraperitoneally for 5 days from the third day of CAWS injection (MZB group), according to the schecule for treatments such as IgG for patients with KD, and the dosage as described elsewhere [32]. Mice for the control group were intraperitoneally treated with 0.2 ml of Dulbecco's phosphate-buffered saline (PBS). After 35 days, the mice were sacrificed by carbon dioxide asphyxiation; autopsy was performed to obtain plasma, and hearts were fixed with 10% neutralized formalin. For a positive control, treatment with intraperitoneal human IgG (Kenketsu Glovenin I, a kind gift of Nihon Pharmaceutical Co. Ltd., Tokyo, Japan) was performed at a dose of 400 mg/mouse/day, or for a negative control saline containing 0.1% glucose (SG) was injected for 5 days according to the same procedures as described elsewhere (IgG group) [33]. The start date of the drug administration was based on the results that the administration from the third experimental day had been the most effective to suppress the development of vasculitis.

Histological evaluation

The fixed hearts were embedded in paraffin and sectioned. To observe the histological changes in the coronary arteries and the aorta in detail, 20 to 30 horizontal step sections per mouse were made every 20 μm. Hematoxylin and eosin (H&E)-stained sections were prepared by using routine techniques for examination by light microscopy [31]. First, we investigated the incidence of mice with panvasculitis in each group. Panvasculitis was defined as inflammation of all layers of the walls of the coronary arteries and/or the aorta. Then, for quantitative evaluation of vascular inflammation, we divided the area of the aortic root and coronary arteries into five segments and graded the intensity of inflammation in each segment as follows: score 3, panvasculitis; score 2, inflammation involving the tunica intima and adventitia; score 1, inflammation localized to the tunica intima; and score 0, no inflammatory cell infiltration in the vascular wall. A section with the severe inflammation was observed in each segment. The scope of inflammation was defined as the number of segments evaluated as score 1 or more in each mouse, and the severity of the arteritis was defined as the average score of the five segments in each mouse.

Measurement of cytokines and chemokines with Bio-Plex

Cytokines and chemokines in the plasma of mice autopsied were measured by a Bio-Plex system. An aliquot of serum (12 μl) collected from peripheral blood and diluted 4-fold with the dilution solution was measured for concentration of cytokines by the 23-Plex kit using Bio-Plex 200 according to the manufacturer's protocol and analyzed by the Bio-Plex Luminex 100 XYP instrument (Bio-Rad, Hercules, California, USA). We assayed the following 23 cytokines and chemokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, eotaxin, G-CSF, GM-CSF, INF-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α as estimated with a single assay to a single standard curve described in the kit instructions. Concentrations of cytokines and chemokines were calculated using Bio-Plex Manager 3.0 software (Bio-Rad, Tokyo) with a five-parameter curve-fitting algorithm applied for standard curve calculations [34].

Statistical analysis

Fisher's exact probability test was used to analyze the differences in the incidence of arteritis among the groups. The data on the scope and severity of the arteritis and cytokine/chemokine levels were analyzed using the two-sample *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Histological evaluation of panvasculitis in treatment with MZR

Panvasculitis developed in the coronary arteries and the aortic root, and histology was similar to that previously described [33]. Specifically, vascular changes were classified as proliferative inflammation that consisted mainly of large mononuclear cells such as histiocytes and fibroblasts and of a small number of neutrophils. The normal structure of the arteries was completely destroyed, and the internal elastic lamina, external elastic lamina, and smooth muscle layer of the tunica media were severely damaged. However, fibrinoid necrosis was not observed in any of the mice. In addition, the histology of panvasculitis was similar in the three groups (Figure 1).

Decrease of coronary arteritis by treatment with MZR

Panvasculitis of the coronary arteries and the aortic root was observed in 5 of 5 mice (100%) in the nontreated control group. On the other hand, the incidence of panvasculitis in the MZR group was 3 of 6 mice (50%), and the IgG group as an effective control showed 3 of 7 (43%) (Figure 2A). In addition, the number of segments evaluated as score 1 or more in each MZR group was decreased compared with the nontreated control group ($P = 0.06$), and the scope of inflammation in IgG groups was significantly lower than in the control group ($P < 0.05$) (Figure 2B). Furthermore, the severity of the arteritis, i.e., the scores of each of five segments in the mice in the MZR and IgG groups, was significantly lower than in the nontreated control group ($P < 0.01$) (Figure 2C).

Reduction of inflammatory cytokines and chemokines by treatment with MZR and IgG

Inflammatory cytokines IL-1α, TNF-α, chemokines KC, MIP-1α, GM-CSF, and Th2, and cytokine IL-13 in plasma of mice, which were inoculated with CAWS in the control group, were elevated (Figure 3). However, in the MZR group, plasma levels of inflammatory cytokines IL-1α ($P < 0.01$) and TNF-α ($P < 0.05$), and chemokines KC ($P < 0.01$), MIP-1α ($P < 0.01$), and GM-CSF ($P < 0.05$) were significantly suppressed (Figure 3A). Inversely, the MCP-1 level increased with MZR treatment (Figure 3A). On the other hand, IL-1α ($P < 0.05$), TNF-α ($P < 0.05$), IL-10 ($P < 0.05$), and IL-13 ($P < 0.01$) were suppressed by administration of IgG (Figure 3B).

Furthermore, we analyzed levels of cytokines/chemokines in plasma, which were related with suppression of the development of coronary arteritis by treatment with MZR. As shown in Figure 4A, the suppression levels were almost the same in all plasmas of MZR-treated mice. These results are not the same as those in the IgG

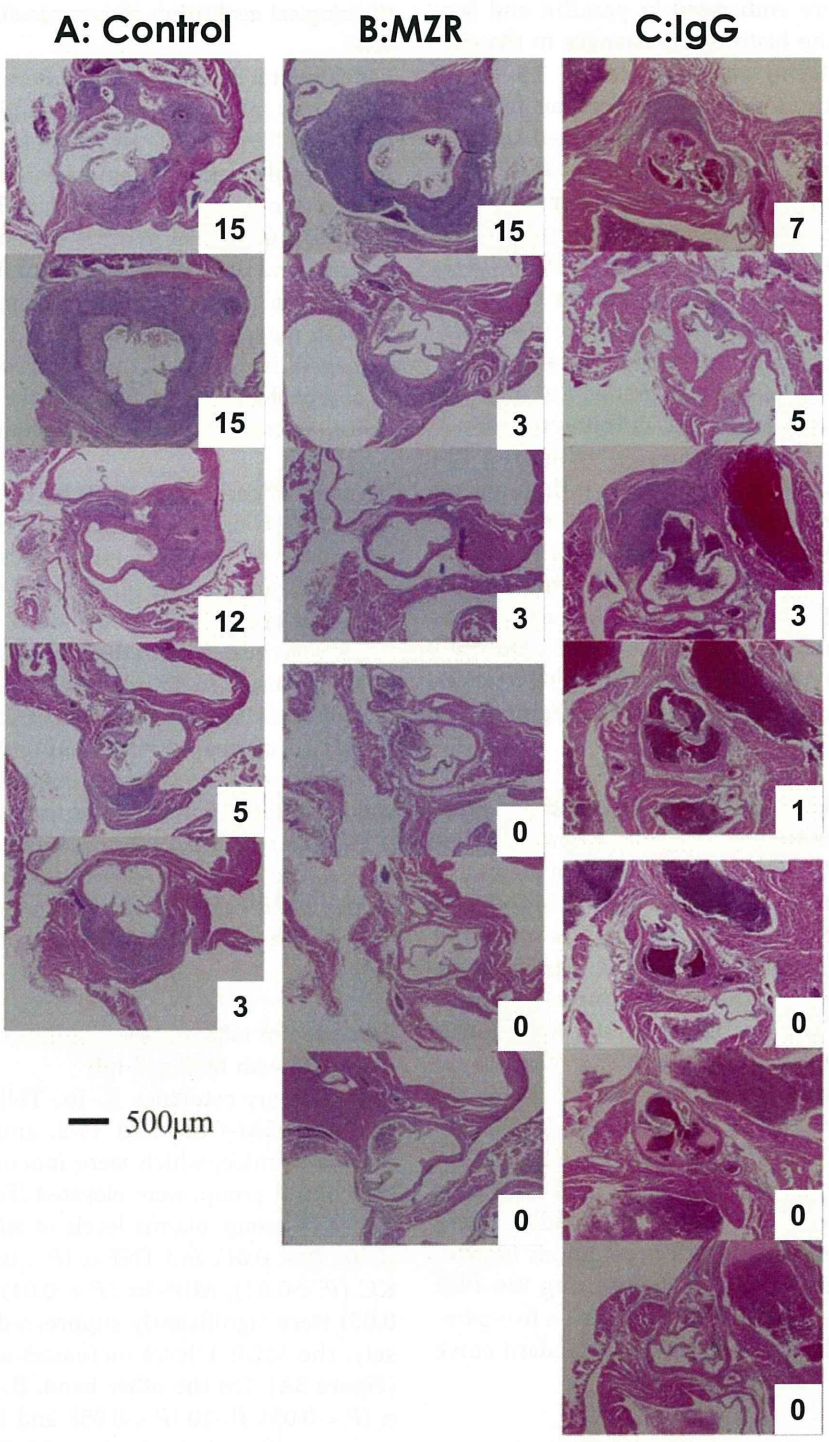

Figure 1 Histological observations of coronary arteritis induced by treatment with MZR and IgG. A, Control (PBS); B, MZR group; C, IgG group. Each micrograph represents an individual mouse. H&E stain, Bar: 500 μm. Numbers (white) are coronary arteritis score.

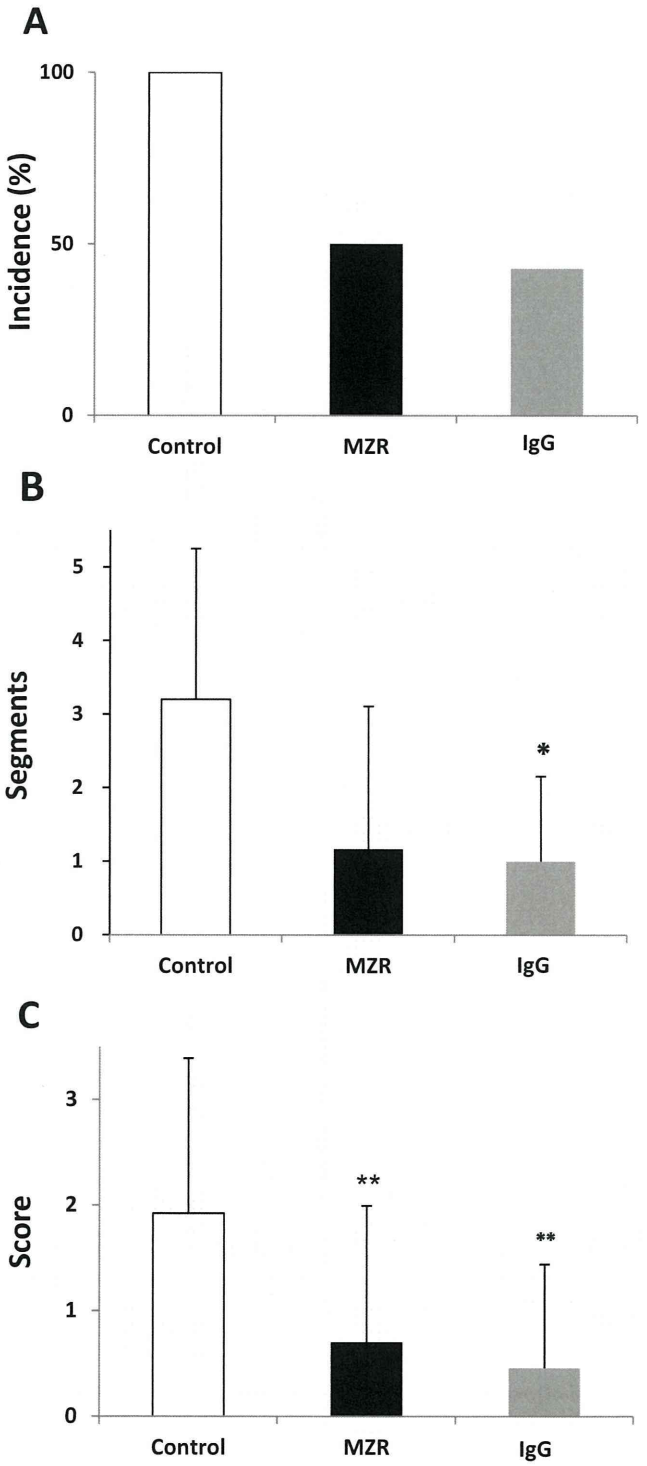

Figure 2 Decrease of incidence of panvasculitis, segment score, and severity score of coronary arteritis by treatment with MZR and IgG. A, incidence of development of panvasculitis; B, scope as number of segments with inflammation evaluated as score 1 or more at aortic root and coronary arteries; C, severity score of each segment. Data are expressed as mean ± SD of results from three individuals. *$P < 0.05$ and **$P < 0.01$ (Student's *t*-test).

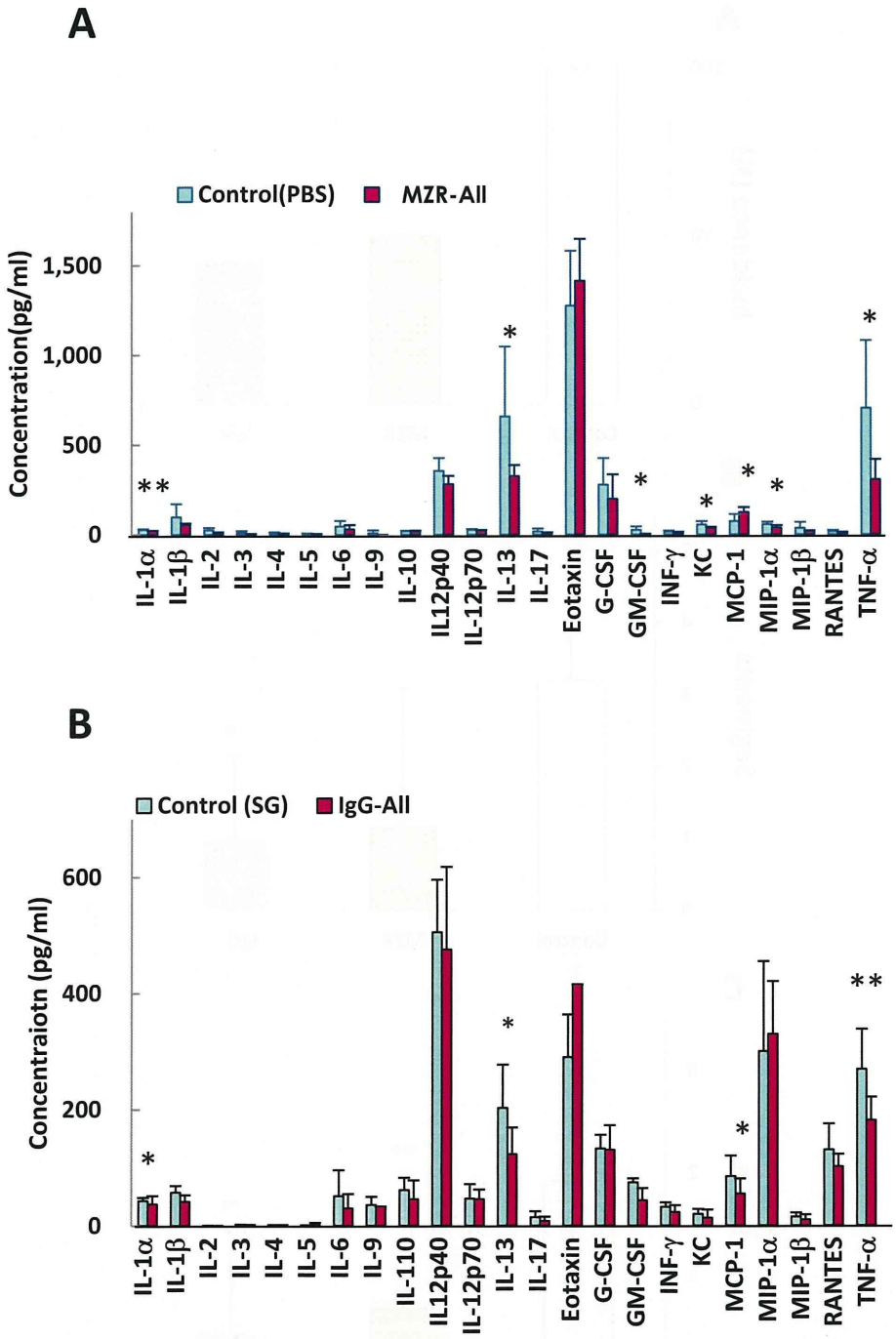

Figure 3 Reduction and enhancement of cytokines and chemokines by treatment with MZR and IgG. A, MZR treatment; B, IgG treatment. SG: saline including 0.1% glucose. Data are expressed as mean ± SD of results from three individuals. *$P < 0.05$ and **$P < 0.01$.

group, showing good response for suppression of the development of coronary arteritis (Figure 4B).

Discussion

Decrease of coronary arteritis by treatment with MZR

We here have shown the efficacy of MZR on vascular inflammation by using a KD vasculitis mouse model to develop alternative treatments for KD patients who are nonresponsive to IVIg treatment. The results here show that the incidence, scope, and degree of inflammation of the coronary arteries and the aortic root were suppressed by MZR administration. Coronary arteritis in this CAWS-induced vasculitis mouse model is also suppressed after administration of IVIg [33]. Furthermore,

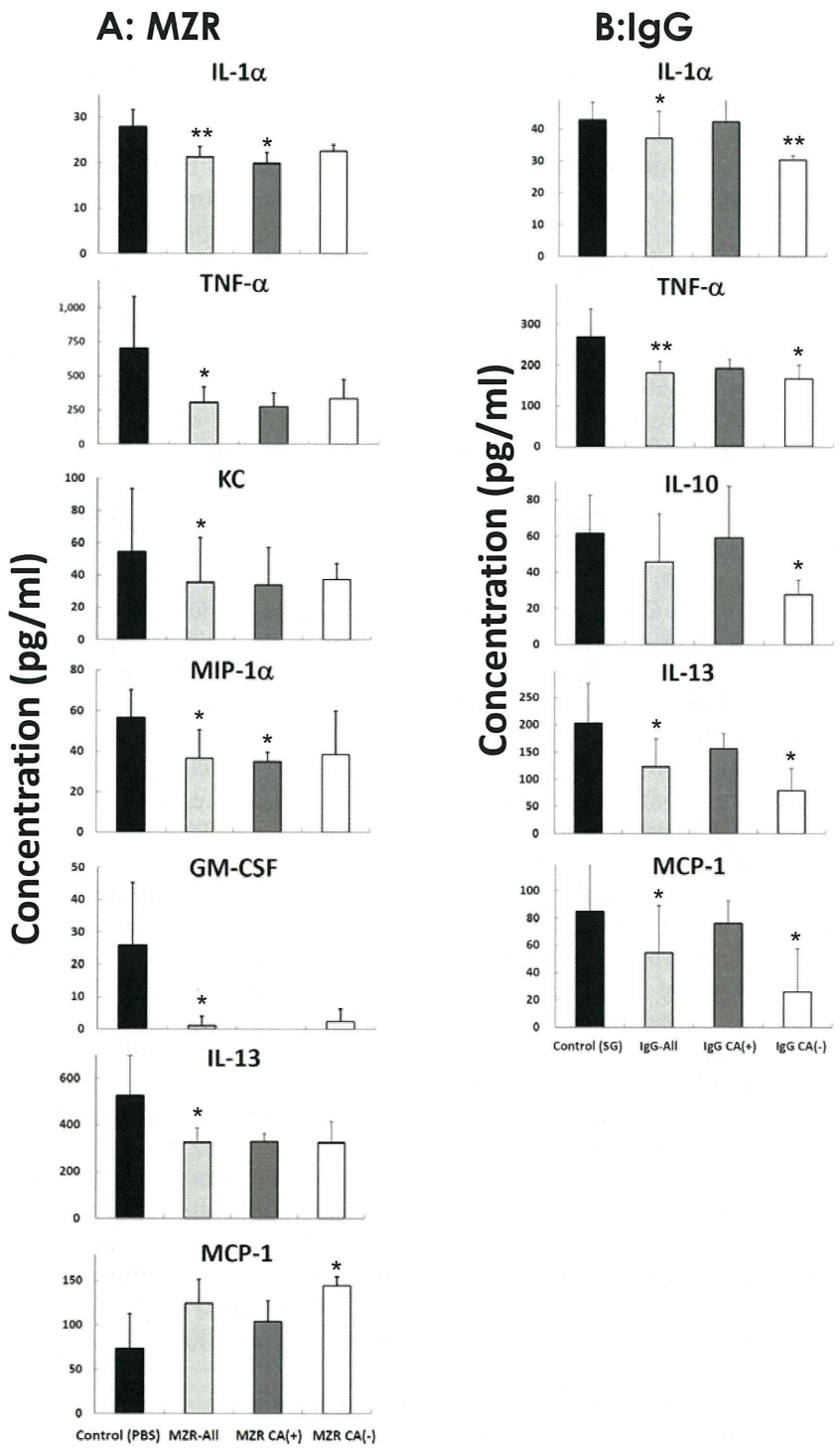

Figure 4 Reduction and enhancement of cytokines and chemokines in coronary arteritis treated with MZR and IgG. A, MZR treatment; B, IgG treatment. CA(+): coronary arteritis score > 0, CA(-): coronary arteritis score = 0, SG: saline including 0.1% glucose. Data are expressed as mean ± SD of results from three individuals. *$P < 0.05$ and **$P < 0.01$.

we have also demonstrated that the anti-TNF-α therapy that has been shown to be effective in treating some children unresponsive to IVIg therapy also dramatically suppresses the development of vasculitis in this mouse model of KD (manuscript in preparation). Thus this mouse model appears to be valuable for evaluation of alternative therapies for KD arteritis.

Reduction of inflammatory cytokines and chemokines by treatment with MZR

Some cytokines and chemokines such as IL-1β, IL-2, sIL-2R, IL-4, IL-6, IL-8, IL-10, IL-12, IL-15, RANTES, MCP-1, M-CSF, G-CSF, and MIPs are elevated in the blood of patients with acute-phase KD. Some elevated cytokines and chemokines are decreased by IVIg treatment in the acute phase, when it is effective [35]. On the other hand, IL-6, TNF-α, IL-4, and IL-12 were increased in the plasma of the KD mouse model induced with *C. albicans*-derived substances (CADS) [36]. Moreover, IL-6 and IFN-γ in splenocytes administered CAWS in C57BL/6 mice were elevated [37]. With IVIg treatment of KD model mice induced with CAWS, elevated proinflammatory cytokines IL-1α, TNF-α, IL-10, IL-13, and MCP-1 were decreased in our data. Furthermore, chemokines IL-1α, TNF-α, KC, GM-CSF, IL-13, and MIP-1α in plasma of autopsied mice were decreased in the MZR treatment group in the present study. The results with MZR treatement show similar effects as well as IgG treatment for KD model mice. However, supression levels of IL-1α, TNF-α, IL-10, IL-13, and MIP-1α in the recovery group from the coronary arteritis (CA(-)) in the MZR group differed from those in the IgG group. Levels in the recovery group (CA(-)) after MZR treatment were not suppressed, whereas those in the CA (-) group after IgG treatment were suppressed in the present study, which suggests that MZR may have a stronger effect than a high dose of IgG (400 mg/kg/day for 5 days). Because these cytokines/chemokines decrease slightly after MZR treatment, they may have a role in the development of coronary arteritis in the KD model.

Effective treatment with MZR of model mice for KD induced by CAWS

In the present study, MZR treatment of the KD model mice significantly suppressed the development of coronary arteritis associated with significant suppression of levels of proinflamatory cytokines and chemokines in plasma. These results suggest association of the suppression of lymphocyte proliferation with MZR [38]. The mode of action of MZR is that it mainly blocks immunosuppression related to lymphocyte proliferation through inhibition of purine synthesis [32,39,40]. In the present study, the incidence of panarteritis decreased to half, and both the scope and severity of inflammation were limited after administration of MZR. In addition to the lymphocyte action, these observations suggest that MZR may act on functions of monocytes/macrophages and neutrophils, which are mainly involved in the development of inflammation, resulting in the possible suppression of coronary arteritis through suppression of proinflammatory cytokines and chemokines released from these cells. Indeed, recently, MZR acted to inhibit functions of lymphocytes as well as those of macrophages, such as migration and production of Nitrous Oxide Systems (NOS), IL-1β, and TNF-α in a dose-dependent manner [41,42]. Furthermore, in the mixed lymphocyte reaction method (MLR) of human peripheral blood mononuclear cells, the IC_{50} is 1 μg/ml [43]. In addition, MLR of T-cells in human peripheral blood, which are stimulated with anti-CD3 monoclonal antibody, shows an IC_{50} of less than 1 μg/ml for MZR and also phorbol myristate stimulation less than 5 μg/ml [44]. In addition, MZR also inhibits activation of M1 macrophages [42], which are classified as inflammatory, showing tissue injury and activation with IFN-γ. In the present study, suppression profiles of proinflammatory cytokines and chemokines by MZR treatment of KD model mice seem to be associated in the literature with those in the M1 macrophage. Therefore, the effect of MZR on KD model mice may be to inhibit the proliferation of lymphocytes and activation of macrophages and neutrophils associated with elevation of proinflamatory cytokines and chemokines.

Based on these observations, suppression of development of coronary arteritis associated with suppression of proinflammatory cytokines and chemokines by MZR treatment for the KD model mice suggests that MZR may be useful for patients with KD in the acute phase. MZR has been used as therapy for kidney transplantation, lupus nephritis, nephrotic syndrome, and rheumatoid disease with few side effects [24]. Furthermore, MZR has been used as maintenance treatment for ANCA-associated vasculitis, frequently relapsing nephrotic syndrome, and purpura nephritis [29,30]. Clinical use will be recommended for immune dysfunctions when the safety of long-time use becomes known. Therefore, MZR is a possible therapy for patients with KD who are nonresponsive to IVIg.

Conclusions

MZR treatment suppressed not only the incidence, range, and degree of vasculitis, but also inflammatory cytokines and chemokines in the plasma of the KD vasculitis model mice. It appears likely that MZR may prove to be a useful for alternative treatment for KD.

Abbreviations used

ANCA: anti-neutrophil cytoplasmic autoantibody; CAWS: *Candida albicans* water-soluble fractions; H&E: