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Susceptibility Loci to Coronary Arteritis in Animal Model of Kawasaki Disease Induced with *Candida albicans*-Derived Substances

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***Abstract*: We have established an animal model of coronary arteritis which is histopathologically similar to that observed in cases of Kawasaki disease (KD), is a well-known childhood vasculitis syndrome. Coronary arteritis in this mouse model has been induced by intraperitoneal injection of *Candida albicans*-derived substances (CADS). Arteritis varied by mouse strain with the highest incidence by 71.1% (27/38) found in C3H/HeN mice, but absent in CBA/JN mice (0%, 0/27), suggesting association of genomic background to develop the disease. The present study aims to elucidate the susceptibility loci associated with coronary arteritis by using this animal model. The association of the onset of arteritis with polymorphic microsatellite markers between the two strains was examined using one hundred and fifteen of N1 backcross progeny [(CBA×C3H)F1×C3H]. Based on our analysis, arteritis-susceptibility loci with suggestive linkage were mapped on *D1Mit171* and *D1Mit245* (map position 20.2 cM) on chromosome 1 (P=0.0019). These loci include several kinds of inflammatory cytokine receptors, such as interleukin 1 receptor and tumor necrosis factor receptor. We also found the cytokine response against CADS, levels of inflammatory cytokines interleukin-1β, tumor necrosis factor-α, and interleukin-6 in sera increased within 24 hr after CADS injection. Our results may indicate based on genomics that ligand-receptor interaction between these inflammatory cytokines and the receptors of these cytokines may affect the onset of arteritis.**

***Key words*: Kawasaki disease, Arteritis, *Candida albicans*, Interleukin 1 receptor, Chromosome mapping**

Kawasaki disease (KD) is an acute febrile mucocutaneous syndrome with systemic vasculitis mainly affecting infants and small children. The principal symptoms of KD are fever, congestion of ocular conjunctivae, reddening of lips and oral mucosa, swelling and reddening of palms and soles followed by peeling of skin, swelling of cervical lymph nodes coincidentally with systemic vasculitis (9). Inflammation of medium-sized muscular arteries, especially the coronary artery, is commonly associated with this disease. Ischemic heart disease with thrombotic occlusion, originating from coronary arteritis is a severe complication of KD. Histopathologically, it was reported that arteritis defined as 'productive granulomatous inflammation' was typical in KD cases (15, 19). This type of inflammation consists of dense infiltration of both neutrophils and histiocytes accompanied with a few lymphocytes. Mechanisms of developing arteritis in the patients with KD

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Abbreviations: CADS, *Candida albicans* derived substances; cM, centi-morgan; ELISA, enzyme-linked immunosorbent assay; EvG, Elastica van Gieson; HE, hematoxylin and eosin; IFN-γ, interferon-γ; IL, interleukin; *Il1r1*, interleukin-1 receptor type 1; *Il1r2*, interleukin-1 receptor type 2; KD, Kawasaki disease; MCLS-6, mucocutaneous lymphnode syndrome-6; MPO, myeloperoxidase; MPO-ANCA, myeloperoxidase-antineutrophilic cytoplasmic antibody; PCR, polymerase chain reaction; QTL, quantitative trait of loci; TNF-α, tumor necrosis factor α; *Tnfrsf1b*, TNF receptor superfamily member 1b; *Tnfrsf8*, TNF receptor superfamily member 8; *Tnfrsf9*, TNF receptor superfamily member 9.

remain to be determined; however, there are some reports that coronary arteritis is affected by genetic polymorphism of several kinds of inflammatory cytokines, such as tumor necrosis factor α (TNF-α) (8), and interleukin-6 (IL-6) (7). Appropriate animal models of KD will allow for the clarification of the mechanisms governing the development of arteritis, and possibly, specific treatments for this disease. One of the animal models of arteritis that exist is the MRL/lpr mouse model. It is the standard animal model for studying systemic lupus erythematosus, with a common affliction to spontaneous arteritis. In MRL/lpr mice, some genes associated with arteritis have been elucidated (5). Recently, it was reported that arteritis in different tissues were under the control of different susceptibility loci (21).

Some infectious microorganisms, such as *Staphylococcus aureus* (11), *Streptococcus sanguis* (16), *Streptococcus pyogenes* (22), and *Rickettsia* (6) have been considered likely etiology candidates for this disease, though the primary causes remain unclear. These microorganisms are considered to act as the initial trigger for the development of arteritis in the patients with KD. Therefore, the initial trigger by an infectious microorganism is necessary for ideal model of KD to induce arteritis. However, this spontaneous arteritis model may not be well suited as an animal model for KD. On the other hand, Murata (13) has established a unique arteritis model that has been evaluated as an animal model of KD. In this model, arteritis induction is ascertained by injecting mice with alkaline extract of *Candida albicans* as an experimental arteritis. It should be noted that the quantity of this yeast was observed to be in elevated in stool samples of KD patients (14). The histology of this experimental arteritis model is similar to that of an autopsy case of KD (2). In this model, genetics in mice may have an influence on the development of arteritis. It was shown that the incidence of coronary arteritis varied by mouse strain, with the C3H/HeN mice having the highest incidence and coronary arteritis being absent in CBA/JN strains (20).

To identify susceptibility loci to the coronary arteritis, we analyzed coronary arteritis in [(CBA/JN×C3H/HeN)×C3H/HeN]N1 backcross progeny. The evidence presented herein shows that the susceptibility loci are linked to genes of several inflammatory cytokine receptors found in the coronary artery.

Materials and Methods

Chemicals. Sabouraud-2% dextrose broth (MERCK, Darmstadt, Germany) was used as culture medium. Sodium chloride, potassium hydroxide, acetic acid, ethanol, acetone, diethyl ether (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and *n*-octyl alcohol (Kanto Chemical Co., Tokyo) were used for polysaccharide extraction from the cell wall of *C. albicans*.

Genomic DNA was isolated from whole blood obtained from the tail of animals using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). FAM labeled primers for microsatellite markers were purchased from SIGMA Genosys Japan (Ishikari, Japan). Amplification and labeling of each microsatellite locus were performed by using Z-*Taq* polymerase (TaKaRa, Kyoto, Japan).

Animals. Mice, CBA/JNCrj (CBA/JN) and C3H/HeNCrj (C3H/HeN), were purchased from Charles River Japan (Astugi, Japan). Using C3H/HeN and CBA/JN strains, (C3H/HeN female×CBA/JN male)F1, (CBA/JN female×C3H/HeN male)F1, and [(CBA/JN×C3H/HeN)F1×C3H/HeN]N1 were prepared. N1 backcross progeny, 4-week-old males, were used for the linkage analysis (n=115). These mice were housed in a specific pathogen-free animal quarter and cared for under strict ethical guidelines.

Preparation of alkaline extract of C. albicans *(CADS).* CADS were prepared as follows. *C. albicans* (strain MCLS-6) isolated from the feces of patients with typical Kawasaki disease, was cultured in Sabouraud's dextrose medium with 2% glucose at 37 C. After a 72-hr incubation period, the yeast was harvested by centrifugation and extracted sequentially with boiling water, 0.1 M, and 0.5 M potassium hydroxide. After neutralization with acetic acid and dialysis against distilled water for 3 days, the extract was precipitated with ethanol. Four milligrams of the CADS, suspended in 0.2 ml of phosphate-buffered saline without calcium and magnesium (PBS(−)), were prepared as the inoculants.

Experimental schedule. Inoculation was conducted as described in the previous procedures (13). Namely, mice were injected once daily with 0.2 ml of inoculate intraperitoneally for 5 consecutive days during the first and fifth week. Each mouse was sacrificed with carbon dioxide asphyxiation at the ninth week and autopsied.

Histopathological evaluation of arteritis. The following visceral organs were obtained for histopathological examination: heart, aorta, kidney, lung, liver, pancreas, spleen, thymus, testis, muscle of hind leg, and spine. These specimens were fixed in 10% formalin and embedded in paraffin. Hematoxylin and eosin (HE) and Elastica van Gieson (EvG) stains were performed by routine histological techniques. Arteritis in individual mice was determined using light microscopy. A mouse with inflammation involving all layers of coronary artery and/or the aortic root was considered positive for coronary arteritis and used for linkage analysis.

Microsatellite markers. Two hundred and fifty-six markers (Fig. 1) were used for the linkage analysis between C3H/HeN and CBA/JN mice. Amplification and labeling of specific microsatellite loci were performed by using the polymerase chain reaction (PCR) with FAM labeled primers. Amplified DNA was analyzed with automated fragment analyzer ABI3700 and genotyped by Genescan software (Applied Biosystems, Japan).

Linkage analysis. Genotype distribution was compared among affected and non-affected N1 mice. Since the trait distribution was similar, we performed non-

Chromosome 1 (53)									
	D1Mcg101	D1Mcg2	D1Mcg3	D1Mcg4	D1Mcg6	D1Mit120	D1Mit121	D1Mit160	D1Mit160
	D1Mit161	D1Mit167	D1Mit17	D1Mit171	D1Mit180	D1Mit19	D1Mit200	D1Mit211	D1Mit230
	D1Mit24	D1Mit242	D1Mit245	D1Mit251	D1Mit296	D1Mit3	D1Mit303	D1Mit316	D1Mit318
	D1Mit321	D1Mit322	D1Mit363	D1Mit372	D1Mit373	D1Mit374	D1Mit380	D1Mit410	D1Mit427
	D1Mit429	D1Mit430	D1Mit431	D1Mit432	D1Mit465	D1Mit518	D1Mit52	D1Mit52.2	D1Mit520
	D1Mit58	D1Mit64	D1Mit66	D1Mit67	D1Mit7	D1Mit70	D1Mit73	D1Mit75	
Chromosome 2 (25)									
	D2Mit106	D2Mit110	D2Mit139	D2Mit166	D2Mit169	D2Mit194	D2Mit200	D2Mit206	D2Mit226
	D2Mit255	D2Mit258	D2Mit265	D2Mit304	D2Mit305	D2Mit307	D2Mit311	D2Mit413	D2Mit443
	D2Mit456	D2Mit457	D2Mit496	D2Mit51	D2Mit6	D2Mit91	D2Mit92		
Chromosome 3 (11)									
	D3Mit200	D3Mit203	D3Mit230	D3Mit28	D3Mit29	D3Mit3	D3Mit305	D3Mit323	D3Mit361
	D3Mit46	D3Mit90							
Chromosome 4 (37)									
	D4Mit116	D4Mit122	D4Mit126	D4Mit134	D4Mit146	D4Mit169	D4Mit18	D4Mit180	D4Mit181
	D4Mit190	D4Mit203	D4Mit219	D4Mit225	D4Mit226	D4Mit227	D4Mit234.2	D4Mit251	D4Mit255
	D4Mit26	D4Mit27	D4Mit272	D4Mit285	D4Mit310	D4Mit33	D4Mit331	D4Mit336	D4Mit348
	D4Mit354	D4Mit357	D4Mit42	D4Mit43	D4Mit45	D4Mit65	D4Mit71	D4Mit81	D4Mit84
	D4Nds3								
Chromosome 5 (22)									
	D5Mit10	D5Mit101	D5Mit108	D5Mit115	D5Mit13	D5Mit134	D5Mit23	D5Mit233	D5Mit239
	D5Mit254	D5Mit26	D5Mit291	D5Mit297	D5Mit314	D5Mit338	D5Mit348	D5Mit371	D5Mit406
	D5Mit425	D5Mit65	D5Mit79	D5Mit93					
Chromosome 6 (4)									
	D6Mit345	D6Mit366	D6Mit8	D6Mit254					
Chromosome 7 (5)									
	D7Mit232	D7Mit259	D7Mit27	D7Mit39	D7Nds5				
Chromosome 8 (3)									
	D8Mit14	D8Mit224	Mt2(D8Mit15)						
Chromosome 9 (3)									
	Cyp1a2(ch#9)		D9Mit2	D9Mit279					
Chromosome 10 (16)									
	D10Mit115	D10Mit134	D10Mit15	D10Mit150	D10Mit186	D10Mit20	D10Mit209	D10Mit214	D10Mit230
	D10Mit261	D10Mit266	D10Mit282	D10Mit297	D10Mit313	D10Mit36	D10Mit61		
Chromosome 11 (3)									
	D11Mit157	D11Mit2	Hoxb(Ch#11)						
Chromosome 12 (4)									
	D12Mit158	D12Mit190	D12Mit231	D12Mit292					
Chromosome 13 (9)									
	D13Mit110	D13Mit186	D13Mit24	D13Mit253	D13Mit26	D13Mit283	D13Mit35	D13Mit48	D13Mit69
Chromosome 14(3)									
	D14Mit2	D14Mit95	D14Nds5						
Chromosome 15(5)									
	D15Mit234	D15Mit29	D15Mit34	D15Mit6	D15Mit90				
Chromosome 16 (7)									
	D16Mit110	D16Mit13	D16Mit211	D16Mit5	D16Mit57	D16Mit88	D16Mit94		
Chromosome 17 (11)									
	D17Mit11	D17Mit119	D17Mit152	D17Mit155	D17Mit176	D17Mit21	D17Mit221	D17Mit266	D17Mit51
	D17Mit52	D17Mit96							
Chromosome 18 (3)									
	D18Mit3	D18Mit40	D18Mit60						
Chromosome 19 (7)									
	D19Mit10	D19Mit10	D19Mit128	D19Mit18	D19Mit8	D19Mit85	D19Mit90		
Chromosome X (25)									
	DXMit119	DXMit121	DXMit143	DXMit149	DXMit154	DXMit156	DXMit16	DXMit189	DXMit197
	DXMit199	DXMit236	DXMit248	DXMit249	DXMit31	DXMit5	DXMit54	DXMit55	DXMit64
	DXMit67	DXMit73	DXMit74	DXMit84	DXMit89	DXMit95	DXMit99		

Fig. 1. A list of markers examined difference between C3H and CBA mouse in a total 256 markers.

parametric statistical analysis for establishing genetic linkage. Contingency tables consisting of affected and non-affected C3H/C3H and C3H/CBA strains were constructed, and chi square (χ^2) tests were performed with one degree of freedom. As recommended by Lander and Kruglyak, $P<0.0034$ ($\chi^2>8.58$) were the thresholds for suggestive linkage (10).

Production of inflammatory cytokines after exposure to CADS. To clarify the inflammatory cytokine response against CADS, we also examined the sequential change of serum cytokines after intraperitoneal injection of CADS. Twenty milligrams of CADS suspended in 0.2 ml of PBS(−) was injected intraperitoneally to C3H/HeN. Sera were obtained from sacrificed mice at each time ($N=5$) for 14 days after injection of CADS and then frozen at −80 C. Serum cytokines, such as interleukins IL-1β, IL-4, IL-6, IL-12, TNF-α, and IFN-γ were measured by using ELISA assay kits: IL-1β, IL-4, IL-6, IL-12, and TNF-α (Genzyme, Mass., U.S.A.), and IFN-γ (Pierce ENDOGEN, Qld, Australia).

Results

Histological Observations of Arteritis

Table 1 shows the incidence of vasculitis in the coronary artery and/or the aortic root in (CBA/JN×C3H/HeN)F1, (C3H/HeN×CBA/JN)F1, and [(CBA/JN×C3H/HeN)×C3H/HeN]N1 was 0%, 16.7%, and 20.7% respectively, while that in C3H/HeN parents was 71.1% (27/38), but in CBA/JN absence (0%, 0/27). Most cases of vasculitis were observed in the aortic root and/or the coronary artery (Fig. 2). All layers of these vessels showed severe inflammation, which is defined as 'productive granulomatous inflammation, but fibrinoid necrosis was rarely determined.' Intima showed various degrees of fibrocellular thickening associated with the lumen of coronary artery became stenotic. In addition to the disruption of internal and external elastic laminas, smooth muscle cells in media deteriorated from severe inflammation. Furthermore, the destruction of the normal structure of the coronary artery in some cases caused aneurismal dilatation. However, neither thrombotic occlusion nor myocardial infarction was observed. Histological differences of arteritis between N1 and C3H/HeN was not elucidated. Arteritis in other visceral organs such as renal artery, testicular artery, and abdominal aorta were rarely detected.

Linkage Analysis with Chromosome Mapping

Two hundred and fifty-six microsatellite markers were tested to segregate loci by original parental strains (Fig. 1). However, most markers were the same sequence length polymorphism between C3H/NeN and CBA/JN. Only 48 markers were selected for the linkage analysis (Table 2). Genome-wide interval mapping analysis between coronary artery and genetic markers for the identification of susceptibility loci was performed by using χ^2 test as described in "Materials and Methods." The markers on the chromosome 1 showed the association even though possibility on other chromosome loci may exist. Two of 11 markers on chromosome 1, *D1Mit171* and *D1Mit245* around 20.2 cM revealed suggestive linkage with P value of 0.0019 (Table 3). The other markers on chromosome 1 did not indicate the association. Based on the suggestive level of *D1Mit171* and *D1Mit245*, this region is thought to influence to the development of coronary arteritis. On the other chromosomes, the marker, *D4Mit285*, showed low probability of 0.017, but was not in the scope to designate an association.

Table 1. Affected rate of coronary arteritis after 9 weeks challenge with CADS

Mice	Affected rate (%)
C3H/HeN	71.1 (27/38)
CBA/JN	0 (0/27)
(C3H male×CBA female) F1	0 (0/9)
(C3H female×CBA male) F1	16.7 (1/6)
(CBA female×C3H male) F1×C3H	20.7 (24/115)

CBA/JN and C3H/HeN (CBA/JN×C3H/HeN) and N1 backcross progeny between F1 and C3H/HeN [(CBA/JN×C3H/HeN)×C3H/HeN] were prepared.

Circulation of Inflammatory Cytokines after Exposure to CADS

Sequential changes of inflammatory cytokines IL-12, IL-1β, TNF-α, IL-6, IFN-γ, and IL-4 in serum for 14 days after intraperitoneal injection of CADS were measured by ELISA assay. Both IL-1β and IL-12 levels in serum increased at 1 hr after injection of CADS, and then decreased gradually, but IL-12 did not decrease like profile of IL-1β (Fig. 3a). After increases of IL-1β and IL-12, levels of TNF-α and IL-6 peaked at 3 hr after the injection, and then restored to baseline by 24 hr (Fig. 3b). Levels of IFN-γ gradually increased over the same period, but no change in IL-4 level was noted (Fig. 3c).

Discussion

Some infectious microorganisms have been implicated in the etiology of KD, though primary causes remain an enigma (6, 11, 16, 22). These candidates of etiology may act as initial trigger to induce arteritis. The spontaneous arteritis model may not be well suited for study-

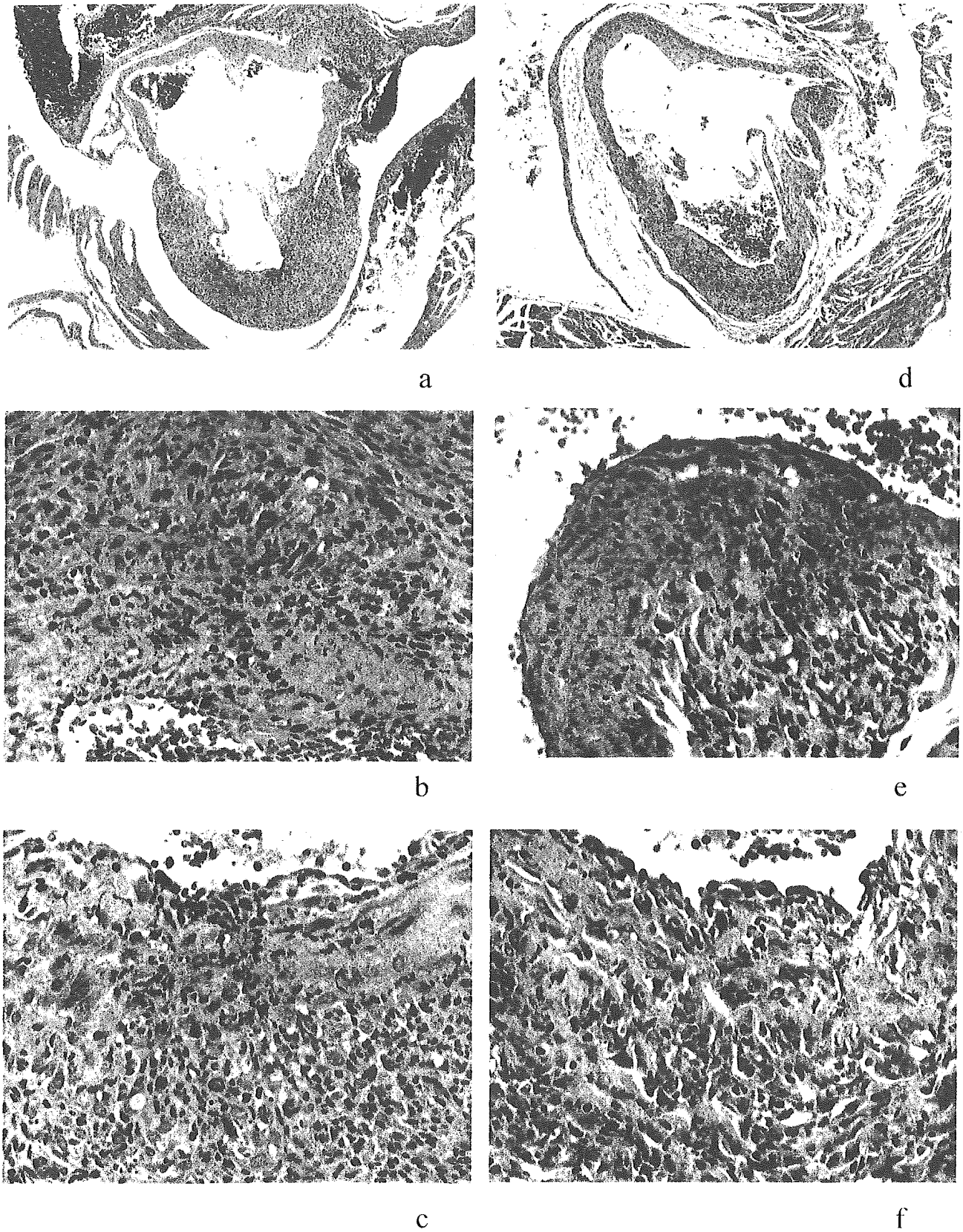

Fig. 2. Histological feature of coronary arteritis in N1 and C3H/HeN. (a) Vasculitis at the coronary artery and aortic root in N1 mouse (HE stain, ×40), (b) coronary arteritis in N1 mouse (HE stain, ×400), (c) aortitis in N1 mouse (HE stain, ×400), (d) coronary arteritis and aortitis in C3H/HeN (HE stain, ×40), (e) coronary arteritis in C3H/HeN (HE stain, ×400), (f) aortitis in C3H/HeN (HE stain, ×400).

Table 2. A list of the 48 markers used for the linkage analysis from 256 candidates for markers

Chromosome	Symbol	Position (cM)	Chromosome	Symbol	Position (cM)
1	D1Mit374	19.0	6	D6Mit345	46.0
1	D1Mit171	20.2	7	D7Mit232	26.8
1	D1Mit245	20.2	8	D8Mit224	17.0
1	D1Mit75	32.1	8	Mt2	45.0
1	D1Mit380	36.9	8	D8Mit14	67.0
1	D1Mit251	38.1	9	D9Mit2	17.0
1	D1Mcg3	38.9	9	Cyp1a2	31.0
1	D1Mcg6	39.9	9	D9Mit279	67.0
1	D1Mit7	41.0	10	D10Mit214	19.0
1	D1Mit200	80.0	11	Hoxb	56.0
1	Tgfbm2	106.3	12	D12Mit231	48.0
2	D2Mit92	41.4	13	D13Mit110	47.0
2	D2Mit206	51.4	14	D14Mit2	5.0
2	D2Mit311	83.1	14	Nfl	28.7
2	D2Mit456	86.3	15	D15Mit6	13.7
2	D2Mit265	105.0	16	D16Mit5	38.0
2	D2Mit200	107.0	17	D17Mit96	54.6
2	D2Mit457	108.0	18	D18Mit60	16.0
3	D3Mit90	4.6	19	D19Mit128	10.9
3	D3Mit200	77.3	19	D19Mit10	47.0
3	D3Mit323	84.9	X	DXMit74	20.0
4	D4Mit272	21.9	X	DXMit16	37.0
4	D4Mit285	71.0	X	DXMit121	67.0
4	D4Mit357	81.5			
5	D5Mit101	81.0			

Table 3. A list of markers that exhibited distribution disequilibrium from the 2×2 χ^2 based on a ratio of affected C3H/C3H:C3H/CBA to non-affected

Chromosome	Distance (cM)	Marker	Affected / Non-affected	χ^2	Probability
1	19.0	D1Mit374	18:6 / 34:45	7.52	0.0061
	20.2	D1Mit171	19:5 / 34:45	9.62	0.0019[a]
	20.2	D1Mit245	19:5 / 34:45	9.62	0.0019[a]
	32.1	D1Mit75	18:6 / 33:46	8.13	0.0044
	38.1	D1Mit251	17:7 / 30:49	8.01	0.0046
	38.9	D1Mcg3	15:9 / 31:48	4.03	0.0447
	39.9	D1Mcg6	15:9 / 31:48	4.03	0.0447
	41.0	D1Mit7	15:9 / 31:48	4.03	0.0447
4	71.0	D4Mit285	7:17 / 45:34	5.69	0.0171

[a] Suggestive linkage.

ing KD, because it requires an initial trigger from some infectious microorganisms to induce arteritis. On the other hand, our model requires injection of CADS to induce arteritis. This model is very useful for the study of the pathogenesis of arteritis in KD for two main reasons: 1) both the histological features and distribution of arteritis are similar to that of KD, and 2) infectious agents are required to induce the development of arteritis. The mechanisms of developing arteritis in patients with KD are still unclear; however, several reports have

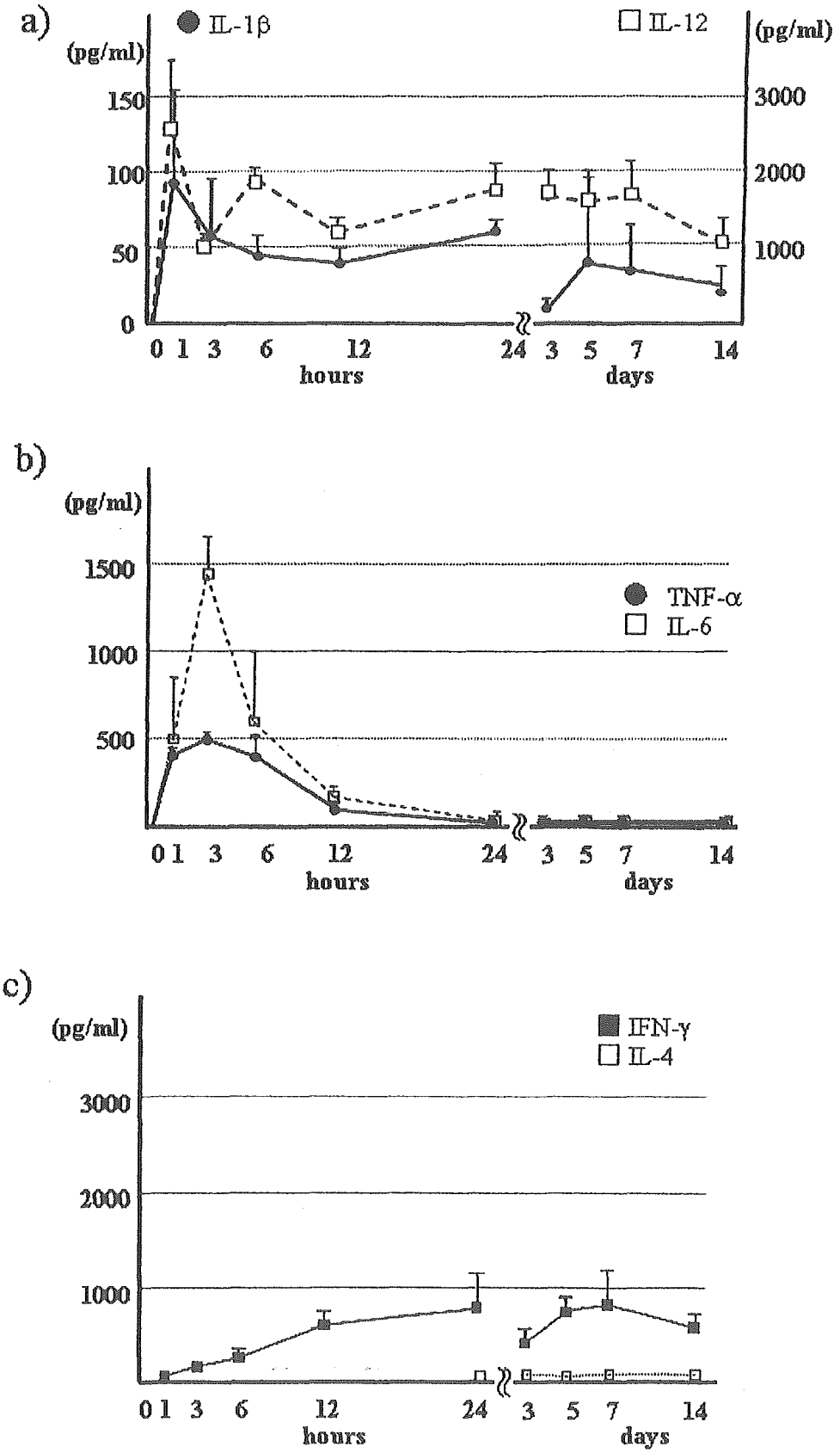

Fig. 3. Time course of serum cytokines after intraperitoneal injection of CADS. (a) Time course of serum IL-1β and IL-12 after injection of CADS, (b) time course of serum TNF-α and IL-6 after injection of CADS, (c) time course of serum IFN-γ and IL-4 after injection of CADS.

discussed the role of inflammatory cytokines in patients with a genetic predisposition to developing coronary arteritis (7, 8). The animal model is considered to be very useful for clarifying the pathogenesis of arteritis in these patients and may allow for the identification of specific treatments for the disease. With the observed differences in the incidence of arteritis in C3H/HeN mice and CBA/JN mice, it can be derived that genetic differences of the mouse strains does influence arteritis. Therefore, it was considered that C3H/HeN may express or attenuate the expression of genes that govern the susceptibility to coronary arteritis.

In the present study, we showed that the loci governing suggestive susceptibility to coronary arteritis were situated on chromosome 1 even though possibility on other chromosome loci may exist. Two of 11 markers on chromosome 1, *D1Mit171* and *D1Mit245* (map position 20.2 cM), appeared to be involved in the susceptibility loci to the development of coronary arteritis. This chromosomal region influences the IL-1 receptors types 1 and 2 (*Il1r1* and *Il1r2*, 19.5 cM). In addition, our results here revealed that IL-1β in sera rapidly increased after intraperitoneal injection of CADS. These findings suggest a ligand-receptor interaction between IL-1β and the IL-1 receptor, which may affect the onset of arteritis. It has been reported that IL-1β regulates vascular damage *in vitro*. Specifically, IL-1β directly injures endothelial cells; however, mechanisms of endothelial cell injury are unclear. Interestingly, an indirect role of IL-1β in the regulation of neutrophil-mediated killing of endothelial cells has been reported (3, 4, 12, 18). Adhesive interaction between activated neutrophils and endothelial cells was facilitated by exposure to IL-1β and superoxide anion, produced by activated neutrophils, and subsequently damaged neighboring endothelial cells (1).

One of the peculiar histological features of arteritis in this model was the severe neutrophilic infiltration observed in the afflicted artery, suggesting an important role of neutrophil activation in the development of arteritis. In addition, it has been reported that the specific antigen to autoantibodies, myleoperoxidase-anti-neutrophil cytoplasmic antibody (MPO-ANCA), targeting its antigen MPO were closely related to the development of coronary arteritis using MPO-deficient mice (17). It is considered that coronary arteritis in this model must be genetically regulated by interactions between neutrophils and endothelial cells modulated by IL-1β. Furthermore, the allelic polymorphism of both *Il1r1* and *Il1r2* and the functional relevance of their polymorphism may be a necessity.

On the other hand, *D4Mit285* (map position 71.0 cM) on chromosome 4 showed negative effect against affectability to coronary arteritis, however this marker showed a probability of 0.017. Several genes related to inflammation are coded around *D4Mit285* on chromosome 4, including TNF receptor superfamily member 1b, 8, and 9 (*Tnfrsf1b*, *Tnfrsf8*, and *Tnfrsf9*) (75.5 cM). The functional relevance of this is seen with the rapid increase of TNF-α following elevation of IL-1β after exposure to CADS. There are also studies that claim IL-1β and TNF-α can induce endothelial cell injury by activated neutrophils (3, 4, 12, 18). Therefore, some of these genes are considered to be protective against the development of coronary arteritis, even in the susceptible C3H/HeN strain. However, the marker *D2Mit265* ($\chi^2=3.61$, $P=0.058$) on chromosome 2, on which IL-1β is located, did not indicate a significant difference. Th-1 cytokines, such as IL-12 and IFN-γ were also produced by exposure of CADS. It was considered that Th-1 type immunity might have an influence on the development of arteritis in this model; however, we have no direct evidence in the present study to determine whether or not the genes of these cytokines affected the coronary arteritis outcome, since the number of microsatellite markers was insufficient to examine these genomic regions.

The difference in the incidence of coronary arteritis between C3H/HeN and CBA/JN may be attributed to differences in the regulation of genes encoding inflammatory cytokines. It may be concluded from the present study that the development of coronary arteritis is multi-factorial and controlled with cumulative effects of these multiple gene loci in this mouse model.

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Induction of Coronary Arteritis with Administration of CAWS (*Candida albicans* Water-Soluble Fraction) Depending on Mouse Strains

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ABSTRACT

The intraperitoneal administration of CAWS (water-soluble extracellular polysaccharide fraction obtained from the culture supernatant of *Candida albicans*) to mice induces coronaritis similar to Kawasaki disease. We analyzed differences in the production of cytokines involved in the occurrence of coronary arteritis among mouse strains, C3H/HeN, C57BL/6, DBA/2 and CBA/J that were injected with CAWS at 4 mg/mouse for 5 consecutive days in the first week and the fifth week of administration. The incidence of arteritis was 100% in C57BL/6, C3H/HeN and DBA/2 mice, but only 10% in CBA/J mice. The coronary arteritis observed in DBA/2 mice

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was the most serious, with several mice expiring during the observation period. The CAWS-sensitive strains revealed increased levels of IL-6 and IFN-γ during the course of a specific response to CAWS by spleen cells. In contrast, IL-10 levels were observed to increase markedly in CAWS-resistant CBA/J mice, but not the CAWS-sensitive strains. However, TNF-α levels were more elevated only in DBA/2 mice. The difference in disease development and cytokine production strongly suggests that the genetic background of the immune response to CAWS contributes to the occurrence of coronary arteritis.

Key Words: *Candida albicans*; Induction of arteritis; Polysaccharide; DBA/2 mice.

INTRODUCTION

Kawasaki disease, also referred to as acute febrile mucocutaneous lymph node syndrome or MCLS, was first reported by Kawasaki in 1967.[1,2] A disease of unknown cause, it affects mainly children aged 4 and under. The number of patients diagnosed with Kawasaki disease annually is roughly 10,000. The patients present with systemic coronary arteritis, and the greatest concern with this symptom is the occurrence of coronary arteritis or coronaritis, which occurs as a sequela in nearly 10% of all patients.[3,4] Moreover, in the case of the formation of giant coronary aneurysms, complications involving myocardial ischemia and myocardial disorders caused by vascular occlusion due to thrombus formation may arise. In actuality, sudden death due to myocardial infarction occurs in several percent of Kawasaki disease patients. Although the occurrence of such coronary artery disorders has decreased with the introduction of γ-globulin therapy, the mechanism of their occurrence along with the pharmacological mechanism of the treatment is unknown.[5,6]

Murata et al.[7,8] reported that Kawasaki-disease-like coronary arteritis was induced specifically at the origin of the coronary arteries in mice administered an alkaline extract of *C. albicans* isolated from patients (CADS) with Kawasaki disease. Ishida-Okawara et al.[9] have demonstrated that mice with coronary arthritis induced with a CADS injection show an increase in anti-myeloperoxidase (MPO)-specific anti-neutrophil cytoplasmic antibody (MPO-ANCA) in their serum. In addition, MPO was identified to be the antigen to MPO-ANCA using MPO-deficient mice.[9] These findings show the substances including β-glucan derived from *C. albicans* might relate to coronary arteritis.

The number of opportunistic infections is on the rise accompanying the proliferation of highly advanced medical treatment. *Candida* infections are frequently observed in patients in high-risk groups.[10,11] As the chemotherapeutic agent for mycoses is completely different from that for bacterial infections, early diagnosis is critical.[12,13] Since fungal cell wall contains β-glucan as its main component, β-glucan is detected in the blood of patients with deep mycoses. Thus, measurement of β-glucan in the blood is widely used for the early diagnosis of deep mycoses.[14,15] In this study, we cultured *Candida* spp. in completely synthetic media, obtained the water-soluble polysaccharide fraction released into the culture supernatant (*Candida albicans* water-soluble fraction or CAWS), and performed various analyses on that fraction. As a result, CAWS was found to be composed of mannoprotein and a β-glucan complex and to activate the limulus G factor, to exhibit acute lethal toxicity in the case of

intravenous administration, and to activate vascular endothelial cells, platelets and lymphocytes.[16-18] In addition to CADS, other substances in the soluble fraction may cause the development of coronary arteritis in mice.

In the present study, the incidence of coronaritis was found to be higher when CAWS was administered than when the conventional alkaline extract was administered, and a difference in the incidence was observed among mouse strains. We also analyzed the correlation of cytokine production in the development of coronary arteritis induced by CAWS.

MATERIALS AND METHODS

Mice

Male C3H/HeN and DBA/2 mice were purchased from Japan SLC, whereas male C57BL/6 and CBA/J mice were purchased from Charles River Japan. The mice were housed in a specific pathogen-free (SPF) environment and were used in the study at 5–14 weeks of age.

Organisms

Candida albicans strain IFO1385 was purchased from the Institute for Fermentation, Osaka (IFO), stored at 25°C on Sabauroud's agar (Difco, USA) and passaged once every three months.

Preparation of CAWS

CAWS was prepared from *C. albicans* strain IFO1385 in accordance with conventional methods.[18] The procedure used is as follows: 5 L of medium (C-limiting medium) was added to a glass incubator and cultured for 2 days at 27°C while supplying air at a rate of 5 L/min and rotating at 400 rpm. Following the culture, an equal volume of ethanol was added and after the mixture was allowed to stand overnight, the precipitate was collected. The precipitate was dissolved in 250 mL of distilled water, ethanol was added and the mixture was allowed to stand overnight. The precipitate was collected and dried with acetone to obtain CAWS.

Administration Schedule for Induction of Coronary Arteritis

CAWS (0 or 4 mg/mouse) was administered intraperitoneally for 5 consecutive days to each mouse in week 1. In week 5, CAWS (0 or 4 mg/mouse) was again administered in the same manner as that in week 1, after which the mice were sacrificed in week 9. The hearts of the animals were fixed with 10% neutral formalin and prepared in paraffin blocks. Tissue sections were stained with Hemotoxylin-Eosin (HE) stain. Cells were prepared from the spleen and cultured. Cells were also prepared from peritoneal exudative cells (PECs) and from the thymus, and enumerated. Liver weight was measured.

Preparation of Mouse Serum

The mice were anesthetized with chloroform and then sacrificed after which blood was drawn from the heart. After the blood samples had been left to stand for 60 minutes at room temperature and then for 60 minutes at 4°C, they were separated by centrifugation at 15,000 rpm × 10 minute, and the resulting supernatant was used as the serum. All the samples were stored at −25°C or lower.

CAWS-Specific Reaction in the Isolated Spleen Cells

The mice were anesthetized and then sacrificed, after which the spleen was excised. After teasing using a mesh in RPMI-1640 medium, the tissue was separated by centrifugation at 1200 rpm × 5 minute, and the resulting cells were treated with ACK-lysing buffer (NH_4Cl 8.20 g/L, $KHCO_3$ 1 g/L, EDTA·2Na 37.2 mg/L). After two washes with RPMI medium, the spleen cells were counted to adjust the cell density and then used after being suspended in RPMI medium with 10% FCS (fetal calf serum). The spleen cells were adjusted to 1×10^7 in RPMI 1640 medium containing 10% FCS and 500 µl aliquots were added to each well of a 48-well plate. Following the addition of CAWS (0, 2.5, 5 or 10 µg/ml), the cells were culture for 48 hours in a 5% CO_2 incubator at 37°C. The cytokine level of the culture supernatant was determined by Enzyme-Linked Immuno Sorbent Assay (ELISA) as described below.

Measurement of IL-1β, IL-4, IL-10 and IL-12

The IL-1β level was measured using a Mouse IL-1β ELISA Kit (Biosource International). Levels of IL-4, IL-10 and IL-12 were measured using Mouse IL-4, IL-10 and IL-12 $O_{PT}EIA^{TM}$ Kits (Pharmingen).

Measurement of IL-6

An ELISA 96-well plate (Sumitomo Bakelite) was coated with rat anti-mouse IL-6 mAb (Pharmingen) using 0.1 M bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. After a wash with Phosphate buffered saline with 0.05% Tween 20 (PBST), the antibody was blocked for 40 minutes at 37°C with BPBST. This was followed by the addition of standard and sample (50 µL), incubation for 40 minutes at 37°C and washing with PBST. Fifty microliters of a secondary antibody in the form of biotinylated rat anti-mouse IL-6 mAb (1/2000; Pharmingen) was then added, and after incubation for 40 minutes at 37°C and a wash with PBST, peroxidase-conjugated streptavidin (1/10000; Zymed Laboratories, Inc.) was added. This was followed by incubation for 40 minutes at 37°C and washing with PBST. Subsequently, 50 µL of peroxidase substrate (TMB microwell peroxidase substrate system, KPL Inc.) was added to generate color, and absorbance was measured as previously described. Recombinant mouse IL-6 (Pharmingen) was used as the standard.

Measurement of IFN-γ

An ELISA 96-well plate (Sumitomo Bakelite) was coated with rat anti-mouse IFN-γ monoclonal antibody (mAb; Pharmingen) using 0.1 M $NaHCO_3$ (pH 8.2) and

incubated overnight at 4°C. After a wash with 0.05% Tween-PBS (PBST), the antibody was blocked for 40 minutes at 37°C with 0.5% BSA (bovine serum albumin)-PBST (BPBST). This was followed by the addition of standard and sample (50 μL), incubation for 40 minutes at 37°C and washing with PBST. Fifty microliters of a secondary antibody in the form of biotinylated rat anti-mouse IFN-γ (1/1000; Pharmingen) was then added, and after incubation for 40 minutes at 37°C and a wash with PBST, peroxidase-conjugated streptavidin (1/2000; Pharmingen) was added. This was followed by incubation for 40 minutes at 37°C and washing with PBST. Subsequently, color was generated using peroxidase substrate (TMB microwell peroxidase substrate system, KPL Inc.). After termination of the reaction with 1 M phosphoric acid, absorbance (OD 450/Ref. 630) was measured. Recombinant mouse IFN-γ (Pharmingen) was used as the standard.

Measurement of TNF-α

An ELISA 96-well plate (Nunc) was coated with rat anti-mouse TNF-α mAb (1/500; Pharmingen) using 0.1 M NaH_2PO_4– 0.1 M Na_2HPO_4 buffer (pH 6.0) and incubated overnight at 4°C. After a wash with PBST, the antibody was blocked for 60 minutes at room temperature with BPBST. This was followed by the addition of standard and sample (50 μL), incubation for 3 hours at room temperature and washing with PBST. Fifty microliters of a secondary antibody in the form of biotinylated rat anti-mouse TNF-α mAb (1/1000; Pharmingen) was then added, and after incubation for 60 minutes at room temperature and a wash with PBST, horseradish-peroxidase-conjugated streptavidin (1/1000; Pharmingen) was added. This was followed by incubation for 30 minutes at room temperature and washing with PBST. Subsequently, color was generated and absorbance was measured as previously described. Recombinant mouse TNF-α (Pharmingen) was used as the standard.

Measurement of Anti-CAWS Antibody Titer

An ELISA 96-well plate (Nunc) was coated with CAWS using 0.1 M bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. After a wash with PBST, the antibody was blocked for 60 minutes at 37°C with BPBST and again washed with BPST. A serum sample diluted with BPBST (50 μl) was added, and incubation continued for another 60 minutes at 37°C. After a wash with PBST, 50 μL of peroxidase-conjugated goat anti-mouse IgG + IgM Ab (1/5000; Wako) was added and reaction was allowed to proceed for 60 minutes at 37°C. Color was generated and absorbance was measured as previously described. Furthermore, color generation was stopped after 10 minutes.

Measurement of Antibody Subclass

An ELISA 96-well plate (Nunc) was coated with CAWS using 0.1 M bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. After a wash with PBST, the antibody was blocked for 60 minutes at 37°C with BPBST and again washed with PBST. A serum sample (50 μL) diluted with BPBST was added and incubation continued

for another 60 minutes at 37°C. After a wash with PBST, 50 μL of biotin-conjugated anti-mouse IgG1 (1/1000)-IgG2a (1/3000)-IgM (1/5000) Ab or peroxidase-conjugated anti-mouse IgE Ab (1/500) was added and the reaction was allowed to proceed for 60 minutes at 37°C. With respect to the biotin-conjugated antibody, after a wash with PBST, horseradish-peroxidase-conjugated streptavidin (1/5000; Pharmingen) was added, and incubation was continued for 60 minutes at 37°C. Color was generated and absorbance measured as previously described. Furthermore, color generation was stopped after 10 minutes.

Test for Significant Difference

Tests for significant differences in this study were performed using Student's t-test and values of $P<0.05$ were judged significant.

RESULTS

Development of Coronary Arteritis Induced with CAWS and Difference Among Strains

Administration of CAWS induced the development of coronary arteritis (Fig. 1). The arteritis-inducing activity of CAWS was compared among four mouse strains in accordance with the method of Murata et. al.[7,8] CAWS was dissolved in physiological saline to a concentration of 4 mg/0.2 mL, administered intraperitoneally to each mouse for five consecutive days, and again administered for five consecutive days in week 5, after which the mice were sacrificed in week 9. The tissue sections in the ninth week were prepared from the origin of the coronary arteries of the coronary aorta and stained with HE. The coronary arteritis induced by CAWS was accompanied by hypertrophy of the tunica intima, the rupture of elastic fibers and a diffuse invasion by lymphocytes, histiocytes, fibroblasts, smooth muscle cells and eosinophils of vascular endothelial cells and the regions surrounding blood vessels (Fig. 1). On the basis of such characteristics, the coronary arteritis induced by CAWS was presumed to be the so-called proliferative granulomatous coronary arteritis, and is clearly different from fibrinoid arteritis. Differences in the incidence of coronary arteritis were observed among the mouse strains. The coronary arteritis was observed in all mice of the DBA/2, C57BL/6 and C3H/HeN strains. It was observed to cover nearly the entire periphery of the vessels in DBA/2 mice, and those mice were considered to demonstrate the most virulent form of coronary arteritis (data not shown). On the other hand, CBA/J mice exhibited the lowest incidence of coronary arteritis among the four strains tested (10%), with few sites where coronary arteritis occurred.

Comparison of Survival Rates Among CAWS-Administered Mice

Among the mice administered CAWS according to the coronary arteritis induction protocol, only DBA/2 mice exhibited high mortality. Therefore, the disease

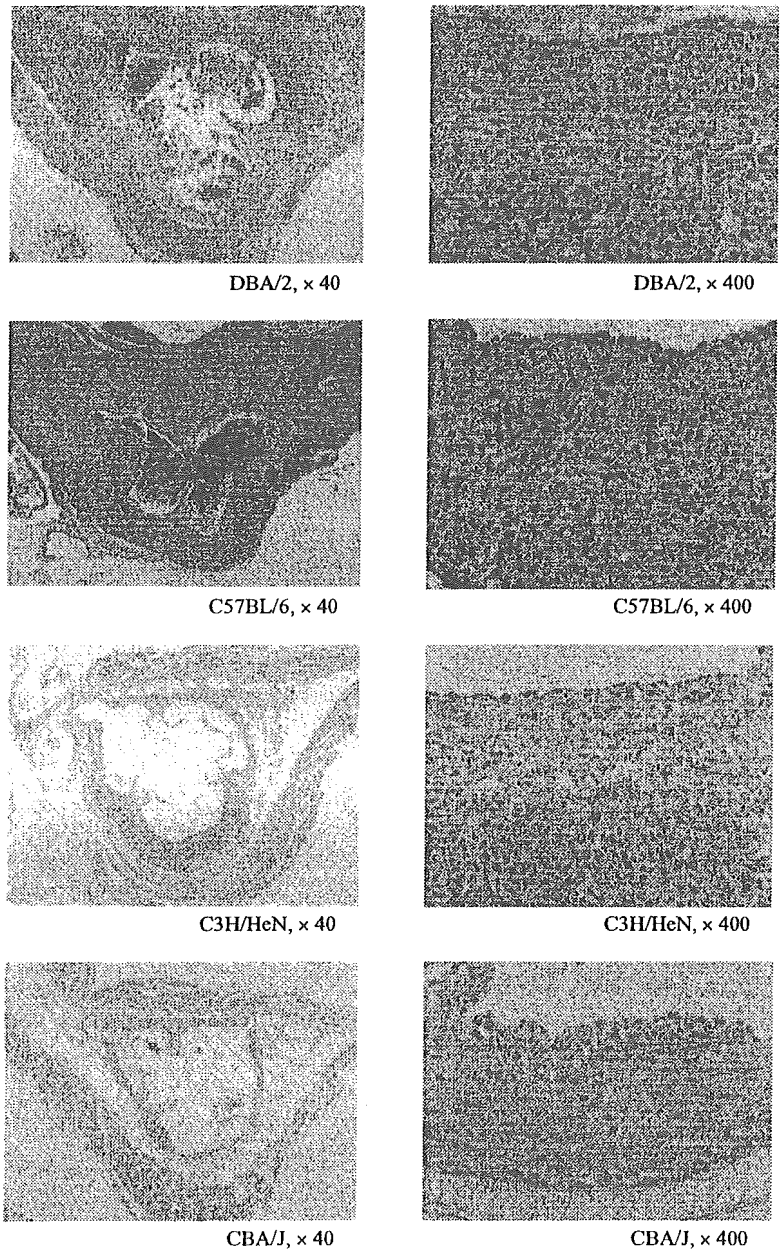

Figure 1. Histological observations of coronary arteritis. CAWS (4 mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, mice were sacrificed and prepared sections stained with the hematoxylin-eosin method.

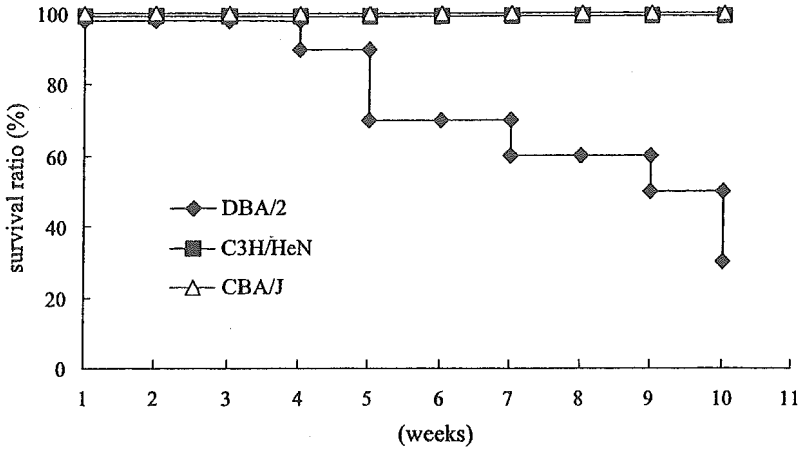

Figure 2. Survival ratio of CAWS-administered mice. CAWS (4 mg/mouse) was administered i.p. to DBA/2, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. Survival had been observed for ten weeks. (N = 10).

course was examined in DBA/2 mice. DBA/2 mice were confirmed to expire beginning in the fourth week after the start of administration. The number that expired gradually increased and by the ninth week when the mice were assessed for coronary arteritis, the survival rate had dropped to 50%, and ultimately decreased to 30% (Fig. 2).

Tissue sections were prepared from the hearts of expired DBA/2 mice and observed microscopically using the HE stain. In the DBA/2 mice that expired due to administration of CAWS (n = 3), prominent neutrophil and histiocyte invasion was observed, along with the disappearance of striated muscle (Fig. 3A), and in some of the tissue, fibrosis appeared to occur in addition to cellular invasion (Fig. 3B and C); namely, the cause of death was suggested to be myocardial infarction. On the basis of these findings, it was suggested that the most virulent form of coronary arteritis was induced in DBA/2 mice, which resulted in the occurrence of cardiac ischemia that ultimately led to myocardial infarction.

Change of Cell Number in Organs on CAWS-Administration

As we observed a high incidence of cornory arteritis induced with CAWS in three strains, the increase in the numbers of immune cells was determined, along with the weight of the liver. These measurements were made in mice administered CAWS in accordance with the coronary arteritis ind ıction protocol. In the spleen, although splenomegaly was observed in C3H/HeN, DBA/2 and C57BL/6 mice, significant increases in cell counts were exhibited by only C3H/HeN and DBA/2 mice (Fig. 4A). There were no changes in the thymus cell count in any mouse strains (Fig. 4B). In the case of PECs, although the changes were not significant, all strains exhibited an increase (Fig. 4C). There were no changes in liver weight (Fig. 4D).

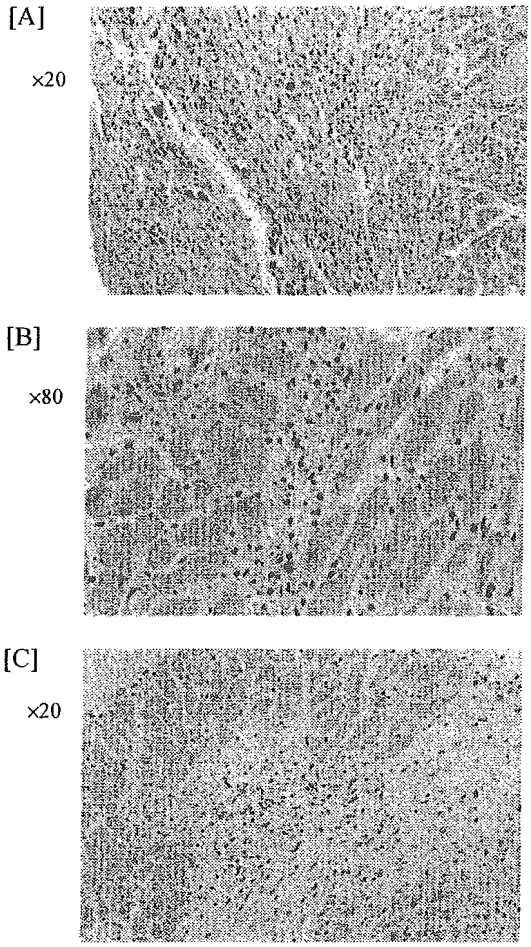

Figure 3. Histological analysis of dead DBA/2 mice administered with CAWS. [A, B, C] CAWS (4mg/mouse) was administered i.p. to DBA/2 mice for five consecutive days in the 1st and 5th week. Thereafter, the hearts of dead mice were stained with hematoxylin-eosin.

Cytokine Production by Spleen Cells of CAWS-Administered Mice on Stimulation with CAWS

As splenocyte counts in DBA/2 mice were high, we examined the production of cytokines in response to CAWS. Spleen cells of mice administered CAWS to induce coronary arteritis were prepared at a concentration of 1×10^7 cells/ml, and cultured for 48 hours in a 5% CO_2 incubator at 37° for observing cytokine production. Following the culture, IL-1β, IL-4, IL-6, IL-10, IL-12, IFN-α, and TNF-α levels in the culture supernatant were measured by ELISA. The strains with severe coronary arteritis, C3H/HeN, DBA/2 and C57BL/6 mice, showed IL-1β and IL-6 production by spleen cells in the CAWS -administered group (Fig. 5). In contrast, IL-10 was significantly produced

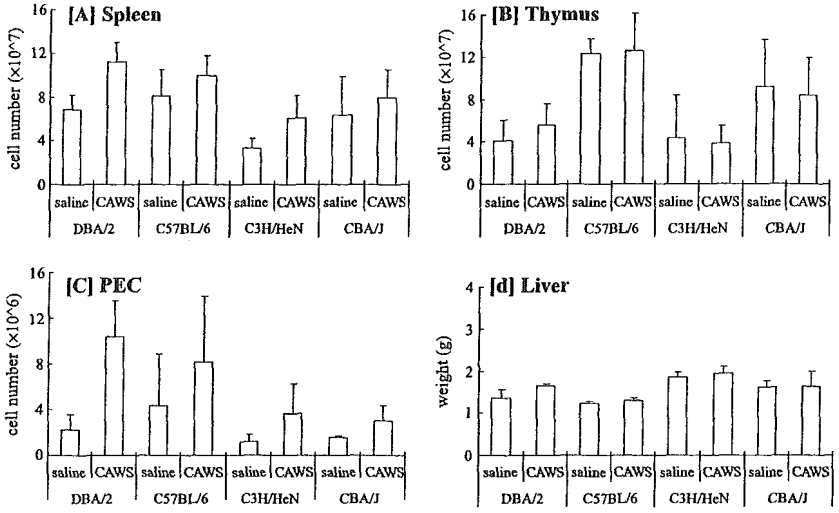

Figure 4. Cell number in peripheral blood and organ weight from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, the internal organs were collected from each mouse. Total cell number was counted with a hemocytometer and organ weight was measured with a analytical balance. The results show the mean ± standard deviation (S.D.). *; $P<0.05$ compared with the control using Student's *t-test*. [A]: Spleen, [B]: Thymus, [C]: PEC, [D]: Liver.

by spleen cells of the CBA/J mouse, which is the strain resistant to the coronary arteritis induced by CAWS.

In order to observe CAWS-specific reactions, the spleen cells of mice administered CAWS were stimulated with CAWS (0, 2.5, 5 or 10 μg/ml) and cultured for 48 hours in a 5% CO_2 incubator at 37°C. IFN-γ, IL-6 and IL-10 levels correlated well with the degree of coronary arteritis induced by CAWS (Fig. 6). IFN-γ and IL-6 production in DBA/2 and C57BL/6 mice tended to increase during the CAWS-specific response in the CAWS groups as compared with the saline groups, but no response was observed in C3H/HeN and CBA/J mice. IL-10 production was particularly enhanced in CBA/J mice treated with CAWS and slightly increased in the saline group, but no significant increase in production was observed even with the administration of CAWS in DBA/2 and C57BL/6 mice, and only a slight increase in C3H/HeN mice. Levels of other cytokines were not correlated with the coronary arteritis caused by CAWS. The amount of IL-4 production was small in all strains, but increased somewhat in CBA/J and C3H/HeN mice. IL-12 was hardly detected in any of the strains.

Measurement of Anti-CAWS Antibody Titer

Serum was obtained from mice administered CAWS in accordance with the coronary arteritis induction protocol, and anti-CAWS antibody in serum was detected with anti-mouse IgG + IgM Ab. Anti-CAWS antibodies were detected in all of the

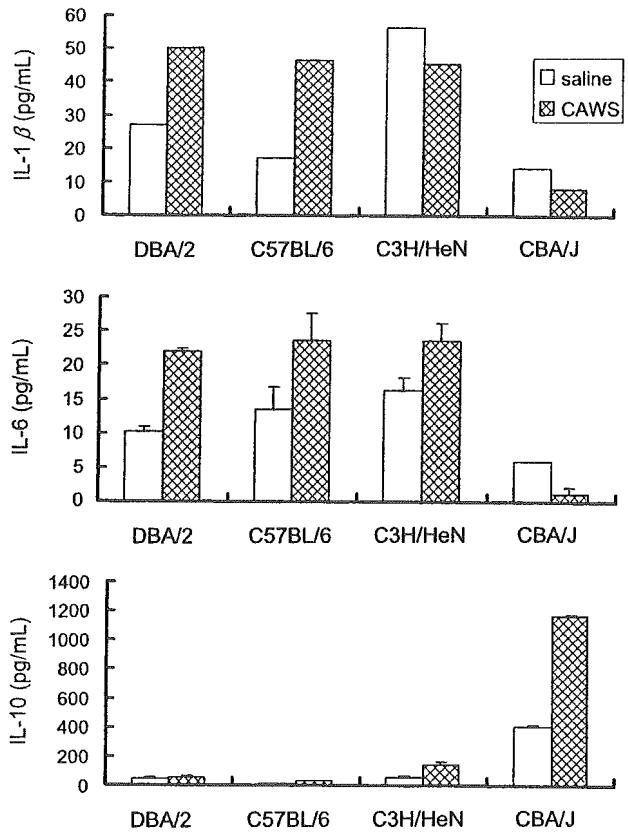

Figure 5. Cytokine production in culture supernatants of splenocytes in vivo from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, splenocytes were collected from each mouse. The splenocytes were cultured for 48 hour at a density of 1×10^7 cells/ml. The culture supernatants were collected and the level of each cytokine was measured by ELISA. The data shows one of four (C3H/HeN and CBA/J), three (DBA/2) or two (C57BL/6) experiments performed with similar results. The results show the mean ± standard deviation (S.D.).

mice, and the titers were extremely high (Fig. 7). Although the IgM titer varied depending on the mouse strain, there was no relationship between the IgM and the incidence of coronary arteritis (Fig. 8). Little IgE was detected in any of the strains. Conversely, IgG1 was detected at high levels in all the strains. CBA/J mice exhibited the lowest IgG2a titers.

On the basis of the above results, the antibodies produced in the form of anti-CAWS antibodies consisted mainly of IgG, followed by IgM. With respect to IgG2a production, although the titers were low in CBA/J and C57BL/6 mice, as the levels in CBA/J mice were roughly half those in C57BL/6 mice, and when considering the results of cytokine production in the spleen, it is possible that CBA/J mice exhibit Th2 bias.