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pean and North American investigators have examined the treatment response and prognostic factors in patients with ANCA-associated renal vasculitis (9-13). The patients described in these studies (9-13) were relatively younger than those in our previous study (1), and half of them were PR 3-ANCA positive. On the other hand, few studies have addressed the prognosis of patients with MPO-ANCA-associated renal vasculitis in Japan, except for one questionnaire.

We conducted a population-based survey of PRV in Miyazaki Prefecture between 2000 and 2004 (1) based on the sub-classification of ANCA-associated vasculitides by the European Systemic Vasculitis Study Group (EUVAS) (16-18). Among 56 identified patients, 91% were MPO-ANCA positive and none had Wegener's granulomatosis or Churg-Strauss syndrome. Here, we examined the prognosis of these patients with respect to mortality and renal survival (outcome) and prognostic factors.

Subjects and Methods

Patients

Fifty-six patients (24 males and 32 females; age 70.4 ± 10.9 years, mean ± SD), with onset of new PRV between January 2000 and December 2004, were enrolled in this study. We defined PRV according to the EUVAS (European Systemic Vasculitis Study Group) criteria (1, 16-18). Table 1 shows the inclusion criteria. All patients underwent serology tests for PR3-ANCA (nephrosucolor PR3-ANC, Euro Diagnostica, Netherlands), MPO-ANCA (nephrosucolor MPO-ANC II, Nipro, Japan) and anti-glomerular basement membrane antibody (anti-GBM antibody) (nephrosucolor GBM, Euro Diagnostica, Netherlands) using an enzyme-linked immunosorbent assay (ELISA). If the ELISA results were negative, both c- and p-ANCA were further tested using indirect immunofluorescence (IIF). Percutaneous renal biopsy had been performed in 33 of the patients. We excluded patients with anti-GBM antibodies and documented episodes of PRV before 2000. In addition, patients with Henoch-Schönlein purpura, systemic lupus erythematosus or other connective tissue diseases were excluded.

Treatment

All patients received an induction therapy with various doses of corticosteroid, depending on the decision of the attending physician. Among 28 patients with intravenous pulse methylprednisolone (dose, 0.5-1.0 g) for 3 consecutive days, 23 received orally more than 30 mg/day of prednisolone for 4 weeks or more as the following therapy. Cyclophosphamide was administered to 23 patients as initial (n= 16) or additional (n=7) therapy, according to the judgment of the attending physician. Among these 23 patients, only 5 were given cyclophosphamide intravenously (300 mg-800 mg/day × 2-4 courses) and the others orally (0.5-1.5 mg/kg body weight). None had received plasma exchange or large doses of immunoglobulins. No strict protocol was followed as prophylaxis for opportunistic infection.

Table 1. Inclusion Criteria

1. New patients with WG, MPA, CSS, or RLV, with or without histological confirmation *
2. Renal involvement ** with or without other organ involvements, attributable to active WG, MPA, CSS, or RLV
3. Positive serology for ANCA ***

1, 2 and 3 are required.

* Histological confirmation: findings of necrotizing vasculitis and pauci-immune necrotizing, crescentic glomerulonephritis.

** Renal involvement: elevated serum creatinine (>1.3 mg/dl), or hematuria (>30 red blood cells per high power field), or proteinuria (>1 g/24 h), or red cell casts.

*** ANCA negativity is allowed if the disease is confirmed histologically.

WG, Wegener's granulomatosis; MPA, microscopic polyangiits; CSS, Churg Strauss syndrome; RLV, renal limitted vasculitis

Definitions

Acute dialysis therapy was defined when the dialysis therapy was required at the start of immunosuppressive treatment. In accordance with the Japanese system of grading the clinical severity of rapidly progressive glomerulonephritis (7), the scores of clinical findings were evaluated for serum creatinine level, age, existence of lung lesion and serum C-reactive protein (CRP) level (Table 2A), and then clinical disease activity was calculated by adding above-mentioned four scores (Table 2B).

Remission was defined as the stabilization or improvement of renal function and resolution of extrarenal manifestations of systemic vasculitis. Relapse was defined as a rise in creatinine concentration or worsening/new extrarenal manifestations attributable to active vasculitis with a rise in ANCA-titers, and improvement following escalation of immunosuppressive therapy (11, 12).

Follow-up

The patients were followed-up over a median of 24 months (range, 1 - 70 months; mean, 24.8 ± 19.4 months) at either our institution or affiliated hospitals and a single investigator (S.U.) collected follow-up information in December 2005 to maintain consistency.

Statistical analysis

Continuous data are presented as means ± standard deviation (SD). Survival curves were constructed by the Kaplan-Meier method and were compared with the use of the log-rank test. We assessed the impact of covariates for death caused by infection or survival using multivariate logistic regression analysis and the Cox proportional hazards model, respectively. All independent variables are expressed in categorical [gender, acute dialysis, lung lesions, MPO-ANCA (> or < 400 U/l), methylprednisolone pulse therapy, cyclophos-

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Table 2. Clinical Score and Activity

(A) Clinical findings score

Score	SCr *, mg/dl	age, years	lung lesion	CRP *, mg/dl
0	< 3.0	< 60	(-)	< 2.6
1	3.0 - 6.0	60 - 69		2.6 - 10.0
2	≧ 6.0	≧ 70	(+)	≧ 10.0
3	dialysis therapy			

* Measured value at the start of initial treatment.

(B) Clinical disease activity

Activity	Total score
Grade I	0 ~ 2
Grade II	3 ~ 5
Grade III	6 ~ 7
Grade IV	8 ~ 9

phamide therapy] or quantitative (age, serum albumin, immunoglobulin G, CRP) formats. The results of the multivariate analyses are expressed as odds ratio or hazard ratios with 95% confidence intervals (CI), and a P-value. All statistical analyses were based on two-tailed probabilities. A value of $p < 0.05$ was considered significant. SPSS for Windows software (version 11.0J, SPSS JAPAN, Tokyo, Japan) and StatView software (version 5, HULINKS Inc., Tokyo, Japan) were used for statistical analysis. All statistical analyses were performed by a statistician (H. N.).

Results

Admission characteristics

Table 3 summarizes the patients' characteristics upon admission. Nineteen patients (34%) had pulmonary symptoms with abnormal findings on chest-X-ray and CT films (pulmonary hemorrhage 6, interstitial pneumonitis with/without pulmonary hemorrhage 16), 3 (5%) had mononeuritis multiplex, 2 (4%) had gastrointestinal bleeding and 1 had otitis media as organ involvements of systemic vasculitis. None had nasal lesions. They were positive for CRP (5.9 ± 6.1 mg/dl) and surrogate markers of renal vasculitis, such as elevated serum creatinine (3.9 ± 2.7 mg/dl), proteinuria (1.8 ± 2.3 g/day), hematuria, and/or red cell casts. The serum level of albumin (3.0 ± 0.6 g/dl) was decreased, while that of immunoglobulin G was not. Histological signs of pauci-immune, necrotizing and crescentic glomerulonephritis and/or vasculitis were evident in 35 patients, 33 of whom had undergone renal biopsy. Five (9% of all patients) of the 33 biopsied patients were proven C- and P-ANCA negative, whereas the other 51 patients were positive for MPO-ANCA. None of the patients were positive for either C-ANCA or PR3-ANCA.

Table 3. Admission Characteristics

Age, years	70.4 ± 10.9
Male vs. Female	24 vs. 32
Extrarenal systems involved (%)	
Lung	34
Nervous system	5
Gastrointestinal tract	4
Labolatory data	
CRP, mg/dl	5.9±6.1
Serum creatinine, mg/dl	3.9±2.7
Proteinuria, g/day	1.8±2.3
Serum albumin, g/dl	3.0±0.6
IgG, mg/dl	1610±656
MPO-ANCA titer	
< 20 EU/ml (%)	9
20 - 200 EU/ml (%)	38
200 - 640 EU/ml (%)	36
> 640 EU/ml (%)	18
Renal histology (n=33)	
Crescents (% of glomeruli involved)	63±24
Global sclerosis (%)	19±21

(mean±SD)

Prognosis and outcome

Fig. 1 charts the progress of the patients after arrival at our renal unit. At the time of the start of immunosuppressive therapy, 14 patients (25%) required acute dialysis therapy (serum creatinine, 7.7±2.1 mg/dl), of whom renal function recovered in 3 and these patients were taken off dialysis. Eleven patients, of whom 7 were dependent on dialysis, died during the first admission. Forty-two patients (75%) did not require acute dialysis therapy (serum creatinine, 2.9±1.6 mg/dl), and thereafter maintenance dialysis therapy was applied to only 8 patients. None of the patients with serum creatinine levels below 3.5 mg/dl at initial presentation were introduced to dialysis therapy until the last follow-up.

Table 4 shows the characteristics of the patients with or without acute dialysis therapy. Except for serum creatinine and albumin levels, the demographic and laboratory data did not statistically differ between the two groups. Significantly fewer of the patients who were put on acute dialysis therapy received cyclophosphamide compared with the others. The prognosis of these patients was significantly poorer than those without acute dialysis therapy (mortality rate, 71.4%

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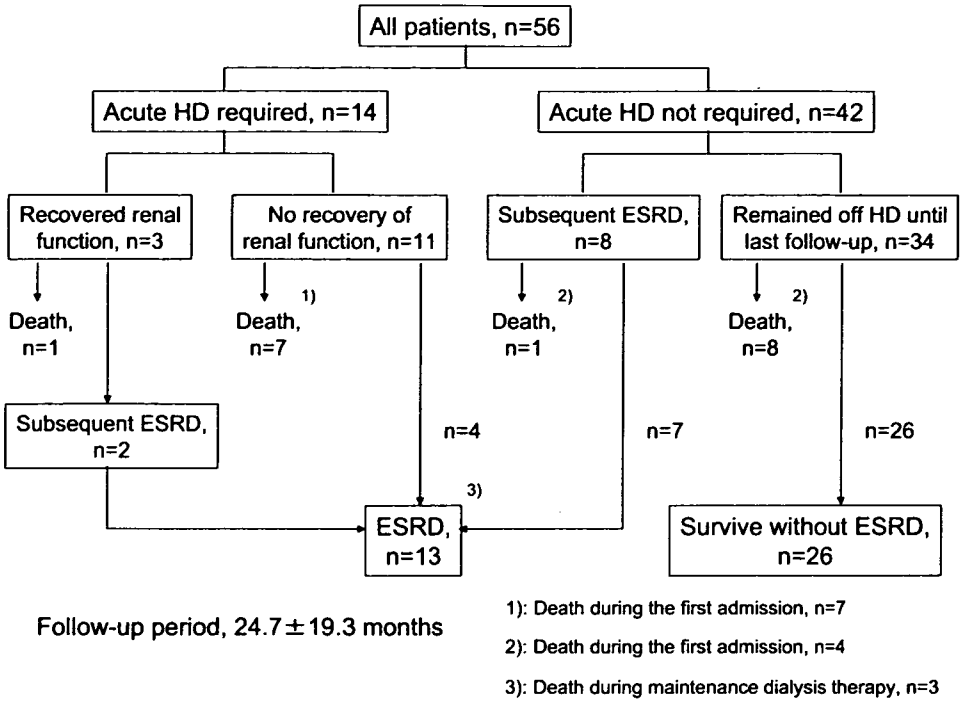

Figure 1. Flow charts indicating renal fate of cohort.

Table 4. Comarison between the Patients with and without Acute Dialysis Therapy

	acute dialysis therapy (+)	acute dialysis therapy (-)	P value
Number	14	42	
Demographic data			
age, years	72.9 ± 9.4	69.7 ± 11.3	0.348
male	9 (64%)	15 (36%)	0.061
presence of lung lesions	6 (43%)	13 (31%)	0.415
follow-up months	17.3 ± 19.4	27.0 ± 19.0	0.103
Labolatory data			
CRP, mg/dl	7.5 ± 4.8	5.3 ± 6.4	0.237
serum creatinine, mg/dl	7.7 ± 2.1	2.9 ± 1.6	< 0.001
serum albumin, g/dl	2.6 ± 0.5	3.1 ± 0.6	0.021
serum immunoglobulin G, mg/dl	1637 ± 549	1602 ± 692	0.884
MPO-ANCA titer ≧ 200 EU/ml	7 (50%)	23 (55%)	0.752
Initial treatment			
methlprednisolone pulse therapy	6 (43%)	23 (55%)	0.537
cyclophosphamide with prednisolone	2 (14%)	21 (50%)	0.018
Prognosis			
death until the last follow-up	10 (71%)	10 (24%)	0.015
death due to infection until 6 months later	4 (29%)	3 (7%)	0.036
renal survival at the last follow-up	0 (0%)	26 (62%)	< 0.001

Contious data were shown as mean±SD.
Data analyses were performed using $\chi 2$ or *Student's t* test.

vs. 23.8%; renal survival rate, 0% vs. 61.9%). Death due to infection within 6 months of starting treatment was significantly frequent in the patients group with acute dialysis therapy (29% vs. 8%, p=0.036).

Table 5 shows the clinical characteristics and prognosis of the patients divided by ANCA-titers, including 5 ANCA-negative patients. The patients with low ANCA-titers (MPO-ANCA titer < 200 EU/ml) were older and had a lower CRP level compared to those with high ANCA-titers, but the difference was not significant (p= 0.100 and 0.076, respectively). The rate of acute dialysis therapy was not different in both groups (26.9% vs. 23.3%). The number of patients receiving methylprednisolone pulse therapy was lower, and the mortality rate higher in the patients with low ANCA-titers than those with high ANCA-titers, but the difference was not significant.

At the final follow-up (24.7 ± 19.3 months), 36 (64%) had survived, of whom 10 (28%) underwent maintenance di-

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Table 5. Clinical Characteristics and Prognosis of the Patients Devided by ANCA-titers

	ANCA titer (EU/ml)		P value
	< 200	≧ 200	
Number	26	30	
Demographic data			
age, years	73.0 ± 7.6	68.3 ± 12.7	0.100
male	10 (38%)	14 (47%)	0.536
presence of lung lesions	9 (35%)	10 (33%)	0.920
follow-up months	25.0 ± 22.0	24.3 ± 17.1	0.898
Labolatory data			
CRP, mg/dl	4.3 ± 4.1	7.2 ± 7.1	0.076
serum creatinine, mg/dl	4.4 ± 2.8	4.0 ± 2.8	0.589
serum albumin, g/dl	3.1 ± 0.6	2.9 ± 0.7	0.242
serum immunoglobulin G, mg/dl	1701 ± 639	1532 ± 673	0.405
Initial treatment			
methlprednisolone pulse therapy	10 (38%)	18 (60%)	0.108
cyclophosphamide with prednisolone	10 (38%)	13 (43%)	0.712
Prognosis			
death until the last follow-up	11 (42%)	9 (30%)	0.338
death due to infection until 6 months later	3 (11%)	4 (13%)	0.840
renal survival at the last follow-up	12 (46%)	14 (47%)	0.969

Contious data were shown as mean±SD.
Data analyses were performed using $\chi 2$ or *Student's t* test.

alysis therapy. The median follow-up of survivors was 28.5 months (31.7±18.7 months). A total of 20 patients (36%) died and their median survival was 5.5 months (range, 1-43 months). Most deaths were indirectly related to vasculitis and seemed to be mostly due to treatment-related infectious complication. Remission of ANCA-associated vasculitis was not achieved in 3 patients, who died of pulmonary hemorrhage, interstitial pneumonitis or convulsions that were probably due to cerebral hemorrhage. Of the 11 patients who died of infection, 7 of them died due to pneumonia and/or sepsis (aspergillus 2, fungus 2, MRSA 1, pneumocystis carini 1, bacteria 4) within 6 months after starting treatment. Other causes of death were neoplasia (n=2), gastrointestinal bleeding (n=2), heart failure and gangrene. The renal survival rate was 72% among the 36 patients who remained alive at the end of follow-up. Vasculitis relapsed in 8 patients (15.1%).

Of the 33 patients (serum creatinine and CRP at admission, 3.6 ± 2.1 mg/dl and 5.5 ± 6.4 mg/dl, respectively) who underwent renal biopsy, 24 (73%) remained alive and 5 (21%) of them had started maintenance dialysis therapy after 26.2 ± 18.7 months. Dialysis was stopped in 3 patients after immunosuppressive therapy, but 2 became repeatedly dependent on dialysis thereafter and one died of gastric cancer.

Prognostic Factors for Survival

Kaplan-Meier analysis revealed that the survival rate was significantly lower in patients who were older, male and with lung lesions or on acute dialysis (P=0.0164, 0.0054, < 0.0001, and 0.0008, respectively; Log-rank test) (Fig. 2). Based on the Japanese system of grading the clinical severity of rapidly progressive glomerulonephritis (7), the survival rate became significantly poorer as the grade increased (P < 0.0001; Log-rank test) (Fig. 3). The survival rates of grades I, II and III + IV at 1 year later were 100%, 80.0% and 50.0% respectively. Among 7 patients who died of infection within 6 months of starting treatment, 6 developed lung lesions as organ involvement associated with systemic vasculitis and 4 underwent acute dialysis therapy. Five (18%) and 4 (29%) patients died of infection within 6 months after starting methylprednisolone pulse or immediate dialysis therapy, respectively, whereas only 2 (7%) and 3 (7%) did so respectively, among those who were not administered with these interventions. Multivariate logistic regression analysis showed that the presence of lung lesions (OR 11.5, 95% CI, 1.10 to 120, P=0.041) was significantly associated, while acute dialysis (OR 7.5, p=0.056) and methylprednisolone pulse (OR 6.8, p=0.137) therapies tended to be associated with death due to infection within 6 months of starting treatment. The Cox proportional hazards model showed that the presence of lung lesions and acute dialysis therapy conferred poorer survival rates (HR, 3.32, 95% CI, 1.14 to 9.71, p=0.028; HR 2.73, 95% CI, 1.03 to 7.23, p= 0.044, respectively). None of age, gender, therapy with methylprednisolone pulse or cyclophosphamide and levels of albumin, CRP or ANCA were significant (Table 6).

Discussion

The ratio of MPO/PR3-ANCA is very high in Japan compared with that of Europe or USA (1-4). However, to our knowledge, no English-language report has addressed the outcome of Japanese PRV patients, except for one questionnaire (8). The characteristics of our patients were as follows: All of those with onset of new PRV in a restricted area over the past 5 years were included; all were closely monitored until follow-up information was collected in De-

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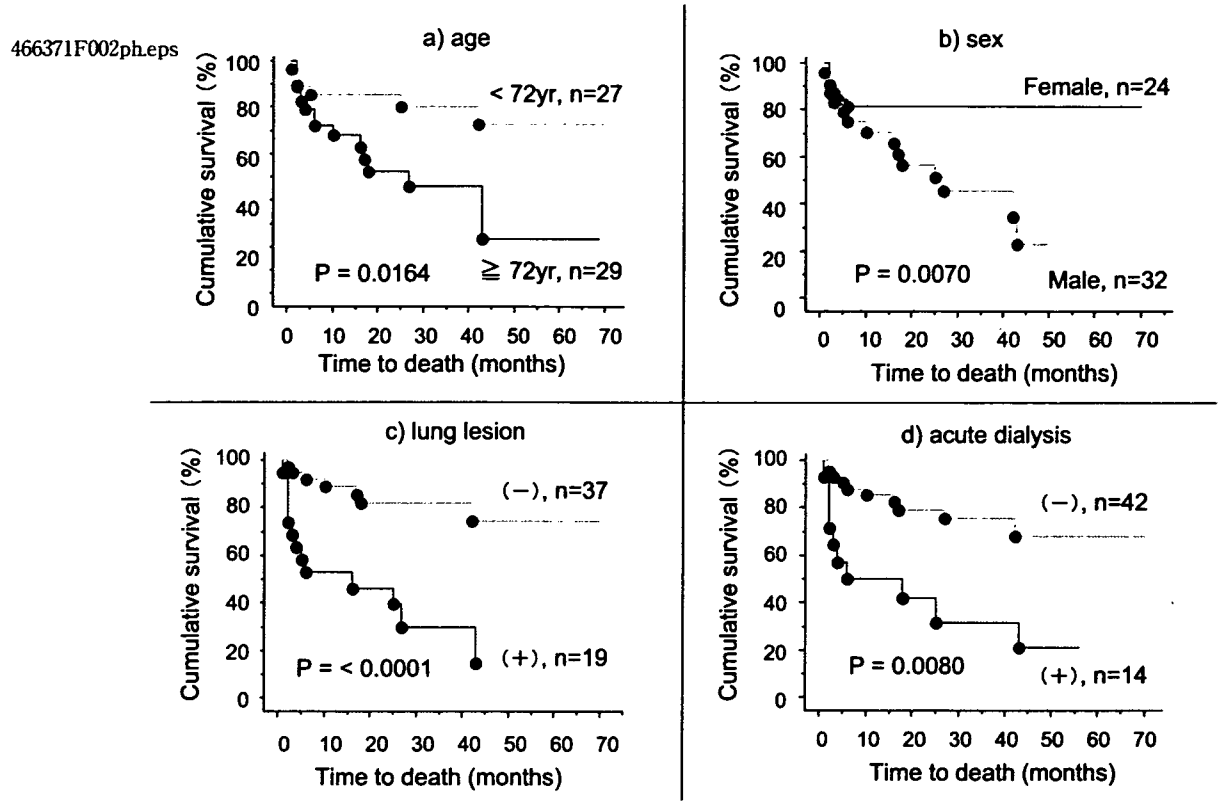

Figure 2. Kaplan-Meier plot indicating unadjusted survival probability according to age (a), gender (b), presence of lung lesions (c) and acute dialysis therapy (d). Differences in survival curves were analyzed by log-rank test.

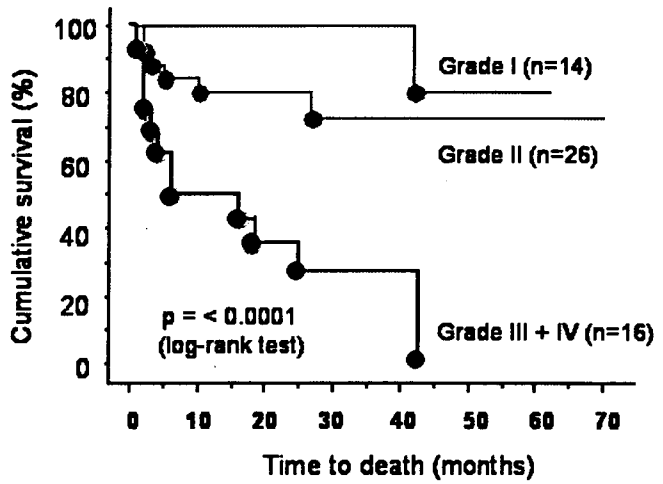

Figure 3. Survival rates based on grading of clinical severity.

cember 2005 (follow-up period, >1 year) or until death; most (91%) were positive for MPO-ANCA; the mean age was >70 years and one-fourth of them required dialysis at the start of immunosuppressive treatment.

We focused on factors predicting the unfavorable outcome of death. In line with other studies including a Japanese nationwide survey (Table 7), Kaplan-Meier analysis of our results revealed that more advanced age, male gender, respiratory tract involvement and advanced renal impairment were significantly associated with a poor cumulative survival rate. The survival rate was significantly poorer with increasing clinical grades based on the system of the Japanese Society of Nephrology (7) that includes serum creatinine and CRP levels, age and lung lesion in scores of clinical severity. In addition, lung involvement and acute dialysis therapy at the start of immunosuppressive therapy conferred poorer survival rates according to the Cox proportional hazard model. The survival rates in our study were identical to those of studies including patients with WG or positive PR3-ANCA, whose life expectancy is considered to be poorer than that of patients with MPA/RLV or who are positive for MPO-ANCA (12, 14). This could be because a high ratio of our patients was elderly and dialysis-dependent. As in other recently published series (10, 19-21) showing that the age of patients with ANCA-associated vasculitis is increasing, the median age of our patients was 72 years and 63% were aged 70 years or older. The 2-year mortality rate of 36% among our patients was similar to the findings of others (9-13) with an older population and severe renal impairment, highlighting the importance of age and renal function for survival. The renal survival rate of 72% among our patients was also similar to those found by others (Table 7), although factors such as mean age, length of follow-up and treatment strategy varied. Renal function tended to recover with aggressive treatment if acute dialysis was not required. Patients with serum creatinine levels below 3.5 mg/dl at initial presentation did not progress to maintenance dialysis therapy. On the other hand, 8 patients (15%) underwent 9

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Table 6. Multivariate Analysis of Factors Conferring Poorer Survival Rates upon PRV Patients using the Cox Proportional-hazards Model

	Hazars ratio	95% CI	P Value
Age (vs. lower than 72 years)	2.13	0.780 – 5.793	0.141
Female (vs. male)	0.55	0.188 – 1.590	0.268
Lung lesions (vs. no lung lesions)	3.32	1.138 – 9.711	0.028
Acute dialysis (vs. no acute dialysis)	2.73	1.027 – 7.231	0.044
Methylprednisolone pulse therapy (vs. no methylprednisolone pulse therapy)	0.97	0.363 – 2.585	0.950

Table 7. Literature Review for Outcome and Factors Predicting Unfavorable Outcome of the Patients with ANCA-associated Primary Renal Vasculitis

Author and year	Number of patients	Mean age, years	Length of follow-up	Cre, mg/dl	Dialysis*	Death	Renal survival	Relapse	Factors predicting unfavorable outcome **
1) Westman et al. [9], 1998	123	61.8	55 months	4.4	-	31%	78%	46%	age, SCr
2) Booth et al. [10], 2003	246	66	-	5.1	-	24%	72%	34%	age, SCr (>200μmol/l), sepsis
3) Little et al. [11], 2004	86	63.5	3.4 years	6.3	55%	38%	63%	19%	Karnofsky performance score
4) Weider et al. [12], 2004	80	63	47 months	4.4	28%	26%	77%	33%	types of immunosuppresion and vasculitis age, SCr, PR3-ANCA
5) Harper et al. [13], 2005	229	65	-	6.2	29%	40%	60%	26%	age, SCr (> 400 μmol/l)
6) Hogan et al. [14], 1996	107	58.8	2.5 years	5.9	29%	15%	58%	29%	pulmonary hemorrhage, C-ANCA
7) Cohen et al. [15], 2000	94	59	33 months	5.0	22%	13%	57%	24%	male, age, lung involvement
8) Yamagata et al. [8], 2004	410	62.6	6 months	4.6	-	25%	71%	-	age, SCr, lung involvement, CRP
9) Present study	56	70.4	25 months	3.9	25%	36%	72%	15%	lung involvement, acute dialysis therapy

1)-5), these studies include WG/CSS in addition to MPA/RLV; 6)-9), these studies include MPA and RLV.
* patients who were dialysis-dependent at presentation; ** factors were determined using multivariate analysis
SCr, serum creacinine; ESRD, end-stage renal disease; MPA, microscopic polyangiitis; RLV, renal limited vasculitis; WG, Wegener's granulomatosis; CSS, Churg-Strauss syndrome

episodes of recurrence, which was less than in other studies. This may be explained by the short follow-up period and the fact that all of our patients were negative PR3-ANCA, because relapse was more common in the patients with PR 3-ANCA/WG compared to those with MPO-ANCA/MPA (10, 22, 23, 24). Since ANCA measurements have become popular for patients with a rapid decline of renal function or advanced renal failure of unknown origin, the amount of ANCA-associated renal vasculitis with both mild and severe renal dysfunction seems to have increased. Further education of general practitioners and physicians about the nature of this rare disease should result in early referral and hence improve outcome.

A major cause of death was indirectly related to vasculitis and mostly consisted of treatment-related infectious complication. Furthermore, 7 of the 11 patients who died due to infectious complications did so within 6 months of starting treatment. The presence of lung involvement of systemic vasculitis (OR, 11.5) was significantly associated with death due to infection. In addition, large doses of steroid (OR, 6.8) or acute dialysis (OR, 7.5) at the start of immunosuppressive therapy tended to be associated with death due to infectious complications. Immune compromised ANCA-positive patients on acute dialysis therapy or immunosuppression with high doses of prednisolone might easily have led towards infection, particularly if lung involvement is present. On the other hand, the selection of treatment modality was dependent on the decision of the attending physician in our study population. For example, the criteria for selection in using cyclophosphamide were not determined in this cohort. In order to avoid and treat opportunistic infection, prophylactic administration of antibiotics with sulphamethoxazole/trimethoprim (25) was not uniformly performed, and the criteria for selection in using various antibiotics and immunoglobulins were not determined. Finally, our study cohort did not receive immunosuppressive therapy in accordance with the treatment guideline shown in EUVAS (26) or Japan (27). In the future, it should be examined whether treatment modality according to these guidelines improves mortality and renal survival rate.

Reports indicate that about 10% of ANCA-negative patients are found among those with pauci-immune crescentic glomerulonephritis or primary renal vasculitis (10, 14, 24, 28). Our study cohort included 5 ANCA-negative patients (9%) with few upper airway symptoms, a shorter prodrome, lower levels of CRP, and a favorable clinical course although renal biopsy specimens revealed extensive crescentic glomerulonephritis (67 ± 24% of glomeruli was involved with crescents). As shown in Table 5, the patients with low ANCA-titers showed higher age and lower CRP levels compared to those with high ANCA-titers, but the difference was not significant. In addition, about half of the MPO-ANCA-positive patients in our series, similar to the reported ratio (9, 12, 14, 15, 29), had no extra-renal involvement, although renal limited vasculitis (RLV) comprises a separate entity within the ANCA-associated vasculitides (23, 28, 29). Further studies might be needed to clarify whether our patients truly did not have ANCA/extrarenal involvement at presentation or later, and whether or not the levels of ANCA titer reflect the difference of disease spectrum including out-

come and prognosis.

In conclusion, a poor survival rate is independently associated with the presence of lung lesions and advanced renal failure at the start of immunosuppressive therapy in patients with PRV. The major cause of death was indirectly related to vasculitis and seemed to be primarily due to complications arising from treatment-related infections. The prognosis of PRV patients should be improved by early disease identification and the application of a treatment protocol that is designed to prevent infectious complications.

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A Murine Model of Vasculitis Induced by Fungal Polysaccharide

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Abstract: CAWS is a mannoprotein-beta-glucan complex obtained from the culture supernatant of the fungal pathogen *Candida albicans*. CAWS exhibits various biological activities, and induces prominent vasculitis of the aortic valve and the coronary arteries in mouse. A significant difference was noted in the susceptibility to and the degree of vasculitis induction among mouse lines. The difference in cytokine production among mouse lines may be strongly related to that difference, namely, IL-6, IFN-γ and TNF-α presumably act as positive factors, and IL-10, as a negative regulator. On the other hand, as a structural component of the inducing substance, the presence or absence of β-1,2-mannose residues was suggested to be closely related to the activity. An understanding of the molecular mechanisms underlying this model could lead to the conquest of many modern diseases. This model is also expected to be useful for the development of new therapeutic drugs for vasculitis and cardiovascular diseases.

Key Words: Murine model, vasculitis, coronary arteritis, remodeling, fungal polysaccharide, CAWS, *Candida albicans*, mannoprotein.

INTRODUCTION

Various functionally differentiated cells and organs build and maintain the structure of multicellular organisms. Blood vessels have the important function of linking cells and organs together and enabling exchange of substances and information, while at the same time, blood vessels themselves are an aggregate of functionally differentiated cells, and blood vessel formation and structural maintenance are regulated by various factors. Many lifestyle diseases and intractable diseases involve vascular lesions. Therefore, the creation and analysis of animal models of vasculitis are important from various perspectives.

In the course of biological evolution, microorganisms were the first to appear and spread on Earth and there is no inconsistency in presuming that animals were equipped from the beginning with a defense system against these microorganisms. Infectious diseases are normally triggered by various microorganisms, but in the present, immune compromised hosts increased because of, i.e., sophisticated medical treatment and spreading of HIV infection, and thus infections caused by higher microorganisms such as fungi are on the rise. We analyzed the host defense system against the fungal pathogen *Candida albicans* from various perspectives, and discovered that the extracellular polysaccharide fraction CAWS induces lethal vasculitis in mouse. We outline herein new findings of the pathology and the molecular mechanisms behind it.

LIMULUS FACTOR G-ACTIVATING CAWS OF PATHOGENIC FUNGAL ORIGIN

Beta-glucan (BG) is often detected in the blood of patients with deep mycosis and is widely used as an early auxiliary diagnostic tool for mycosis [1-10]. There is no doubt that the appearance of BG in the blood is a result of partial lysis of the fungal cell wall. However, its overall structure is unknown due to its extremely low (pg level) concentration in the blood. In addition, BG may have various biological activities. In an effort to clarify these points, we focused on *C. albicans*, the fungal pathogen most often encountered in clinical practice, and analyzed preparations of the extracellular polysaccharide fraction CAWS. CAWS is an extracellular polysaccharide fraction derived from the culture of *C. albicans* in a completely synthetic medium, which has a yield of approximately 80 mg/L, sugar content of 70%, protein content of 10%, and mannose and glucose as its main sugars (M/G ratio=6.3±1.3 from *C. albicans* IFO 1385 derived CAWS). CAWS reacts with serum factors against *Candida* cell wall mannan, and with limulus derived G factor (limulus G factor, factor G), which points to its close relationship with BG in the blood of patients with deep mycosis. CAWS was prepared without specifically segregating all the macromolecular glycoproteins released from the fungus, thus, its structural details remain unclear. Nevertheless, it is presumed to be a conjugate containing mannoprotein, β-1,6-glucan and β-1,3-glucan (Fig. 1). CAWS used in various experiments always contains less than 1 ng/mg endotoxin.

VASCULITIS MODEL SIMILAR TO KAWASAKI DISEASE

Kawasaki disease (KD) is a pediatric disease of unknown cause, and is accompanied by acute fever. KD sometimes results in coronary aneurysms and other fatal sequelae [11-15]. High-dose infusion of immune globulin has been used as standard treatment, however, the risks presented by such biologics have prompted a search for better treatment strategies [16-21]. The analyses of animal models are important for the development of new treatment strategies and so far, piglet, canine, rabbit, murine and other models have been proposed and analyzed (reviewed in ref. 22). The canine

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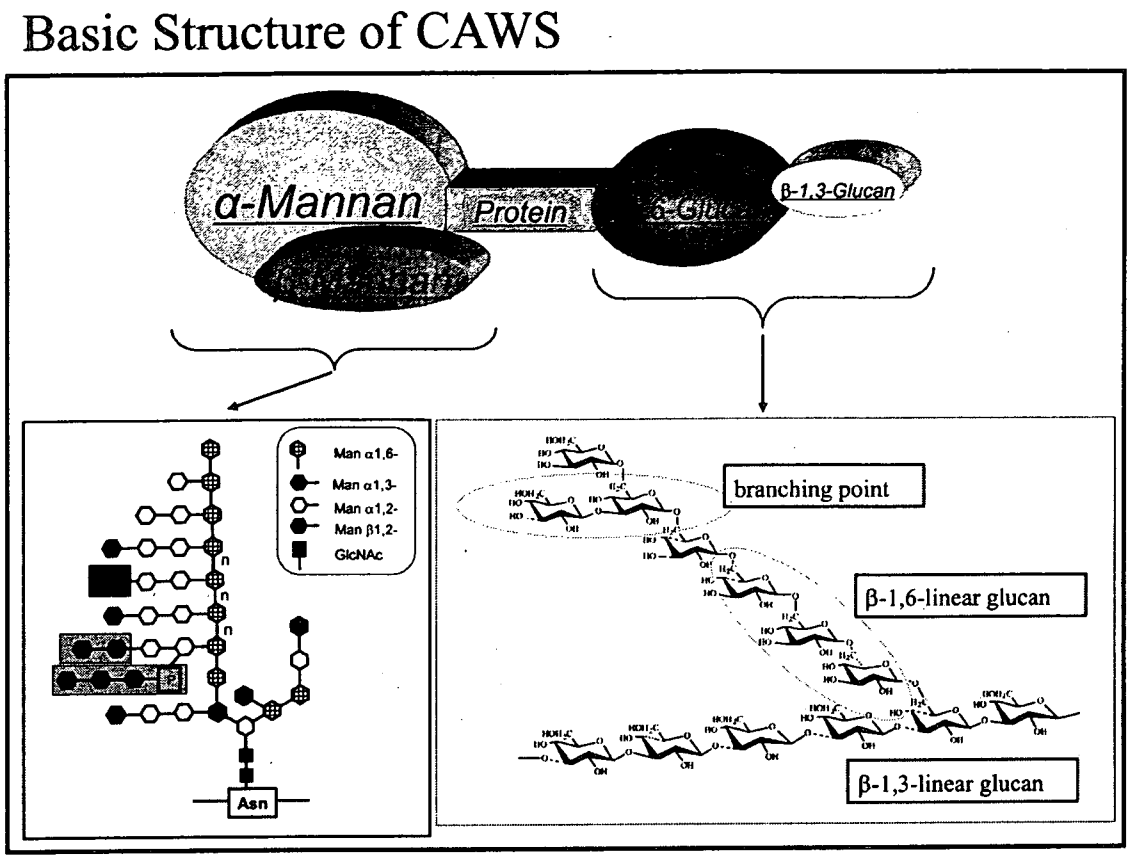

Fig. (1). Basic structure of CAWS

Beta-mannan component is strongly influenced by culture conditions (see ref. 32). Residues of beta-mannan are shaded in the figure. Proposed structure of mannan moiety is deduced from Klis's review (ref. 53.55.56). Proposed structure of glucan moiety is deduced from our study (ref. 72).

model is autoimmune, while the other models are induced. In the piglet and rabbit models, KD is induced in association with serum sickness caused by the administration of equine serum. However, many of the reported experiments were conducted independently and only a few studies were designed for the purpose of clarifying the mechanisms of disease or treatment strategies. Studies that have been actively pursuing these goals, using murine models of vasculitis induction, include those of Lehman *et al.* using *Lactobacillus casei* cell wall [23-27] and ours using *Candida* components [28-44].

L. casei is used for various purposes in experimental medical research, e. g., induction of experimental rheumatic arthritis. *L. casei* induced vasculitis in many mouse lines including nude mouse. Whereas C3H/HeJ mice that had a mutation in TLR4 were resistant, TLR4-KO mice on C57Bl/6 background were susceptible. IFNγ-KO mice were also susceptible. On the other hand, MyD88-KO and TLR2-KO mice were reported to be resistant [23]. *L. casei* acts as a superantigen and has been implicated in the mechanism of the disease, however, the details are not clear. A comparison of available literature showed a significant difference from the histological images of CAWS-induced vasculitis. In the analysis of the pathology of vasculitis and cardiovascular disease and the development of new treatment strategies, an understanding of both models and their comparison are desirable.

MODEL OF VASCULITIS INDUCED BY *CANDIDA* COMPONENTS

The development of animal models of *Candida* component induced vasculitis has its origin in the study of Murata and Naoe (Toho University School of Medicine), who found that the extract of *C. albicans* (CADS) isolated from the feces of children with KD induced coronary arteritis similar to KD in mice [28, 29]. According to the prototype protocol, vasculitis was induced by intraperitoneally injecting CADS for 5 consecutive days during week 1 and week 5, and specimens were collected and examined on week 9. In 50-60% of mice, arteritis and coronary arteritis were detected. As CADS is a crude polysaccharide fraction, it was further fractionated to determine the active substance, and it became clear that the mannan fraction was the most active (unpublished results). CADS-induced vasculitis differed between lines, with C3H/HeN mice showing approximately 70% susceptibility and CBA/JN mice showing resistance. Cross-breeding of the two lines and analysis of the genetic polymorphisms responsible for the susceptibility of the resulting

F2 generation, showed that the genes responsible for susceptibility were positioned on D1Mit171 and D1Mit245 (map position 20.2 cM) of chromosome 1, where the IL1 receptor and the TNF receptor were positioned [30].

With this as background, it was shown that in a similar protocol, CAWS induced arteritis with a similar mechanism. Interestingly, the rate of occurrence of CAWS-induced vasculitis was much higher than that of CADS-induced vasculitis, leading us to conclude that CAWS-model was appropriate for quantitative analysis. The sites of vasculitis induction have not been thoroughly analyzed, although the aortic valve and the roots of the coronary arteries have been repeatedly shown to be the common sites of vasculitis induction.

SCREENING FOR CAWS ACTIVITY

As indicated above, the forefront of CAWS-related research has focused so far on its usefulness in the early diagnosis of deep mycosis, however, CAWS-induced vasculitis has also sparked interest in CAWS as a fungal toxin. Therefore, to further investigate the mechanism of vasculitis induction by CAWS, we attempted to screen its activity.

Intravenous injection into mice *in vivo* showed acute lethal toxicity. The reaction varied among mouse lines, with induction noted in ICR, C57Bl/6, KSN nu/nu (removal of thymus), C3H/HeN, C3H/HeJ (TLR4 mutation), and WBB6F1-w/w/^{W} (mast cell_deficient) mice, and no induction in DBA/2 (complement C5 deficient) mice [41]. Moreover, lethality was prevented by the administration of β-2 stimulators and survival was improved by subsequent administration of epinephrine. The concomitant use of antihistamine agents, antiserotonin agents, or anti-PAF agents also improved survival [43]. Administration of the antisense peptide of complement C5 also improved survival. [44]. On the other hand, myelosuppression resulting from the administration of cyclophosphamide or damage of the macrophage system after treatment with carrageenan or gadolinium chloride did not affect lethality (unpublished results). Together, the results indicate that acute lethality is mediated mainly by the activation of the complement or by smooth muscle contraction. Also, in mice that did not expire after the first administration of CAWS, subsequent administration did not result in lethality, indicating that a certain kind of tolerance had developed. The acute lethality caused by CAWS is similar to the anaphylactoid reaction caused by O9LPS of *E. coli* origin, which has an O antigen polysaccharide having high mannan content, and the tolerance induction showed cross reactivity between CAWS and O9LPS [34]. The anaphylactoid reaction caused by O9LPS is dependent on muramyldipeptide (MDP) administration, whereas the acute lethality caused by CAWS is not, suggesting a different mechanism of action.

Observation of the levels of fluctuation of cytokines and chemokines in blood after a single administration of CAWS showed that minutes to hours after administration, the levels of IL-1, IL-6, IL-10, IL-12, and MIP-2 increased transiently [31]. At the same time, the production of soluble ICAM-1 that indicates vascular endothelial damage also increased. These showed that at the whole body level, the administration of CAWS induced significant increases in cytokine levels in blood. The symptoms resembled the acute-stage symptoms of vasculitis.

In a mouse *in vitro* evaluation system that uses spleen cells, at low concentrations of CAWS, the production of IL-6, IFN-γ, etc. showed a slight increase [36, 37]. At high concentrations, cellular damage was observed and the mitogen activities of LPS and ConA were suppressed. Moreover, in the macrophage cell line RAW264.7, suppression of cell proliferation and of cytokine production was observed [40]. The addition of CAWS to a dendritic cell culture prepared from mouse myeloid cells cultured in the presence of both GM-CSF and IL-4, increased the expression of B7-1 or MHC and the production and activation of IL-6, IFN-γ, TNF-α, etc (see Fig. 7).

In a human *in vitro* evaluation system, complement activation through the lectin pathway was induced and anaphylatoxin was produced, PBMCs were cultured in the patient's own serum and stimulation with CAWS led to an increase in the production of IL-8, TNF-α, etc [40]. Platelet-rich plasma showed a mild increase in platelet agglutination, and thrombomodulin production by human umbilical vein endothelial cells (HUVECs) was decreased.

The above results demonstrate that CAWS shows various biological activities not only *in vivo*, but also in cell lines cultured *in vitro*.

IMMUNOLOGICAL MECHANISM OF VASCULITIS INDUCTION

A comparison of CAWS-induced vasculitis among mouse lines showed that C3H/HeN, C3H/HeJ, DBA/1, DBA/2, A/J, CBA/N, C57Bl, AKR, and BALB/c mice were highly susceptible, while CBA/j mice were resistant. The degree of lesions varied among the highly susceptible lines, with most DBA/2 mice expiring during the observation period, suggesting the development of serious cardiac disease [36, 37]. No difference between sexes was observed (unpublished results). The degree of lesions and lethality were more prominent in younger specimens (unpublished results). According to standard protocol, a dose of 4 mg/mouse was used, but in DBA/2 mice, even a reduced dose of 250μg led to vasculitis induction and lethal toxicity (Fig. **2**. modified from ref. 32). Lower the dose required longer time period to induce lethal toxicity (Fig. **2**). Histological characteristics of CAWS-vasculitis was shown in Fig. **3**. Aorta with aortic valves were stained with HE and examined microscopically with x20, x40, x200, and x400 magnifications. Elastic fibers were clearly stained with parallel orientation in the control group, however, the fibers were partly appeared in the CAWS group. In the CAWS group significant thickening of the wall was seen with large number of leukocytes with disorganization. Observation of the time course of vasculitis induction in DBA/2 mice showed that around 3 days after the last administration, no prominent changes in the histological images of blood vessels were noted, however, after approximately 10 days, localized proliferative changes and rupture of elastic fibers were observed (Fig. **4**). Moreover, when vasculitis had developed sufficiently and mice started expiring 4 weeks after the last administration, a significant increase in cardiac weight was observed (unpublished results). Therefore, the vasculitis and the resulting lethality can be divided roughly into the initial induction stage, the vasculitis formation stage and the late stage of cardiomegaly (Fig. **5**).

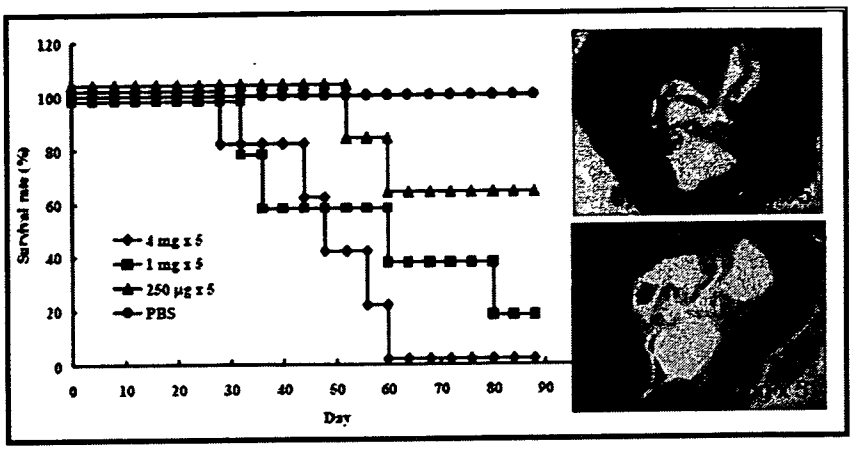

Fig. (2). Lethal toxicity of CAWS-induced vasculitis in DBA/2 mice and histological images of vasculitis (HE staining)
CAWS was administered intraperitoneally to DBA/2 mice in doses of 4 mg. 1 mg or 250 μg for 5 consecutive days. and survival rates were compared. The images below show the aortic valve (HE staining) at the time of death. Modified from data presented in Ref 32. Histological images between 4mg. 1mg. and 250μg of CAWS were not significantly changed. Only survival of the mice were different and higher the dose shorter the lifespan.

The administration of CAWS to DBA/2 mice led to modification of the whole immune system, prominent splenomegaly, and increases in neutrophil and macrophage ratios [36, 37]. The splenomegaly persisted even 5 weeks after the last administration. Even in the absence of stimulation, spleen cells that were cultured *in vitro* immediately after CAWS administration showed an increase in the production of IFN-γ and IL-6 in the supernatant and the release of myeloperoxidase. Serum MPO-ANCA levels also increased. These observations indicated that the activation of neutrophils in the spleen was sustained. Even for highly susceptible lines, for example, in C3H/HeN mice that showed a relatively mild degree of vasculitis, no IFN-γ production was observed immediately after CAWS administration. Nine weeks after the first administration, secondary CAWS stimulation led to an increase in IL-6, IFN-γ, and TNF-α production in the vasculitis-induced lines, while in the resistant CBA/j line, IL-10 production was increased. IL-10 is well known as an immunosuppressant, and may account for the resistance to CAWS-induced vasculitis. From these results, it can be concluded that the induction of vasculitis passes through the early, middle and late stages in which various factors interact in a synergistic or antagonistic fashion to form the pathology.

CHARACTERISTICS OF FUNGAL POLYSACCHARIDE REACTIVITY IN DBA/2 MICE

Harada *et al.* compared the reactivity of spleen cells in various mouse lines towards BG of fungal origin and showed a marked induction of IFN-γ production only in DBA mice

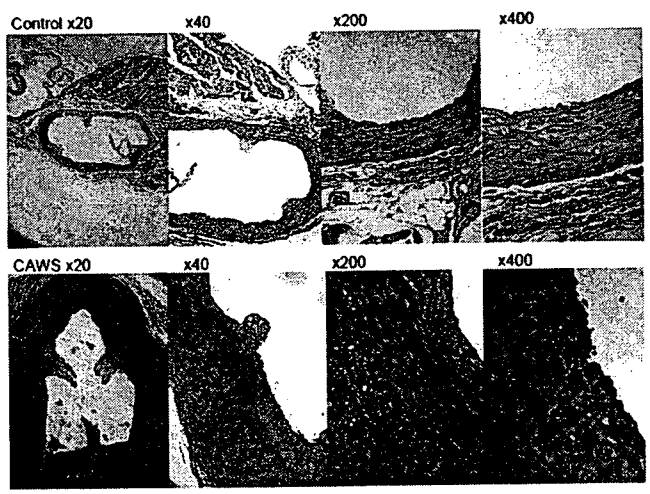

Fig. (3). Histological images of aorta obtained from control and CAWS-administered DBA/2 mice (HE-staining). Microscopic images of aorta and aortic valve were shown with various magnifications (x20. x40. x200. x400). Upper pictures. control. lower pictures. CAWS administered DBA/2 mice. Characteristic feature of CAWS-vasculitis is enlarged size of aorta. thickening wall. infiltration of large number of leukocytes. and degradation of elastic fiber.

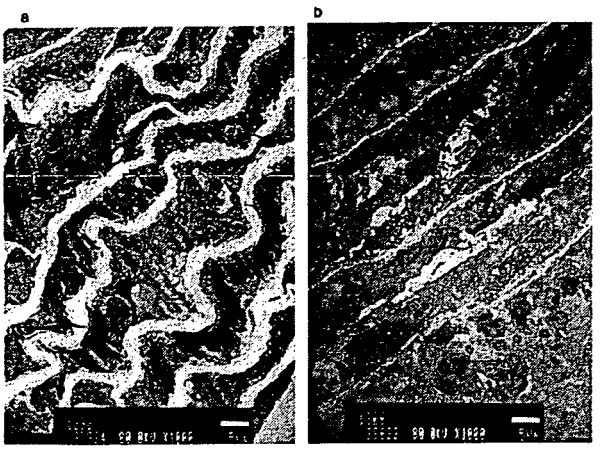

Fig. (4). Electron microscope image of CAWS-induced vasculitis in DBA/2 mice

An electron microscope image (x1000) of the aorta 5 days (a) and 14 days (b) after CAWS administration. Kinetics of histological changes by HE- staining is shown in ref. 36. Image of 5 days is indistinguishable to that of the control mouse. Prominent cell infiltration and rupture of elastic fibers were observed in 14 days.

[45-49]. An increase in the production of other cytokines such as GM-CSF, TNF and IL-12 was also observed. Moreover, the production of each of these cytokines was inhibited by the addition of antibodies against GM-CSF. In contrast, when spleen cells from other mouse lines were stimulated with BG in the presence of GM-CSF, IFN-γ production was induced. From the above, it can be presumed that GM-CSF is a key cytokine that controls the reactivity of DBA mice towards BG.

Moreover, analysis of the cellular interactions resulting from BG stimulation of cytokine-producing spleen cells showed that the production of individual cytokines was intricately regulated, and the production of GM-CSF and IFN-γ required the simultaneous culture of adherent and nonadherent cells. However, in the presence of GM-CSF, the production of TNF and IL-12 was induced only in adherent cells (Fig. 6). In a similar experiment with CAWS, we noted a dose-dependent production of GM-CSF by spleen cells from DBA/2 mice, and spleen cells stimulated with CAWS in the presence of GM-CSF showed an increase in the production of IFN-γ, IL-6 and TNF-α, etc (Fig. 7).

Bone marrow derived dendritic cells (BMDC) were prepared *in vitro* culture in the presence of GM-CSF and IL-4. BG is known to react with BMDC strongly to produce cytokines and is dectin-1 dependent [50]. Reactivity of CAWS to BMDC was tested and it induced significant concentration of cytokine similar to BG (Fig. 7). These results again strongly suggest that GM-CSF production is the rate-limiting step in CAWS reactivity.

MANNAN STRUCTURE OF CAWS AND VASCULITIS INDUCTION ACTIVITY

CAWS is an extracellular polysaccharide fraction that does not undergo a stringent refinement process. The mannan component of CAWS is considered to be important for vasculitis induction, however, it has not been analyzed sufficiently as a structural requirement. On the other hand, *Candida* mannan has been extensively studied from various per-

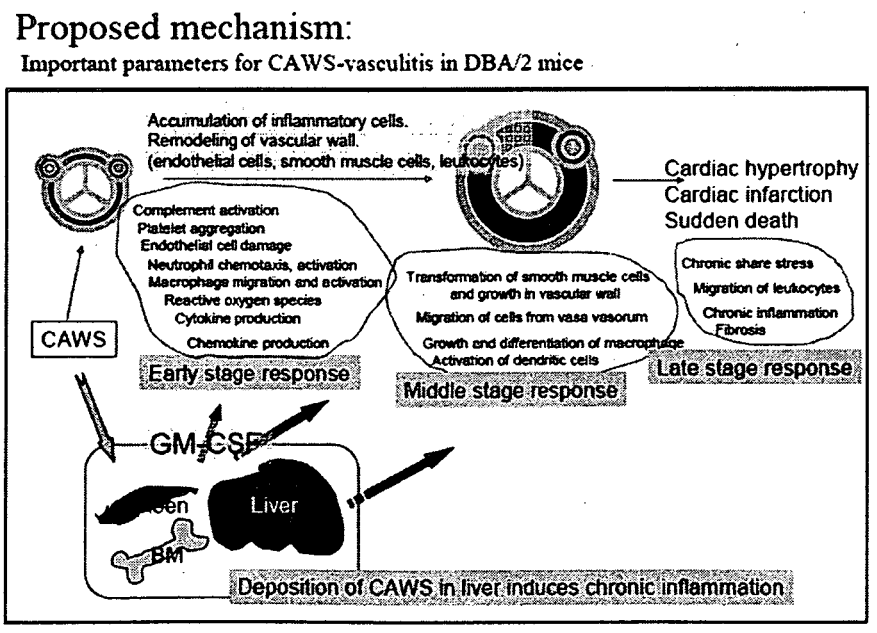

Fig. (5). Proposed mechanism on the development of CAWS-vasculitis in DBA/2 mice

The vasculitis induced by CAWS in DBA/2 mice develops through early, middle and late stages, and each stage is regulated by different factors. The most relevant events are presented schematically.

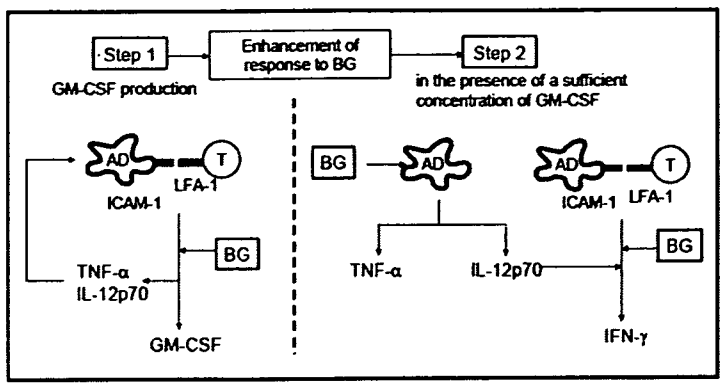

Fig. (6). Proposed scheme of cellular interactions leading to cytokine production. induced by a fungal polysaccharide in spleen cells of DBA/2 mice

The figure outlines the cellular responses involved in cytokine production. triggered by β-glucan stimulation of spleen cells of DBA/2 mice *in vitro*. This scheme was deduced from the series of Harada's study (see references 45-49).
BG. fungal polysaccharide. AD. adherent cell. T. mature T cell

spectives as it is related to pathogenicity [51-59]. Identifying the fungal species using serum factor in diagnosis is also easy. The main structure of *Candida* mannan has α-1,2-, α-1,3-, and α-1,6-linkages, but partially existing β-1,2-linked mannose residues are closely related to cell adhesion and pathogenicity, as reported in numerous papers.

CAWS is a polysaccharide fraction released from *Candida* cultured in a completely synthetic medium, and is produced in large quantities in a jar fermenter. So far, a completely synthetic medium has been used, enabling appropriate circulation and stirring during culture. When we examined the changes in pH during culture, it became clear that the pH shifts towards the acidic side during culture [32]. As the *Candida* mannan structure is influenced by changes in pH and temperature, we cultivated *Candida* under controlled pH and temperature conditions, isolated the released polysaccharide fraction, and examined its vasculitis induction activity. The results showed that CAWS had no vasculitis induction activity at 27℃-pH 5 (CAWS-27-5) or 27℃-pH 7 (CAWS-27-7). Under these conditions, the acute lethal toxicity of CAWS was also significantly diminished (Fig. 6, modified from ref. 32). On the other hand, cultivation at 37℃ (CAWS-37) or pH 2 (CAWS-27-2) led to vasculitis induction. Furthermore, when we examined the structural details of these polysaccharide fractions using serum factor, it became clear that the fractions that had increased reactivity to

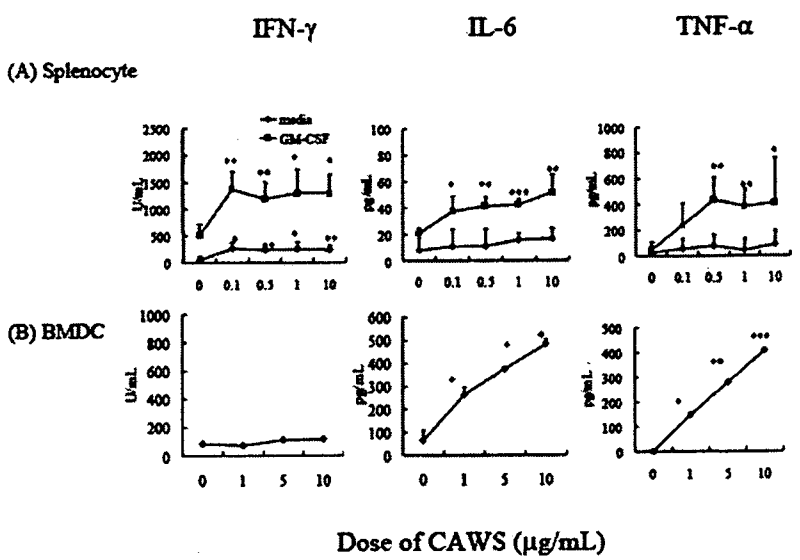

Fig. (7). Effect of GM-CSF on the cytokine production of splenocytes and bone marrow derived dendritic cells (BMDC) stimulated by CAWS. Preparation of splenocytes and BMDC were followed by Harada's procedure shown in reference 45-49. Splenocytes and BMDC from DBA/2 mice were cultured in 24-well flat-bottomed plates and stimulated with saline or CAWS. Spleen cell culture was conducted in the presence or absence of recombinant GM-CSF. Concentrations of cytokines were measured using ELISA. All mAbs and corresponding recombinant cytokines used were purchased from PharMingen.

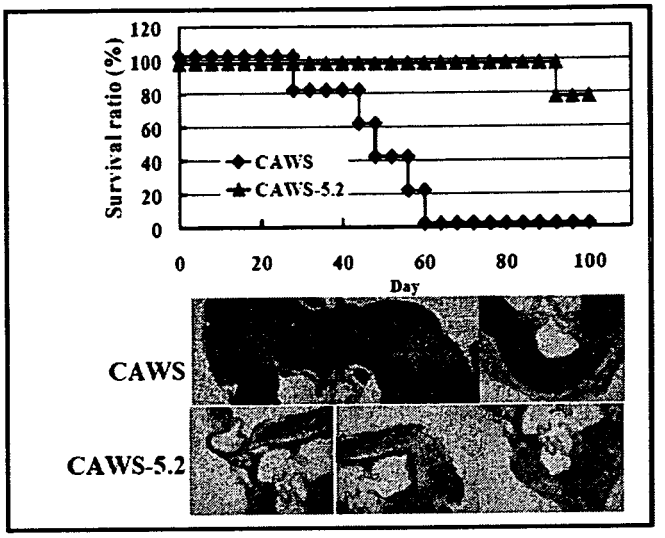

Fig. (8). Vasculitis and lethal toxicity induced by CAWS produced under controlled culture conditions in DBA/2 mice

CAWS produced under standard conditions and that produced at 27℃ (pH 5.2) . designated as CAWS-5.2. were compared in terms of vasculitis induction activity and lethal toxicity. Lethal toxicity was appeared only by CAWS. Picutures of aorta with valve and coronary artery were shown. Vasculitis of aorta and coronary artery were severe in the case of CAWS (upper pictures). Left picture shows aorta and left cusp of coronary artery. Middle shows coronary artery. Right shows aorta with valve. Lower picture shows those of CAWS-5.2. Only slight remodeling of aortic wall was seen in left and middle pictures. Right picture shows aorta with valve and weak vasculitis were induced. Vasculitis and aortic remodeling induced in CAWS-5.2 are not sufficient to induce strong lethal toxicity. Modified from data presented in Ref 32.

serum factor 4, 5 and 6 did not induce vasculitis. These factors recognize β-1,2-linkages, which strongly suggests that β linkages inhibit the vasculitis induction activity of CAWS.

CONCLUSIONS

CAWS is a strong inducer of vasculitis in mouse. The presence or absence of vasculitis induction and its degree vary greatly among mouse lines, suggesting that multiple genetic backgrounds of the host are related to its pathology. From the perspective of cytokine production, it is presumed that IL-6, IFN-γ and TNF-α act as positive factors, and IL-10 acts as a negative factor. On the other hand, as a structural component of the inducing substance, mannoprotein of cellular wall origin is considered to play an important role, changes in cultivation conditions strongly influence the induction of vasculitis, and immunochemical analysis suggests that the presence or absence of β-1,2-mannose residues is closely related to the activity [57-59]. These findings show that CAWS-induced vasculitis occurs only under specifically controlled conditions determined by both the host and the inducing substance. It is difficult to say, however, that the mechanism of vasculitis induction and its treatment strategies have been fully understood or standardized. Our model aims to contribute to the resolution of these issues.

Vasculitis is inflammation of vessel walls. In 1997, Jennettes reviewed in New England Journal of Medicine that it is categorized to several patterns, such as large-vessel vasculitis, medium-sized-vessel vasculitis, and small-vessel vasculitis [60]. These vasculitis includes individual, specific vasculitis, such as Giant-cell arteritis, Takayasu's arteritis, Kawasaki's disease, Wegener's granulomatosis, Goodpasture's syndrome, and so on. Antinueutrophil cytoplasmic antibody (ANCA) is strongly associated with some diseases. Categorize vasculitis is important for accurate diagnosis. Animal models for vasculitis has been developed, such as MLR, SCG, MPO-KO [61-63]. Each disease as well as animal models show characteristic vasculitis and MLR and SCG induced small vessel vasculitis related to ANCA-vasculitis. In contrast, CAWS-vasculitis was mainly induced around aorta, aortic valve and coronary artery. Microscopic examination of kidney tissues of CAWS- administered mice seldom detected vasculitis. Thus, histological characteristics of CAWS-vasculitis is significantly different from the above models. Significant remodeling by CAWS of the arterial wall and more susceptible of young animals to the lethal toxicity suggested that CAWS-vasculitis is a model for KD.

Many modern diseases such as diabetes, hypertension and hyperlipidemia, are related to vascular pathologies [64-71]. Elucidation of the molecular mechanism of this model is expected to help conquer these diseases. CAWS-induced vasculitis has already been replicated in multiple institutions and the induction has been achieved universally and stably. It may be a very suitable model for the development of new diagnostic and treatment strategies.

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ABBREVIATIONS

ANCA = Anti-neutrophil cytoplasmic antibody

B7-1 = Also called as CD80

BG = Beta-glucan

CADS = Extract of *Candida albicans*

CAWS = *Candida albicans* water soluble mannoprotein-beta-glucan complex

GM-CSF = Granulocyte macrophage colony stimulating factor

HUVEC = Human umbilical vein endothelial cells

ICAM = Intercellular adhesion molecule

IFN-γ = Interferon gamma

IL- = Interleukin-

KD = Kawasaki disease

KO = Knockout mouse

LPS = Lipopolysaccharide

MDP = Muramyldipeptide

MHC = Major histocompatibility complex

MIP = Macrophage inflammatory protein

MPO = Mycloperoxidase

MyD88 = Myeloid differentiation factor 88

PAF = Platelet activating factor

PBMC = Peripheral blood mononuclear cells

TLR = Toll like receptor

TNF-α = Tumor necrosis factor α

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The influence of culture conditions on vasculitis and anaphylactoid shock induced by fungal pathogen *Candida albicans* cell wall extract in mice

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Abstract

To explore whether *Candida* cell wall mannan is responsible for induction of vasculitis similar to Kawasaki syndrome and anaphylactoid shock in mice, we examined the biological effects of various mannan structures from *Candida* cell wall extracts prepared using various culture conditions. Intraperitoneal injection of 3 of 4 *Candida* cell wall extracts dramatically induced coronary arteritis and acute anaphylactoid shock in mice; only the cell wall extract derived from YPD medium culture at 27 °C had no toxic effect. It is of note that these biological effects depended on culture conditions around the cells such as culture temperature and media. These conditions lead to the structural rearrangement of cell wall mannan as confirmed by reactivity against antisera and NMR spectroscopy. Since the expression of β-1,2-linked mannan varies dramatically between biologically active and inactive mannan, β-1,2-linked mannan might negatively affect *Candida* cell wall extract-induced coronary arteritis and acute anaphylactoid shock in mice. Our findings indicate that *Candida* cell wall mannan might contribute to coronary arteritis and acute shock, and that an alteration of mannan structure could be responsible for *Candida* pathogenicity.

Keywords: Anaphylactoid shock; *Candida albicans*; Culture conditions; Mannan; Vasculitis

1. Introduction

Kawasaki syndrome (KS) is a systemic childhood vasculitis that can result in aneurysms of the coronary arteries [1,2]. The diagnosis of KS is based entirely on clinical features. For classic KS, individuals must have a fever for more than 5 days and either meet at least 4 of 5 criteria—(1) bilateral conjunctivitis, (2) erythema of the mouth or pharynx, strawberry tongue, or stomatitis, (3) polymorphous rash, (4) erythema or edema of the hands or feet, and (5) nonsuppurative cervical lymphadenopathy—or meet at least 3 of these criteria and have evidence of coronary artery abnormalities. Incomplete or atypical KS, in which these criteria are not fully met, can also occur and result in aneurysms of the coronary arteries. Laboratory findings are nonspecific, and there are no diagnostic tests for KS.

The etiology of KS remains mostly unknown despite numerous efforts. However, many recent studies reported that KS might be triggered by a response to an infectious agent, e.g. fungi, bacteria, virus, etc. [3–5]. Moreover, invasive *Candida* infection in neonates can result in mycetoma of the right atrium and cause Candidal endocarditis [6]. Pathogenic fungi including *Candida albicans* can also induce septic shock. *Candida*-induced septic shock is as much 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 most common microbes causing bloodstream infections in immunocompromised or intensive-care patients [7]. Invasive mycoses including candidiasis are increasingly becoming associated with the use of immunosuppressive

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therapies and immunodeficiency associated with human immunodeficiency virus infection [8].

Murata et al. [9] reported that an extract of *C. albicans* isolated from a KS patient induced coronary arteritis in mice when repeatedly injected intraperitoneally. The arteritis was granulomatous with no fibrinoid changes and was similar to that found in KS patients [10]. We have also reported that *C. albicans* water-soluble fraction (CAWS), a water-soluble polysaccharide fraction secreted into culture media by *C. albicans* was composed of a mannoprotein-β-glucan complex [11] and strongly caused vasculitis similar to KS [12,13], leading to production of cytokines by leukocytes, platelet aggregation [14], and acute anaphylactoid shock in mice [15]. In addition, we recently found a relationship between *C. albicans* culture conditions and induction of several biological effects of CAWS including acute anaphylactoid shock, coronary arteritis, and complement activation [16]. Specifically, culture conditions resulting in a β-1,2-mannosyl linkage within the mannan moiety of CAWS significantly reduced the biological effects described above.

Analysis of the mechanism by which carbohydrate components derived from *C. albicans* could induce vasculitis similar to KS and anaphylactoid shock in mice could indicate the etiology of *Candida* infection. *Candida* cell wall carbohydrates, especially mannan, that are located in the outermost cell layers are well known to contribute to antigenicity and pathogenicity [17–19]. β-1,2-Linked mannooligosaccharides are now thought to be a particularly important component for the pathogenicity of *C. albicans*; they are likely responsible for tumor necrosis factor-α (TNF-α) secretion from various cells [20,21], as well as the adherence of *Candida* cells to macrophages [22,23] and epithelial cells [24]. Since numerous studies have reported that *Candida* cell wall mannan is altered by various culture conditions such as growth temperature [25], pH [26], and osmotic pressure, we postulated that these structural rearrangements could be crucial for the biological effects of *Candida* cell wall mannan. Thus, further examination of the molecular structure of cell wall mannan is important for understanding the mechanisms underlying its biological effects. These studies will be useful for establishing effective therapeutic strategies for these diseases.

In the present study, we examined whether *Candida* cell wall mannan is responsible for induction of vasculitis similar to KS and anaphylactoid shock in mice. Furthermore, we verified our speculation that the expression of alternate β-mannosyl residues in the *Candida* cell wall after various environmental changes could reduce the induction of vasculitis and anaphylactoid shock in mice. We obtained cell wall extracts from *C. albicans* with or without β-mannosyl residues by altering the culture conditions, and we then compared their biological activity.

2. Results

2.1. Chemical compositions of cell wall extracts

The chemical compositions of cell wall extracts from *C. albicans* NBRC 1385 are summarized in Table 1. All extracts were mainly composed of carbohydrates (41.7–56.9%) and proteins (16.7–33.6%). The monosaccharide content of the cell wall extracts was determined by GLC analysis. Mannose and glucose were both detected. The extracts from cells cultured in natural media (HWE-Y27; 22.0:1.00, HWE-Y37; 11.2:1.00) showed higher counts than extracts from completely synthetic media (HWE-C27; 0.556:1.00, HWE-C37; 0.828:1.00). These analyses reveal that the cell wall extracts contained the mannoprotein–glucan complex, yet no endotoxin contamination (maximum 12.4 ng/mg) was detected.

2.2. Coronary arteritis induced by Candida cell wall extracts derived from various culture conditions

We first examined the coronary arteritis induction activity of *Candida* cell wall extracts and then studied the influence of culture conditions. Fig. 1 shows hematoxylin–eosin (HE) staining of the aorta in hot water cell wall extract (HWE)-administered DBA/2 mice. As revealed by histological examination, intraperitoneal injection of HWE-induced coronary arteritis in DBA/2 mice. Interestingly, injection

Table 1
Chemical composition of cell wall extracts from *Candida albicans*

Culture media	Culture temperature (°C)	Extraction method	Yield (%)	Total carbohydrate (%)	Total protein (%)	Elemental analysis (C:H:N)	Sugar composition (Man:Glc)	Endotoxin content (ng/mg)
C-limiting medium	27	Hot water	21.0	50.3	20.7	40.8:7.09:4.20	0.556:1.00	N.D.
		Alkaline	14.6	45.9	16.7	41.0:7.04:5.41	2.52:1.00	N.D.
	37	Hot water	24.2	54.4	30.7	43.1:6.96:4.91	0.828:1.00	5.20
		Alkaline	14.2	41.7	17.1	42.9:6.76:4.90	2.17:1.00	3.00
YPD medium	27	Hot water	19.6	53.6	28.2	42.7:6.99:5.72	22.0:1.00	N.D.
		Alkaline	24.0	48.7	20.1	43.8:7.08:6.55	8.94:1.00	N.D.
	37	Hot water	26.9	43.3	33.6	43.9:6.90:8.39	11.2:1.00	12.4
		Alkaline	19.2	56.9	22.3	42.8:6.86:4.38	8.05:1.00	N.D.

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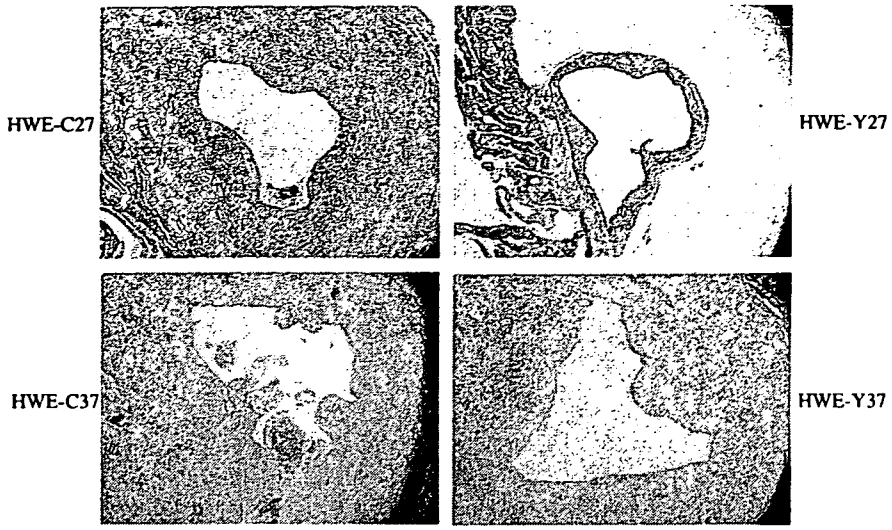

Fig. 1. Histological examination of hot water extract-induced coronary arteritis in mice. HWE-C27, HWE-C37, HWE-Y27, and HWE-Y37 (4 mg/mouse) were administered i.p. to DBA/2 mice for 5 consecutive days in the 1st week. Five weeks later, the hearts of DBA/2 mice were fixed in buffered formalin solution, embedded in paraffin, thin-sectioned, and stained with hematoxylin–eosin.

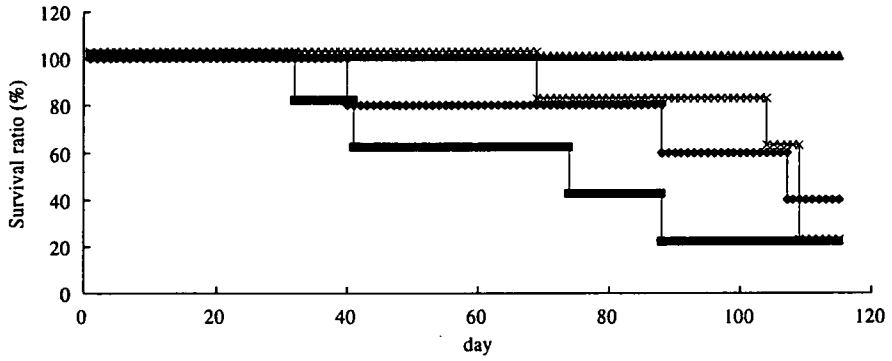

Fig. 2. Survival time of hot water extract-administered mice. HWE-C27 (♦), HWE-C37 (■), HWE-Y27 (▲), and HWE-Y37 (×) (4 mg/mouse) were administered i.p. to DBA/2 mice for 5 consecutive days in the 1st week. Survival was observed for 18 weeks.

of HWE derived from C-limiting medium at 27 °C (HWE-C27), HWE derived from C-limiting medium at 37 °C (HWE-C37), and HWE derived from YPD medium at 37 °C (HWE-Y37) resulted in severe induction of coronary arteritis, whereas HWE derived from YPD medium at 27 °C (HWE-Y27) did not have any effect. Coronary arteritis was also examined in terms of the survival rate. As shown in Fig. 2, all of the mice administered with HWE gradually died except for those treated with HWE-Y27. These effects mirrored those observed for the induction of coronary arteritis by alkaline cell wall extracts in mice (data not shown). These studies show that the environmental conditions around *Candida* cells control the ability of *Candida* cell wall extracts to induce of coronary arteritis.

2.3. *Rapid anaphylactoid shock induced by Candida cell wall extracts derived from various culture conditions*

We next examined another typical biological effect exhibited by *Candida* cell wall mannan and found that HWE-C27, HWE-C37, and HWE-Y37 administration resulted in acute anaphylactoid shock in ICR mice, whereas HWE-Y27 showed less of an effect (Table 2). These effects matched those observed for induction of acute anaphylactoid shock by alkaline cell wall extracts in mice (data not shown). These results indicate that environmental conditions are also important for the induction of rapid anaphylactoid shock.

2.4. *The reactivity of cell wall extracts to Candida serum factors*

To understand the different biological effects of cell wall extracts derived from various culture conditions, we next examined the structural differences between their mannan residues. Table 3 shows the reactivity of cell wall extracts to *Candida* serum factors, which consist of rabbit polyclonal antibodies against *Candida* cell wall mannan. The reactivities of sera nos. 4, 5, and 6 against cell wall extracts derived from YPD medium were significantly higher than

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Table 2
Ability of crude HWE to induce rapid anaphylactoid shock in ICR mice

Dose (mg/kg)	Incidence	Anaphylactoid shock score[a]	Mortality
HWE-C27			
0	0/4	0	0/4
4	2/4	2	0/4
8	3/4	4	2/4
16	4/4	4	3/4
HWE-C37			
0	0/4	0	0/4
4	3/4	3–4	0/4
8	3/4	4	2/4
16	4/4	4	3/4
HWE-Y27			
0	0/4	0	0/4
4	0/4	0	0/4
8	1/4	1	0/4
16	2/4	1–4	1/4
HWE-Y37			
0	0/4	0	0/4
4	3/4	2–3	0/4
8	3/4	3–4	1/4
16	4/4	4	4/4

Indicated dose (mg/kg) was i.v. administered to mice ($n = 4$). Mortality was monitored within 1 h. */*, number of mice, dead/total.

[a]The scoring of the shock was as follows: 0, no symptoms of shock; 1, staggering; 2, crawling and prostration; 3, prostration and weak convulsions; 4, prostration and strong convulsions.

Table 3
The reactivity of cell wall extract to *Candida* serum factors

Culture media	Culture temperature	Extraction method	Serum factors		
			4	5	6
C-limiting	27	Hot water	1.620	0.403	0.757
Medium	37	Hot water	0.610	0.157	0.159
YPD	27	Hot water	2.522	2.356	2.171
Medium	37	Hot water	1.560	0.614	1.204

Each value was expressed by optical density at 450 nm.

reactivities against extracts from C-limiting medium. Furthermore, culturing at 37 °C reduced the reactivity against nos. 5 and 6 when compared to 27 °C. These results mirrored those observed for the reactivity of alkaline cell wall extracts (data not shown). It is therefore likely that the specific epitopes of no. 5 (linear β-1,2-linked mannooligosaccharides) and no. 6 (non-reducing end β-1,2-mannosides linked α-mannooligosaccharides) (Table 4) were contained in cell wall extracts from *C. albicans* that did not generate acute anaphylactoid shock or coronary arteritis.

2.5. NMR analyses of cell wall extracts

For further structural characterization, we next analyzed samples using NMR spectroscopy. Fig. 3 shows the 1D-^{1}H nuclear magnetic resonance (NMR) spectra of cell wall

Table 4
Chemical structure of antigenic factors, 4, 5, and 6

4	Manα1 ↓6 Manα1-2Manα1-3Manα1-2Manα1-2Manα1-2Man
5	Manβ1-2Manβ1-2Manβ1-2Manβ1-2Manβ1-2Manβ1-2Man
6	Manβ1-2Manβ1-2Manβ1-2Manα1-2Manα1-2Manα1-2Man

Chemical structure of antigenic factors used in study. Man; mannose.

extracts. Each spectrum contained too many signals in the anomeric region (4.4–5.8 ppm). Thus, we could not completely assign the signals using this technique. However, it is apparent that the carbohydrates of cell wall extracts from *C. albicans* were altered depending upon both temperature and medium. In addition, we further examined samples using ^{1}H, ^{13}C-hetero nuclear single quantum coherence (HSQC) spectra to detect any structural differences between biologically active structures. Fig. 4 shows overlaid HSQC (black) spectra of HWE-C27, (green) HWE-C37, (red) HWE-Y27, and (blue) HWE-Y37. The overlaid HSQC spectra show 34 signals in their anomeric region (δ_H 4.4–5.8 ppm, δ_C 94–105 ppm) that were arbitrarily labeled nos. 1–34 as described in Table 5. On the basis of their observed chemical shifts and $^3J_{H1,H2}$, nos. 1–30 were assigned to mannose residues; in addition, nos. 31–34 were assigned to glucose residues. 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. From the observed $^1J_{H1,C1}$ obtained from ^{1}H, ^{13}C-HSQC spectra without decoupling during acquisition, 19 of 31 mannose residues were assigned to α-mannose, 11 of 31 mannose residues were assigned to β-mannose, and all glucose residues were assigned to β-glucose (Table 5). We next examined samples using 2D-total correlation spectroscopy (TOCSY) spectra to determine the linkage types of each residue by the method of Shibata et al. [27]. The findings are described in Table 5. It is of note that HWE-Y27, which did not exhibit any biological effects, had more signals than the other samples; 14 signals were exclusively present in HWE-Y27. Interestingly, all 14 signals were related to β-mannosyl residues within both acid-labile and acid-stable regions. No other differences were identified.

2.6. Effect of mild acid hydrolysis on anaphylactoid shock

To examine the involvement of β-1,2-linked mannooligosaccharides on anaphylactoid shock in mice, we evaluated the effect of mild acid hydrolysis, which cleaves the phosphodiester linkage of the acid-labile region [28]. The results showed that the acid-labile region was eliminated by mild acid hydrolysis; this effect was confirmed by the disappearance of the 5.55 ppm signal in the 1D-^{1}H NMR spectrum. However, we were not able to observe any modification of biological effect (Table 6).

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