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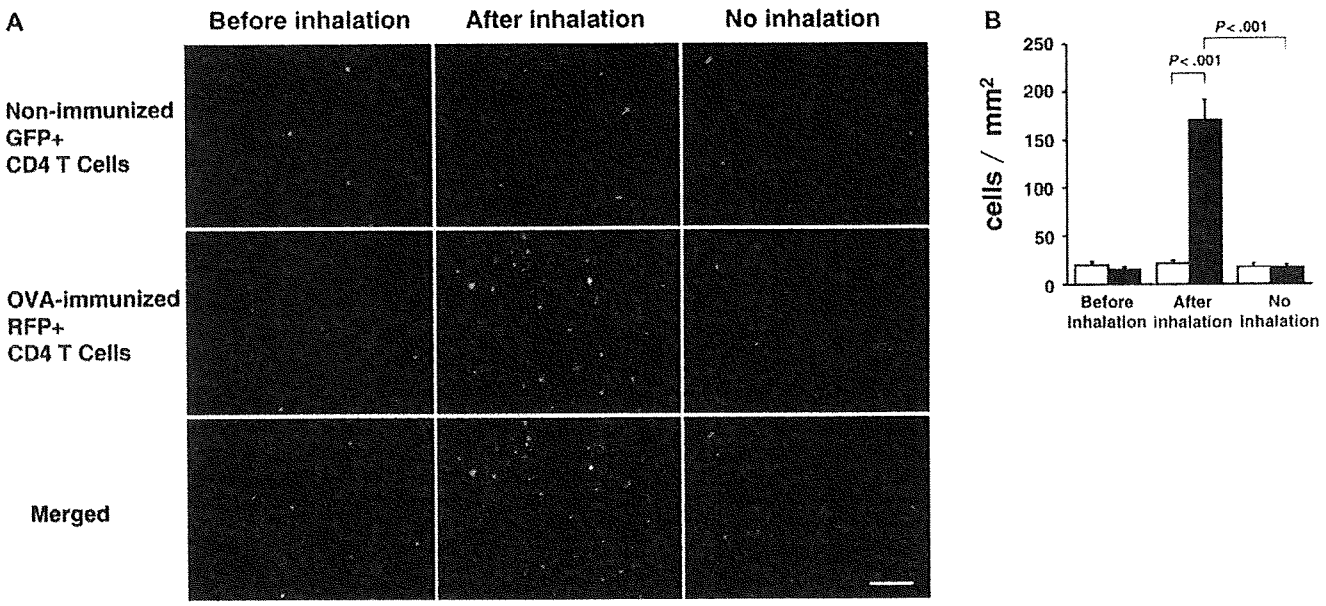


**FIG E1.** Color -coded fluorescence imaging of CD4 T-cell infiltration into the lung during ovalbumin (OVA)-induced allergic asthma. **A,** Color-coded images of CD4 T cells with the opposite color to the experiment shown in Fig 1, *B.* Splenic CD4 T cells from OVA-primed RFP Tg and nonprimed GFP Tg mice were injected into normal recipient mice. $GFP^+$ and $RFP^+$ CD4 T cells were monitored in the excised lung as in Fig 1, *B. Bar,* 100 μm. **B,** Summary of the accumulation of fluorescent cells of *A.* Data are from 15 fields from 3 mice with SD. *Open bar,* GFP; *closed bar,* RFP. $P < .001$ by the Student's *t* test.

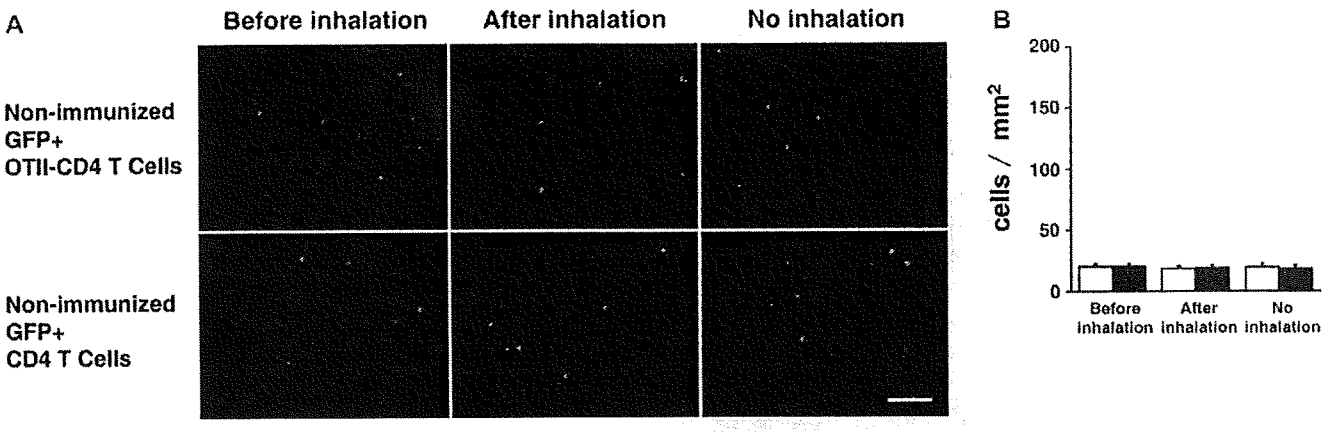


**FIG E2.** Fluorescence imaging of naive OT II–CD4 T and CD4 T cells in the lung. **A,** Images of naive OT II–CD4 T and CD4 T cells. Unprimed $GFP^+$ OT II–CD4 Tand CD4 T cells were monitored in the lung before ovalbumin inhalation and 24 hours after ovalbumin inhalation by using the OV100 Small Animal Mouse Imaging System. *Bar,* 150 μm. **B,** Summary of the accumulation of fluorescent cells of *A.* Data are from 15 fields from 3 mice with SD. *Open bar,* OT II–CD4 T cells; *closed bar,* CD4 T cells.

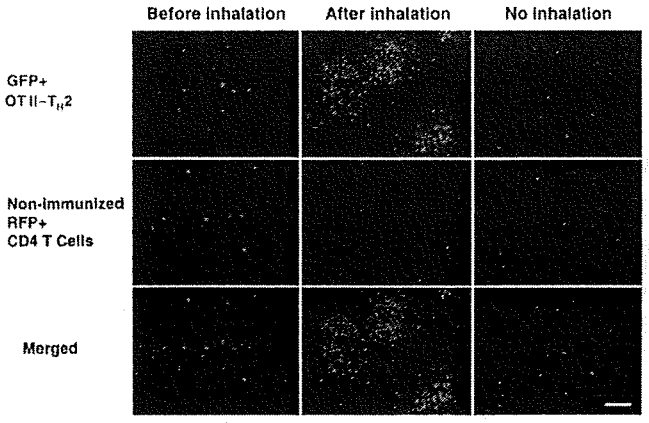


**FIG E3.** Color -coded fluorescence imaging of OT II-$T_H2$ cells and CD4 T cells in the lung. Primed $GFP^+$ OT II-$T_H2$ cells and $RFP^+$ CD4 T cells from nonprimed RFP Tg mice were monitored before ovalbumin inhalation and 24 hours after ovalbumin inhalation by using the OV100 Small Animal Mouse Imaging System. *Bar,* 150 μm. The results are representative of 3 experiments.

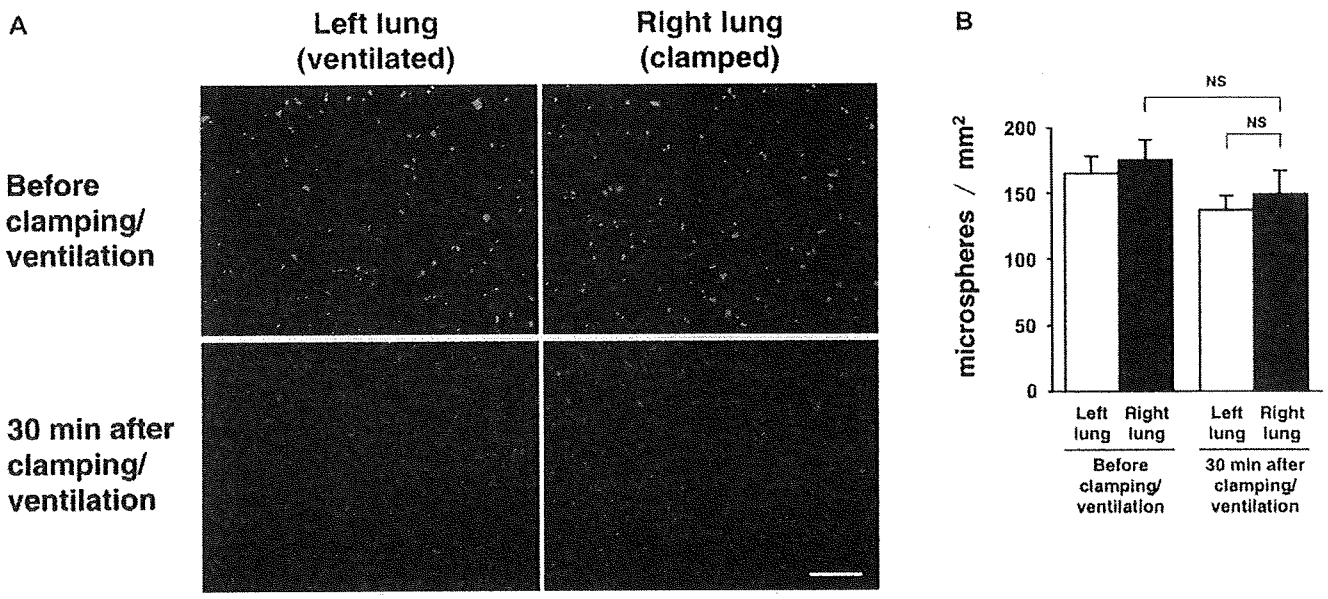


**FIG E4.** Measurement of blood flow in the clamped right lung and the ventilated left lung. **A,** Assessment of blood flow using fluorescent microspheres. Yellow-green fluorescent microspheres (10 μm, Invitrogen) were infused before ventilation and red fluorescent microspheres (10 μm, Invitrogen) infused 30 minutes after ventilation. The excised lungs were assessed by using the OV100 Small Animal Mouse Imaging System. *Bar,* 150 μm. **B,** Summary of the number of fluorescent microspheres. Data are from 9 fields from 3 mice with SD. *Open bar,* left lung; *closed bar,* right lung *NS,* not significant.

**VIDEO E1-E6.** Dynamic real time cellular imaging of ovalbumin-specific $GFP^+$ OT II-$T_H2$ cell migration into the lung after ovalbumin inhalation in living mice. Ovalbumin-specific $GFP^+$ $T_H2$ cell migration and accumulation in the lung before ovalbumin inhalation (Videos E1 and E2 [high magnification]) and after ovalbumin inhalation: 6 hours (Video E3), 12 hours (Videos E4 and E5 [high magnification]), and 21 hours (Video E6). Individual cells were imaged every 5 seconds. Videos are at ×225 real-time.

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ORIGINAL ARTICLE

# Administration of human immunoglobulin suppresses development of murine systemic vasculitis induced with *Candida albicans* water-soluble fraction: an animal model of Kawasaki disease

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**Abstract** We investigated the inhibitory effect of human immunoglobulin (h-Ig) on the development of coronary arteritis in a murine model of vasculitis induced with a *Candida albicans* water-soluble fraction (CAWS). CAWS was intraperitoneally injected to C57BL/6 mice for 5 days. Then h-Ig was administered according to various schedules. The animals were sacrificed in week 5, and the status of vasculitis in the coronary arteries and the aortic root was investigated histologically. The groups in which h-Ig was administered for 5 days from day 3 and from day 5 of the experiment showed a significant reduction in the incidence of panvasculitis. In addition, the scope and severity of the inflammation of the aortic root and the coronary arteries were reduced in both groups. In the group administered h-Ig for 5 days from day 1 and the group administered a high dose of h-Ig once on day 1 or day 3, no suppression of development of vasculitis was observed. The h-Ig acted by suppressing the generation and progression of vasculitis in this CAWS-induced murine vasculitis model.

**Keywords** *Candida albicans* · Immunoglobulin · Kawasaki disease · Mouse · Systemic vasculitis

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## Introduction

Kawasaki disease (KD) is an acute febrile illness that manifests mainly in infancy and early childhood [1]. The most important complication of KD is coronary arteritis, which leads to formation of aneurysms. KD has attracted special interest because it is most frequently attributed to ischemic heart disease caused in children by thrombosed coronary aneurysms [2]. Although many studies have been performed, the etiology of KD is not well understood. Today, immunoglobulin (Ig) is administered to 85% of children with acute-stage KD in order to prevent coronary aneurysms in Japan [3]. Our pathological study showed that coronary arteritis began 6–8 days after the onset of KD and immediately progressed to inflammation of all layers of the artery (panvasculitis) by the 10th day of disease [4]. These findings suggest that treatment of acute-stage KD patients should be completed by the 10th day of disease.

We established an interesting systemic vasculitis mouse model in which coronary arteritis was induced by injection of a *Candida albicans* (*C. albicans*) alkaline extract (CADS) [5, 6]. This is a useful animal model of KD, since it exhibits many histological similarities to KD [6, 7]. On the other hand, Ohno et al. reported unique biologic characteristics for substances obtained from the *C. albicans* water-soluble fraction (CAWS) [8–10]. Studies in mice

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demonstrated that CAWS induced vasculitis at a clearly higher incidence than CADS, and that the vasculitis was histologically similar to that induced by CADS [11, 12].

The objective of this study was to clarify, histologically in the murine vasculitis-induced model, whether administration of Ig at the time of generation and progression of inflammation, which is the same as the therapeutic strategy for KD, was able to suppress that inflammation.

## Materials and methods

### Animals

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

### Organisms

*C. 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 3 months.

### Preparation of *C. albicans* water-soluble fraction (CAWS)

CAWS was prepared from *C. albicans* strain IFO1385 in accordance with the reported method [10]. Briefly, 5 l medium (C-limiting medium) was added to a glass incubator, and culture was conducted for 2 days at 27°C while supplying air at a rate of 5 l/min and stirring at 400 rpm. Following culture, an equal volume of ethanol was added. After allowing this to stand overnight, the precipitate was collected. After dissolving the precipitate in 250 ml distilled water, ethanol was added and the mixture was allowed to stand overnight. The precipitate was collected and dried with acetone to obtain CAWS.

### Preparation of h-Ig

h-Ig derived from donated blood from healthy humans was kindly provided by Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan).

### Induction of vasculitis and administration of h-Ig

CAWS (0.4 mg/mouse) was injected intraperitoneally to the mice in a volume of 0.2 ml on each of five consecutive days in week 1. At 6 h after the CAWS administration, a syringe pump (Harvard, model 11) was used to administer h-Ig dissolved in saline into the tail vein of the mice over a period of 8 min. The h-Ig dosage and number of doses are shown below for each of the treatment groups.

Group 1 Control (no h-Ig).
Group 2A h-Ig (400 mg/kg) was administered for 5 days beginning from day 1 of the experiment.
Group 2B h-Ig (400 mg/kg) was administered for 5 days beginning from day 3 of the experiment.
Group 2C h-Ig (400 mg/kg) was administered for 5 days beginning from day 5 of the experiment.
Group 3A High-dose h-Ig (2000 mg/kg) was administered once, on day 1 of the experiment.
Group 3B High-dose h-Ig (2000 mg/kg) was administered once, on day 3 of the experiment.

In week 5, the mice were killed by carbon dioxide asphyxiation. Autopsy was performed, and the hearts were fixed in 10% neutral formalin.

### Histological evaluation

The fixed hearts were embedded in paraffin and sectioned. In order to observe the histological changes in the coronary arteries and the aorta in detail, step sections in the horizontal direction were made every 20 μm. Hematoxylin and eosin (H&E)-stained sections were prepared using routine techniques for examination by light microscopy [7].

First, we investigated the incidence of mice with panvasculitis in each group. Panvasculitis was defined as inflammation of all layers of the walls of the coronary arteries and/or the aorta.

Then, for quantitative evaluation of vascular inflammation, we divided the area of the aortic root and coronary arteries into five segments and graded the intensity of inflammation in each segment as follows: score 3, panvasculitis; score 2, inflammation involving the tunica intima and adventitia; score 1, inflammation localized to the tunica intima; and score 0, no inflammatory cell infiltration in the vascular wall (Fig. 1). The severity of the arteritis in each mouse was defined as the sum of the scores for the five segments.

### Peripheral WBC count

Blood samples were collected at the time the animals were sacrificed, and the white blood cell (WBC) counts were determined. The counts were compared among the various treatment groups.

### Statistical analysis

Fisher's exact probability test was used to analyze the differences in the incidence of arteritis among the groups.

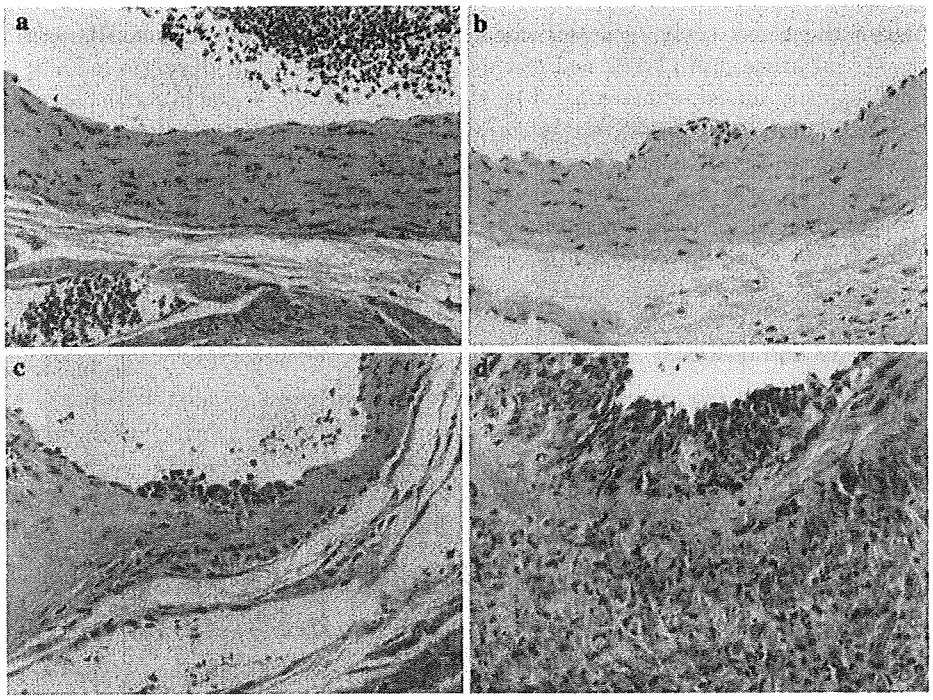



**Fig. 1** Grading applied for vascular lesions. **a** Score 0: normal artery. **b** Score 1: inflammatory cells attachment in the intima. **c** Score 2: inflammation localized in the intima and adventitia. **d** Score 3: panvasculitis. (H&E stain, original magnifications, 400×)

The data on the severity of the arteritis and the peripheral WBC count were analyzed using the two-sample $t$-test. A value of $p < 0.05$ was considered statistically significant.

## Results

### Incidence of mice with panvasculitis

Panvasculitis of the coronary arteries and the aortic root was observed in 32 of the 37 mice (86.5%) in group 1. Group 2B and group 2C showed panvasculitis in 8/20 mice (40%) and 5/10 mice (50%), respectively. The incidences of panvasculitis in these two groups were significantly reduced compared with group 1.

On the other hand, the incidence of panvasculitis was not significantly reduced in group 2A, group 3A or group 3B in comparison with group 1. The incidences of panvasculitis in those three groups were 7/10 mice (70%), 8/10 mice (80%), and 7/10 mice (70%), respectively (Fig. 2).

### Scope and severity of inflammation

The distribution and score of the inflammation in each segment in each group are shown in Table 1. The number of segments evaluated as score 1 or more in each of group 2B and group 2C was significantly decreased compared with group 1. Furthermore, the severity of the arteritis, i.e., the sum of the scores for the five segments in the mice, in group 2B and group 2C was significantly lower than in group 1. These results suggested that the area

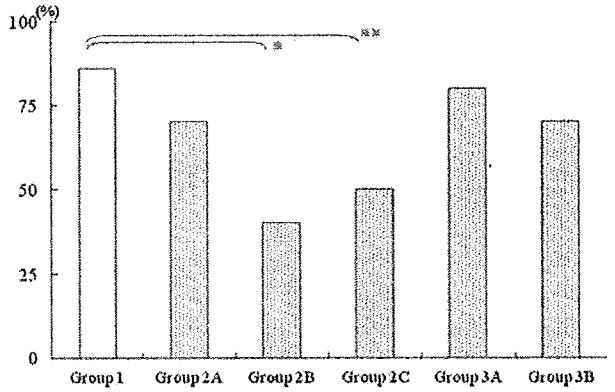


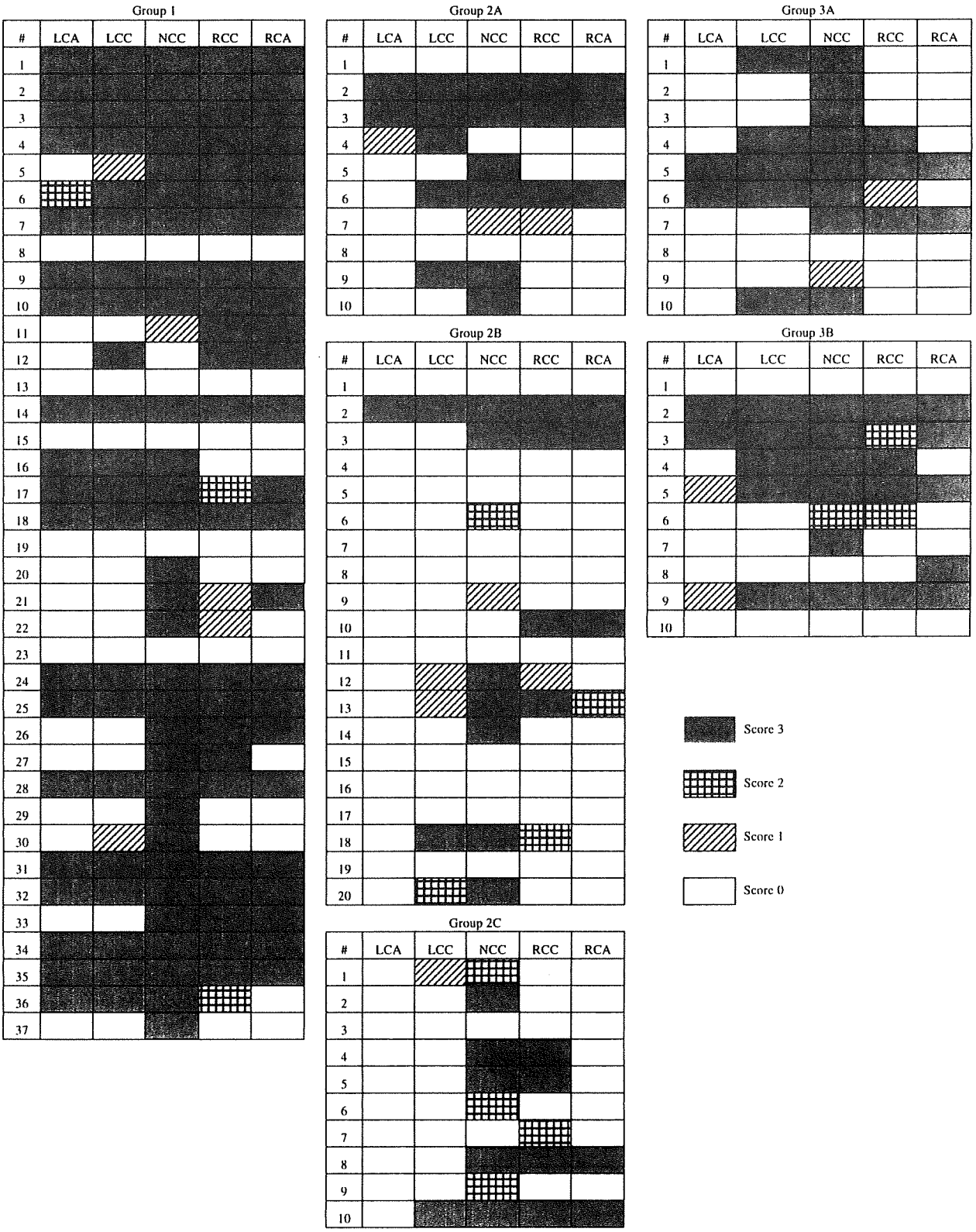

**Fig. 2** Incidence of development of panvasculitis. Incidence of development of panvasculitis is significantly decreased in group 2B and group 2C compared with the control group (group 1). $*p < 0.01$, $**p < 0.05$

and the degree of inflammation were reduced in these two groups. However, there were no significant differences in the area and the degree of inflammation among group 2A, group 3A, group 3B, and group 1 (Table 2).

### Histology of panvasculitis

Panvasculitis had developed in the coronary arteries and the aortic root, and histologically the changes were classified as granulomatous proliferative inflammation. The normal structure of the arteries was completely destroyed, and the internal elastic lamina, external elastic lamina, and the smooth muscle layer of the tunica media were severely

**Table 1** Distribution and severity of inflammation at aortic root and coronary arteries in each group




*LCA* left coronary artery, *RCA* right coronary artery, *LCC* left coronary cusp, *NCC* noncoronary cusp, *RCC* right coronary cusp

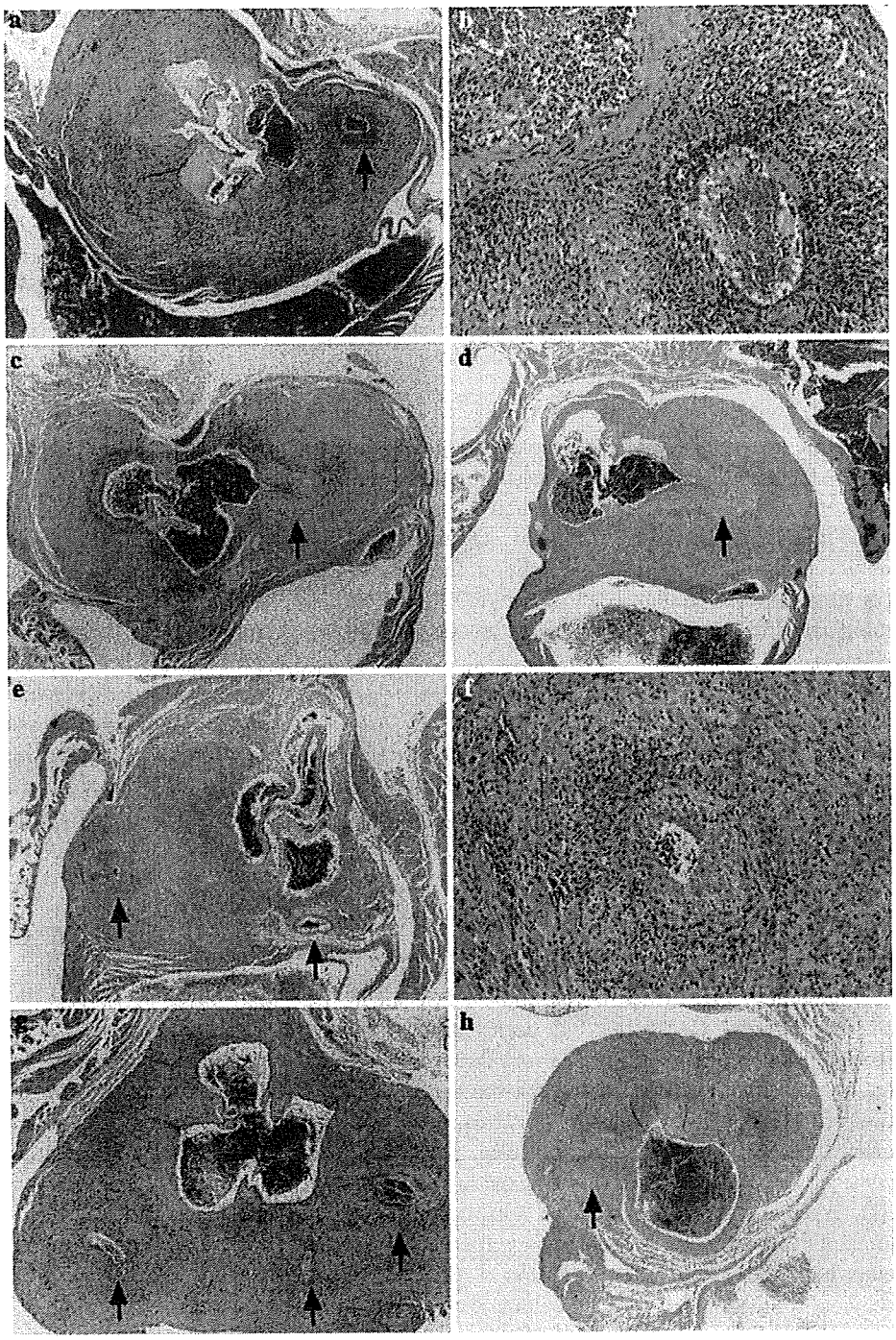



**Fig. 3** Comparison of histology of vascular lesions in the aortic root and coronary arteries. Inflammatory cells infiltrated the aortic wall and coronary arteries (*arrows*), but no apparent histological differences were noted between the experimental groups. **a** Aortic root and **b** coronary artery in a mouse from group 1, the control. **c** Group 2A: administered h-Ig for 5 days from the 1st experimental day. **d** Group 2B: administered h-Ig for 5 days from the 3rd experimental day. **e** Aortic root and **f** coronary artery in a mouse of group 2C: administered h-Ig for 5 days from the 5th experimental day. **f** Group 3A and **g** group 3B: administered high-dose h-Ig on day 1 and day 3 of the experiment (H&E stain; original magnifications, **a**, **c**, **d**, **e**, **g**, and **h**: 40×; **b** and **f**: 200×)

that is localized in the tunica intima. This change is seen in the arteries of mice 1–3 days after the administration of CAWS, corresponding to the 6th to 8th experimental day. Next, the inflammatory cell infiltration progresses to include the adventitia by the 10th experimental day. Finally, the inflammation in the tunica intima and the adventitia spreads to include the tunica media, resulting in full development of panvasculitis by the 12th experimental day. Severe and extensive inflammation is observed in the arteries of mice on the 14th and 28th experimental days (papers in preparation). On the other hand, histological examination shows that the coronary arteritis in KD starts 6–8 days after the onset of disease. Panvasculitis is formed by the 10th day of disease [4]. Therefore, it is necessary to complete the h-Ig treatment by the 10th day after the onset of KD to prevent development of coronary aneurysms. In

**Table 2** Scope and severity of inflammation at aortic root and coronary arteries

| | Scope[a] | | Severity[b] | |
|---|---|---|---|---|
| | Segments/mouse (mean ± SD) | *p* | Score/mouse (mean ± SD) | *p* |
| Group 1 | 3.38 ± 1.90 | | 9.78 ± 5.75 | |
| Group 2A | 2.20 ± 1.90 | ns | 6.00 ± 5.80 | ns |
| Group 2B | 1.25 ± 1.60 | <0.001 | 3.15 ± 4.30 | <0.001 |
| Group 2C | 1.70 ± 1.20 | <0.01 | 4.70 ± 3.70 | <0.05 |
| Group 3A | 2.20 ± 1.50 | ns | 6.20 ± 4.60 | ns |
| Group 3B | 2.70 ± 2.20 | ns | 7.40 ± 6.00 | ns |

[a] Number of segments with inflammation evaluated as score 1 or more per mouse

[b] Sum of the score of five segments per mouse

damaged. The cells in the lesions consisted mainly of large mononuclear cells, such as histiocytes, fibroblasts, etc., and neutrophils. Scattered lymphocytes and plasma cells were also observed. However, fibrinoid necrosis was not observed in any of the mice. In addition, the histology of panvasculitis was similar in all of the groups, and no histological differences were detected among the groups (Fig. 3).

Peripheral WBC count

The WBC count in blood samples collected from animals at the time of sacrifice was not significantly increased in any of the groups compared with group 1 (Table 3).

## Discussion

We previously established a vasculitis mouse model by administration of CADS that had been isolated from the feces of a patient with KD [5]. We used that model to carry out various studies [6, 7, 13] and showed that administration of CADS induced systemic vasculitis, including coronary arteritis, in about 70% of mice. This model is considered to be highly useful as an experimental model of KD, especially in view of the fact that it induces granulomatous inflammation of the coronary arteries [7]. On the other hand, Kurihara and Uchiyama et al. purified soluble cellular components from the culture supernatant of a standard strain of *C. albicans* (CAWS) and reported on various biological activities shown by CAWS [8–10]. Both CADS and CAWS contain mannoprotein–$\beta$-glucan complexes derived from the cellular components of *C. albicans*. We carried out vasculitis-induction experiments using CAWS, and the results were surprising. In all of the experimental mice, CAWS caused panvasculitis that was histologically similar to panvasculitis caused by CADS, and CAWS showed extremely potent vasculitis-inducing activity [11]. In this manner, a murine model of vasculitis induced by CAWS was developed, and this model has made it possible to induce vasculitis more efficiently than with prior experimental models and allows various experiments to be easily carried out. Okawara et al. [14] recently reported that CAWS injection caused neutrophil activation, followed by complement activation and production of proinflammatory cytokines, chemokines, and granulocyte colony-stimulating factor (G-CSF), which may be involved in the development of coronary arteritis.

h-Ig is currently in wide use as a high-dose intravenous therapy for acute-stage KD, and its efficacy has been established [15, 16]. In addition, in recent years intravenous administration of h-Ig has been tested in the treatment of other necrotizing vasculitic disorders [17–19]. The efficacy of h-Ig in the treatment of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis has gained particular attention [20, 21]. It is also known that the myeloperoxidase (MPO)–ANCA titer increases in some acute-stage KD patients [22]. As noted earlier, the present murine model of vasculitis shows many histological points of similarity with KD. Moreover, in vasculitis-inducing experiments using CADS, the serum MPO–ANCA titer was found to be significantly elevated in mice that had developed vasculitis. In addition, it was elucidated that the incidence of vasculitis is reduced in MPO-deficient mice, and it was surmised that MPO-ANCA is involved in the development of vasculitis in this murine model [23].

On the basis of that background, we investigated the effect of h-Ig on CAWS-induced vasculitis in mice. The results in each experimental group were rated as showing suppression or no suppression. The results indicate that there is an optimum timing for Ig administration to inhibit the development of vasculitis because the total dosage of h-Ig was the same in all groups.

In our experimental model, the initial step in the vascular changes consists of inflammatory cell infiltration

**Table 3** Peripheral blood WBC counts

| | WBC count ($\times 10^2$cells/μl) | |
|---|---|---|
| | Mean ± SD | *p* |
| Group 1 | 79 ± 27 | |
| Group 2A | 112 ± 25 | ns |
| Group 2B | 94 ± 19 | ns |
| Group 2C | 82 ± 19 | ns |
| Group 3A | 87 ± 19 | ns |
| Group 3B | 101 ± 35 | ns |

accordance with the treatment strategy for acute stage of KD, h-Ig was administered between the 6th and 10th days of the experimental period in this study, corresponding to the period of development from endarteritis to panvasculitis. As a result, the incidence of arteritis, the scope and severity of inflammation in arteries, were suppressed in groups 2B and 2C. Thus, we interpret these results as indicating that, in the present murine experimental system, h-Ig acted mainly by interfering with the development of inflammation and progression to panvasculitis.

There was a tendency for the arteritis to be suppressed in group 2A, although the difference from group 1 was not statistically significant. The half-life of h-Ig is assumed to be about 7 days. It is likely that Ig was not effective in fully suppressing the generation and progression of arteritis in group 2A because the timing of h-Ig administration was too early and the h-Ig was metabolized. Concerning groups 3A and 3B, in which the incidence of panvasculitis was not significantly reduced, we speculated that the Ig administration had been too early to inhibit the inflammation, that the inflammation recurred in spite of the fact that it had been inhibited once, or that the CAWS (mannoprotein–β-glucan complexes) had not been degraded immediately and was taken up by the reticuloendothelial system and caused persistent inflammatory stimulation.

The mechanism by which high-dose h-Ig improves the vasculitis of KD is unknown. The possibilities include blockade of immunological activation or the inflammatory response directed at vascular walls, saturation of Fc receptors on the endothelial cells, and provision of a specific antibody that counteracts an as-yet unidentified causal agent of KD [24]. On the other hand, it has been shown that MPO possesses an epitope that is closely related to the severity of vasculitis in MPO-ANCA-positive patients; that is, it has been elucidated that the MPO-ANCA that is elevated in the serum of MPO-ANCA-positive vasculitis patients is a monoclonal antibody that shows highly specific binding to the risk epitope of MPO [25–27]. In this connection, it is noted that the h-Ig used in our present experiments was derived from the blood of healthy adult volunteer donors and contained polyclonal MPO-ANCA. There is a possibility that the polyclonal MPO-ANCA acted competitively with monoclonal MPO-ANCA, which is high in pathogenicity, to inhibit vasculitis.

This animal model may provide important information on evaluating the therapeutic effect of new drugs for vasculitis diseases. Actually, we have been analyzing the potential effectiveness of anticytokine agents by using this animal model. However, since the experimental schedule has been based on that used for KD treatment, in future studies it will be necessary to alter that experimental schedule in order to evaluate the effectiveness of h-Ig in other arteritis diseases such as ANCA-associated vasculitis. Ig should be administered after arteritis has already developed, and then the anti-inflammatory effect of h-Ig in reversing that arteritis should be determined.

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**Conflict of interest statement** None.

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# A Comparison of the Clinical Features of ANCA-Positive and ANCA-Negative Idiopathic Pulmonary Fibrosis Patients

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**Key Words**
Idiopathic pulmonary fibrosis · Microscopic polyangiitis · Myeloperoxidase-antineutrophil cytoplasmic antibody · Proteinase 3-antineutrophil cytoplasmic antibody

**Abstract**
***Background:*** The existence of antineutrophil cytoplasmic antibody (ANCA)-positive pulmonary fibrosis (PF) has recently been recognized. ***Objectives:*** The aim of this study was to clarify whether there is any difference in the clinical features between ANCA-positive PF and ANCA-negative PF. ***Methods:*** A retrospective study was carried out on 53 patients with idiopathic PF whose myeloperoxidase (MPO)- and proteinase 3 (PR3)-ANCA levels were measured. After dividing the patients into ANCA-positive and ANCA-negative PF, we compared their symptoms, pulmonary function tests, chest CT findings, bronchoalveolar lavage findings, the effects of therapy and survival rates. ***Results:*** 17 of the 53 patients with idiopathic PF were MPO-ANCA-positive, 2 were PR3-ANCA-positive and 34 were negative for both ANCA types. Lactate dehydrogenase was lower in ANCA-positive PF than in ANCA-negative PF. However, there was no significant difference in the symptoms, lung function tests, CT findings and bronchoalveolar lavage findings. Corticosteroid therapy tended to be more effective in ANCA-positive PF, but the overall survival rate in ANCA-positive PF was not different from that in ANCA-negative PF. However, the low-titer (<50 EU) group showed better survival than the high-titer (≥50 EU) group in ANCA-positive PF. ANCA titers of the patients who developed microscopic polyangiitis were higher than of those who did not develop it in ANCA-positive PF. ***Conclusions:*** Our studies showed little difference in the clinical features between ANCA-positive and ANCA-negative PF. However, it may be important to measure ANCA when idiopathic PF is diagnosed, because a high titer of ANCA suggests a poor prognosis and the association with microscopic polyangiitis.


## Introduction

At present, idiopathic interstitial pneumonias are classified into 7 groups by the American Thoracic Society/European Respiratory Society (ATS/ERS) consensus classification. Idiopathic pulmonary fibrosis (IPF) has a high frequency and a poor prognosis among the idiopathic interstitial pneumonias [1]. In 1990, Nada et al. [2] reported 3 cases in the Mayo Clinic that had been initially diagnosed as IPF but which were later found to be pulmonary-renal syndrome. They pointed out that pulmonary fibro-

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sis (PF) was a partial lesion of microscopic polyarngiitis (MPA), since small vessel vasculitis was observed in 2 cases of PF with antineutrophil cytoplasmic antibodies (ANCA). This was the first report suggesting the relationship between PF and ANCA.

ANCA is an autoantibody described in 1982 by Davies et al. [3] in patients with segmental necrotizing glomerulonephritis. Two main patterns of ANCA staining are recognized by indirect immunofluorescence microscopy: cytoplasmic and perinuclear. The primary target antigen is proteinase 3 (PR3) for cytoplasmic ANCA-positive patients and myeloperoxidase (MPO) for most perinuclear ANCA-positive patients. Because these target antigens have diverse specificities, the 2 ANCA patterns also seem to be associated with different clinical syndromes [3–6]. Cytoplasmic ANCA has a high sensitivity and specificity for patients with Wegener's granulomatosis [7], whereas perinuclear ANCA is most often associated with syndromes characterized by small-vessel vasculitis, such as MPA, pauci-immune glomerulonephritis or Churg-Strauss syndrome [8–9]. Perinuclear ANCA is also present in other conditions, such as inflammatory bowel diseases (e.g. Crohn's) and autoimmune diseases [10]. ANCA have been recognized as important markers for the diagnosis and monitoring of these diseases [11].

In ANCA-associated systemic vasculitis, particularly in MPO-ANCA-positive cases, rapidly progressing glomerulonephritis is frequently observed and, therefore, the clinical studies have mainly been focused on renal lesions. However, it has become clear that pulmonary lesions such as alveolar hemorrhage or PF appear during the observation of renal lesions [12]. On the other hand, we occasionally encounter PF patients manifesting ANCA-positive vasculitis and renal lesions during the follow-up periods of PF. However, no comparison of the clinical features of ANCA-positive and ANCA-negative PF has been made. Therefore, the aim of this study was to examine whether there was any difference in clinical features between ANCA-positive and ANCA-negative PF. The effects of corticosteroid therapy and prognosis were also evaluated.

## Methods

*Patients*

There were 173 patients with IIP who were admitted to the Respiratory Division of Tokyo Women's Medical University Hospital between January 1999 and December 2006. From these we retrospectively selected 53 patients (29 males and 24 females) whose ANCA were measured and who fulfilled the criteria of clinically defined IPF based on the ATS/ERS consensus classification [1]. The decision to measure ANCA had been made by referring doctors as ANCA had not been measured consecutively in our hospital during the period in question. The remaining 120 patients who did not receive ANCA measurement consisted mainly of IPF and nonspecific interstitial pneumonia (NSIP), but they were excluded from this study. Patients with other inflammatory diseases, like Crohn's disease, were not included. We classified the 53 patients as ANCA-positive PF or ANCA-negative PF and then compared their clinical symptoms, chest CT scan images, pulmonary function tests, bronchoalveolar lavage (BAL) findings, the effects of therapy and survival rates. In the ANCA-positive PF group, 4 MPA patients were included because they had initially been diagnosed as having IPF and had renal lesion later (2 months, 5 months, 1 year and 4 years) and, as a result, fulfilled MPA criteria. Serum MPO-ANCA and PR3-ANCA (healthy control values <10 EU) obtained on the same day were measured with an ELISA [13]. Concerning clinical symptoms, multiple answers were counted separately. For this retrospective study, we had obtained prior approval from the institutional review boards for accessing the patients' medical records, and patient confidentiality was maintained.

*Chest CT Scans*

High-resolution CT scan images of the chest were evaluated at the time of hospitalization. CT scans were performed with an Aquilion 16, Asteion (Toshiba Medical Systems, Tokyo, Japan), ProSeed SA and LightSpeed Ultra 16 (Geyms, Tokyo, Japan). Routine scanning of the entire lung was carried out (10-mm section thickness) followed by an additional thin-section CT (1.0-mm section thickness) of the parenchymal abnormalities. Thin-section CT images were reconstructed with a high spatial frequency algorithm, and were printed with a fixed window setting. Two expert observers conducted a consensus reading of the CT images.

*Pulmonary Function Tests*

At the time of hospitalization, basic pulmonary function tests (vital capacity, $FEV_1$) for 34 cases and $D_{LCO}$ for 24 cases were performed to evaluate the restrictive impairment of the lung and the disease severity in IPF [14]. These tests were measured according to the standard methods with a Chestac-9800 (Chest Co. Ltd., Tokyo, Japan) and a Fudac-77 (Fukuda Denshi Co. Ltd., Tokyo, Japan).

*Bronchoalveolar Lavage*

The bronchofiberscope was inserted into the tracheobronchial tree via the oral route and wedged into the middle lobe or lingual. A sterile 0.9% saline solution was instilled 3 times, 50 ml each time, and recovered by gentle suctioning into siliconized containers. The recovered BAL fluid sample was cytocentrifuged and separated into the cells and the supernatant. May-Grünwald-Giemsa staining was used for differential cell counting. CD4/CD8 was calculated with flow cytometry. The results of differential cell counts in BAL fluid were classified as BAL lymphocytosis (lymphocytes >20% of total white blood cells) or BAL neutrophilia (neutrophils >5% of total white blood cells) [15].

*Laboratory Tests*

White blood cell counts in blood, C-reactive protein (CRP) and lactate dehydrogenase in serum were measured. As markers of activity of pulmonary fibrosis, levels of KL-6 and surfactant

**Table 1.** The comparison of clinical features between ANCA-positive PF patients and ANCA-negative PF patients

| | ANCA-positive (n = 19) | ANCA-negative (n = 34) | p value |
|---|---|---|---|
| Age, years | 69 (52–80) | 72 (48–86) | 0.1215 |
| Age <60 years, % | 31.5 | 11.7 | 0.0823 |
| Sex (M:F) | 7:12 | 18:16 | 0.2011 |
| History of smoking, % | 47.4 | 60.6 | 0.1741 |
| Hypertension, % | 15.8 | 23.3 | 0.3849 |
| Symptoms | | | |
| Dyspnea | 73.7 | 79.4 | 0.7953 |
| Cough | 21.1 | 32.4 | 0.2924 |
| Fever | 31.6 | 17.6 | 0.2047 |
| Bloody sputum | 5.3 | 2.9 | 0.5929 |
| Weight loss | 5.3 | 3.0 | 0.5929 |
| Laboratory findings | | | |
| White blood cells, n/ml | 9,660 (1,370–17,800) | 6,820 (1,090–20,000) | 0.0663 |
| Serum creatinine, mg/dl | 0.75 (0.51–8.76) | 0.83 (0.46–2.48) | 0.2540 |
| CRP, mg/dl | 2.59 (0.04–22.4) | 2.05 (0.08–19.34) | 0.4039 |
| Lactate dehydrogenase, IU/l | 226 (138–442) | 286 (175–802) | 0.0329* |
| KL-6, U/ml | 848 (181–3,013) (n = 17) | 924.5 (345–6,200) (n = 28) | 0.3490 |
| Surfactant protein D, ng/ml | 189.5 (39.2–829) (n = 12) | 242 (97.7–912) (n = 23) | 0.7890 |
| Surfactant protein A, ng/ml | 102 (27.4–179) (n = 5) | 108 (49.2–176) (n = 10) | 0.9025 |
| $AaDO_2$, mm Hg | 25.2 (14–102.3) | 31.2 (7.8–289) | 0.2839 |
| Pulmonary function tests | | | |
| Percentage vital capacity | 86.0 (61–108) (n = 13) | 74.5 (43.5–116) (n = 23) | 0.9213 |
| Percentage $D_{LCO}$ | 41.4 (13.4–72) (n = 12) | 40.7 (20–80.25) (n = 16) | 0.0858 |
| BAL findings | (n = 13) | (n = 15) | |
| Macrophages, % | 67.4 (25.9–97.8) | 84.4 (22.2–90.8) | 0.8719 |
| Neutrophils, % | 3.8 (0.2-60) | 2.7 (0.6–44.9) | 0.4375 |
| Neutrophilia (>5%), % | 40.0 | 33.3 | 0.3800 |
| Lymphocytes, % | 10.3 (1.8–55.8) | 8.4 (2.5–67.8) | 0.7999 |
| Lymphocytosis (>20%), % | 20.0 | 26.7 | 0.5691 |
| Eosinophils, % | 1.76 (0–32.2) | 1.76 (0.1–7.3) | 0.1227 |
| CD4/CD8 | 0.83 (0.05–1.61) | 0.54 (0.15–1.38) | 0.6225 |
| Corticosteroid ± CPA therapy | 94.7 (n = 18) | 64.7 (n = 22) | 0.0378* |
| Improvement | 83.3 (n = 15) | 54.5 (n = 12) | 0.0896 |

Unless otherwise stated, the data are shown as medians with ranges in parentheses or as simple percentages. CPA = Cyclophosphamide. * $p < 0.05$.

proteins A and D in serum were measured with an ELISA [16–17]. As for renal lesions, urinalysis and a serum creatinine test were performed. From arterial blood gas data, $AaDO_2$ was calculated.

*Statistical Analysis*

Data are shown as medians and ranges. The Mann-Whitney U test was used to compare nonparametric data between the 2 groups. Fisher's exact probability test was used to compare dichotomous variables between the 2 groups. The Wilcoxon signed-ranks test was used for the comparison before and after corticosteroid treatment. Survival curves were obtained using Kaplan-Meier's method. The significance was obtained by a log-rank test. $p < 0.05$ was considered statistically significant.

## Results

Nineteen (35.8%) patients were ANCA-positive from the selected 53 patients, and the remaining 34 (64.2%) were negative for both ANCAs (simply defined as ANCA-negative). Of the 19 ANCA-positive patients, there were 17 MPO-ANCA-positive and 2 PR3-ANCA-positive. There was no patient who was positive for both types of ANCA.

The median age was 69 years among ANCA-positive PF patients, and 72 years among ANCA-negative PF. There was no significant difference in age between the 2 groups, as shown in table 1. However, the frequency of

**Table 2.** The comparison of chest CT findings between ANCA-positive PF patients and ANCA-negative PF patients

| | ANCA-positive (n = 15) | ANCA-negative (n = 31) | p value |
|---|---|---|---|
| Subpleural predominance | 73.3 (11) | 80.6 (25) | 0.5730 |
| Peribronchovascular predominance | 26.7 (4) | 25.8 (8) | 0.9503 |
| Reticular | 100 (15) | 96.8 (30) | 0.4818 |
| Honeycombing | 73.3 (11) | 54.8 (17) | 0.2283 |
| Ground-glass opacity | 80.0 (12) | 64.5 (20) | 0.2846 |
| Consolidation | 0 (0) | 16.1 (5) | 0.0994 |
| Nodules | 0 (0) | 9.7 (3) | 0.2127 |
| Cysts | 26.7 (4) | 9.7 (3) | 0.1326 |
| Pleural effusion | 0 (0) | 3.2 (1) | 0.4818 |
| Traction bronchiectasis | 80.0 (12) | 87.1 (27) | 0.5299 |
| Extent limited in lower lung fields | 26.7 (4) | 38.7 (12) | 0.4214 |

The data are percentages with numbers in parentheses.

middle-aged patients (<60 years) tended to be higher in ANCA-positive PF ($p = 0.0823$). As for clinical symptoms, dyspnea was the most common symptom, observed in 73.7% of ANCA-positive PF and 79.4% of ANCA-negative PF cases. The frequency of cough was 21.1% in ANCA-positive PF and 32.4% in ANCA-negative PF cases. Fever was relatively common in ANCA-positive PF. However, there was no significant difference in any of the symptoms between the 2 groups. As for laboratory findings, white blood cell levels tended to be higher in ANCA-positive PF ($p = 0.0663$), but lactate dehydrogenase levels were significantly lower ($p = 0.0329$). KL-6 and surfactant protein D levels, which are serological markers of PF activity, were not significantly different. As for pulmonary function tests, percentage vital capacity and percentage $D_{LCO}$ were not significantly different between the 2 groups.

BAL was performed in 13 patients with ANCA-positive PF and in 15 patients with ANCA-negative PF. The neutrophil fraction of BAL was 3.8% in ANCA-positive PF and 2.7% in ANCA-negative PF, while the lymphocyte fraction was 10.3 and 8.4%, respectively. There was no significant difference in BAL findings between the 2 groups.

Table 2 shows the CT findings in 15 patients with ANCA-positive PF and 31 with ANCA-negative PF. Honeycombing was observed in 73.3% of ANCA-positive PF cases and in 54.8% of ANCA-negative PF. Ground-glass opacity was seen in 80.0% of ANCA-positive PF and 64.5% of ANCA-negative PF patients. Among 4 patients with MPA, 2 showed honeycombing. The reticulation and honeycombing limited to the lower lung fields was observed in 26.7% of ANCA-positive PF and 38.7% of ANCA-negative PF cases. Overall, there was no significant difference in the CT scan images between the ANCA-positive PF and the ANCA-negative PF patients.

As for the therapy, 18 patients (94.7%) in the ANCA-positive PF group and 22 patients (64.7%) in the ANCA-negative PF group received corticosteroids during hospitalization (tables 1, 3). Of these patients, 3 were treated with a combination therapy of cyclophosphamide and 6 received a methylprednisolone pulse therapy in the ANCA-positive PF group. In 1 untreated patient (patient 17), the ANCA level was 14 EU, and a careful follow-up was performed with urinalysis. The ANCA-negative patients not treated with corticosteroid were given other supportive therapy, such as antibiotics and oxygen. Improvement in chest CT or chest X-ray after the corticosteroid and cyclophosphamide therapy was observed in 15 patients (83.3%) in the ANCA-positive PF group, whereas it was observed in 12 patients (54.5%) in the ANCA-negative PF group. Thus, the corticosteroid therapy tended to be more effective in the ANCA-positive PF patients ($p = 0.0896$). Changes in ANCA, CRP and KL-6 before and after the therapy in 7 MPO-ANCA-positive patients (patients 1, 3, 4, 6, 7, 12 and 14) are shown in figure 1. Although both ANCA and CRP declined significantly after the therapy in all 7 patients (ANCA $p < 0.05$, CRP $p < 0.05$), KL-6 increased in 4 patients.

The comparison between the patients with MPA and those without MPA in the ANCA-positive PF group is

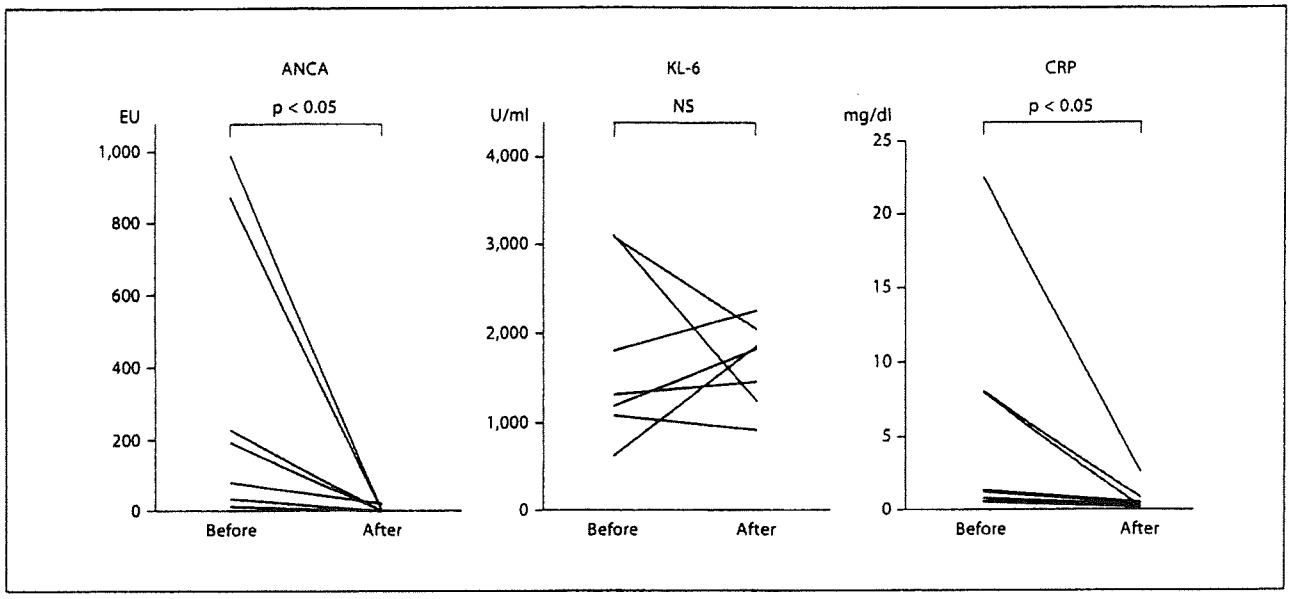


**Fig. 1.** Changes in ANCA, KL-6, and CRP before and after corticosteroid therapy in 7 patients with MPO-ANCA-positive PF. After steroid therapy, ANCA and CRP levels decreased ($p < 0.05$), but KL-6 levels did not significantly change. NS = Not significant.

**Table 3.** Clinical courses in ANCA-positive PF

| Patient No. | Sex | Age years | ANCA type | AH | Renal dys-function | Steroid pulse | PSL mg | CPA | HD | ANCA titer | ANCA titer after treatment | Change in CT scan | Time to end point months | Cause of death |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1[1] | M | 59 | MPO | + | + | + | 50 | + | + | 923 | <10 | - | 1† | renal failure, bleeding |
| 2 | M | 74 | MPO | - | - | + | 60 | + | - | 10 | - | unchanged | 3† | infection, shock |
| 3 | F | 59 | MPO | + | - | + | 40 | + | - | 34 | <10 | improved | 2[2] | |
| 4[1] | F | 54 | MPO | + | + | + | 40 | - | - | 812 | <10 | improved | 90 | |
| 5[1] | F | 69 | MPO | - | + | - | 40 | - | - | 185 | - | improved | 3† | renal failure |
| 6[1] | M | 73 | MPO | - | + | - | 30 | - | - | 181 | <10 | improved | 37 | |
| 7 | M | 52 | MPO | - | - | - | 60 | - | - | 79 | 17 | improved | 5† | infection |
| 8 | M | 64 | MPO | - | - | - | 30 | - | - | 68 | 31 | improved | 15† | infection, relapse |
| 9 | M | 72 | MPO | - | - | - | 30 | - | - | 32 | <10 | improved | 36 | |
| 10 | F | 77 | MPO | - | - | - | 30 | - | - | 10 | - | improved | 43 | |
| 11 | F | 69 | MPO | - | - | - | 30 | - | - | 354 | - | improved | 1[2] | |
| 12 | M | 73 | MPO | + | - | - | 40 | - | - | 212 | <10 | improved | 15 | |
| 13 | F | 64 | MPO | - | - | - | 30 | - | - | 20 | - | improved | 16 | |
| 14 | M | 73 | MPO | - | - | - | 25 | - | - | 12 | <10 | improved | 2[2] | |
| 15 | M | 80 | MPO | - | - | - | 20 | - | - | 98 | - | unchanged | 1† | infection |
| 16 | F | 52 | MPO | - | - | - | 12 (DEX) | - | - | 18 | - | - | 42 | |
| 17 | F | 56 | MPO | - | - | - | - | - | - | 14 | - | unchanged | 22 | |
| 18 | F | 70 | PR3 | - | - | + | 50 | - | - | 18 | <10 | improved | 5[2] | |
| 19 | F | 71 | PR3 | - | - | + | 30 | - | - | 15 | - | improved | 34 | |

AH = Alveolar hemorrhage; PSL = predonisolone; DEX = dexamethasone; CPA = cyclophosphamide; HD = hemodialysis.
[1] Patient had MPA. [2] Patient was transferred. † Patient died.

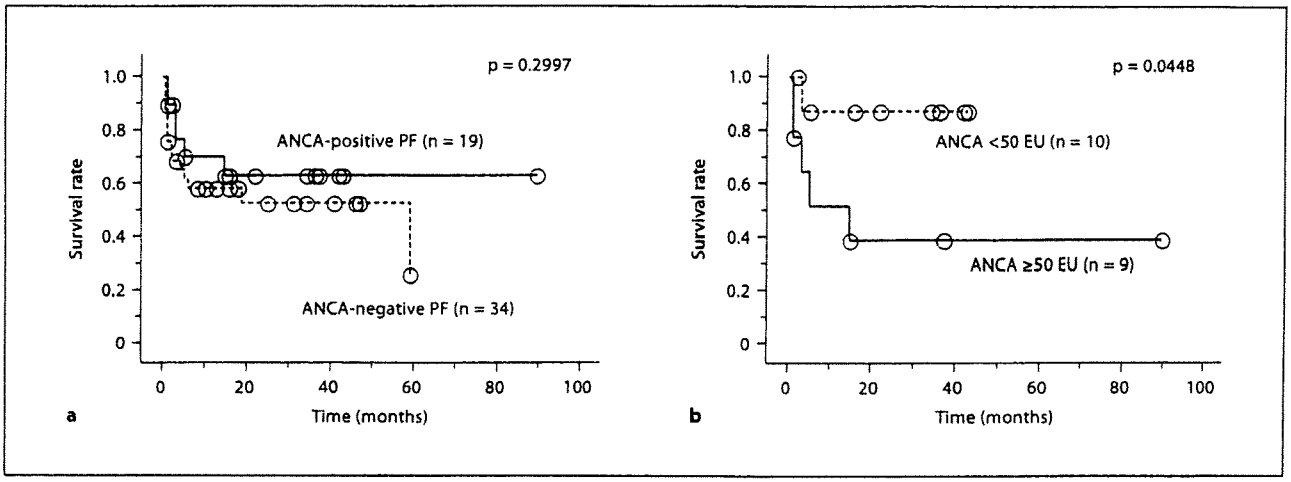


**Fig. 2.** Kaplan-Meier's survival curves. **a** ANCA-positive PF vs. ANCA-negative PF. **b** The low-titer group (ANCA <50 EU) vs. the high-titer group (≥50 EU).

**Table 4.** The comparison of laboratory data between the patients with MPA and those without MPA in the ANCA-positive PF group

| | MPA+ (n = 4) | MPA− (n = 15) | p value |
|---|---|---|---|
| ANCA titer, EU | 499 (181–923) | 33 (12–354) | 0.0093* |
| CRP, mg/dl | 12.03 (7.9–22.4) | 0.87 (0.04–15.75) | 0.0215* |
| Serum creatinine, mg/dl | 0.99 (0.78–8.76) | 0.69 (0.51–0.89) | 0.0143* |

Data are shown as medians with ranges in parentheses. * $p < 0.05$.

shown in table 4. ANCA titers, CRP levels and serum creatinine levels were significantly higher in the patients with MPA than in the patients without MPA (ANCA $p < 0.01$, CRP $p < 0.05$, serum creatinine $p < 0.05$).

Six patients in the ANCA-positive PF group died during the observation period (1–90 months) of this study, and 4 patients were lost to follow-up (table 3). Two of 4 patients with MPA died early of renal failure (patients 1 and 5). The remaining 2 patients recovered from renal damage after corticosteroid therapy and survived (patient 4 and 6). Two patients whose CT findings were unchanged after the therapy died of infection (patients 2 and 15). Two patients with an insufficient decrease in ANCA died of infection and relapse of lung lesion (patients 7 and 8). On the other hand, 15 patients in the ANCA-negative group died during the observation period (1–58 months) and 12 patients were lost to follow-up. The causes of death were infection in 11 patients, acute exacerbation of IPF in 2 patients, lung cancer in 1 patient and hepatic failure in 1 patient. The mean survival time in the ANCA-negative PF group was 32.5 months.

As for the survival rate, there was no significant difference between ANCA-positive PF and ANCA-negative PF ($p = 0.2997$; fig. 2a). However, when the patients with ANCA-positive PF were divided into the low-titer group (ANCA; <50 EU) and the high-titer group (≥50 EU), survival in the low-titer group was significantly better than that in the high-titer group ($p = 0.0448$; fig. 2b) and tended to be better than that in ANCA-negative PF ($p = 0.0720$).

## Discussion

Interstitial pneumonia is a lung disease associated with various causes (e.g. drugs, collagen disease, radiation). However, most of them are cryptogenic [1, 18]. Since ANCA became measurable, the existence of ANCA-pos-