for 2 consecutive days or 2 g/kg/d for 1 day), whereas patients in the IVIG + PSL group received IVIG plus PSL (2 mg/kg/d, in 3 divided doses) given by intravenous injection until the fever resolved, and then orally until C-reactive protein (CRP) levels normalized (<0.5 mg/dL). After CRP normalized, doses of PSL were tapered over 15 days in 5-day steps (2 mg/kg/d for 5 days, 1 mg/kg/d for 5 days, and 0.5 mg/kg/d for 5 days). These methods of administration were based on our previous report.[5] Patients also received aspirin (30 mg/kg/d). The dose of aspirin was reduced to 5 mg/kg/d after normalization of CRP.

Clinical Outcomes and Stratification Using a Risk Score

The patient was considered afebrile when body temperature remained below 37.5°C for more than 24 hours. Nonresponse to initial treatment was defined as the patient having fever that persisted for more than 24 hours after completion of initial IVIG. Recurrence was defined as recrudescent fever associated with KD symptoms 24 hours after an afebrile period. These patients were defined as having treatment failure. CAA, detected by 2-dimensional echocardiography, were defined as present if the internal lumen diameter reached 3 mm in a child less than 5 years old, or at least 4 mm in a child aged 5 years or more; if the internal diameter of a segment was at least 1.5 times as large as that of an adjacent segment; or if the lumen was irregular. We stratified KD patients according to a risk scoring system developed to predict IVIG unresponsiveness. This risk score was based on a multiple logistic regression analysis, including 750 consecutive KD patients given IVIG. Seven variables were included in the risk score (Table 1). Point scores and cut-off values for each variable were as follows: 2 points each for sodium 133 mmol/L or less, 4 or less days of illness before initial treatment, aspartate aminotransferase (AST) 100 IU/L or more, percentage of white cells representing neutrophils at least 80%; and 1 point for platelet count 30.0 × 10^4/mm^3 or less, CRP at least 10 mg/dL, and age 12 months or less. If a laboratory test was performed twice or more before primary therapy, the highest value was chosen for % neutrophils, AST, and CRP, while the lowest value was chosen for platelet count and sodium. We considered KD patients as high risk when they had risk scores of 5 points or more.

Statistical Analysis

All analyses were carried out by means of the SPSS statistical package program (version 16.0J; SPSS Japan, Tokyo). Data were presented as mean ± SD for continuous variables or as a percentage for categorical variables. Patients who had missing values were excluded from this study. We compared clinical and coronary outcomes by dividing both treatment groups into high- and low-risk patients. Categorical data were compared between the IVIG group and the IVIG + PSL group using Fisher exact test. Two-sample t tests were used for analysis of normally distributed continuous variables, and the Mann-Whitney U test was used for continuous variables with a non-normal distribution. Normality was determined with the Kolmogorov-Smirnov algorithm. In high-risk patients, multiple logistic regression analysis was performed and odds ratios were adjusted for sex and score points. For all analyses, a 2-sided P value less than 0.05 was considered to indicate statistical significance.

TABLE 1. Risk Score for Prediction IVIG Unresponsiveness

Variable	Cut-Off Point	Score Point
AST	100 IU/L or more	2
Sodium	133 mmol/L or less	2
Illness days of initial treatment	4 or previous	2
Neutrophils	80% or more	2
CRP, (mg/dL)	10 mg/dL or more	1
Age in months	12 or less	1
Platelet counts	300,000 /mm^3 or less	1

RESULTS

Characteristics and Laboratory Findings of Patients

During the study period, 1123 KD patients were admitted and treated at our hospitals. Eight KD patients who presented with CAA at admission and 109 KD patients who had missing values were excluded from this study. Thus 1006 KD patients (IVIG group n = 896, IVIG + PSL group n = 110) were analyzed in this study. Of the 110 subjects in the IVIG + PSL group, 90 patients were participants in a previously reported randomized trial.[9] The other 20 patients were administered IVIG + PSL according to their pediatrician's selection except for one patient whose parents requested treatment with IVIG + PSL. Table 2 shows the baseline characteristics and laboratory findings of IVIG and IVIG + PSL groups. The IVIG + PSL group had significantly lower sodium, earlier illness days of initial treatment, and higher CRP and score points.

Univariate Analyses of Clinical Outcomes

Using the risk score, 298 patients (33.3%) in the IVIG group and 48 patients (43.6%) in the IVIG + PSL group were classified as high-risk patients (P = 0.034). In low-risk patients, clinical course and coronary outcomes did not show significant differences between IVIG and IVIG + PSL groups (Table 3). Only 5 patients in the IVIG group showed coronary dilation at 1 month, without giant coronary aneurysms exceeding 8-mm diameter. Table 4 summarizes clinical and coronary outcomes in high-risk patients. Treatment failure and nonresponse to initial treatment were less frequent in the IVIG + PSL group (20.8% and 8.3%, respectively) than in the IVIG group (51.7% and 47.3%, respectively; $P < 0.001$ both for comparison of treatment failure and for comparison of nonresponse to initial treatment). Similarly, the incidence of CAA before 1 month of treatment was significantly less in the IVIG + PSL group (3 patients, 6.3%; P = 0.037) than in the IVIG group (54 patients, 18.1%).

TABLE 2. Baseline Characteristics Both IVIG and IVIG + PSL Group

Variable	IVIG Group (n = 896)	IVIG + PSL Group (n = 110)	P
Male, n (%)	512 (57.1)	70 (63.6)	0.22
Age in months	30.3 ± 22.3	30.6 ± 23.9	0.87
Illness days of initial treatment	4.8 ± 1.4	4.5 ± 1.4	0.02
Past history of Kawasaki disease, n (%)	30 (3.3)	4 (3.6)	0.78
White blood cell counts, (×10^3/mm^3)	14.8 ± 5.0	14.9 ± 4.4	0.91
Neutrophils, (%)	68.8 ± 14.9	70.7 ± 15.0	0.20
Platelet counts, (×10^4/mm^3)	34.4 ± 10.7	36.1 ± 10.3	0.10
AST, (IU/L)	116 ± 222	127 ± 238	0.65
Sodium, (mmol/L)	134.6 ± 2.8	133.9 ± 3.0	0.01
CRP, (mg/dL)	8.6 ± 5.2	9.7 ± 5.2	0.05
Score points	3.5 ± 2.4	4.3 ± 2.5	0.002

cally, these proteins regulate adhesion of neutrophils and monocytes to endothelial cells and are implicated in their transmigration into the vessel wall (25). MRP8/MRP14 is believed to have an important functional role in vasculitis syndromes and serves as a marker identifying patients at risk for developing CAL during acute phase of KD (8). Interestingly, MRP8/MRP14 has recently been identified as novel endogenous ligands of TLR4, which directly promote inflammation. MRP14 $-/-$ mice are protected from LPS-induced shock due to a lack of TNF-α production compared with wild-type mice indicating that MRP8/MRP14 act up-stream of TNF-α in the inflammatory cascade (12). This hypothesis is supported by our data showing that blockade of TNF-α efficiently inhibits down-stream pathways of systemic inflammation, such as the expression of IL-6, but does not completely block expression of local proinflammatory mechanisms. Whether this lack of action has negative consequences with respect to the long-term prognosis of KD-patients after infliximab treatment cannot be answered by this relatively small cohort but requires a prospective follow-up study including a larger number of patients.

Another member of the S100 family, S100A12, is also released by neutrophils, binds to the RAGE on endothelial cells and leukocytes, and induces cell activation and cytokine production through the nuclear factor-kappa-B signaling pathway (26) (Fig. 3). Transcript and protein levels of all three of these S100 proteins are increased in circulating leukocytes and in serum during the acute phase of KD (8–11,15,23). Taken together, these observations support the hypothesis that MRP8/MRP14 heterodimers and S100A12 participate directly in the pathogenesis of the coronary artery vasculitis in KD and contribute to endothelial cell damage and transmigration of leukocytes into the arterial wall independent of TNF-α activity. Moreover, during acute KD, sRAGE may block the binding of S100A12 to RAGE on the cell surface by neutralizing this proinflammatory protein. The fact that concentrations of MRP8/MRP14 and S100A12 did not change during infliximab treatment indicates that MRP8/MRP14 and S100A12 maintain signaling of local vascular injury despite the blockade of TNF-α. Lower levels of sRAGE detected in KD patients might thus confer susceptibility to hyperinflammation (27). Lower levels of sRAGE may reflect insufficient compensation of S100A12 because of the occurrence of dramatic and strong inflammation in refractory KD patients during infliximab treatment.

VEGF enhances proliferation and migration of endothelial cells in collaboration with nitric oxide and may contribute to later vascular remodeling after the acute phase of KD (7). Because systemic overproduction of VEGF has been demonstrated in acute KD, VEGF is considered to be involved in the pathophysiology of KD, especially in the development of CAL (28,29).

This study shows that serum IL-6 and sTNFRI levels dramatically decreased after treatment and correlated with serum CRP levels and fevers; however, the serum levels of VEGF, MRP8/MRP14, and S100A12 remained high after infliximab treatment, especially in patients with CAL. In contrast, in IVIG responders all cytokines decreased markedly after IVIG treatment. These data indicate that proinflammatory cytokines decrease in response to infliximab treatment, but VEGF and local inflammatory proteins of the DAMP-family such as MRP8/MRP14 and S100A12, which were reported to be important factors in development of CAL during acute stage of KD, do not. Thus, it seems that infliximab is effective for the suppression of systemic inflammation, but could not completely block the local vasculitis in KD.

We have now successfully treated eight of the 11 refractory KD patients. Three patients who did not respond to infliximab treatment needed additional therapy. Four patients developed CAL despite infliximab therapy: of note, these patients were treated later than the patients without CAL and had signs of CAL before treatment. Histopathological studies have shown that transient infiltration by granulocytes occurs in the very early stage of acute KD, before infiltration of mononuclear cells, suggesting granulocytes act as a trigger in the pathogenesis of CAL (30). In light of our data, to prevent the progression of CAL and to prevent the secretions of MRP8/MRP14 and S100A12 from inflammatory cells such as neutrophils and monocytes, early administration of infliximab, or combination therapy with IVIG may lead to the successful cessation of the inflammatory response and vasculitis.

Study limitations. Limitations of this study include its retrospective nature, small number of patients, and the administration of multiple different therapies after the first IVIG infusion failure. Different IVIG preparations were used at different centers and concomitant or sequential antiinflammatory therapies administered to several of these patients precluded a final assessment of the effect of infliximab infusion. In conclusion, in this study, we show for the first time that inflammatory cytokines are up-regulated and changed dynamically during Infliximab treatment in refractory KD patients. Infliximab was effective for suppression of systemic inflammation, but could not completely block the local vasculitis in refractory KD patients. The early administration of infliximab or combination therapy with IVIG might be recommended for refractory KD patients.

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tributed to the therapeutic benefit, gained from adding PSL in high-risk patients.

On the other hand, the IVIG + PSL group was at significantly higher risk of relapse despite having a lower risk of overall treatment failure in high-risk patients. In the IVIG + PSL group, 6 of 10 patients relapsed at the steroid tapering phase. Although further examinations are needed to confirm this, we speculate that patients who relapse in the IVIG + PSL group have a severe form of KD, and the relapse is coupled with steroid tapering.

As our simple risk score predicts IVIG unresponsiveness with high sensitivity and specificity, patients in the low-risk group are unlikely to benefit from the addition of corticosteroids. This is important, given that corticosteroids may be associated with many potential adverse reactions.[9–11] Furthermore, epidemiologic and clinical features of KD suggest an infectious etiology,[31] and in general corticosteroid therapy can aggravate infectious processes. This study did not have sufficient statistical power to assess either the likelihood of adverse effects or the effectiveness of therapies in the low-risk group. Therefore, considering the balance between benefit and risk, only KD patients at high risk for nonresponse to IVIG should be given both corticosteroids and IVIG, as part of primary therapy. In addition, a randomized controlled trial suggested the usefulness of corticosteroid therapies in children at the highest risk for resistance to IVIG.[32]

There were several limitations to the study. First, our initial dose of aspirin, which is commonly used in Japan, is lower than that used in the United States. Second, Japanese Ministry of Health criteria used to diagnose CAA might underestimate the true incidence of CAA in patients with KD,[33] though it is a simple set of criteria that are easy to use in clinical settings. Third, this was a retrospective study not a randomized controlled study. A residual confounding effect was not completely ruled out, though we adjusted odds ratios of treatment for sex and for the risk score associated with clinical and coronary outcomes.

In conclusion, IVIG plus PSL might improve coronary and clinical outcomes in patients at high risk of IVIG failure as defined by our simple risk score. Future controlled, randomized clinical trials will define the benefit of this stratification and the role of IVIG + PSL therapy in high-risk KD patients with IVIG failure.

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clones were HLA-DR-restricted $CD4^+$ helper T cells; these features were consistent with previously reported MPO-reactive T-cell lines obtained from a patient with MPO-ANCA-associated vasculitis, although the clonality of those lines was not evaluated (17). Analysis of the TCR β chain of the MPO-specific T-cell clones revealed the presence of several distinct CDR3 amino acid motifs in association with the recognition of epitopes on both the light and heavy chains of MPO.

The MPO-specific T-cell clones derived from MPA patients and healthy individuals had common characteristics, including a $CD4^+CD8^-$ helper phenotype and restriction by HLA-DR, and some of them shared CDR3 amino acid motifs in the TCR β chain. Thus, there was no difference in properties determined during T-cell development between the MPO-specific T cells derived from the patients and those from healthy individuals. An analogous phenomenon has been reported for several other autoreactive T-cell clones, including those to myelin basic protein in patients with multiple sclerosis, to topoisomerase I in patients with scleroderma, and to β_2-glycoprotein I in patients with antiphospholipid syndrome (15, 16, 18). Taken together, it is likely that T cells autoreactive to MPO are a component of the T-cell repertoire of some healthy individuals.

However, the cytokine expression profiles of the MPO-specific T-cell clones were apparently different: the clones derived from MPA patients had a Th1 phenotype, while those from healthy individuals were Th0. In this regard, the MPO-reactive T-cell lines established from an MPO-ANCA-positive patient reported by Yoshida *et al.* also represented a Th1 cytokine expression profile (17). A difference in cytokine expression profiles between patients and healthy individuals was also shown for T-cell clones autoreactive to topoisomerase I (14). The mechanism responsible for this difference is unclear, but it is possible that autoreactive T cells in patients are activated due to ongoing or recent antigenic stimulation and differentiate into Th1 cells, whereas those in healthy individuals remain immature Th0 cells, due to a lack of appropriate antigenic stimulation.

The CDR3 amino acid motifs of the TCR β chain unique to MPO-specific T cells might be a potential selective therapeutic target for ANCA-associated vasculitis. TCR-based immunotherapy, such as TCR vaccination, is an effective treatment for various autoimmune diseases in animal models, and active investigations into its appropriateness for clinical applications are on-going (19).

In summary, we have identified characteristics unique to autoreactive T cells against MPO by generating MPO-specific T-cell clones. This information may be useful in elucidating the pathogenesis of ANCA-associated vasculitis and in developing selective immunotherapy for this intractable disease.

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Characterization of autoreactive T-cell clones to myeloperoxidase in patients with microscopic polyangiitis and healthy individuals

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ABSTRACT

Objective. *To characterize autoreactive T cells against myeloperoxidase (MPO) by generating antigen-specific T-cell clones from patients with microscopic polyangiitis (MPA) and healthy individuals.*

Methods. *Peripheral blood T cells from five patients with MPA and MPO-anti-neutrophil cytoplasmic antibodies (ANCAs) and from three healthy donors were used to establish MPO-specific T-cell clones by repeated stimulation with recombinant MPO fragments, followed by limiting dilution. The MPO-specific T-cell clones were subjected to analyses for CD4/CD8 phenotype, human leukocyte antigen (HLA) class II restriction, T-cell receptor (TCR) β-chain gene usage, complementarity-determining region 3 (CDR3) amino acid sequences, and cytokine expression profiles.*

Results. *We successfully generated seven MPO-specific T-cell clones, five from the patients and two from healthy donors. Two clones recognized the light chain of MPO and five recognized the heavy chain. All the clones were HLA-DR-restricted $CD4^+CD8^-$ helper T cells. The T-cell clones shared TCR β CDR3 amino acid motifs, depending on their MPO epitope: AGxxN was used by clones recognizing the light chain and TGxS or QGxE by those recognizing the heavy chain, whether the cells were derived from MPA patients or healthy subjects. However, the cytokine expression profiles of the patients' clones were skewed towards the Th1 phenotype, whereas the healthy individuals' clones remained Th0.*

Conclusions. *We have characterized MPO-reactive T cells in detail. This information may be useful for elucidating the mechanism of ANCA production and for developing selective therapeutic strategies for MPO-ANCA-associated vasculitis.*

Introduction

Anti-neutrophil cytoplasmic antibodies (ANCAs) with a specificity for myeloperoxidase (MPO) are detected in patients with microscopic polyangiitis (MPA) or with Churg-Strauss syndrome (1). MPO-ANCAs are thought to contribute directly to the pathogenic process of vasculitis by binding to target antigens expressed on the surface of primed neutrophils and monocytes, leading to the release of oxygen metabolites and proteinases (2-4). These responses induce injury and activation of the endothelium, which also plays an important role in development of vasculitis (5).

MPO-ANCAs are predominantly of the high-affinity IgG isotype (6), suggesting that their production results from a T-cell-dependent isotype switch and affinity maturation. In addition, treatment with T-cell-directed agents, such as cyclosporine A, reduces the titer of ANCAs and induces remission in some patients with ANCA-associated vasculitis (7, 8). Finally, Xiao *et al.* showed that lymphocytes derived from MPO-deficient mice that were pre-immunized with mouse MPO directly induced pauci-immune crescentic glomerulonephritis and pulmonary vasculitis in recombinase-activating gene-2-deficient mice, as well as in wild-type mice (9). These findings strongly suggest that ANCA production requires an antigen-specific collaboration between T and B cells. Thus, although autoreactive T cells that recognize MPO are a potential target for selective immunotherapy for MPO-ANCA-associated vasculitis, their characteristics are largely unknown. Recently, we identified $CD4^+$ T cells autoreactive to multiple epitopes on MPO in patients with MPO-ANCA-associated vasculitis and in healthy individuals (10). In this study, we successfully established MPO-specific T-cell clones from MPA patients and healthy individuals and characterized their phenotypes, including their T-cell receptor (TCR) and cytokine expression profiles.

Materials and methods

Human subjects

Five patients with MPA (P4, P5, P7, P8, and P13) and three healthy donors (HD1-3) were selected from the subjects examined in our previous study (10), based on the capacity of their peripheral blood T cells to respond to at least one recombinant MPO fragment and the availability of peripheral blood samples. All the MPA patients fulfilled the Chapel Hill criteria (11), and were positive for MPO-ANCA, as determined

TABLE 3. Clinical and Coronary Outcomes Among Low-Risk Patients

Variable	IVIG Group (n = 598)	IVIG + PSL Group (n = 62)	*P*
Treatment failure, n (%)	57 (9.5)	5 (8.1)	1.00
Nonresponse to initial treatment, n (%)	40 (6.7)	2 (3.2)	0.42
Relapse, n (%)	19 (3.2)	3 (4.8)	0.45
CAA until 1 mo, n (%)	14 (2.3)	0 (0.0)	0.38
CAA at 1 mo, n (%)	5 (0.8)	0 (0.0)	1.00

TABLE 4. Clinical and Coronary Outcomes Among High-Risk Patients

Variable	IVIG Group (n = 298)	IVIG + PSL Group (n = 48)	*P*
Treatment failure, n (%)	154 (51.7)	10 (20.8)	<0.001
Non-response to initial treatment, n (%)	141 (47.3)	4 (8.3)	<0.001
Relapse, n (%)	17 (5.7)	7 (14.6)	0.06
CAA until 1 mo, n (%)	54 (18.1)	3 (6.3)	0.04
CAA at 1 mo, n (%)	25 (8.4)	2 (4.2)	0.40

TABLE 5. Sex- and Risk Score Point-Adjusted Odds Ratios and 95% Confidence Intervals of Clinical and Coronary Outcomes Among High-Risk Patients

Variable	IVIG Group (n = 298)	IVIG + PSL Group (n = 48)	
		Adjusted OR	95% CI
Treatment failure	1.00 (reference)	0.18	(0.08–0.39)
Nonresponse to initial treatment	1.00 (reference)	0.07	(0.02–0.20)
Relapse	1.00 (reference)	2.75	(1.07–7.08)
CAA until 1 mo	1.00 (reference)	0.25	(0.07–0.85)
CAA at 1 mo	1.00 (reference)	0.41	(0.09–1.81)

OR indicate odds ratio.

Multivariate Analyses of Clinical Outcomes in High-Risk Group

Table 5 shows the sex- and risk score point-adjusted odds ratio of each of the end points. The IVIG + PSL group was at significantly lower risk of treatment failure (adjusted odds ratio 0.17; 95% confidence interval [CI], 0.08–0.39), nonresponse to initial treatment (adjusted odds ratio 0.07; 95% CI, 0.02–0.20), and CAA until 1 month of treatment (adjusted odds ratio 0.25; 95% CI, 0.07–0.85), despite having a significantly higher risk of relapse (adjusted odds ratio 2.75; 95% CI, 1.07–7.08).

DISCUSSION

The current study shows that risk-based stratification might guide decision making concerning initial treatment for KD. Among patients with high scores for risk of treatment failure with IVIG monotherapy, KD patients assigned to the IVIG + PSL treatment group were more likely to respond to therapy and avoid CAA. On the other hand, among patients with a low-risk of IVIG treatment failure, coronary and clinical outcomes were similar between the treatment-defined groups. These data suggest that primary therapy with a combination of IVIG and PSL might be a better therapeutic option than IVIG alone for this more severe form of KD. Patients with low risk of treatment failure might fare equally well with IVIG alone, thus limiting potential toxicities or adverse events due to steroid complications.

Stratification of primary therapy in KD patients was attempted in Japan in the 1990s. Harada[15] developed a risk score for prediction of coronary aneurysms for use when a child first presented with KD. Accordingly, at centers in Japan adopting this scoring system, IVIG was given to children fulfilling 4 of the following criteria: white blood cell count over 12000/mm^3; platelet count below 350000/mm^3; CRP exceeding 3+ by a semiquantitative analysis; hematocrit below 35%; albumin below 3.5 g/dL; age not greater than 12 months; and male gender. However, the influence of the Harada score in selecting KD patients who require IVIG has waned in Japan because IVIG is now given to almost all KD patients, as first-choice primary therapy. In addition, the American Heart Association and the American Academy of Pediatrics have recommended that all patients diagnosed with KD should be treated with IVIG, given the limited ability of scoring systems to predict CAA before initiation of IVIG.[16]

Pathologically, an affected coronary artery shows an influx of neutrophils in the early stage of lesion development (7–9 days after onset), followed by rapid transition to large mononuclear cells as well as lymphocytes (predominantly CD8$^+$ T cells) and IgA plasma cells.[17,18] Destruction of the internal elastic lamina followed by fibroblast proliferation occurs at this stage to form a coronary aneurysm. These findings underscore the importance of treating inflammation and vasculitis as soon as possible, before pathologic changes become irreversible. Recent clinical trials have focused on additional rescue therapy for KD patients who fail to respond to initial IVIG rather than primary therapy in KD. These rescue therapies have included corticosteroids,[19–21] tumor necrosis factor α blockade,[22] cyclosporine,[23] or plasma exchange.[24] Although these therapies were reported to bring about improvement in symptoms without significant worsening of adverse effects, no report concluded that these additional rescue therapies ultimately reduced the occurrence of CAA. Because IVIG nonresponders were mostly identified 24 to 48 hours after completion of the initial course of IVIG, rescue therapies generally were initiated 2 to 3 days after diagnosis of KD. Such a delay in administration of rescue therapies would permit initiation of CAA development. Because many studies have indicated that most KD patients with CAA are IVIG nonresponders,[12,13,25–26] the predictive identification of IVIG nonresponders permitting use of the additional intensive primary therapy, for IVIG plus corticosteroids should improve overall clinical and coronary outcomes.

Although we found addition of corticosteroids to IVIG primary therapy to benefit high-risk patients, the mechanism by which combining PSL with IVIG reduces the incidence of CAA in high-risk KD patients remains unclear. At this point, we suspect that rapid down-regulation of cytokine secretion by corticosteroid treatment might benefit high-risk patients because many investigators have concluded that various proinflammatory cytokines might take part in the pathogenesis of KD.[27,28] We previously reported that adding corticosteroids to IVIG when treating KD children rapidly ameliorated symptoms while reducing circulating cytokines, including interleukin IL-2, IL-6, IL-8, and IL-10.[29] Lin et al[30] reported that KD patients with CAA were found to have more abundant circulating pro-inflammatory cytokines, including IL-6, IL-8, and tumor necrosis factor α than patients with normal coronary arteries. Production of CRP occurs almost exclusively in hepatocytes as part of the acute-phase response upon stimulation by IL-6, tumor necrosis factor α, and IL-1β, produced at sites of inflammation. Although further studies are needed, these findings strongly support our inference that cytokine down-regulation con-

from patients with MPA and two from healthy donors (Table I). We failed to obtain a T-cell line from one healthy donor. The clonality of each line was confirmed by verifying that each one had only one functionally rearranged TCR β chain. The patterns of reactivity with MalBP and MPO fragments of representative T-cell clones are shown in Figure 1. Two clones derived from MPA patients recognized MPO-L, and the remaining five (three from MPA patients and two from healthy donors) recognized MPO-HII. All the MPO-specific T-cell clones had a $CD4^+CD8^-$ helper phenotype and were restricted by HLA-DR.

TCR β chain

The Vβ-Jβ-Cβ gene rearrangement, CDR3 amino acid sequence, and CDR3 length in the TCR β chain of each MPO-specific T-cell clone are summarized in Table I. There was no common Vβ or Jβ gene segment shared by the MPO-specific T-cell clones. Regarding the CDR3 amino acid sequence, both of the T-cell clones that recognized MPO-L had the amino acid motif AGxxN. In contrast, the TGxS or QGxE motif was found in the TCR Vβ chain of the T-cell clones that recognized MPO-HII. Since MPO-HII encompassed a region spanning 256 amino acid residues, it is likely that two different epitopes are present in this region, in association with different TCR β CDR3 motifs.

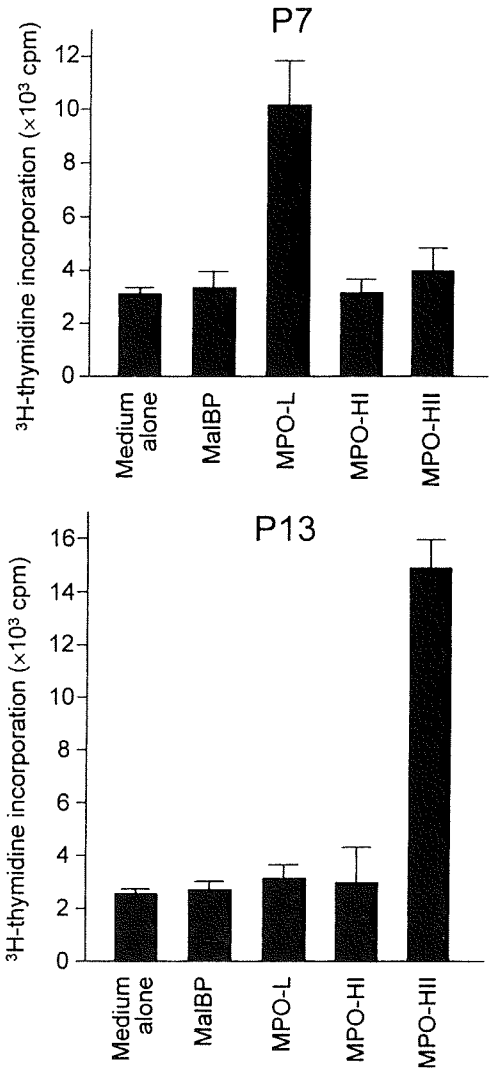
Cytokine expression profiles

Individual MPO-specific T-cell clones expressed different sets of cytokines, while all the clones expressed IFN-γ and TGF-β (Figure 2). Notably, the MPO-specific T-cell clones derived from healthy donors expressed IL-4 in combination with IFN-γ, a pattern consistent with a Th0 phenotype. In contrast, none of the clones derived from patients with MPA expressed IL-4, and their cytokine expression pattern was consistent with a Th1 phenotype.

Discussion

We successfully established MPO-specific T-cell clones from the peripheral blood of MPA patients and healthy individuals. All the MPO-specific T-cell

Fig. 1. Reactivities to MalBP and MPO fragments of MPO-specific T-cell clones established from two patients with MPA (P7 and P13). T-cell clones were cultured with or without MPO-L, MPO-HI, MPO-HII, or MalBP, and irradiated autologous Epstein-Barr virus-transformed lymphoblastoid B cells for 3 days, then the T-cell proliferation was measured by ^{3}H-thymidine incorporation. All cultures were carried out in triplicate, and the results shown are the mean and standard deviation. Representative results obtained from three independent experiments are shown.



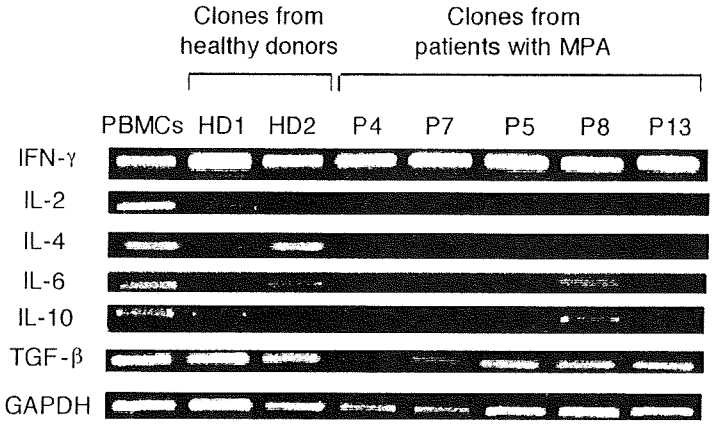


Fig. 2. Cytokine expression patterns of MPO-specific T-cell clones, determined by PCR. MPO-specific T-cell clones derived from MPA patients and healthy donors were subjected to PCR to detect the expression of IFN-γ, IL-2, IL-4, IL-6, IL-10, TGF-β, and GAPDH. PMA/ionomycin-treated PBMCs were used as a control for individual cytokine expression. The PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide. Concordant results were obtained in two additional experiments.

Table 1 Diagnosis and disease activity

	Age	Sex	Year of admission	Serological analysis						Diagnosis	Grade	BVAS	Lung lesions	Lung XP/CT	Renal bx	Lesions
				MPO/ ANCA	(EU)	PR3/ ANCA	(EU)	a-GBM	(EU)							
Group I																
1	59	M	1996	+	100/<10	−		−		RLV/HCV	1		−		+	C/F Cres, Pim
3	58	M	1999	+	24/<10	−		−		MPA	2	15	+	RS		
4	84	M	1999	+	660/12	−		−		MPA	3	20	+	RS/EP		
9	65	F	1996	+	831/12	−		−		MPA/HCV/LC	2		−		+	C/F Cres, Pim
12	42	F	1998	+	142/<10	−		−		MPA	2		−			
17	76	F	2003	+	510/<10	−		−		RLV	2		−			
21	74	M	2005	+	640/<10	−		−		RLV	1		−			
22	68	M	2005	+	138/<10	−		−		RLV	1		−			
23	56	F	2006	+	142/<10	−		−		MPA	2	16	+	PH/RS/NO/CV		
24	55	M	2006	+	144/<10	−		−		RLV	1		−			
25	72	F	2006	+	88/<10	−		−		RLV	2		−			
Ave	64.45															
SD	11.9															
Group II																
2	75	M	1997	+	24/<10	+	24/<10	−		PN/RA	3	14	+	RS/PL/GC		
5	69	M	2000	+	175/<10	+	12/<10	−		MPA	4	27	+	RS		
6	63	M	2004	+	920/69	+	24/<10	−		MPA	1		−		+	C Cres, Pim
Ave	69															
SD	6															
Group III																
8	70	M	2002	+	208/<10	−		+	660/<10	RA	2		−			
14	42	F	2003	−		−		+	20/<10	GP s/o	2		−		+	C Cres, linear IgG
18	53	F	2003	+	91/<10	−		+	300/<10	MPA	2	30	+	PH/RS/PL/NO		
20	70	F	2004	+	42/<10	−		+	132/<10	MPA	3	25	+	PH/RS		
21	63	F	2005	−		−		+	117/<10	RLV	3		−			
26	93	M	2006	+	232/<10	−		+	24/<10	MPA	3	27	+	RS/EP/PH		
Ave	65.17															
SD	17.4															
Group IV																
7	42	M	2003	−		−		−		MPA	2	18	+	PH/RS/PL	+	C Cres, IgG±
10	49	F	1998	−		−		−		HSP	2		−			
11	57	F	1998	−		−		−		SLE	2		−			
13	38	F	2001	−		−		−		IgAN	1		−		+	C Cres, IgA3+

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ORIGINAL ARTICLE

Clinicoepidemiological manifestations of RPGN and ANCA-associated vasculitides: an 11-year retrospective hospital-based study in Japan

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Abstract Antineutrophil cytoplasmic antibody (ANCA)-associated small-vessel vasculitides are major causes of rapidly progressive glomerulonephritis (RPGN). Although recent papers suggest differences in clinicoepidemiological manifestations of ANCA-associated vasculitis between Japan [microscopic polyangiitis (MPA) ≫ Wegener's granulomatosis (WG)] and Europe (WG ≫MPA), little is known about the prevalence and serological pattern. We retrospectively analyzed 27 RPGN patients who were admitted in our hospital over the past 11 years and who could be basically followed for more than 1 year, concerning the incidence of ANCA-related vasculitis, the presence of (MPO)/proteinase 3 (PR3)-ANCA and their clinical outcomes. As there were no PR3-ANCA single positive and/or WG patients, all patients were serologically divided into four groups; Groups I: MPO-ANCA single-positive patients ($N = 11$), II: MPO-ANCA and PR3-ANCA double-positive patients ($N = 3$), III: anti-glomerular basement membrane antibody (anti-GBM Ab)-positive patients ($N = 6$), and IV: all negative patients ($N = 7$). Patients in Groups II/III showed more severe manifestation at admission. However, in Group I, only 36.3% patients avoided death and/or dialysis-dependent end-stage renal disease. Most patients in Group IV were women (85.7%), and 50% of these patients was diagnosed as having rheumatic diseases. Every patient in Groups I–III was treated with oral corticosteroid and/or methylprednisolone pulse therapy. Most patients treated with immunosuppressants showed severe prognosis because of frequent recurrences of vasculitis and infectious episodes after repeated and prolonged treatments with immunosuppressants. Present analysis further confirms the epidemiological and serological differences in ANCA-related RPGN between Japan and Europe, and reinforced the fact that ANCA-associated vasculitis is the most serious causal disease for RPGN.

Keywords MPO-ANCA · PR3-ANCA · Microscopic polyangiitis (MPA) · Wegener's granulomatosis

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Introduction

More than 80% of patients with active, untreated, necrotizing small-vessel vasculitis associated with an absence or paucity of immunoglobulin (Ig) deposition in vessel walls have circulating antineutrophil cytoplasmic antibody (ANCA) [1]. The major clinicopathological expressions of ANCA-associated small-vessel vasculitis are Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), Churg–Strauss syndrome, and renal-limited vasculitis

Table I. Vβ-Jβ-Cβ gene rearrangement, CDR3 amino acid sequence, and CDR3 length of the TCR-β chain used by MPO-specific T-cell clones.

Antigen	Donor	CD4/CD8 phenotype	HLA class II restriction	Gene segment*			CDR3 amino acid sequence†			CDR3 length (amino acids)
				Vβ	Jβ	Cβ	Vβ	N-Dβ-N-Jβ	Jβ	
MPO-L	P4	$CD4^+/CD8^-$	DR	2.1	1.5	1	CS	ARVP**AGI**SNQPQHF	GDGTRLSIL	14
	P7	$CD4^+/CD8^-$	DR	7	2.4	2	CASS	QDL**AGG**ENIQYF	GAGTRLSVL	12
MPO-HII	P5	$CD4^+/CD8^-$	DR	4	2.2	2	CS	VDTGASGELFF	GEGSRLTVL	11
	P8	$CD4^+/CD8^-$	DR	1	1.5	1	CASS	ATGASNQPQH	GDGTRLSIL	10
	P13	$CD4^+/CD8^-$	DR	2.3	1.1	1	CS	ASLQGTEAFF	GQGTRLTVV	10
	HD1	$CD4^+/CD8^-$	DR	22.1	1.6	1	CAS	RTGQSTSPLH	GNGTRLTVT	10
	HD2	$CD4^+/CD8^-$	DR	12.2	1.4	1	CA	AQKRQGDEKLFF	GSGTQLSVL	12

*Determined based on the nucleotide sequence of the TCRβ PCR products.
†The AGxxN motif in the TCR used by MPO-L-specific T cell clones and the TGxS and QGxE motifs in the TCR used by MPO-HII-reactive T-cell clones are shown as bold, underlined, and doubled-underlined text, respectively.

by a commercially available enzyme-linked immunosorbent assay kit (MBL, Nagoya, Japan). All blood samples were obtained after the subjects gave their written informed consent, as approved by the Institutional Review Board.

MPO-specific T-cell lines

We used three recombinant MPO fragments, expressed as maltose-binding protein (MalBP) fusion proteins, for T-cell stimulation. These included MPO-L, the entire 112 amino acid (AA) light chain; MPO-HI, AA 1-227 of the heavy chain; and MPO-HII, AA 212-467 of the heavy chain (10). Antigen-specific T-cell lines were generated according to a previously described method (12) with some modifications. Briefly, peripheral blood mononuclear cells (PBMCs) were cultured in complete medium supplemented with 8% autologous heat-inactivated plasma and one of the recombinant MPO fragments (10 μg/ml) that had been known to be capable of stimulating T cells in our previous study (10), in the presence of a monoclonal antibody (mAb) to CD29 (2.5 μg/ml) (clone MAR4; BD Pharmingen, San Diego, CA, USA). This procedure improves the viability of T-cell blasts by suppressing antigen-induced cell death *in vitro* (13). After the second stimulation, T-cell blasts were cloned by limiting dilution at 0.3 cells/well, and the cells in growth-positive wells were expanded and maintained in cultures that included interleukin (IL)-2 and irradiated autologous Epstein-Barr virus-transformed lymphoblastoid B cells, and were repeatedly stimulated with the antigen. The specificity of each T-cell line was evaluated by a proliferation assay, in which MalBP and a series of MPO fragments were used as the antigen (10). T-cell lines responsive to one of the MPO fragments, but not to MalBP, were regarded as MPO-specific T-cell lines.

CD4/CD8 phenotype

The surface expression of CD4 and CD8 was assessed by flow cytometry using a fluorescein isothiocyanate-conjugated anti-CD4 mAb and a phycoerythrin-conjugated anti-CD8 mAb (12).

Human leukocyte antigen (HLA) class II restriction

The HLA class II restriction of individual T-cell lines was determined by examining the inhibitory effect of anti-HLA-DR, anti-HLA-DQ, and anti-HLA-DP mAbs on MPO-induced T-cell proliferation (10).

Preparation of complementary DNA (cDNA)

MPO-specific T-cell lines or PBMCs were stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/ml) and ionomycin (1 μg/ml) for 3 days. The $CD4^+$ T cells were isolated by incubation with anti-CD4 mAb-coupled magnetic beads (Dynal Biotech ASA, Oslo, Norway) (14). The total RNA was extracted using a phenol/guanidine isothiocyanate extraction procedure (Isogen; Nippon Gene, Tokyo, Japan) and subjected to reverse transcription with oligo-dT priming, to generate cDNAs.

Analysis of the TCR β chain

The TCR β gene usage of individual MPO-specific T-cell lines was analyzed by polymerase-chain reaction (PCR) using a panel of Vβ region primers corresponding to Vβ1-24, in combination with a Cβ region primer, as described previously (15, 16). The PCR products were directly sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The complementarity-determining region 3 (CDR3) was defined as the region starting from the amino acid residue after the CASS sequence of each Vβ segment and ending with the last amino acid before the GxG box in the Jβ region.

Cytokine expression profiles

The gene expression of interferon (IFN)-γ, IL-2, IL-4, IL-6, IL-10, transforming growth factor (TGF)-β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was evaluated by PCR using a panel of specific primers, as described elsewhere (14). PBMCs that were stimulated with PMA and ionomycin were used as a control for individual cytokine expression. The PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide.

Results

Establishment of MPO-specific T-cell clones

We successfully generated a total of seven T-cell lines specific to MPO: five

into four groups, as follows: Group I: MPO-ANCA single-positive patients ($N = 11$, male:female = 6:5), Group II: MPO-ANCA and PR3-ANCA double-positive patients ($N = 3$, M:F = 3:0), Group III: anti-GBM -Ab-positive patients ($N = 6$, M:F = 2:4) (including MPO-ANCA-positive patients: $N = 4$, M:F = 2:2), and Group IV: all MPO-/PR3-ANCA and anti-GBM-Ab-negative patients ($N = 7$, M:F = 1:6, systemic lupus erythematosus: 3, rheumatoid arthritis: 1, MPA: 1, IgA nephropathy: 1, Henoch-Schonlein purpura: 1).

Statistical analyses

The significance of differences in age between each group (Table 1) was assessed by paired Student's t test using StatView statistical software (Hulinks, Tokyo, Japan).

Results

The number of RPGN patients treated in our hospital has been increasing since 2003 (Fig. 1). Although there was no clear difference in incidence between male and female patients (M:F 12:15), onset age of male RPGN patients ($N = 12$, 67.5 ± 13.55) was older than that of female patients ($N = 15$, 57.6 ± 10.91) ($P < 0.05$).

Serological analyses revealed no PR3-ANCA single-positive patients with RPGN (Table 1). Consistent with this result, WG patients were not included in this study. The age at first medical examination or admission (years old), serum Cr (mg/dl), CRP (mg/dl) and hemoglobin (Hb) (g/dl) were examined. The average age of patients in Group IV (54.3 ± 11.9) was significantly lower than those in other groups ($p < 0.05$) (Table 1). Serum Cr at admission in Group III was higher than that of the other groups (p value; Group III vs. Groups I, II and IV; 0.31, 0.06, and 0.15) (Table 2). There was no clear difference in anemia at admission among the groups (Table 2). CRP at admission in Group IV tended to be low, whereas in other groups, it varied widely (Table 2). Serological changes of MPO- and PR3-ANCA and a-GBM Ab at admission or just before admission/final data after treatments (EU) in each patient is summarized in Table 1.

The severity of RPGN was evaluated by a grading score [9], as shown in Tables 1 and 2. Average grades in each group are shown in Fig. 2 (Groups I vs. II vs. III vs. IV; 1.73 ± 0.65, 2.67 ± 1.53, 2.50 ± 0.55, and 1.57 ± 0.53). Higher grades at admission were observed in Groups II and III (Fig. 2).

Nine of 27 patients (six men: 66.67 ± 16.06 years old; grade 1: 0, grade 2: 4, grade 3: 4, grade 4: 1) showed abnormal shadows suggesting interstitial pneumonitis on X-ray and CT scan analyses summarized in Table 1. Fifty-six percent of those patients showed abnormal shadows indicating alveolar hemorrhage. Only one Group IV (serologically negative) patient had interstitial pneumonitis with alveolar hemorrhage. Average Birmingham Vasculitis Activity Score (BVAS) for patients with lung lesions was 21.33 ± 6.0 at admission. Their BVAS were correlated with grading scores [9] (grade 2: 19.75 ± 6.95, grade 3: 21.5 ± 5.80, grade 4: 27). In particular, three MPO-ANCA/anti-GBM Ab double-positive cases in Group III showed high BVAS (25, 27, and 30) at admission, with severe lung lesions (Table 1). In addition to interstitial pneumonitis, these patients showed vasculitis-related severe eye lesions and cardiovascular complications or stroke with visual disturbance.

We performed renal biopsies in seven patients (grade 1: 4, grade 2: 3) (Table 1). Patients with IgA nephropathy, lupus nephritis, and Goodpasture syndrome were included. All patients showed cellular and fibrous cellular crescents in >50% of glomeruli. Pauci-immune patterns in immunofluorescence analysis were observed in three patients (Group I/grade 1: 1, Group I/grade 2: 1, Group II/grade 1: 1), whereas one patient in Group III showed cellular crescents with linear IgG deposition in glomeruli. One patient in Group II/grade 2 showed 75% crescent formation (15/20 glomeruli) with not only perinuclear ANCA (P-ANCA) but also anti-hepatitis-C-virus (HCV) antibody and cryoglobulin. The systemic lupus erythematosus (SLE) patient (Group IV/grade I) showed cellular crescent formation with glomerular C1q deposition.

Patient and renal prognoses were divided into four groups: survival, dialysis-dependent end-stage renal disease (ESRD) alone, patient death alone, and ESRD/patient death. The prognosis for each grade of patients is shown in Fig. 3a, whereas the prognosis of each group of patients is summarized in Fig. 3b and Table 2. In MPO-ANCA single-positive Group I, only four patients did die or enter ESRD (4/11; 36.4%). Three patients in Group I who died had sepsis or lethal gastrointestinal bleeding with or without colonic penetration (Table 2). In the other groups, all patient deaths occurred within 1 year after onset. The major causes of patient death were severe infections and subsequent disseminated intravascular coagulation (DIC). Although all cases of death in Group III were due to pneumonia, one death, in a patient 93 years old, was based on aspiration pneumonia (Table 2). One patient in Group IV died because of cerebral hemorrhage.

All patients in Groups I, II, and III were treated with oral corticosteroid and/or methylprednisolone pulse therapy. Hemodialysis therapy was introduced directly without therapy by steroid or immunosuppressants in two of seven patients in Group IV. Methylprednisolone pulse therapy was used for most patients in Groups II and III (Group I: 46%, Group II: 100%, Group III: 83.3%, and Group IV:

cases did not include patients with RPGN due to WG or PR3-ANCA single-positive RPGN. This finding appears to be consistent with the results of the survey and support previous reports that incidence of MPO-ANCA-positive vasculitis and/or glomerulonephritis in Japan is much higher than that of PR3-ANCA-positive patients [7–10]. This tendency is reversed in European countries [3, 4, 12, 13, 21]. Although previous papers discussed human leukocyte antigen (HLA) allele frequency and environmental factors in relation to the discrepancy [14–17, 22, 23], underlying mechanisms remain unclear. At least, MPO-ANCA ELISA commercially available in Japan, including the method used in our study, exhibited high sensitivity and specificity for diagnosing ANCA-associated vasculitides and provided similar diagnostic value to those in Europe [24]. The rate of MPO-/PR3-ANCA double-positive cases in our study was 11.1% (3/27). Although the data was for a small number of patients only, this rate was higher than that in the results of nationwide Japanese surveys (3.8%) [10]. All three patients in our study were elderly men with a high level of severity. Although false positivity of PR3-ANCA in MPO-ANCA-positive patients should be carefully discussed and further study with more patients is required, this is interesting concerning the background of Japanese RPGN patients showing PR3-ANCA. At least in our study, this aging factor may influence the high grading scores, although there were only three patients.

A Japanese nationwide RPGN survey revealed that the primary disease of RPGN was anti-GBM nephritis in 6.5% (median age 54) [9, 10], Whereas reports from the USA and European countries showed that 12–20% of RPGN patients had anti-GBM Ab [24–26], suggesting that the frequency of anti-GBM-Ab-associated RPGN may be lower in Japan than in Western countries. However, in our study, frequency of RPGN with anti-GBM Ab was 22.2% (6/27), similar to that in Western countries. It is well known that RPGN with anti-GBM Ab frequently shows a severe clinical course with poor prognosis [9–11]. In fact, patients in our analysis showed higher Cr, CRP, and severity at admission and high mortality (50%). Five of six patients suffered ESRD and/or death. On the other hand, both MPO-ANCA single-positive (Group I) and serologically negative (Group IV) groups showed lower severity at admission. In MPO-ANCA single-positive patients, 10 /11 patients showed grades 1 or 2 at admission. However, as 7/11 patients in this group died or entered ESRD, there was a discrepancy in the initial grade and prognosis. Analysis of correlation between grade and prognosis revealed that all patients with grade 3 suffered ESRD and/or death, whereas grade 2 patients showed a highly variable prognosis, suggesting that careful treatment and evaluation of prognosis are required in grade 2 patients. However, grade 2 patients included those who had already been treated with steroid pulse and immunosuppressants in another hospital and then transferred to our hospital in severe condition. This initial treatment may influence the discrepancy of actual severity and grading score. Therefore, the reason about 40% patients in grade 2 died may be partly due to this initial bias.

Major causes of patient death (5/8 patients) were infectious complications, including DIC. This lethal infection was mainly linked to pneumonia by opportunistic pathogens, including *Pneumocystis carinii*, *Candida albicans*, and cytomegalovirus. This result was consistent with that of the nationwide survey [9–11]. Eighty percent (4/5) of patients who died due to severe infection had received immunosuppressants. Moreover, 80% of patients who were treated with the immunosuppressants in addition to steroid therapy died by infection-related causes. However, we found that most of these patients on immunosuppressants showed high disease activity with frequent recurrence of vasculitides. Therefore, one major reason they died due to opportunistic infections despite prophylactic pretreatments may be partly due to repeated and prolonged treatments with immunosuppressants because of recurrences. Immunosuppressive treatments may improve patient/renal survival rates [27–29] but are still closely linked to immunocompromised status, leading to lethal infectious complications, particularly in older patients and patients with strong activity of vasculitis.

This analysis further confirms clinicoepidemiological differences between Japanese and European patients with ANCA-related RPGN. In addition, the findings emphasize that ANCA-associated vasculitis is the most important causal disease for RPGN and has an extremely serious prognosis.

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Conflict of interest statement None.

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Table 1 continued

	Age	Sex	Year of admission	Serological analysis					BVAS	Lung lesions	Lung XP/CT	Renal bx	Lesions
				MPO/ ANCA	(EU)	PR3/ ANCA	(EU)	a-GBM (EU)	Diagnosis	Grade				
15	70	F	2002	–		–		–	RA/Cryogl	1	–		
16	58	F	2003	–		–		–	SLE	2	–		
19	66	F	2004	–		–		–	SLE	1	–	+	C Cres, C1q 1+
Ave	54.3	(*$P < 0.05$, vs. Groups I–III)											
SD	11.9												

MPO/ANCA myeloperoxidase antineutrophil cytoplasmic antibody, *EU* equivalent unit, *PR3/ANCA* proteinase 3 antineutrophil cytoplasmic antibody, *a-GBM* antiglomerular basement membrane, *BVAS* Birmingham Vasculitis Activity Score, *XP/CT* X-ray/computed tomography, *bx* biopsy, *MPA* microscopic polyangiitis, *RLV* renal limited vasculitis, *HCV* hepatitis C virus, *RA* rheumatoid arthritis, *LC* liver cirrhosis, *PN* polyarteritis nodosa, *GP* Goodpasture syndrome, *HSP* Henoch-Schönlein purpura nephritis, *SLE* systemic lupus erythematosus, *IgAN* immunoglobulin A nephropathy, *Cryogl* cryoglobulin, *RS* reticular shadow, *EP* emphysema, *PH* pulmonary hemorrhage, *NO* nodular opacity, *CV* cavitation, *PL* pleuritis, *GC* granulomatous change, *C Cres* cellular crescent, *F Cres* fibrous crescent, *Pim* pauci-immune, *Ave* average, *SD* standard deviation, + positive, – negative

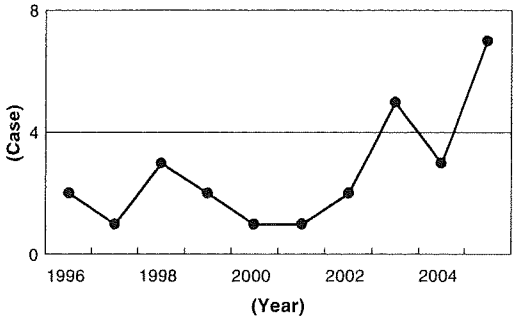

Fig. 1 Annual numbers of rapidly progressive glomerulonephritis (RPGN) patients who were admitted to Division of Nephrology, Juntendo University Hospital, from 1996 to 2006 and could be followed for more than 1 year

57%). Four of six patients in Group III received plasmapheresis therapy. The use of methylprednisolone pulse therapy increased with severity (grade 1: 50%, grade 2: 61.5%, grade 3: 80%, and grade 4: 100%). Only patients in grades 2 (30.7%) and 4 (100%) were treated with immunosuppressants, mainly with cyclophosphamide (Table 2). Eighty percent of patients treated with immunosuppressants died. These patients had strong disease activity and thus showed frequent recurrences of vasculitis and episodes of opportunistic infections, including *Candida albicans* and cytomegalovirus after treatments, even with prophylaxis treatments (Table 3).

Discussion

This study evaluated serological and prognostic outcomes in 27 patients with RPGN at the Division of Nephrology, Juntendo University Hospital, in the past 11 years. Although the average age of all RPGN patients was around 60 years (62.4 ± 13.3), that of male patients was >5 years older than that of female patients. However, this was partly due to the fact that serologically negative patients (M:F = 1:6) included relatively young women with rheumatic diseases and primary glomerulonephritis. In fact, the average age of ANCA-positive cases (66.6 ± 12.2) was older than that of the serologically negative group (54.3 ± 11.9).

Although we did not change the method for measuring ANCA and anti-GBM Ab in the study period, RPGN patients mainly with ANCA-associated vasculitis increased after 2003. In particular, in 2005, we had seven patients. This increment was consistent with the nationwide tendency [9, 10], suggesting that the increasing incidence may be partly due to an aging society, increased opportunity for serological measurement of MPO-/PR3-ANCA, and an increase in referral rates from home doctor to tertiary center hospitals such as university hospitals, based on an

(RLV). The incidence of WG among the ANCA-associated small-vessel vasculitides is higher than that of MPA and/or RLV in northern Europe [2–6], whereas conversely, the incidence of MPA/RLV is higher than that of WG in Japan [7]. Two nationwide Japanese surveys demonstrated that the number of patients with MPA and/or RLV is sixfold higher than those with WG in Japan [7–11]. In addition, it is also known that the presence of myeloperoxidase (MPO)-ANCA/proteinase 3 (PR3)-ANCA among Japanese patients with ANCA-associated vasculitides differs from that in European countries [4, 5, 12, 13]. Therefore, recent clinical studies indicate that ANCA-associated systemic vasculitides differ epidemiologically and serologically between Japanese and European countries [7, 11]. However, underlying mechanisms to explain the clinicoepidemiological difference remain unclear.

Vasculitis is a pathological process characterized by inflammation and necrosis of blood-vessel walls. In the kidney, vasculitis preferentially affects the small vessels (arterioles, capillaries, and venules). Therefore, microscopic hematuria with or without proteinuria is consistent with renal vasculitis. Over the past decade, clinical and experimental studies have provided compelling evidence that ANCA is a primary pathogenic factor in renal vasculitis, mainly by augmenting leukocyte–endothelial interactions [14–17]. The renal features of ANCA-associated systemic vasculitis include oliguria, microscopic hematuria, and proteinuria. Its central pathological feature is a pauci-immune focal segmental fibrinoid necrosis with extracapillary proliferation that may become crescentic glomerulonephritis with the accumulation of macrophages and epithelial cells in Bowman's space. This histopathological hallmark is frequently associated with rapid deterioration of renal function, clinically diagnosed as rapidly progressive glomerulonephritis (RPGN). Recent studies revealed an increasing incidence of ANCA-associated vasculitides in the older population [3, 18]. Therefore, RPGN patients with ANCA-associated vasculitides may show poor prognosis. In fact, although the prognosis of patients with RPGN is regarded as having improved over the past 20 years [14–17, 19], the prognosis of older patients with RPGN and their long-term renal survival is still a serious concern [11, 15, 20].

In this study, we retrospectively evaluated RPGN patients over the past 11 years who could be followed for more than 1 year or died within 1 year after onset, and we analyzed the incidence of ANCA-related vasculitis in RPGN, presence of MPO/PR3-ANCA, and their clinical outcomes. This hospital-based analysis may aid the understanding of clinicoepidemiological differences and serve as a baseline for future therapeutic approaches to ANCA-associated systemic vasculitides.

Materials and methods

Patients and assessment of disease manifestation

Twenty-seven patients with RPGN who were admitted to the Division of Nephrology of Juntendo University Hospital from April 1996 to December 2006 and could be followed for at least for 1 year after first admission ($N = 20$) or died within 1 year after onset ($N = 7$) were enrolled in this study. Renal involvement with features of glomerulonephritis, including erythrocyturia, erythrocyte cylindria, and glomerular proteinuria was seen in all patients. Patients with rapid aggravation of renal dysfunction with >30% rise in serum creatinine (Cr) levels over several days to a few months were defined as having RPGN [9, 10]. The hospital Ethical Committee approved the study design.

Age, gender, blood pressure, complete blood count (CBC), and serum markers such as C-reactive protein (CRP), Cr, MPO/PR3-ANCA and antiglomerular basement membrane (anti-GBM) antibody, and urinalysis were assessed at onset and admission and followed for >1 year. For evaluation of lung lesions, chest/abdominal X-rays and/or computed tomography (CT) scans were also examined and followed. Renal biopsies were performed in some patients (three men, four women; age 52.57 ± 12.34 years) who were in relatively good condition at admission. Correlation between clinical outcomes with or without each treatment, such as hemodialysis, plasmapheresis, steroid (oral corticosteroid and/or methylprednisolone pulse therapy), and immunosuppressants (mainly cyclophosphamide), and these clinical markers was evaluated. As most patients treated with immunosuppressants were followed not only by nephrologists but also by rheumatologists, indication of immunosuppressants was typically based on the clinical manual or guidelines of the Committee for Intractable Vasculitides in Japan, Ministry of Health, Labor, and Welfare, Japan. In addition, clinical severity of RPGN in each case was graded by the grading score of the Committee for Guidelines on Diagnosis and Therapy of Rapidly Progressive Glomerulonephritis in Japan, Special Study Group on Progressive Glomerular Disease, Ministry of Health, Labor and Welfare, Japan [9] (Tables 1 and 2). Patients were graded as follows: grade 1: 29.7% ($N = 8$), grade 2: 48.1% ($N = 13$), grade 3: 18.5% ($N = 5$) and grade 4: 3.7% ($N = 1$).

Serological tests for MPO-ANCA, PR3-ANCA, and anti-GBM antibody (anti-GBM Ab) were conducted using enzyme-linked immunosorbent assay (ELISA). MPO or PR3 were immobilized on microplates as antigens for each ELISA. Normal ranges in each MPO-, PR3-ANCA and anti-GBM Ab titers were settled under 10, 10, and 10 EU, respectively. All RPGN patients were serologically divided

Table 2 continued

	Diagnosis	Grade	s-Cr (mg/dl)	Hb (g/dl)	CRP (mg/dl)	Urinalysis prot (g/day, g/gCr)	Treatment			Prognosis			Causative bacteria/virus	Prophylaxis treatment
							Hema	Steroid/ pulse	ImSup	HD	ESRD	Death	Cause of death	
Group IV														
7	MPA	2	4.29	10	1.2	2.2	+	+						ST/AMPB
10	HSP	2	13.29	6.6	0.3	4.35	+	+		+	+			–
11	SLE	2	6.29	8.6	16.9	0.6	+	+	+ CY	+				ST/AMPB
13	IgAN	1	2.01	10	0.1	3.3	+	+						–
15	RA/Cryogl	1	1.84	10.9	0.2	4.85	+			+	+			–
16	SLE	2	4.95	6.9	1.2	1.4	+	+		+	+	+	Stroke	ST
19	SLE	1	1.94	8.7	0.8	1.5	+	+						ST
		Ave	4.9	8.8	3.0									
		SD	4.1	1.6	6.2									

MPA microscopic polyangiitis, *RLV* renal limited vasculitis, *HCV* hepatitis C virus, *RA* rheumatoid arthritis, *LC* liver cirrhosis, *PN* polyarteritis nodosa, *GP* Goodpasture syndrome, *HSP* Henoch-Schönlein purpura nephritis, *SLE* systemic lupus erythematosus, *IgAN* immunoglobulin A nephropathy, *Cryogl* cryoglobulin, *s-Cr* serum creatinine, *Hb* hemoglobin, *CRP* C-reactive protein, *prot* protein, *Hema* hematuria, *ImSup* immunosuppressant, *HD* hemodialysis, *ESRD* end-stage renal disease, *ND* not done, *CY* cyclophosphamide, *AZ* azathioprine, *CA* cyclosporine, *Col Penet* colonic penetration, *GIB* gastrointestinal bleeding, *Pnm* pneumonia, *DIC* disseminated intravascular coagulation, *UTI* urinary tract infection, *CMV* cytomegalovirus, *ST* sulfamethoxazole trimethoprim, *AMPB* amphotericin B, *GCV* ganciclovir

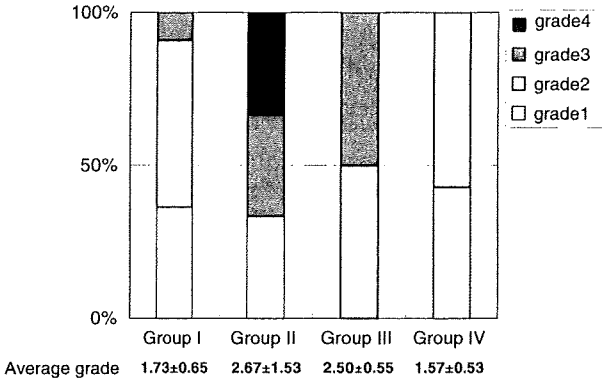

Fig. 2 Clinical grades in severity of each group at admission

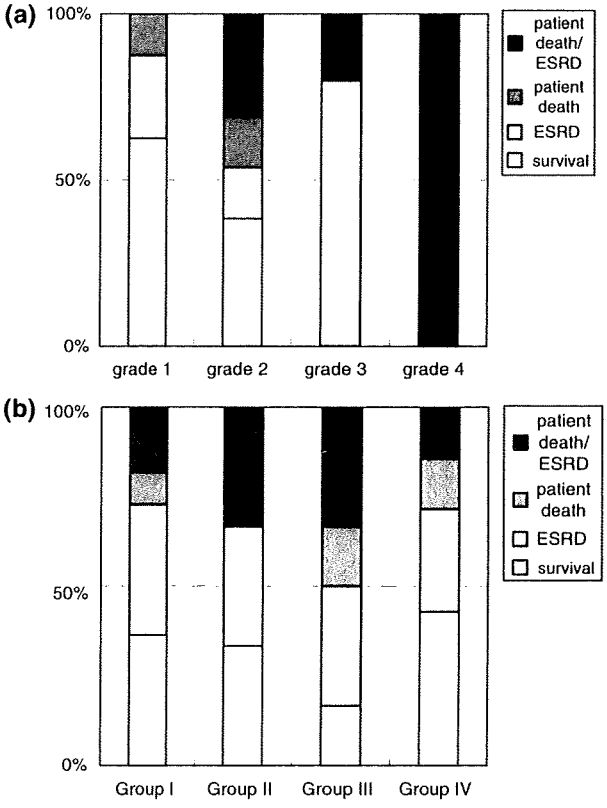

Fig. 3 Prognosis of each grade (**a**) or group (**b**). *ESRD* dialysis-dependent end-stage renal disease

improved recognition of RPGN and ANCA-associated vasculitis by general practitioners and family physicians [10]. Indeed, the Japan RPGN Registry Group first published Japanese guideline for RPGN in 2002 [9, 11].

The ratio of the number of patients with ANCA-associated vasculitis among all RPGN patients was 74% (20/27), slightly higher than results of nationwide Japanese RPGN surveys (MPO-ANCA 58.7%; PR3-ANCA 51.7%; MPO-ANCA 3.2%, and PR3-ANCA 3.8%) [9, 10]. Our

orrhages. In Japan, RPGN therapy guidelines have not included apheresis. However, as some randomized controlled trials (RCT) and case series reported the usefulness of apheresis, we have conducted investigations retrospectively for 10 years and studied renal and life prognoses in our hospital.

Patients and Methods

1. Patients

Sixty-four patients, who were diagnosed with RPGN clinically based on the WHO definition, among all patients hospitalized in the Division of Nephrology during the 10 years from 1995 to 2005, were study subjects. Renal biopsy was performed in 36 cases of RPGN patients. Those with therapies started from the time of admission, RPGN recrudescence, collagenosis, drug-induced RPGN, and subjects not showing crescent formation, despite renal biopsy, were excluded.

2. Treatment regimen

All 39 patients, who became subjects, were on steroids except one who was included in the apheresis group. Some received steroids pulse therapy, while others did not. In each group the rate of patients who received steroid pulse therapy against those who did not receive it did not show statistical difference (6/9 patients of the former group received steroid pulse therapy, while 17/30 patients of the latter received it; $p=0.59$). As for steroids, both prednisolone and methyl-prednisolone were used. Converted into methyl-prednisolone, a total dosage for 8 weeks was calculated, and the average daily dose per 1 kg of patient weight was studied. Two types of apheresis were conducted double membrane filtration and single plasma exchange. For the double membrane filtration on a plasma separator, the plasma flow was achieved with an Asahi Medical Co., Limited device and an Evaflux 2A (Kuraray Medical Corp) was used secondarily. The fluid substitution in one session of double membrane filtration was 8% albumin 500 ml (a blood cleaner KPS 8800CE or KM 8800), and plasma flow was used for simple plasma exchanges to achieve exchanges of 46.5 ± 5.4 ml/kg with fresh frozen plasma. There were 2-7 sessions, or 3.4 ± 1.4 times sessions on average, per case.

3. Patient follow up

At the time of admission, serum Cr, serum total protein and albumin, urea nitrogen, CRP, proteinuria level, uro-hematid numbers, 24 hours creatinine clearance as the glomerular filtration rate, and serum ANCA antibody titer (ELISA Method, Biocarb Diagnostics, Sweden) were evaluated. As for serum Cr, proteinuria and serum ANCA titers, the amounts measured 8 weeks after starting therapy were compared. In addition, to judge the initial therapy efficacy, renal and life prognoses were studied.

4. Statistical analysis

For the statistical analysis, each index was expressed using the average±standard deviation. Student's t-tests were used to determine the significance of differences between two groups. The rate of patients with or without steroid pulse therapy was analyzed using the χ^2 test. In order to examine if apheresis is independent indicator to predict the renal outcome, multivariate analyses were performed using JMP version 5.1 (SAS for Windows, Cary, NC, USA) as the software. The dependent variable were serum Cr values and independent variables were age, sex, proteinuria, MPO-ANCA titer, Cr, CRP, total steroid doses and apheresis. Because renal biopsy was not performed in all patients, pathological findings were not included in the independent variables. P value <0.05 was considered significant for every test.

Results

The ages of the 64 RPGN patients were 16-81, males 39, and females 25. The 24 excluded cases were 6 RPGN with recurrences, 8 who already had a therapy history on admission, 2 drug induced disease, and 2 with acute glomerulonephritis. There was one case each of end-stage renal failure, diabetes mellitus, focal segmental gomerulosclerosis, membranous nephropathy, hypohydremia, mixed connective disease and tubular disease. The 39 RPGN patients were 28-79 years of age (average 62.3 ± 12.9), males 17 and females 22. Renal biopsy was conducted on 27/39 (62.5%). Apheresis was carried out in 9 of the 39, including 5 double filtration plasmapheresis cases and 4 simple plasma ex-

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Table 2 Treatment profiles and prognosis

	Diagnosis	Grade	s-Cr (mg/dl)	Hb (g/dl)	CRP (mg/dl)	Urinalysis prot (g/day, g/gCr)	Treatment			Prognosis				Causative bacteria/virus	Prophylaxis treatment
							Hema	Steroid/ pulse	ImSup	HD	ESRD	Death	Cause of death		
Group I															
1	RLV/HCV	1	4.69	10.4	0.3	2	+	+				+	Col Penet./GIB		ST/AMPB
3	MPA	2	16.74	8.5	1.9	0.79	+	+		+	+				ST
4	MPA	3	5	7.6	1.3	2.3	+	+		+	+				ST
9	MPA/HCV/LC	2	3.19	6.2	0.3	4.8	+	+							–
12	MPA	2	3.16	8.3	33.5	ND	+	+	+ CY	+	+	+	Sepsis/DIC	*S. aureus*/ *P. aeruginosa*	ST/AMPB
17	RLV	2	4.85	9.4	1.2	3.5	+	+		+	+	+	Col Penet/Pnm	*P. aeruginosa*	ST
21	RLV	1	8.84	8.5	1.8	6.1	+	+		+	+				ST
22	RLV	1	1.77	10.9	0.2	ND	+	+							–
23	MPA	2	5.54	7	4.3	2.1	+	+							ST/AMPB
24	RLV	1	11.02	7.3	0.9	3.4	+	+		+	+				ST
25	RLV	2	3.68	7.9	0.1	ND	+	+							ST
		Ave	6.23	8.36	4.16										
		SD	4.4	1.4	9.8										
Group II															
2	PN/RA	3	5.01	6.1	17.5	0.3	+	+		+	+				ST
5	MPA	4	4.18	8.9	16.7	1.3	+	+	+ CY	+	+	+	UTI/DIC/GIB	*P. fluorescans*/ *C. albicans*	ST/AMPB
6	MPA	1	4.18	10.8	2.3	0.83	+	+							–
		Ave	4.46	8.60	12.17										
		SD	0.48	2.36	8.55										
Group III															
8	RA	2	5.74	9.3	6.3	0.59	+	+	+ CY			+	Pnm/DIC	*P. carini*/ *C. albicans*	ST/AMPB
14	GP s/o	2	8.68	10	6.9	3.84	+	+							ST/AMPB
18	MPA	2	8.77	6.5	1.3	ND	ND	+	+ CY/CA/AZ	+	+	+	Pnm/DIC/GIB	CMV/ S epidermidis	ST/AMPB/GCV
20	MPA	3	8.95	10	0.6	4.64	+	+		+	+				ST
21	RLV	3	6.3	8.8	23	0.06	+	+		+	+				ST
26	MPA	3	3.25	8.7	5.7	1.5	+	+		+	+	+	Aspiration Pnm		ST/AMPB
		Ave	6.95	8.88	7.30										
		SD	2.3	1.3	8.1										

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Original

Short-Term Effects of Apheresis on Renal Function and Proteinuria in the Treatment of Rapidly Progressive Glomerulonephritis

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For rapidly progressive glomerulonephritis (RPGN), immunosupressants such as steroids constitute a major therapy, and apheresis is done on a case by case basis. The aim of the present study to assess the efficacy of apheresis in the treatment of RPGN. We investigated the clinical course of 39 patients with RPGN during the 10 years from 1995 to 2005, retrospectively. They were divided into two groups, apheresis group and non-apheresis group. All patients except one were administrated steroids. Nine patients in apheresis group were carried out double filtration plasmapheresis or single plasma exchange and there were 2-7 sessions per case. There were no statistically significant differences in the renal function as estimated by serum creatinine levels or inflammatory reactions. The average amounts of proteinuria were 3.4±2.5 g/day (0.38-6.44 g/day) in the apheresis group and 1.5±1.4 g/day (0.12-5.58 g/day) in the non-apheresis group ($p=0.007$). However, total steroid dosages were 0.49±0.22 mg/kg/day in the apheresis group and 0.63±0.16 mg/kg/pay in non-apheresis group, indicating a significantly smaller steroid dose in the apheresis group ($p=0.04$). Finally, we found better renal prognosis and mortality in the apheresis group. These results suggest that apheresis has an advantage in the treatment of RPGN patients in terms of the improvements of renal and life prognosis.

Key words: rapidly progressive glomerulonephritis, plasmapheresis, ANCA, vasculitis, proteinuria

Introduction

Rapidly progressive gromerulonephritis (RPGN) is defined by the World Health Organization (WHO) as a hematuria crisis of sudden or insidious onset, proteinuria, anemia and progressive renal insufficiency. Generally speaking, from a histological perspective, nephritides forming necrotizing crescents, cellular crescent formation of multiple glomeruli are common[1]. RPGN is a clinical syndrome, and according to the classification by Couser et al[2], the basal disease is classified into 3 types, i.e. Type I of glomerular basement membrane antibody type, Type II of RPGN immunocomplex type, and Type III of RPGN pauchi-immune type.

In Japan, according to research on progressive renal insufficiency by the Ministry of Health, Labour and Welfare published in 1999, the pauci-immune type shows frequent serum antineautrophil cytoplasm autoantibodies (ANCA)-positivity (39.6%), anti- glomerular basement membrane (GBM) antibody type (6.5%), immunocomplex type (3.4%), microscopic polyangitis (17.8%), Wegener's granulomatosis (2.5%), and systemic lupus erythematosus (5.9%) respectively. However RPGN in the US and Europe has higher rates of anti-GBM vasculitis somewhat different from ours[3].

For this disease, immunosuppressants such as steroids constitute a major therapy, and apheresis is done on a cases by case basis. In Europe, apheresis is recommended for cases with anti-GBM antibodies, ANCA positive cases with high serum creatinine (Cr) levels, and cases with pulmonary hem-

Table 4 Studies of the efficacy of apheresis therapy in RPGN identified by a through literature search

Author	Year	Type of study	Case	Patient's disease	Apheresis	Results
Mauri[10]	1985	RCT	22	CN	PE	Benefit on serum creatinine
Glockner[7]	1988	RCT	26	CN	PE	No benefit for renal function and survival
Rifle[11]	1990	RCT	14	unknown	unknown	Benefit on renal survival
Pusey[6]	1991	RCT	48	WG, MPA, CN	PE	Benefit on renal function, but no benefit on survival
Cole[8]	1992	RCT	32	CN	PE	No benefit for renal survival and survival
Furuta[12]	1998	RCT	24	CN	LCAP	Benefit on serum creatinine
Matic[19]	2001	case series	3	WG 2, Anti GBM 1	IA	Benefit
Zauner[9]	2002	RCT	23	RPGN	PE	No benefit
Frasca[14]	2003	NRCT	26	WG, MPA	PE	Benefit on death
Nakamura[15]	2004	NRCT	22	MPO-ANCA AD	DFPP	Benefit on death
Hasegawa[16]	2006	case series	21	MPO-ANCA AD	GCAP, LCAP	Benefit for complications, renal function recovered in 76.5%
Jayne[13]	2007	RCT	137	ANCA AD (WG, CN)	PE	Benefit on renal survival

RCT: randomized controlled trial, NRCT: non-randomized controlled trial, CN: crescentic glomerulonephritis without Anti-GBM nephritis, WG: Wegener's granulomatosis, MPA: microscopic polyangitis, RPGN: rapidly progressive gromerulonephritis without Anti-GBM nephritis, MPO-ANCA AD: MPO-ANCA associated disease, PE: single plasma exchange, LCAP: leukocytapheresis, IA: immunoadsorption, DFPP: double filtration plasmapheresis, GCAP: granulocytapheresis.

search. Pusey et al[6] evaluated 48 cases and divided them into a pharmacotherapy group and a group receiving a combination of plasma exchange and drugs, based on renal functions. Although the rate of weaning from dialysis was high in the apheresis group, life prognosis was poorer than that of the pharmacotherapy group. Glocker et al[7] reported RCT in 26 cases and who showed no significant difference in death rates from dialysis weaning by plasma exchange. The report by Cole et al[8] started that the rate of dialysis weaning was same and that there were 2 deaths in the plasma exchange group but none in the control group. Zauner et al[9] reported that the HD weaning rate was the same. However, Mauri et al[10] reported in 22 RCT cases that there was a significant improvement in renal functions of cases with serum Cr levels exceeding 800 μmol/L. Rifle et al[11] and Furuta et al[12] reported in 24 RCT cases that serum Cr levels had decreased significantly with lymphocytapheresis. Jayne et al[13] reported that the rate of dialysis weaning was better in the plasma exchange group. In case series, Frasca et al[14] studied 26 ANCA positive cases and reported that the rate of dialysis weaning was better during the acute phase and there were fewer deaths in the plasma exchange group. Nakamura et al[15] reported a reduction in urinary podocytes and no deaths in the plasma exchange group. Hasegawa et al[16] conducted granulocytapheresis and leukocytaphereis on 21 MPO-ANCA positive cases, and serum Cr levels improved in 13 of the 17 responsive cases. As shown here, many reports describe improvements in renal functions while discussing evaluations of prognosis.

In our retrospective studies, we found improvements in both life and renal prognosis with apheresis therapy, finding not reported previously. Considering the state usually requiring hemodialysis as complete renal failure, the number of such cases was significantly reduced in the apheresis group. As for life prognosis, the long-term prognosis was not evaluated (On-dialysis cases or patients who changed hospitals due to moving could not be traced.). However, the evaluation done after 8 weeks, when a stable state had returned, after surviving the acute phase with the highest death rate for vasculitis, is considered to be quite valuable. In fact, we studied traceable cases and found that patients were off dialysis and alive for at least 2 years afterwards.

We studied creatinine clearance, and serum Cr levels for the evaluation of renal functions on admission in both groups, albumin levels for the evaluation of nutrition, hematuria for the evaluation of vasculitis, and CRP and leukocyte levels for the evaluation of inflammatory state, We detected no differences between the two groups. Their systemic states were considered to be similar. Only the