Original Paper

nephron
Clinical
Practice

Nephron Clin Pract 2006;102:c35–c42
DOI: 10.1159/000088313

Received: February 11, 2005
Accepted: May 30, 2005

Clinical Efficacy of Intravenous Immunoglobulin for Patients with MPO-ANCA-Associated Rapidly Progressive Glomerulonephritis

Toshiko Ito-Ihara[a] Takahiko Ono[a] Fumiaki Nogaki[a] Katsuo Suyama[a]
Mari Tanaka[b] Satomi Yonemoto[b] Atsushi Fukatsu[a] Toru Kita[a]
Kazuo Suzuki[c] Eri Muso[b]

[a]Department of Nephrology and Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, [b]Division of Nephrology, Kitano Hospital, Tazuke Kofukai Foundation, Medical Research Institute, Osaka, and [c]Biodefense Laboratory, National Institute of Infectious Diseases (NIID-NIH), Tokyo, Japan

Key Words
Intravenous immunoglobulin · MPO-ANCA · Tumor necrosis factor-α

Abstract

Background: To determine whether intravenous immunoglobulin (IVIg) can control disease activity in patients with myeloperoxidase-antineutrophil cytoplasmic antibody (MPO-ANCA)-associated rapidly progressive glomerulonephritis (RPGN). ***Methods:*** Twelve patients with serologically and histologically confirmed MPO-ANCA-associated RPGN (7 men, 5 women; mean age 71 ± 3 years) received IVIg (400 mg/kg/day) alone for 5 days. The effects of IVIg were evaluated by white blood cell counts, serum C-reactive protein levels, Birmingham Vasculitis Activity Score, rate of change in reciprocal creatinine (1/Cre), and plasma tumor necrosis factor-α levels after IVIg administration. Corticosteroids with or without cyclophosphamide were commenced after IVIg. ***Results:*** After IVIg treatment, a significant decrease was observed in white blood cell count ($p < 0.05$), C-reactive protein values ($p < 0.001$), and Birmingham Vasculitis Activity Score ($p < 0.001$) concomitant with the amelioration of systemic symptoms. The rate of change in 1/Cre significantly improved ($p < 0.05$). Plasma tumor necrosis factor-α levels that were significantly elevated in patients before IVIg compared with normal controls ($p < 0.0001$), rapidly declined after IVIg with a significant reduction ($p < 0.05$). Three months post-treatment with IVIg, all patients showed improvement of disease without serious infectious complications. ***Conclusion:*** IVIg is a potential component of remission induction therapy for patients with MPO-ANCA-associated RPGN.

Introduction

Antineutrophil cytoplasmic antibody (ANCA)-associated rapidly progressive glomerulonephritis (RPGN), which occurs in Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA) [1], leads to renal failure through systemic vasculitis and diffuse crescentic glomerulonephritis. Since crescent formation has features of delayed-type hypersensitivity and is accompanied by the presence of T cells, macrophages, and fibrin in the glomerular lesion [2], high-dose corticosteroids and cyclophosphamide (CYC) are standard treatment for ANCA-associated RPGN; however, such immunosuppressive therapy is often complicated by severe infection in elderly patients [3]. Therefore, to induce early remission of

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2006 S. Karger AG, Basel
1660-2110/06/1021-0035$23.50/0

Accessible online at:
www.karger.com/nec

Eri Muso, MD, PhD
Division of Nephrology, Kitano Hospital
Tazuke Kofukai Foundation, Medical Research Institute
2-4-20, Ohgimachi, Kita-ku, Osaka 530-8480 (Japan)
Tel. +81 6 6312 8824, Fax +81 6 6312 8867, E-Mail muso@kitano-hp.or.jp

the disease including renal insufficiency and to avoid fatal side effects, it is important to establish a therapeutic regimen that can maintain the immune potency of such patients.

Intravenous immunoglobulin (IVIg) has been advocated as a safe and effective treatment for other immune-mediated diseases, such as Kawasaki disease, idiopathic thrombocytopenic purpura, Guillain-Barré syndrome, and chronic inflammatory demyelinating polyneuropathy. European investigators have recently shown that IVIg is clinically useful and safe when administered in conjunction with immunosuppressive drugs, helps suppress disease activity for at least 1 year, and consequently reduces the total dose of immunosuppressive agents in patients with ANCA-associated vasculitis, mainly WG with or without RPGN [4, 5]. Accumulating evidence suggests that it works in multiple phases of immune response; neutralization of circulating pathogenic antibodies, Fc receptor modulation and blockade, or suppression of antibody-dependent cellular toxicity, natural killer cell function, autoantibody production, and complement activation [6]. In addition, Guillain-Barré syndrome patients who received IVIg showed clinical recovery in parallel with reduction in serum levels of tumor necrosis factor-alpha (TNF-α), suggesting an important role of IVIg in inhibiting cytokine activity [7].

Here we report a study evaluating the effectiveness of IVIg as an initial treatment, preceding corticosteroids with or without CYC, in 12 patients with MPO-ANCA-associated RPGN. Since it remains unclear whether IVIg is independently effective [8], we investigated the potential immunomodulatory effect unique to IVIg by comparing renal function, clinical score (Birmingham Vasculitis Activity Score, BVAS), and circulating TNF-α levels before and after IVIg treatment. Since MPO-ANCA-associated RPGN is more common than those related to PR3-ANCA in Asia, which clearly contrasts with the incidence in Western countries, we were able to recruit sufficient numbers of patients with MPO-ANCA-associated RPGN to examine the results statistically. This is the first report of the effects of IVIg in MPO-ANCA-specific RPGN patients.

Patients and Methods

Patients

Twelve consecutive patients with MPO-ANCA-associated RPGN (7 men and 5 women; mean age 72 years; range 57–83 years), who were admitted to the Nephrology Department of Kyoto University Hospital and Kitano Hospital between January 2001 and February 2003, were enrolled in this study (table 1). All patients were diagnosed as having MPA because of elevated serum MPO-ANCA as well as characteristic pathology observed in the renal biopsy specimen before treatment. In all patients, the disease was confirmed based on the definition of MPA described by the Chapel Hill Consensus Conference [9]. Renal involvement was seen in all patients. Patient with rapid aggravation of renal dysfunction with more than 30% rise in serum creatinine (Cre) levels were defined as having RPGN. All except 1 (patient No. 2) were newly diagnosed patients who had been transferred from other hospitals due to the onset of RPGN. Patient 2 had previously demonstrated MPO-ANCA-associated RPGN and recovered after 6 years of treatment with prednisolone and CYC. He again developed fever, arthralgia, and myalgia with elevation of white blood cells (WBC), C-reactive protein (CRP), Cre (33% rise) and MPO-ANCA levels, leading to a diagnosis of MPA recurrence. All patients provided written informed consent for renal biopsy as well as treatment according to the protocol. The hospital Ethical Committee approved the study design.

Histological Evaluation

All renal biopsy specimens showed MPA. Hematoxylin and eosin, periodic acid-Schiff, periodic acid silver-methenamine, Masson trichrome, and elastica van Gieson stain were performed. Direct immunofluorescence studies were performed using frozen sections of renal tissue. Histological activity was assessed as follows: active crescent formation (%) = number of glomeruli with cellular and fibrocellular crescent formation/number of glomeruli without global sclerosis × 100. Each biopsy specimen was scored by two pathologists independently. If there was disagreement between the scores, patients were re-examined by the team to determine a final diagnosis by consensus.

Treatment Protocols

After serum MPO-ANCA, WBC, CRP, renal biopsy, and BVAS were all evaluated to establish a definite diagnosis and activity grading, IVIg was administered intravenously as an initial treatment for 5 consecutive days (400 mg/kg/day). Patients 1–10 received freeze-dried sulfonated human normal immunoglobulin (Kenketsu Venilon-I, Teijin Co., Ltd, Tokyo, Japan), and patients 11 and 12 received freeze-dried polyethylene glycol-treated human normal immunoglobulin (Kenketsu Glovenin-I, Nihon Pharmaceutical Co., Ltd, Tokyo, Japan). Both preparations were free from IgG aggregations that can form in prepared solutions containing sucrose. During IVIg treatment, none of the patients received any other immunosuppressive agents, any blood transfusions, or any intravascular volume repletion treatment. Following the post-IVIg treatment evaluation of clinical scores and laboratory data, all patients received immunosuppressive treatment with oral corticosteroids with or without CYC (table 2). Oral corticosteroids (prednisolone 0–1.0 mg/kg/day) were administered dependent on disease severity and patient age. Methylprednisolone pulse and oral CYC (25–50 mg/day) were also administered to 3 of 12 and 8 of 12 patients, respectively.

Assessment of Disease Activity

Complete blood count and serum markers such as CRP, Cre, and MPO-ANCA were evaluated at the onset of disease, before, and immediately after (mean 6.3 days, range 0–17 days) IVIg, and 1 and 3 months after IVIg. Disease activity was assessed by BVAS before and immediately after IVIg and 1 and 3 months after IVIg. BVAS consists of 59 predefined items derived from clinical, radio-

Table 1. Characteristics of the 12 patients (M/F = 7/5)

Patient	Age	Sex	Data before treatment								
			WBC/µl	CRP mg/l	Cre µmol/l	MPO-ANCA, EU	Active crescents, %	BVAS	extrarenal manifestations	pulmonary involvement	latent and antibiotics resistant infections
1	82	F	7,000	80	283	239	81	19	S, F, E		HBV carrier
2	75	M	9,600	178	106	435	0	23	S, F, A, C, N		MAC, *K. pneumoniae*
3	61	F	8,100	43	126	244	71	15	S, F		
4	82	M	9,400	104	417	159	71	14	S, A		
5	64	F	12,100	154	737	276	81	19	S, F, L	infiltrate	
6	59	M	10,200	139	389	140	90	15	S, F		*Aspergillus*
7	83	F	4,700	1	258	617	60	20	S, F, Ab		
8	82	F	14,700	113	210	306	38	21	S, F, N		
9	57	M	10,700	99	357	980	64	19	S, A, N		
10	62	M	10,100	101	732	370	80	25	S, L, N	nodules	
11	75	M	9,300	60	1,012	82	33	27	S, E, L	infiltrate	HBV carrier, MRSA, *P. aeruginosa*
12	67	M	11,900	68	401	1,740	78	19	S, L	hemoptysis	MRSA
Mean	71		9,820	95	419	466	62	20			
Reference range			3,500–9,100	<3	<106	<20		0			

WBC = White blood cell count; CRP = C-reactive protein; Cre = serum creatinine; MPO-ANCA = myeloperoxidase antineutrophil antibody; BVAS = Birmingham Vasculitis Activity Score; S = systemic symptom (malaise, myalgia, weight loss); F = fever; A = arthralgia; C = cutaneous; E = ear-nose-throat; L = lung; Ab = abdomen; K = kidney; N = neuropathy; HBV = hepatitis B virus; MAC = *Mycobacterium avium* complex; *K. pneumoniae* = *Klebsiella pneumoniae*; MRSA = methicillin-resistant *Staphylococcus aureus*; *P. aeruginosa* = *Pseudomonas aeruginosa*.

Table 2. Treatment and outcomes after IVIg treatment

Patient	Initial immunosuppressive treatment just after IVIg				Treatment after 3 months			
	mPSL pulse	PSL dosage mg/kg/day	CYC dosage mg/kg/day	dialysis	PSL dosage mg/kg/day	CYC dosage mg/kg/day	dialysis	Cre µmol/l
1	–	0.3	0.7	–	0.3	0	–	124
2	–	0.7	0.4	–	0.3	0.4	–	88
3	–	1.0	1.0	–	0.5	1.1	–	92
4	–	0.5	–	HD	0.7	–	–[1]	308
5	–	1.0	1.0	–	0.6	1.1	–	204
6	1 g, 3 days	0.7	0.8	–	0.3	0	–	177
7	–	0.5	1.3	–	0.3	0	–	203
8	–	0.6	–	–	0.4	–	–	87
9	1 g, 3 days	0.7	0.7	–	0.2	1.7	–	141
10	–	0.8	0.8	–	0.4	0.8	–	353
11	–	0	–	HD	0.5	–	HD	723[2]
12	0.5 g, 3 days	0.5	–	–	0.5	–	–	131
Mean		0.6	0.8[3]		0.4	0.6[3]		173[4]

mPSL pulse = Methylprednisolone pulse therapy; PSL = prednisolone; CYC = cyclophosphamide; Cre = serum creatinine; HD = hemodialysis.

[1] Cessation of HD; [2] Cre level before a hemodialysis; [3] mean CYC dose of 8 patients; [4] patient 11 was excluded.

logic, and laboratory evaluations in 9 organs systems. Each organ system carries a weight (ranging from 0 to 12), and an item is positively scored if the investigator considers it present and caused by active vasculitis. The maximal score is 63 with higher scores indicating more active disease [10].

Evaluation of the Progression Rate of Renal Dysfunction: Rate of Change in 1/Cre

To determine whether rapid progression of renal failure was occurring in these patients, the rate of change in reciprocal Cre (1/Cre) levels (dl/mg/day) was compared before and after IVIg treatment [11]. Briefly, Cre levels (mg/dl) were evaluated at five time points as follows: the first visit to the primary care physician with initial symptoms (Cre1 at time 1 [T1]), admission to the hospital (Cre2 at time 2 [T2]), transfer to the nephrology unit (Cre3 at time 3 [T3]), just before IVIg treatment (Cre4 at time 4 [T4]), and after IVIg treatment without receiving other immunosuppressive treatment (Cre5 at time 5 [T5]). The unit of time was 1 day. The largest value among (1/Cre4-1/Cre1)/(T4-T1), (1/Cre3-1/Cre1)/(T3-T1), and (1/Cre2-1/Cre1)/(T2-T1) was regarded as a rate of change in 1/Cre before IVIg and compared with (1/Cre5-1/Cre4)/(T5-T4). Only patient 11 was excluded from this evaluation because he was already undergoing permanent hemodialysis before IVIg because of rapidly deteriorating renal function.

Measurement of Plasma Cytokines

Venous blood samples were drawn from patients before and after IVIg, and before initiating immunosuppressive therapy. Plasma samples were stored at −80°C until use. Plasma samples were available for 9 patients, in whom cytokine levels were compared before and after IVIg. According to the manufacturer's instructions, the following cytokines were measured: TNF-α, interleukin (IL)-6, IL-8, IL-1β using Human Cytokine UltraSensitive ELISA kit (Biosource International, Camarillo, Calif., USA). Absolute values of these cytokines were also measured using the blood samples from 12 normal controls, and compared with those of the 9 patients. An average of +2 standard deviations (SD) for each cytokine level in the 12 normal controls was considered the upper limit of the normal range.

Statistical Analysis

The significance of differences between pre- and post-IVIg values of clinical laboratory data was assessed by paired Student's t test using StatView II software (version 5.0 for Macintosh; SAS Institute Inc., Cary, N.C., USA). To compare the cytokine levels of normal controls to the pre-IVIg cytokine levels of patients, unpaired t test was used. Fischer's exact test was performed to compare BVAS and laboratory data before IVIg with those after 1 and 3 months. A p value <0.05 was considered significant. All data were expressed as mean ± SEM.

Results

Clinical and Pathological Features before IVIg Treatment

Demographic and clinical characteristics and renal histological findings of 12 patients enrolled in this study are summarized in table 1. All patients were clinically diagnosed as having RPGN with micro- or macroscopic hematuria and rapidly worsening renal function. The mean Cre value was 419 μmol/l (range 106–1,012) just before IVIg treatment. The mean BVAS was 20 (range 14–27) before treatment. Laboratory tests demonstrated increased levels of WBC (mean 9,820/μl; range 4,700–14,700), CRP (mean 90 mg/l; range 1.0–178; reference <3), and MPO-ANCA level (mean 466 EU; range 82–1,740; reference <20). Crescentic glomerulonephritis with or without systemic features of MPA was present in all patients. Mean percentage of active crescent formation was 62%. Direct immunofluorescence study demonstrated pauci-immune deposition (scant depositions of immunoglobulins) in all patients.

Clinical Responses

(1) The change in WBC count and CRP value: Total WBC counts were 9,820 ± 740/μl before IVIg and decreased to 7,960 ± 870/μl after IVIg; the pre- and post-treatment levels were significantly different ($p < 0.01$). A significant decrease was also observed in neutrophil, lymphocyte, and eosinophil differential counts: the decrease in neutrophils was the most significant (pre-IVIg 7,950 ± 740/μl; post-IVIg 6,010 ± 800; $p < 0.001$). Mean CRP value was 97 mg/l (range 5–178) at the onset of vasculitis, and 95 mg/l (range 1–178) just before IVIg treatment. Following IVIg, the mean CRP value decreased significantly to 57 mg/l (range 1–124) ($p < 0.001$; fig. 1a).

(2) Rapid effect on renal function: The mean Cre level was 89 μmol/l (range 44–124) at the onset of disease, but increased to 365 μmol/l (range 106–737) in 62 ± 14 days (range 22–185) just before IVIg (fig. 1b). As a sensitive method of detecting rapid changes in renal dysfunction, we calculated the rate of change in 1/Cre before and after IVIg as shown previously [11]. The rate of change in 1/Cre was −0.041 ± 0.020 dl/mg/day before IVIg and increased to 0.007 ± 0.004 dl/mg/day after IVIg ($p < 0.05$).

(3) Temporal profiles of pro-inflammatory cytokine levels before and after IVIg: Before IVIg treatment, the plasma TNF-α levels were significantly elevated in patients compared to normal controls (patients, pre-IVIg 4.23 ± 0.92 pg/ml vs. control, 0.23 ± 0.40 pg/ml; $p < 0.0001$). After IVIg treatment, the plasma TNF-α levels decreased significantly (pre-IVIg 4.23 ± 0.92 pg/ml vs. post-IVIg 2.40 ± 0.53; $p < 0.05$; fig. 2). Plasma IL-6 levels (pg/ml) were significantly higher before IVIg treatment in patients compared to that in normal controls (pre-IVIg 2.75 ± 4.45 vs. control, 0.00 ± 0.00; $p < 0.05$). The plasma IL-6 levels decreased on average after IVIg treatment, but the difference did not reach significance (data not shown).

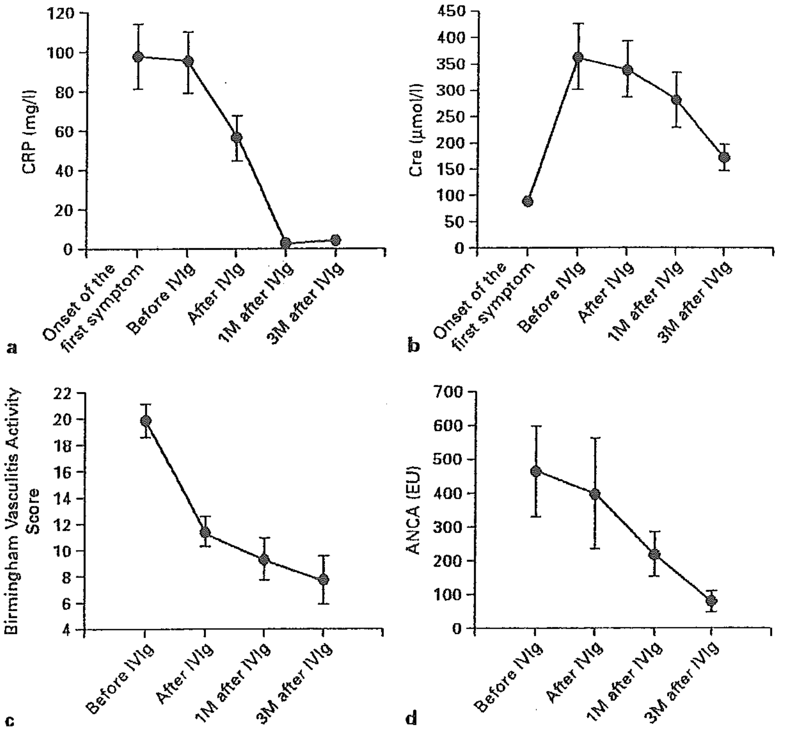


Fig. 1. Three-month follow-up of the patients. **a** Serum C-reactive protein (CRP), mg/l (n = 12). **b** Serum creatinine (Cre) level, µmol/l (n = 11). **c** Birmingham Vasculitis Activity Score (n = 12). **d** Anti-neutrophil cytoplasmic antibody (ANCA) (n = 12). M = Months.

The plasma IL-8 levels of patients before IVIg treatment did not significantly differ from that of normal controls or that after IVIg treatment (data not shown). Some patients showed markedly elevated IL-6 (patient No. 1, 6, 8–10) and IL-8 (patient No. 1, 5, 8–10) levels before IVIg treatment, which decreased after IVIg treatment. The plasma IL-1β levels (pg/ml) of patients before IVIg treatment did not significantly differ from that of normal controls or that after IVIg treatment (data not shown).

(4) BVAS: After IVIg treatment, significant reduction was seen in BVAS (pre-IVIg 20 ± 1; post-IVIg 11 ± 1; $p < 0.0001$; fig. 1c). First, systemic symptoms improved; malaise (8 of 12), myalgia (3 of 3), arthralgia/arthritis (2 of 2), and fever (7 of 9). Before IVIg treatment, hematuria and proteinuria were observed in all patients; rapid aggravation of renal dysfunction with more than 30% rise in Cre was also noted in all patients. Lung involvement was seen in 4 patients; 1 showed nodular lesions, 1 showed hemoptysis and the other 2 showed infiltrative lesions. These lesions improved partially following IVIg.

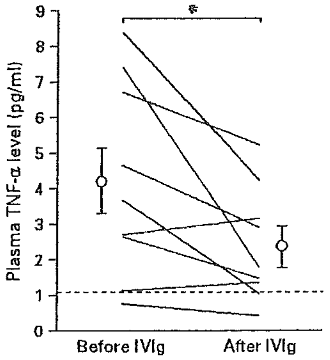


Fig. 2. Plasma TNF-α levels (pg/ml) before and after IVIg (n = 9). The dotted line represents the upper limit of the normal range. * $p < 0.05$ vs. before IVIg.

Immunosuppressive Therapy following IVIg Therapy

Following the 5-day IVIg course, 12 patients were treated as summarized in table 2. After IVIg, 3 patients received steroid pulse treatment and 11 patients received oral steroids with no more than 1.0 mg/kg/day, 6.3 days after IVIg treatment on average (range 0–17). The mean initial dose of oral steroid for 12 patients was 0.6 ± 0.1 mg/kg/day (33.3 ± 4.5 mg/day). Additionally, those who responded inadequately to steroids received CYC unless active infections were concurrent. CYC was administered to 8 patients at a mean dose of 0.8 ± 0.1 mg/kg/day (46.9 ± 3.1 mg/day).

Outcome and 3-Month Follow-Up

There were no disease-related deaths for 3 months after IVIg treatment. Vasculitis recurred 3 months after IVIg treatment in patient 11 who did not receive any immunosuppressive drugs after IVIg treatment because he was a carrier of MRSA and antibiotic-resistant *Pseudomonas aeruginosa*. There was no fatal complication due to infections in any of the patients during the 3-month observation period after treatment.

CRP level continued to decrease and normalized 3 months after IVIg treatment (4.0 ± 2.0 mg/l; $p < 0.0001$ vs. before IVIg; fig. 1a).

As shown in figure 1b, the Cre level began to decrease following IVIg; Cre level was 173 μmol/l (range 88–353; except for patient 11 who was on maintenance hemodialysis) 3 months after IVIg treatment (vs. pre-IVIg; $p < 0.0001$). Among 3 patients (patient No. 5, 10, and 11) whose Cre level exceeded 700 μmol/l before IVIg treatment, only one patient (No. 11) required chronic hemodialysis within 3 months after IVIg treatment. Although hemodialysis was also required for patient 4 within 1 month after IVIg treatment, he could be withdrawn from hemodialysis shortly thereafter. Collectively, the 3-month renal survival rate was 92% in our 12 patients.

The mean BVAS continued to decrease after IVIg treatment (fig. 1c). The mean BVAS was 20 (range 15–27) before IVIg and 11 (range 1–16) immediately after IVIg ($p < 0.0001$); 9 (range 0–19) 1 month after IVIg ($p < 0.0001$ vs. pre-IVIg BVAS), and 8 (range 0–22) 3 months after IVIg ($p < 0.0001$ vs. pre-IVIg BVAS). In particular, urinalysis showed that hematuria and/or proteinuria disappeared in 8 patients, 3 months after IVIg treatment. Systemic symptoms such as body weight loss, and nervous or alimentary tract symptoms also improved at 3 months after IVIg treatment.

The mean MPO-ANCA levels obtained within 8 ± 5 days after IVIg treatment were 401 EU (range 70–990) and those 1 month after IVIg treatment were 218 EU (range 13–640). The titers at these two time points after IVIg treatment were not significantly different from that prior to IVIg treatment (465.7 ± 135.7 EU). The mean MPO-ANCA levels 3 months after IVIg treatment, 78.8 EU (range 0–389), were significantly lower than that prior to IVIg treatment ($p < 0.01$; fig. 1d).

Adverse Drug Reaction

There were no major side effects observed in patients who received IVIg treatment. Patient 4 experienced transient mild hypertension and edema of the extremities during IVIg infusion, but it subsided when the rate of infusion of IVIg was lowered.

Discussion

The present study was conducted to evaluate the safety and efficacy of IVIg as an initial therapy for patients with MPO-ANCA-associated RPGN. All 12 patients with ANCA-associated RPGN enrolled in this study had experienced rapidly deteriorating renal dysfunction with multiorgan involvement. Administration of IVIg for 5 consecutive days led to partial resolution of inflammatory signs and symptoms in parallel with significant decreases in CRP, TNF-α, and BVAS values as well as cessation of progression in renal dysfunction. No life-threatening infections or side effects developed with our regimens including IVIg in all patients, including those older than 80 (patient No. 1, 4, 7, 8) and those with latent, antibiotic-resistant infections (patient No. 1, 2, 6, 11, 12). Clinical improvement was seen in all patients with IVIg for initial therapy followed by immunosuppressants, none of whom died within 3 months. The 3-month renal and patient survival rates were 92 and 100%, respectively, which were more favorable than those previously reported in MPO-ANCA-positive RPGN patients treated with immunosuppressive agents in Japan: 3-month renal and patient survival rates were about 75 and 85%, respectively [12].

Notably, there was a rapid and significant decrease in neutrophil count following IVIg treatment. Activated neutrophils are known to be involved in vasculitis. During the active phase of Kawasaki disease, circulating activated neutrophils increase in number and secrete excessive amounts of autotoxic mediators such as reactive oxygen species and elastase. In this active phase, neutrophil apoptosis is inhibited, resulting in a prolonged lifespan, which then might contribute to the pathogenesis of the vasculitic lesions. High-dose IVIg therapy decreased the number of circulating neutrophils by accelerating their apoptosis in Kawasaki disease and was effective in preventing the development of coronary aneurysm [13]. Similarly, in MPO-ANCA-associated vasculitis, activated neutrophils are involved in renal damage. TNF-α-primed neutrophils undergo accelerated and dysregulated apoptosis, and such apoptotic neutrophils express ANCA antigen on their cell surface in affected organs, where leukocytoclasia can further augment inflammatory injury [14]. Although the precise mechanism by which IVIg affects the apoptosis of neutrophils remains unknown, the rapid decrease of WBC count following IVIg treatment coupled with the significant decrease in CRP suggests accelerated clearance of apoptotic neutrophils by IVIg in patients with MPO-ANCA-associated RPGN in this study.

Our study showed that the plasma TNF-α value significantly decreased following IVIg treatment, suggesting immunomodulatory effect of IVIg. Serum levels of TNF-α were reported to be increased in patients with active vasculitis [15]. In addition, elevation of serum TNF-α was associated with upregulation of TNF-α mRNA at the sites of vasculitis [16]. TNF-α released from activated macrophages following infectious stimuli is known to prime and activate neutrophils. Once activated, neutrophils can attach to the endothelium and further release MPO and reactive oxygen species, ultimately leading to endothelial damage [17, 18]. Consistent with this, Booth et al. [19] recently reported TNF-α blockade with infliximab was effective at inducing remission in 88% of patients with ANCA vasculitis. Their report and our findings suggest that TNF-α may play a key role in ANCA-associated vasculitis. Decrease in the TNF-α value following IVIg suggests that IVIg plays a positive role in disrupting such a vicious inflammatory cycle.

Another possible mechanism of IVIg is that therapeutic concentrations of IgG block Fc receptors on phagocytes and inhibit antibody-dependent cell-mediated cytotoxicity [6] or downregulate the proliferation of activated B and T cells, reducing cytokine production from these immunoeffector cells [20]. Because the latter mechanism requires a substantial time interval, the rapid TNF-α suppression with IVIg observed in this study suggests that IVIg has direct effects on activated macrophages, rather than an effect mediated through T- or B-cell suppression.

IVIg treatment, even without immunosuppressants, has been shown to ameliorate systemic symptoms of active vasculitis [8]. In patients with asthma, IVIg was found to act synergistically with steroids, improved the clinical parameters, and reduced oral corticosteroid requirements and the duration of hospitalization. Such effects are partially mediated by improvement in glucocorticoid-receptor-binding affinity [21]. In the present study, RPGN was significantly improved by a relatively low initial dose of steroid (0.6 mg/kg/day). Our patients, who are relatively old and therefore at higher risk of developing infectious complications after steroid administration, might have benefited from the potential steroid-sparing effect of IVIg.

A major side effect of IVIg is renal dysfunction probably due to hyperosmolarity induced by sucrose contained in immunoglobulin formulations [22]. Therefore, in Europe, such a formulation is used for WG patients without renal involvement, but not for those with renal involvement. For MPA patients with renal involvement, we used immunoglobulin formulation that contained mannitol (Kenketsu Venilon-I) or glucose (Kenketsu Glovenin-I) instead of sucrose because the former two substances are less likely to cause hyperosmolarity. Although we cannot exclude the possibility that intravascular volume repletion with mannitol might have increased tubular flow, there is no convincing evidence for the efficacy of mannitol in RPGN [23]. With our regimen, patients demonstrated improved renal function, supporting the safety of IVIg with mannitol or glucose.

In conclusion, the present study demonstrated the safety and potential efficacy of IVIg as an initial therapy for patients with MPO-ANCA-associated RPGN. Our study is limited by its small size, relatively few severe cases, and non-standardized follow-up protocols. However, our findings suggest that IVIg is potentially effective for treating MPO-ANCA-associated RPGN, either as first-line or adjunctive therapy. Further research into the optimal dose and duration of treatment is required to define the role of IVIg in treatment of MPO-ANCA-associated RPGN.

Acknowledgments

We wish to thank Drs. Ning Liu, Noriyuki Iehara, Hidenori Arai, and Masafumi Ihara for excellent advice. This work was supported by a Research Grant on Pharmaceutical and Medical Safety, Ministry of Health, Labour, and Welfare, through NIID-NIH, Japan.

References

▶ 1 Van der Woude FJ, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, van der Giessen M, van der Hem GK, The TH: Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. Lancet 1985;i:425–429.

▶ 2 Cunningham MA, Ono T, Hewitson TD, Tipping PG, Becker GJ, Holdsworth SR: Tissue factor pathway inhibitor expression in human crescentic glomerulonephritis. Kidney Int 1999;55:1311–1318.

▶ 3 Harper L, Savage CO: ANCA-associated renal vasculitis at the end of the twentieth century – a disease of older patients. Rheumatology (Oxford) 2005;44:495–501.

▶ 4 Jayne DR, Davies MJ, Fox CJ, Black CM, Lockwood CM: Treatment of systemic vasculitis with pooled intravenous immunoglobulin. Lancet 1991;337:1137–1139.

▶ 5 Jayne DR, Chapel H, Adu D, Misbah S, O'Donoghue D, Scott D, Lockwood CM: Intravenous immunoglobulin for ANCA-associated systemic vasculitis with persistent disease activity. QJM 2000;93:433–439.

▶ 6 Kazatchkine MD, Kaveri SV: Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. N Engl J Med 2001;345:747–755.

▶ 7 Sharief MK, Ingram DA, Swash M, Thompson EJ: Intravenous immunoglobulin reduces circulating proinflammatory cytokines in Guillain-Barré syndrome. Neurology 1999;52:1833–1838.

▶ 8 Jayne DR, Lockwood CM: Intravenous immunoglobulin as sole therapy for systemic vasculitis. Br J Rheumatol 1996;35:1150–1153.

▶ 9 Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, Hagen EC, Hoffman GS, Hunder GG, Kallenberg CG, et al: Nomenclature of systemic vasculitides. Proposal of an international consensus conference. Arthritis Rheum 1994;37:187–192.

▶ 10 Luqmani RA, Bacon PA, Moots RJ, Janssen BA, Pall A, Emery P, Savage C, Adu D: Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis. QJM 1994;87:671–678.

▶ 11 Yashiro M, Muso E, Itoh-Ihara T, Oyama A, Hashimoto K, Kawamura T, Ono T, Sasayama S: Significantly high regional morbidity of MPO-ANCA-related angiitis and/or nephritis with respiratory tract involvement after the 1995 great earthquake in Kobe (Japan). Am J Kidney Dis 2000;35:889–895.

▶ 12 Sakai H, Kurokawa K, Koyama A, Shiiki H, Nishi S, Mitarai T, Yokoyama H, Yoshimura A, Yorioka N: Guidelines for the management of rapidly progressive glomerulonephritis. Nippon Jinzo Gakkai Shi 2002;44:55–82.

▶ 13 Tsujimoto H, Takeshita S, Nakatani K, Kawamura Y, Tokutomi T, Sekine I: Intravenous immunoglobulin therapy induces neutrophil apoptosis in Kawasaki disease. Clin Immunol 2002;103:161–168.

▶ 14 Harper L, Ren Y, Savill J, Adu D, Savage CO: Antineutrophil cytoplasmic antibodies induce reactive oxygen-dependent dysregulation of primed neutrophil apoptosis and clearance by macrophages. Am J Pathol 2000;157:211–220.

▶ 15 Arimura Y, Minoshima S, Kamiya Y, Tanaka U, Nakabayashi K, Kitamoto K, Nagasawa T, Sasaki T, Suzuki K: Serum myeloperoxidase and serum cytokines in anti-myeloperoxidase antibody-associated glomerulonephritis. Clin Nephrol 1993;40:256–264.

▶ 16 Noronha IL, Kruger C, Andrassy K, Ritz E, Waldherr R: In situ production of TNF-α, IL-1β and IL-2R in ANCA-positive glomerulonephritis. Kidney Int 1993;43:682–692.

▶ 17 Falk RJ, Terrell RS, Charles LA, Jennette JC: Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. Proc Natl Acad Sci USA 1990;87:4115–4119.

▶ 18 Savage CO, Pottinger BE, Gaskin G, Pusey CD, Pearson JD: Autoantibodies developing to myeloperoxidase and proteinase 3 in systemic vasculitis stimulate neutrophil cytotoxicity toward cultured endothelial cells. Am J Pathol 1992;141:335–342.

▶ 19 Booth A, Harper L, Hammad T, Bacon P, Griffith M, Levy J, Savage C, Pusey C, Jayne D: Prospective study of TNF-α blockade with infliximab in anti-neutrophil cytoplasmic antibody-associated systemic vasculitis. J Am Soc Nephrol 2004;15:717–721.

▶ 20 Kaveri S, Vassilev T, Hurez V, Lengagne R, Lefranc C, Cot S, Pouletty P, Glotz D, Kazatchkine MD: Antibodies to a conserved region of HLA class I molecules, capable of modulating CD8 T cell-mediated function, are present in pooled normal immunoglobulin for therapeutic use. J Clin Invest 1996;97:865–869.

▶ 21 Spahn JD, Leung DY, Chan MT, Szefler SJ, Gelfand EW: Mechanisms of glucocorticoid reduction in asthmatic subjects treated with intravenous immunoglobulin. J Allergy Clin Immunol 1999;103:421–426.

▶ 22 Cayco AV, Perazella MA, Hayslett JP: Renal insufficiency after intravenous immune globulin therapy: a report of two cases and an analysis of the literature. J Am Soc Nephrol 1997;8:1788–1794.

▶ 23 Girbes AR: Prevention of acute renal failure: role of vasoactive drugs, mannitol and diuretics. Int J Artif Organs 2004;27:1049–1053.

Bioimages 12 (2–4): 85–90, December 2004. ©2004 by Bioimaging Society

MPO-ANCA Binding Site on MPO Molecule Estimated from Epitope Mapping Study and Molecular Modeling

Rintaro Suzuki[1], **Kazuo Tomizawa**[2], **Kazuo Suzuki**[2], **Masaru Tanokura**[3*]

[1]National Institute of Agrobiological Sciences, 2–1–2 Kannondai, Tsukuba, Ibaraki 305–8602, Japan, [2]Department of Bioactive Molecules, National Institute of Infectious Diseases, 1–23–1 Toyama, Shinjuku-ku, Tokyo 162–8640, Japan, and [3]Graduate School of Agricultural and Life Sciences, University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan.

Summary

Myeloperoxidase (MPO) has been identified as one of the major target of anti-neutrophil cytoplasmic antibody (ANCA), and ANCA with specificity for MPO is called MPO-ANCA. Binding of MPO-ANCA to MPO is a trigger for many inflammatory diseases, but the details of the interaction between two molecules are unknown. We used the result of an epitope mapping study and molecular modeling techniques to identify the MPO-ANCA binding site of MPO. The structural features of MPO suggest that the most likely region for the interaction on the molecule lies adjacent to the peroxidase active site.

Key words

Myeloperoxidase, MPO-ANCA, epitope mapping, molecular structure

Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) appear in the serum of most patients with inflammatory diseases such as systemic vasculitis, especially microscopic polyangiitis and Wegener's granulomatosis, and pauciimune necrotizing and crescentic glomerulonephritis (van der Woude et al., 1985; Falk and Jennette, 1988; Nolle et al., 1989; Tervaert et al., 1990a; Tervaert et al., 1990b; Lassoued et al., 1991; Arimura et al., 1992; Arimura et al., 1993). ANCAs are specific for antigens which localize in neutrophil granules and lysosomes of monocytes. Myeloperoxidase (MPO, EC 1.11.1.7) is one of the major target antigens of ANCAs (Falk and Jennette, 1988).

MPO is an enzyme which plays a central role in microbicidal activity of neutrophils by producing hypochlorous acid from chloride ions in the presence of hydrogen peroxide (Harrison and Schultz, 1976). However, the hypochlorous acid and various reactive oxygen species generated from the hypochlorous acid are also considered important pathophysiologic factors in the diseases. The extracellular release of MPO has been reported to occur during a number of inflammatory diseases (Weiss, 1989). It has been suggested that the binding of MPO in blood plasma to the cell surface of neutrophil and the succeeding recognition of MPO by ANCA with specificity for MPO (MPO-ANCA) are the triggers of the disease process (Hess et al., 2000). Furthermore, purified anti-MPO IgG itself causes glomerulonephritis in mice (Xiao et al., 2002). Thus MPO-ANCA molecule that is binding to MPO may be the key factor in the onset of diseases.

On the other hand, molecular basis of interaction between MPO and MPO-ANCA is unknown because the amount of MPO-ANCA in sera of patients is not enough for biochemical analysis. MPO-ANCA does not inhibit the peroxidase activity of MPO (Falk et al., 1992; Griffin et al., 1999) while it prevents inactivation of MPO by ceruloplasmin, an inhibitor of MPO (Griffin et al., 1999). Thus the epitope site of MPO-ANCA may not involve the active site of MPO, but may overlap with or juxtapose to the site recognized by ceruloplasmin. However, the structural basis of the interaction between MPO and ceruloplasmin is also unknown.

Mature MPO is a 140 kDa tetramer with two heavy chains and two light chains (Olsen et al., 1986). Each pair of the heavy and light chains results from post-translational excision (Koeffler et al., 1985; Akin and Kinkade, 1986). MPO molecule has two hemes, six glycosylation sites, two chloride ion binding sites, and two calcium ion binding sites. It has been reported that some recombinant fragments of the heavy chain of MPO show high reactivities with the sera of patients with MPO-ANCA-positive diseases (Tomizawa et al., 1998; Fujii et al., 2000). These fragments are expected to correspond to MPO-ANCA binding sites on MPO. To elucidate the molecular basis for these reactivities, we analyzed the crystal structure of MPO molecule and specified the epitope site on the molecule.

---

*To whom all correspondence should be addressed.
E-mail: amtanok@mail.ecc.u-tokyo.ac.jp

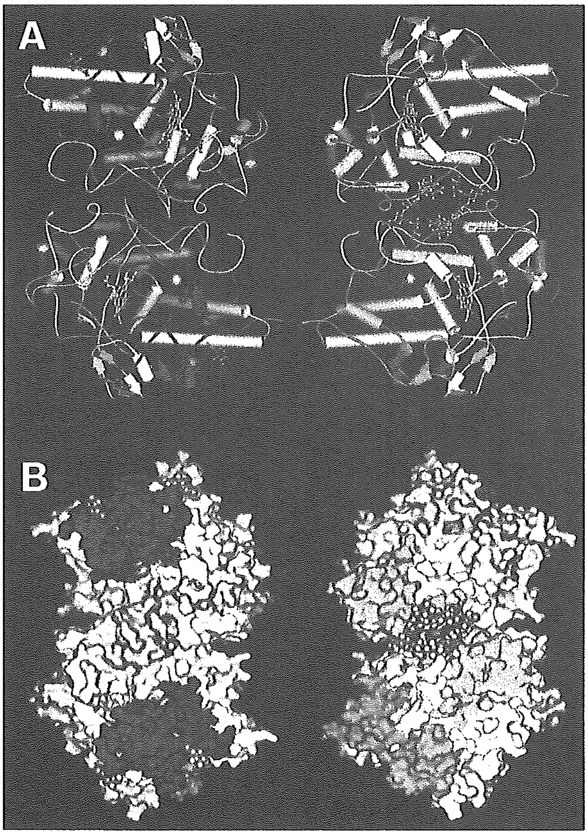


Fig. 1. Distribution of the fragments with high reactivity against the sera of patients on MPO molecule. The fragments Ha, Hb, Hf, and Hg are colored red, blue, green, and magenta, respectively. A. Schematic views of MPO molecule from two opposite directions. The sugar chains (colored by atoms) and the heme molecules (yellow) are displayed in ball-and-stick models. The chloride ions (light blue) are also shown. B. Molecular surface of MPO from the same viewpoint as A. Sugar chains and heme molecules are displayed in space-filling models.

Methods

The coordinates of three-dimensional structure of the human MPO, antibody Fv fragment, and ceruloplasmin were taken from Protein Data Bank (PDB) operated by Research Collaboratory for Structural Bioinformatics (RCSB). The PDB ID of the coordinates of these molecules were 1CXP (Fiedler et al., 2000), 1AR1 (Ostermeier et al., 1997), and 1KCW (Zaitseva et al., 1996). The programs RasMol v2.7.1.1 (Sayle and Milner White, 1995), insightII 98.0 (Accerlys), MolScript v2.1.2 (Kraulis, 1991), and Raster3D v2.6 (Merritt and Bacon, 1997) were used for molecular visualization and modeling. The electrostatic potential of the molecule was calculated with the program GRASP (Nicholls et al., 1991).

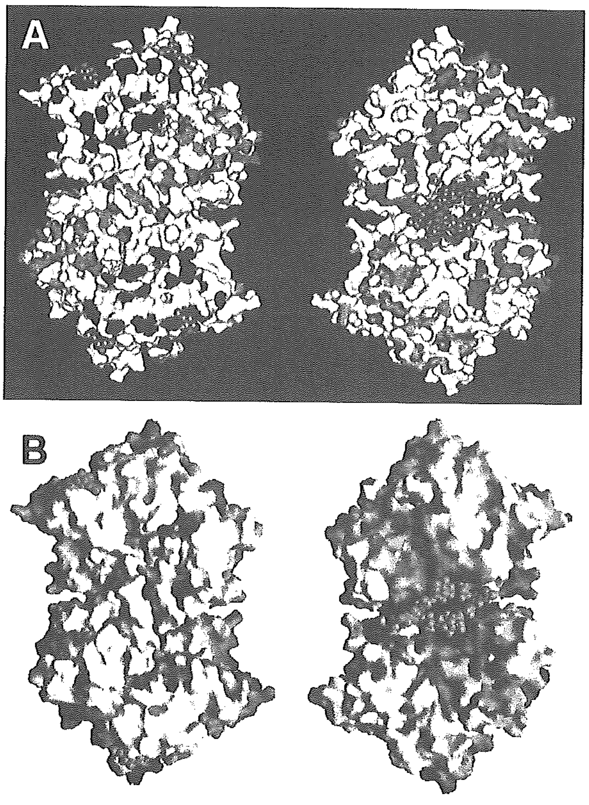


Fig. 2. Residues expected to be involved in interaction. A. Distribution of side-chains of polar and aromatic residues in the fragments with high reactivity against the sera of patients on molecular surface of MPO. Color scheme is the same as in Fig. 1. B. Electrostatic potentials on molecular surface of MPO. Positive and negative charges are colored red and blue, respectively.

Results

Distribution of epitopes on 3D-strcuture of MPO

An epitope mapping study of MPO fragments determined by ELISA has been reported (Tomizawa et al., 1998; Fujii et al., 2000). The reactivities of these fragments against the sera of patients were high at two N-terminal (Ha and Hb) and two C-terminal (Hf and Hg) fragments of heavy chain. The reactivities of fragments Ha and Hb were higher than those of Hf and Hg. These reactivities were correlated with the severity of patients. The fragments of light chain did not show any reactivities. We mapped these fragments with high reactivities on the structure of MPO. The fragments occupy various parts in the molecule (Fig. 1A). The exposed parts of these fragments were also sprinkled over the molecular surface, and they did not assemble together (Fig. 1B). There were four regions where surfaces of two fragments were contiguous with each other. These regions were located adjacent to the heme (Ha and Hb), an N-acetyl-D-glucosamine sugar chain bound to N189 (Hb and Hg), a loop with Arg136 (Ha

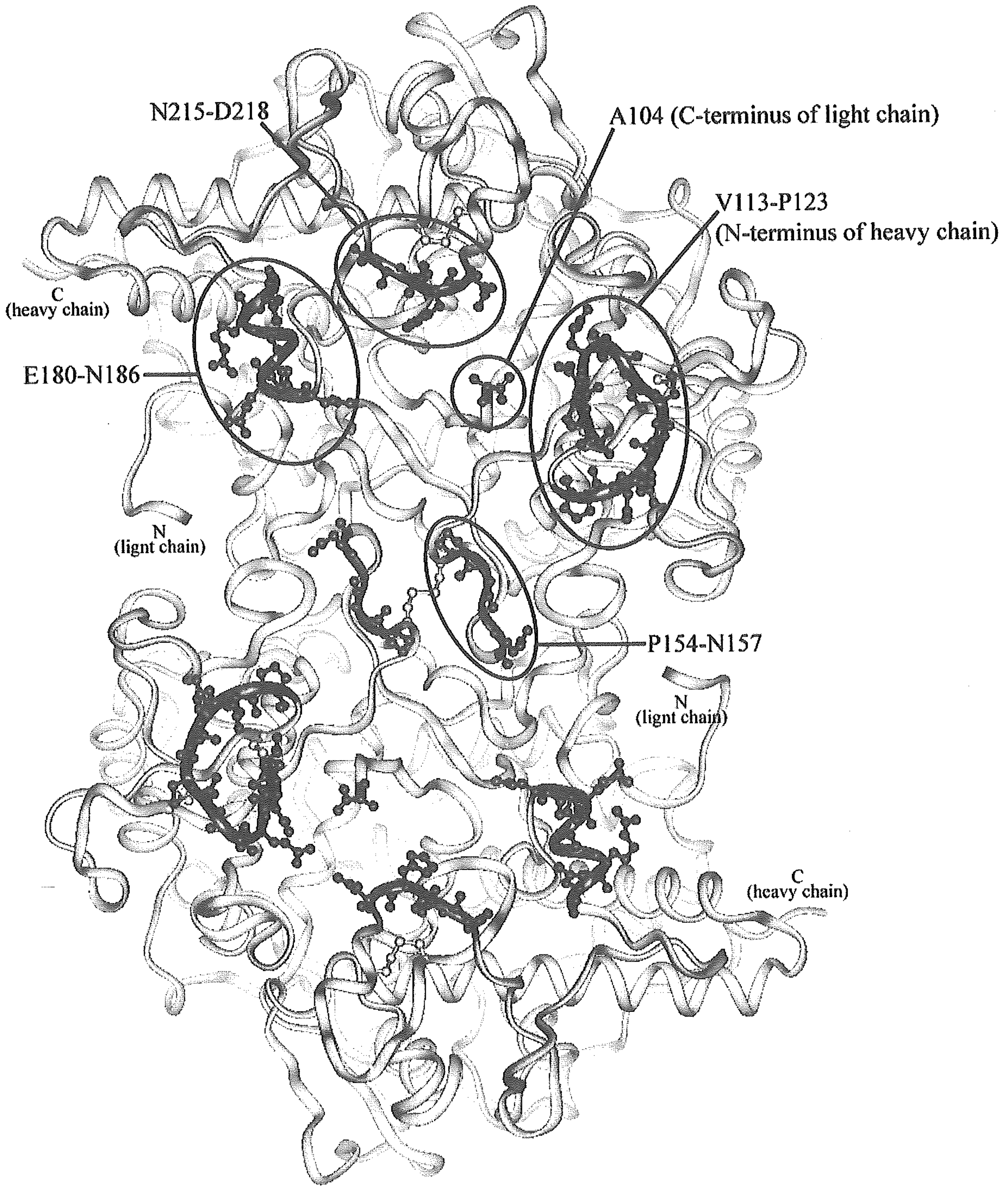
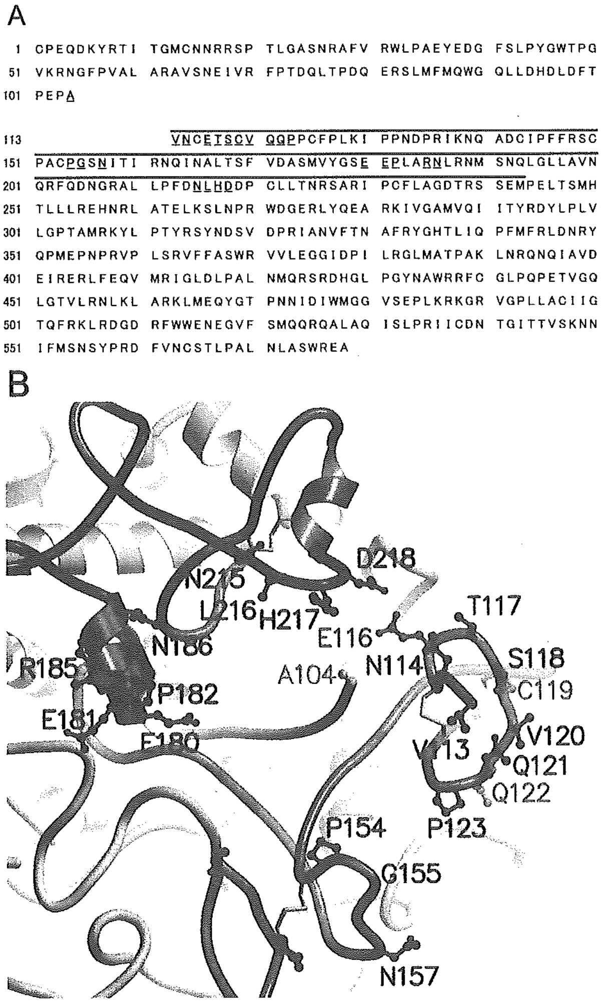


Fig. 3. Exposed residues around possible interaction site. The exposed residues around the region, where the fragments Ha and Hb are contiguous, are shown in gray. Disulfide bonds which stabilize the conformation of exposed residues are shown in white.

and Hf), and the big sugar chain on the opposite side of the active site (Hf and Hg). These are the candidate positions for recognition by MPO-ANCA.

Residues expected to be involved in antibody-antigen interactions

It has been known that the antibody-antigen complexes interact predominantly through polar and aromatic residues (Jackson, 1999). We mapped the side-chains of these residues in the above fragments (Fig. 2A), but



1 CPEQDKYRTI TGMCNNRRSP TLGASNRAFV RWLPAEYEDG FSLPYGWTPG
51 VKRNGFPVAL ARAVSNEIVR FPTDQLTPDQ ERSLMFMQWG QLLDHDLDFT
101 PEPA

113 VNCETSCV QQPPCFPLKI PPNDPRIKNQ ADCIPFFRSC
151 PACPGSNITI RNQINALTSF VDASMVYGSE EPLARNLRNM SNQLGLLAVN
201 QRFQDNGRAL LPFDNLHDDP CLLTNRSARI PCFLAGDTRS SEMPELTSMH
251 TLLLREHNRL ATELKSLNPR WDGERLYQEA RKIVGAMVQI ITYRDYLPLV
301 LGPTAMRKYL PTYRSYNDSV DPRIANVFTN AFRYGHTLIQ PFMFRLDNRY
351 QPMEPNPRVP LSRVFFASWR VVLEGGIDPI LRGLMATPAK LNRQNQIAVD
401 EIRERLFEQV MRIGLDLPAL NMQRSRDHGL PGYNAWRRFC GLPQPETVGQ
451 LGTVLRNLKL ARKLMEQYGT PNNIDIWMGG VSEPLKRKGR VGPLLACIIG
501 TQFRKLRDGD RFWWENEGVF SMQQRQALAQ ISLPRIICDN TGITTVSKNN
551 IFMSNSYPRD FVNCSTLPAL NLASWREA

Fig. 4. Detail of the exposed residues around possible interaction site. A. The amino acid sequence of MPO. The upper sequence is the light chain and the bottom is the heavy chain. The fragments Ha and Hb are indicated by red and blue lines, respectively. The exposed residues are underlined. B. The exposed residues around the possible interaction site are shown in detail. Residues in the fragments Ha and Hb are colored red and blue, respectively. Disulfide bonds which stabilize the conformation of exposed residues are shown in yellow.

these side-chains were disseminated over the surface. We also calculated the electrostatic potential of the surface of MPO to investigate the charge distribution on MPO (Fig. 2B). Both positive and negative charges exist around the active site, while the negative charge dominates around the big sugar chain on the opposite side of the active site.

The possible interaction sites of MPO-ANCA on MPO

Among the four regions where two fragments were adjacent to each other, we focused on the region around the heme. In this region, two fragments with high reactivities, Ha and Hb, were contiguous with each other, and both positive and negative charges are distributed. Exposed residues in this region are A104, V113, N114, E116-P123, P154, G155, N157, E180-P182, R185, N186, and N215-D218 (Figs. 3 and 4). A104 is the C-terminal residue of light chain. The residues V113-P123 constitute an N-terminal loop in the heavy chain. The conformation of the N-terminal loop is stabilized by two disulfide bonds, C115-C125 and C119-C143. P154-N157 contacts with an identical part of the other heavy chain, connected by a disulfide bridge at C153. E180-N186 is an α-helix and N215-D218 has no secondary structure.

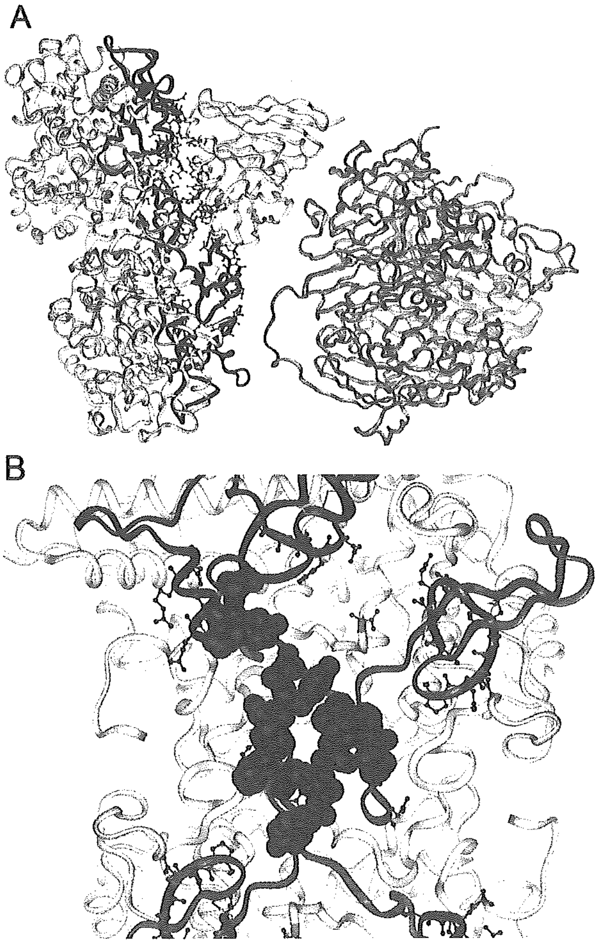


Fig. 5. Modeled interaction between MPO and Fv fragment. The residues that are expected to participate in the interaction are shown. A. Side view of two molecules. The residues in Fv fragment which are close to the original antigen in the PDB entry 1AR1 are colored green. The exposed residues are the same color as in Fig. 4. For the comparison of the molecular size, a ceruloplasmin molecule is also shown (pink). B. Front view of the residues. The residues in Fv fragment are shown in space-filling models.

Possible binding mode between MPO and MPO-ANCA

Surfaces of protrusions made of V113-P123, P154-N157, E180-N186, and N215-D218 are not charged (Fig. 2B). However, there are many polar residues in these regions (Fig. 4). The hollows between these protrusions have charged surfaces (Fig. 2B). These characteristics meet the general interaction pattern of antibody-antigen complexes.

The area of one binding surface on an antibody is about 400 Å^2. This binding surface is not able to cover all protruded residues mentioned above. When the binding mode of an MPO molecule and an antibody Fv fragment are modeled, for example, the central two loops (P154-N157) and an α-helix E180-N186 are covered by a binding surface of Fv fragment (Fig. 5).

While the ceruloplasmin binding site of MPO is unknown, ceruloplasmin is a molecule as large as MPO. Thus the binding of the antibody to MPO from the direction of the active site surface will compete with the binding of the ceruloplasmin.

Discussion

The structural characteristics of MPO and the result of epitope mapping studies suggest that MPO-ANCA recognizes the exposed residues around the active site of MPO. There are three loops and one α-helix around the active site protruding into the solvent. The binding site of an antibody molecule is smaller than the surface around the active site of MPO and one or two of the protruding surfaces may be recognized by one MPO-ANCA molecule. Chloride ion, which is the substrate of MPO, is very small and may slip through the interspace between MPO and MPO-ANCA. Larger molecule such as ceruloplasmin is able to cover the whole active site of MPO and may inhibit the activity of MPO.

If all MPO-ANCA molecules recognize same epitopes, two protruded surfaces on MPO are the interaction site. If there are several types of MPO-ANCA which recognize different epitopes, three or more protruded surfaces are involved in the interaction between MPO and MPO-ANCA. These regions consist of four to eleven residues (Fig. 4A) and the peptides containing these sequences may also be recognized by MPO-ANCA. Thus short peptides with about ten residues are useful for further investigation of the epitope sites and the recognition mechanism of MPO-ANCA.

Acknowledgments

This work was supported in part by a grant from the Ministry of Health, Labour and Welfare, Japan.

Received January 31 2005; revised February 28 2005.

References

Akin, D.T. and Kinkade, J.M.J. (1986). Processing of a newly identified intermediate of human myeloperoxidase in isolated granules occurs at neutral pH. J. Biol. Chem., 261: 8370–8375.

Arimura, Y., Minoshima, S., Kamiya, Y., Nakabayashi, K., Kitamoto, K. and Nagasawa, T. (1992). A case of Goodpasture's syndrome associated with anti-myeloperoxidase antibodies. Intern. Med., 31: 239–243.

Arimura, Y., Minoshima, S., Kamiya, Y., Tanaka, U., Nakabayashi, K., Kitamoto, K., Nagasawa, T., Sasaki, T. and Suzuki, K. (1993). Serum myeloperoxidase and serum cytokines in anti-myeloperoxidase antibody-associated glomerulonephritis. Clin. Nephrol., 40: 256–264.

Falk, R.J. and Jennette, J.C. (1988). Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. N. Engl. J. Med., 318: 1651–1657.

Falk, R.J., Becker, M., Terrell, R. and Jennette, J.C. (1992). Anti-myeloperoxidase autoantibodies react with native but not denatured myeloperoxidase. Clin. Exp. Immunol., 89: 274–278.

Fiedler, T.J., Davey, C.A. and Fenna, R.E. (2000). X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 Å resolution. J. Biol. Chem., 275: 11964–11971.

Fujii, A., Tomizawa, K., Arimura, Y., Nagasawa, T., Ohashi, Y.Y., Hiyama, T., Mizuno, S. and Suzuki, K. (2000). Epitope analysis of myeloperoxidase (MPO) specific anti-neutrophil cytoplasmic autoantibodies (ANCA) in MPO-ANCA-associated glomerulonephritis. Clin. Nephrol., 53: 242–252.

Griffin, S.V., Chapman, P.T., Lianos, E.A. and Lockwood, C.M. (1999). The inhibition of myeloperoxidase by ceruloplasmin can be reversed by anti-myeloperoxidase antibodies. Kidney Int., 55: 917–925.

Harrison, J.E. and Schultz, J. (1976). Studies on the chlorinating activity of myeloperoxidase. J. Biol. Chem., 251: 1371–1374.

Hess, C., Sadallah, S. and Schifferli, J.A. (2000). Induction of neutrophil responsiveness to myeloperoxidase antibodies by their exposure to supernatant of degranulated autologous neutrophils. Blood, 96: 2822–2827.

Jackson, R.M. (1999). Comparison of protein-protein interactions in serine protease-inhibitor and antibody-antigen complexes: implications for the protein docking problem. Protein Sci., 8: 603–613.

Koeffler, H.P., Ranyard, J. and Pertcheck, M. (1985). Myeloperoxidase: its structure and expression during myeloid differentiation. Blood, 65: 484–491.

Kraulis, P.J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst., 24: 946–950.

Lassoued, S., Sixou, L., Oksman, F., Pages, M. and Fournie, A. (1991). Antineutrophil cytoplasmic antibodies and antibodies to myeloperoxidase in rheumatoid arthritis. Arthritis Rheum., 34: 1069–1070.

Merritt, E.A. and Bacon, D.J. (1997). Raster3D photorealistic molecular graphics. Methods Enzymol., 277: 505–524.

Nicholls, A., Sharp, K.A. and Honig, B. (1991). Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. Proteins, 11: 281–296.

Nolle, B., Specks, U., Ludemann, J., Rohrbach, M.S., DeRemee, R.A. and Gross, W.L. (1989). Anticytoplasmic autoantibodies: their immunodiagnostic value in Wegener granulomatosis. Ann. Intern. Med., 111: 28–40.

Olsen, R.L., Steigen, T.K., Holm, T. and Little, C. (1986). Molecular forms of myeloperoxidase in human plasma. Biochem. J., 237: 559–565.

Ostermeier, C., Harrenga, A., Ermler, U. and Michel, H. (1997). Structure at 2.7 Å resolution of the Paracoccus denitrificans two-subunit cytochrome c oxidase complexed with an antibody FV fragment. Proc. Natl. Acad. Sci. U.S.A., 94: 10547–10553.

Sayle, R.A. and Milner White, E.J. (1995). RASMOL: biomolecular graphics for all. Trends Biochem. Sci., 20: 374.

Tervaert, J.W., Goldschmeding, R., Elema, J.D., Limburg, P.C., van der Giessen, M., Huitema, M.G., Koolen, M.I., Hene, R.J., The, T.H., van der Hem, G.K., von dem Borne, A.E. and Kallenberg, C.G. (1990a). Association of autoantibodies to myeloperoxidase with different forms of vasculitis. Arthritis Rheum., 33: 1264–1272.

Tervaert, J.W., Goldschmeding, R., Elema, J.D., van der Giessen, M., Huitema, M.G., van der Hem, G.K., The, T.H., von dem Borne, A.E. and Kallenberg, C.G. (1990b). Autoantibodies against myeloid lysosomal enzymes in crescentic glomerulonephritis. Kidney Int., 37: 799–806.

Tomizawa, K., Mine, E., Fujii, A., Ohashi, Y.Y., Yamagoe, S., Hashimoto, Y., Ishida-Okawara, A., Ito, M., Tanokura, M., Yamamoto, T., Arimura, Y., Nagasawa, T., Mizuno, S. and Suzuki, K. (1998). A panel set for epitope analysis of myeloperoxidase (MPO)-specific antineutrophil cytoplasmic antibody MPO-ANCA using recombinant hexamer histidine-tagged MPO deletion mutants. J. Clin. Immunol., 18: 142–152.

van der Woude, F.J., Rasmussen, N., Lobatto, S., Wiik, A., Permin, H., van Es, L.A., van der Giessen, M., van der Hem, G.K. and The, T.H. (1985). Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. Lancet, 1: 425–429.

Weiss, S.J. (1989). Tissue destruction by neutrophils. N. Engl. J. Med., 320: 365–376.

Xiao, H., Heeringa, P., Hu, P., Liu, Z., Zhao, M., Aratani, Y., Maeda, N., Falk, R.J. and Jennette, J.C. (2002). Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. J. Clin. Invest., 110: 955–963.

Zaitseva, I., Zaitsev, V., Card, G., Moshkov, K., Bax, B., Ralph, A. and Lindley, P. (1996). The X-ray structure of human serum ceruloplasmin at 3.1 angstrom: nature of the copper centres. J. Biol. Inorg. Chem., 1: 15–23.

Clinical Nephrology, Vol. 63 – No. 6/2005 (437-445)

clinical nephrology
Original

ISSN 0301-0430

Epitope analysis of myeloperoxidase-specific antineutrophil cytoplasmic autoantibodies (MPO-ANCA) in childhood onset Graves' disease treated with propylthiouracil

M. Fujieda[1], K. Suzuki[2], H. Sato[3], M. Hattori[4], N. Wada[5], M. Tsuchiya[6], N. Okamoto[7], T. Murata[7], M. Matsudaira[8], M. Shimizu[9], K. Ohta[9], K. Naruse[1], S. Sugihara[10] and H. Wakiguchi[1]

[1]Department of Pediatrics, Kochi University Medical School, Kochi, [2]Department of Bioactive Molecules, National Institute of Infectious Disease, Tokyo, [3]Division of Endocrinology, Chiba Children's Hospital, Chiba, [4]Department of Pediatric Nephrology, Tokyo Women's Medical University, Tokyo, [5]Department of Pediatric Nephrology, Shizuoka Children's Hospital, Shizuoka, [6]Department of Pediatrics and Child Health, Nippon Medical School Hospital, Tokyo, [7]Department of Pediatrics, Osaka Medical College, Osaka, [8]Department of Pediatrics, Nagasaki University, Graduate School of Biomedical Sciences, Nagasaki, [9]Department of Pediatrics, Kanazawa University, Graduate School of Medical Science, Kanazawa, and [10]Department of Pediatrics, Daini Hospital, Tokyo Women's Medical University, Tokyo, Japan

Key words:
antineutrophil cytoplasmic autoantibody – Graves' disease – epitope – myeloperoxidase – propylthiouracil

Abstract. Aim: This study aimed to elucidate the relationship between epitope profiles and clinical manifestations of patients with myeloperoxidase antineutrophil cytoplasmic autoantibodies- (MPO-ANCA) positive childhood onset Graves' disease treated with propylthiouracil (PTU). Methods: Sixteen patients were studied. The patients were grouped into ten without clinical vasculitis and nephritis (non-vasculitis group) and six with biopsy-proven pauci-immune necrotizing crescentic glomerulonephritis (vasculitis group). Epitope analysis was performed on serum samples by an enzyme-linked immunosorbent assay (ELISA) using a panel of recombinant deletion mutants of MPO. Results: The high frequency sites were region upstream of Met^{341} (Ha region) near the N-terminus of the heavy chain, and regions downstream of Gly^{598} (Hf and Hg regions) near the C-terminus. Most patients in the non-vasculitis group had polyclonal MPO-ANCA recognizing both the above linear sites and other epitope sites of the heavy chain of MPO. Only one of ten patients in the non-vasculitis group, and four of six patients in the vasculitis group had MPO-ANCA recognizing only the linear sites of the heavy chain of the MPO molecule (Ha, Hf and/or Hg). Of the four patients in the vasculitis group, two had nephritis, like rapidly progressive glomerulonephritis and one had alveolar hemorrhage. Conclusion: These findings suggest that most patients with childhood onset Graves' disease treated with PTU who manifest no vasculitis have polyclonal MPO-ANCA recognizing both the linear and other epitope sites of the heavy chain of MPO. However, some patients who develop nephritis have MPO-ANCA recognizing only the linear sites of the heavy chain of MPO. This clonality of MPO-ANCA may be a risk factor that induces clinical vasculitis and nephritis in patients treated with PTU. Therefore, patients exposed to PTU should be monitored for MPO-ANCA level and epitopes.

Introduction

Propylthiouracil (PTU) induces myeloperoxidase- (MPO) specific antineutrophil cytoplasmic autoantibody- (ANCA) positive vasculitis. We previously reported a high prevalence of MPO-ANCA positivity in PTU-treated patients with childhood onset Graves' disease [Sato et al. 2000]. However, only a small number of patients with PTU-associated MPO-ANCA-positive glomerulonephritis and vasculitis were identified in a nationwide survey in Japan during 1990 – 1997 [Fujieda et al. 2002]. The issues of whether

Received
April 26, 2004;
accepted in revised form
September 30, 2004

Correspondence to
M. Fujieda, MD
Department of Pediatrics, Kochi University,
Medical School,
Kohasu, Oko-cho,
Nankoku, Kochi
783-8505, Japan
fujiedam@med.kochi-u.ac.jp

Table 1. Patients with childhood onset Graves' disease treated with propylthiouracil.

Case	Sex	Age at study (years)	Duration of PTU[a] therapy (years)	MPO-ANCA[b] (EU/ml)	Anti-TPO[c] (U/ml)	Anti-TG[d] (U/ml)	Recognizing sites
Non-vasculitis group							
1	M	19	6.6	205	23.7	303.0	Ha, Hf, Hg, Hc
2	M	31	17.5	81	6.6	129.0	Ha, Hf, Hg
3	F	15	0.6	24	2.9	< 0.3	None
4	F	8	4.2	71	70.8	11.2	Ha, Hf, Hg, Hb, Hd
5	F	15	5.5	186	47.2	51.7	None
6	F	13	5.5	53	5.6	13.6	Hf, Hg, Hd
7	F	9	2.0	22	110.0	0.7	Ha, Hf, Hg, Hd
8	F	14	3.0	39	26.5	12.3	Ha, Hd
9	F	13	3.0	42	117.0	4.0	Ha, Hf, Hg, Hb, Hd
10	F	11	1.5	57	17.0	<0.3	Ha, Hf, Hg, Hc, Hd
Vasculitis group							
11	M	11	2.0	533	6.8	2.5	Ha, Hf, Hg, Hb, Hd
12	F	17	8.2	298	2.5	2.5	Hf, Hg
13	F	17	1.8	121	5.4	< 0.3	Ha, Hg
14	F	15	4.0	956	< 0.3	<0.3	None
15	F	10	3.0	859	10.3	1.0	Ha, Hg
16	F	15	5.7	35	29.8	25.5	Hg

[a] = PTU: propylthiouracil, [b] = MPO-ANCA, myeloperoxidase-specific antineutrophil cytoplasmic autoantibody, [c] = anti-TPO: anti-thyroid peroxidase antibody, [d] = anti-TG: anti-thyroglobulin antibody.

ANCA plays a role in the induction of vasculitis and whether ANCA can be used as a guide to therapy remain disputable.

Circulating ANCA was first reported in 1982 by Davies et al. [1982] in patients having pauci-immune necrotizing glomerulonephritis with crescent, and is now regarded as a serological marker for that disease together with systemic vasculitis such as Wegener's granulomatosis, microscopic polyangiitis and Churg-Strauss syndrome [Jennette et al. 1994]. ANCA is also detected in a number of vasculitic diseases including drug-induced systemic vasculitis.

Recently, we have established an ELISA for epitope analysis of MPO-ANCA [Otani et al. 1997, Tomizawa et al. 1998] and applied this method to correlate clinical manifestations and epitopes [Fujii et al. 2000]. In the present study, we used this method to examine the relationship between the epitope profile of MPO-ANCA and clinical manifestations in patients with childhood onset Graves' disease treated with PTU.

Materials and methods

Patients

Sixteen Japanese patients (three males and 13 females) with childhood onset Graves' disease and positive MPO-ANCA were studied (Table 1). Graves' disease was diagnosed between 3 and 15 years of age (10.2 ± 3.5 years, mean ± SD) on the basis of clinical features, diffuse goiter, anti-thyroid antibodies and hyperthyroidism. All patients were treated with PTU at an initial dosage of 10 mg/kg/day (maximum dose, 300 mg/day). Of the 16 patients entered in this study, six (Case No. 11 – 16) had biopsy-proven pauci-immune necrotizing crescentic glomerulonephritis (vasculitis group) and 10 never manifested clinical vasculitis and nephritis (non-vasculitis group).

In the non-vasculitis group, all patients were receiving PTU at the time of blood sampling. The duration of PTU therapy was 4.9 ± 4.8 year (range 0.6 – 17.5 years).

In the vasculitis group, all patients except Case No. 12 were switched to methimazole (MMI), and blood sampling was done at the diagnosis of nephritis before steroid or immunosuppressant therapy, or during active disease (proteinuria, over 1 g/day) in Case No. 11 and 15 who were administered oral prednisolone (40 and 20 mg/day, respectively) at the time of blood sampling. The duration of PTU therapy was 4.1 ± 2.5 year (range 1.8 – 8.2 years). Two (Case No. 15 and 16) of the six patients in the vasculitis group had nephritis, like rapidly progressive glomerulonephritis (RPGN, creatinine clearance (C_{cr}), 40.7 and 43.6 ml/min/1.73 m^2, respectively) and one (Case No. 13) had alveolar hemorrhage.

All blood samples were centrifuged immediately, and the sera were stored at –20 °C until assay.

ANCA analysis

All the sera from patients included in this study were screened for ANCA by indirect immunofluorescence microscopy using normal peripheral blood neutrophils, according to the guideline of the First International ANCA Workshop [Wiik 1989].

ANCA were also measured using enzyme-linked immunosorbent assay (ELISA) kits for MPO-ANCA and proteinase 3 (PR3) ANCA, as previously reported [Nagasawa et al. 1995]. For the MPO-ANCA ELISA, briefly the 96-well plates (Nissho Co., Osaka, Japan) were precoated with MPO extracted from human neutrophil cytoplasmic α-granule by Wieslab (Lund, Sweden); 200 μl of 1 : 20 diluted serum was added to each well and incubated for 1 h at 25 °C. After washing, 200 μl/well of diluted alkaline phosphatase-conjugated anti-human IgG was added and left for one h at room temperature. After washing, the substrate was added and the optimal density was read at 405 nm. Level of MPO-ANCA was calculated using a standard curve obtained from three standards (10, 100 and 1 000 ELISA units (EU)/ml). The normal MPO-ANCA level is below 20 EU/ml. The intraassay and interassay coefficients of variability (CV) were 2.5 – 5.9% and 5.6 – 8.1%, respectively [Nagasawa et al. 1995]. The PR3-ANCA ELISA plates were precoated with PR3 extracted from human neutrophil cytoplasmic α-granule (BioCarb Diagnostics, Lund, Sweden). The procedures were similar to those for MPO-ANCA ELISA. The normal PR3-ANCA level is below 10 EU/ml. The intraassay and interassay CV were 1.2 – 4.4% and 3.3 – 6.5%, respectively [Nagasawa et al. 1992].

Anti-thyroid antibody assays

Serum antibodies to thyroid peroxidase (TPO) and thyroglobulin were measured with commercial available radioimmunoassay (RIA) kits (RSR Limited, Cardiff, UK) using purified TPO and thyroglobulin, respectively. The detection limit for both was 0.3 U/ml. The intraassay and interassay CV for anti-TPO antibody were 2.0 – 3.2% and 3.5 – 5.2%, respectively [Hirooka et al. 1992], and those for antithyroglobulin were 3.3 – 4.0% and 4.1 – 5.7%, respectively [Hirooka et al.1992].

Preparation of recombinant deletion mutants of MPO fragments

Recombinant deletion mutants of the heavy chain and light chain of MPO were prepared as previously reported [Otani et al. 1997, Tomizawa et al. 1998]. Briefly, oligonucleotides derived from the cDNA sequences of MPO subunits were synthesized with an OLIGO 1,000 DNA synthesizer (Beckman, CA). Each MPO cDNA [Hashinaka et al. 1988] encoded fragment was amplified by PCR using a cDNA thermal cycler (Perkin Elmer/Cetus) by programmed incubation for 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, and repeated 25 times. The PCR products were digested with restriction enzymes Hind III and Bam H I and inserted into the expression vector pQE (Qiagen Inc., Valencia, CA, USA). Various DNA fragments were inserted between Hind III and Bam H I sites of the plasmid pQE32, pQE30 or pQE32 vectors. The cells were harvested after a further 16-h incubation with 1 mM isopropyl-β -D-thiogalactopyranoside. The 6 × histidine-tagged proteins were purified with a Ni^{2+}-charged nitrito-triacetic acid column (Qiagen

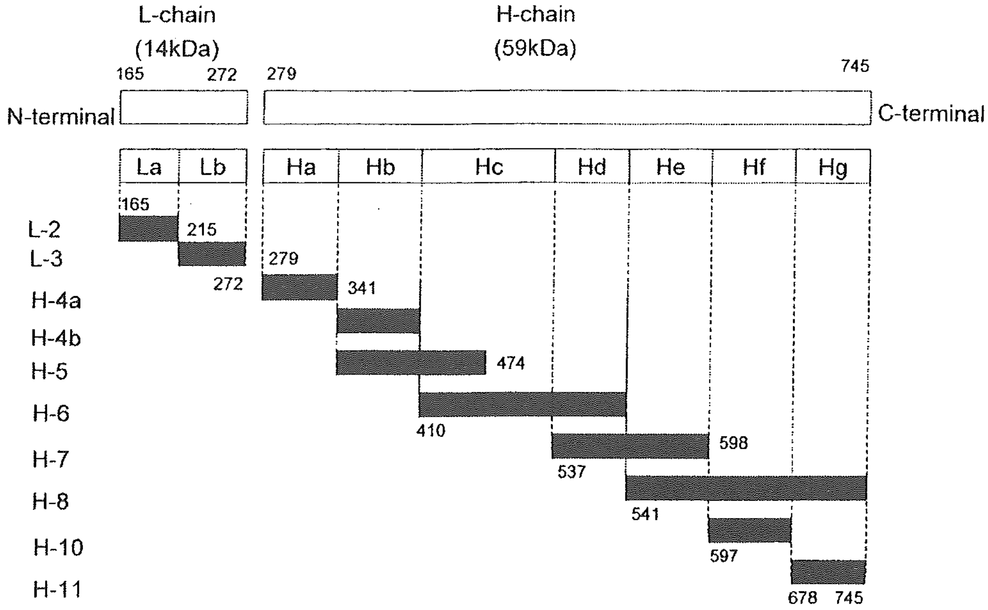


Figure 1. Locations and nomenclature of the recognition sites on both light and heavy chains of myeloperoxidase (MPO). La, Lb, Ha, Hb, Hc, Hd, He, Hf and He are names of the recognition sites on light and heavy chains of MPO. Numbers denote amino acid sequence. L-2, L-3, H-4a, H-4b, H-5, H-6, H-7, H-8, H-10, and H-11 are names of deletion mutants of MPO. L-chain: light chain of MPO, H-chain: heavy chain of MPO.

GmbH, Hilden, Germany) and eluted with 50 ml of a buffer containing 8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris-HCl (pH 4.5). A panel of recombinant fragments was obtained, which covered 10 regions of the MPO molecule (L-2, L-3, H-4a, H-4b, H-5, H-6, H-7, H-8, H-10, and H-11), as shown in Figure 1. The ten fragments cover the whole length of MPO. We classified the light chain into two regions (La and Lb) and heavy chain into seven fragments (from Ha to Hg) according to the recognition sites of the recombinant fragments.

Determination of reactivity of recombinant MPO fragments by ELISA

ELISA was performed as described previously [Tomizawa et al. 1998]. Briefly, each fragment was dissolved in 0.01 M Tris-HCl with 4 M urea and 0.1 M NaH_2PO_4 (pH 7.2). Each protein solution was added to coating buffer (0.015 M sodium carbonate and 0.035 M sodium bicarbonate (pH 9.6)) to a final protein concentration of 10 μg/ml. One hundred Ál aliquots of the mixture were then added to the wells and kept at room temperature overnight. The plate was washed three times with washing buffer (0.0025% Tween 20 (Bio-Rad, Tokyo, Japan) and 0.15 mM sodium azide in phosphate-buffered saline without calcium and magnesium (PBS)). Blocking was carried out with Block Ace (Yukijirushi Nyugyo Co., Ltd., Sapporo, Japan) diluted 1 : 4 with PBS. Then, the patients' sera or rabbit anti-human MPO antibody (Dako, A/S, Glostrup, Denmark) diluted 1 : 500 with dilution buffer (1% bovine serum albumin (Gibco, Rockville, MO, USA) in Tween-Tris-buffered saline) were added. Every plate contained native MPO III (1 μg/well) as a positive control. The plate was washed three times and alkaline phosphatase-conjugated goat anti-human IgG (H + L; ICN ImmunoBiologicals, San Francisco, CA, USA) or alkaline phosphatase-conjugated goat anti-rabbit IgG (H + L; Bio-Rad, Tokyo, Japan) diluted 1 : 6,000 with the dilution buffer was added. The plate was washed three times with washing buffer. As substrate, 100 μl of p-nitrophenylphosphate disodium (Sigma, St. Louis, MO, USA) were added at a concentration of 1 mg/ml dissolved in substrate buffer (50 mM sodium carbonate buffer (pH 9.6) containing 1 mM $MgCl_2$). After incubation for 60 min at room temperature, the plate was measured with double beams at 405 and 650 nm. The relative

Table 2. Relative reactivity to MPO[a] fragments in patients with childhood onset Graves' disease treated with propylthiouracil.

Case	Relative reactivity (%)						
	Ha	Hb	Hc	Hd	He	Hf	Hg
Non-vasculitis group							
1	25	0	25	6	0	37	34
2	35	0	0	3	0	25	25
3	0	0	0	0	0	3	9
4	26	21	16	21	9	34	27
5	2	5	5	7	4	10	11
6	16	16	11	43	16	24	35
7	37	11	0	27	0	22	24
8	23	0	4	22	0	13	6
9	33	24	0	48	3	33	23
10	33	7	25	29	0	32	70
Vasculitis group							
11	45	25	0	47	0	30	25
12	6	0	3	4	0	25	26
13	43	0	0	0	0	0	40
14	0	0	0	0	0	0	2
15	30	0	0	0	0	0	45
16	3	0	2	0	0	0	50

Underline indicates positive result in epitope analysis, defined as a relative reactivity greater than the mean + 3 SD of MPO-ANCA-negative control, [a] = MPO: myeloperoxidase.

reactivity to recombinant MPO fragments was calculated as follow: (F/M – FG/Max FG) × 100 (%), where F is the absorbance of fragments in sample, M is the absorbance of MPO III in sample, FG is the absorbance of fragments of rabbit anti-human MPO antibody, and Max FG is the maximum absorbance of FG. Samples were assessed as positive if the relative reactivity was greater than that of the mean + 3 SD of MPO-ANCA negative control sera (i.e. > 20%). The reliability of this ELISA system was proven by Western blotting in our previous studies [Fujii et al. 2000, Otami et al. 1997, Tomizawa et al. 1998].

Statistical analysis

The levels of ANCA are presented as logarithmic mean and standard deviation range. Statistical analyses were performed by the χ^2 test and nonparametric Mann-Whitney U-test, as appropriate. Statistical calculations were computed using Statview 5.0 (Abacus Concepts, Berkeley, CA, USA). The level of significance was $p < 0.05$. All reported p values were two-tailed.

Results

ANCA analysis in patients with childhood onset Graves' disease treated with PTU

The level of MPO-ANCA was 60 (28 – 126) EU/ml (range: 22 – 205 EU/ml) in the non-vasculitis group and 286 (80 – 1031) EU/ml (range: 35 – 956 EU/ml) in the vasculitis group, with significantly ($p < 0.01$) higher level in the vasculitis group compared to the non-vasculitis group (Table 1). No significant relationship was detected between MPO-ANCA level and the level of each of the antithyroid antibody. No patient had PR3-ANCA (data not shown).

Epitope analysis of the sera from patients

Table 2 shows the relative reactivities to various MPO fragments in the patients. The mean relative reactivity of 10 sera in the non-vasculitis group was 23.0 ± 13.2% (range: 0 – 37%) for Ha, 23.3 ± 11.4% (range:

Table 3. Epitope recognition profile of MPO[a] and clinical findings.

Recognizing sites	Case	CrGN[b]	RPGN[c]	AH[d]
Only linear sites[e]	2	–	–	–
	12	+	–	–
	13	+	–	+
	15	+	+	–
	16	+	+	–
Both linear and other sites	1	–	–	–
	4	–	–	–
	6	–	–	–
	7	–	–	–
	8	–	–	–
	9	–	–	–
	10	–	–	–
	11	+	–	–
None	3	–	–	–
	5	–	–	–
	14	+	–	–

[a] = MPO: myeloperoxidase, [b] = CrGN: crescentic glomerulonephritis, [c] = RPGN: rapidly progressive glomerulonephritis-like glomerulonephritis, [d] = AH: alveolar hemorrhage, [e] = linear sites represent by Ha, Hf, and Hg regions.

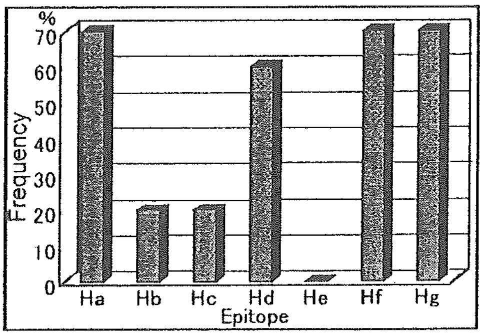


Figure 2a.

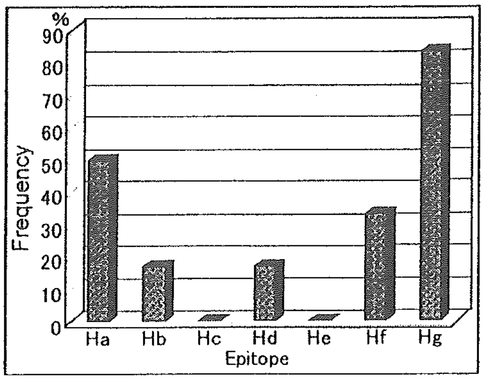


Figure 2b.

Figure 2. Frequencies of recognition sites in patients with myeloperoxidase-specific antineutrophil cytoplasmic autoantibody- (MPO-ANCA) positive childhood onset Graves' disease treated with propylthiouracil. a: patients without vasculitis, b: patients with vasculitis.

3 – 37%) for Hf, and 26.4 ± 18.3% (range: 6 – 70%) for Hg. The mean relative reactivity of 6 sera in the vasculitis group was 21.2 ± 20.6% (range: 0 – 45%) for Ha 31.3 ± 17.5% (range: 2 to 50%) for Hg, 30% (Case 11) and 25% (Case 12) for Hf. There were no differences in relative reactivity for Ha, Hf and Hg (the linear sites) between the two groups, but the relatively reactivities for other epitope sites were higher in the non-vasculitis group compared to the vasculitis group.

In the non-vasculitis group, eight of ten sera reacted with recombinant fragments of the heavy chain of MPO, whereas no serum reacted with the fragments of the light chain (data not shown). The frequencies of site recognition of ten sera were 70% for Ha, 20% for Hb, 20% for Hc, 60% for Hd, 0% for He, 70% for Hf, and 70% for Hg (Figure 2a). However, two sera (Case Nos. 3 and 5) reacted with neither the heavy chain nor the light chain. Only one serum (Case No. 2) recognized only the linear sites near the N-terminus (Ha) and the C-terminus (Hf and Hg), which were reported to be the major MPO epitopes in MPO-ANCA-positive glomerulonephritis [Fujii et al. 2000].

In the vasculitis group, five of six sera reacted with recombinant fragments of the heavy chain of MPO, whereas no serum reacted with the light chain (data not shown). The frequencies of site recognition of six sera were 50% for Ha, 17% for Hb, 0% for Hc, 17% for Hd, 0% for He, 33% for Hf and 83% for Hg (Figure 2b). Only one serum (Case No. 14) reacted with neither the heavy chain nor the light chain. Of six patients, four sera (Case Nos. 12, 13, 15, 16) recognized only the major epitopes (Ha, Hf and/or Hg) and no other epitopes.

The percentage of patients with MPO-ANCA recognizing only the major epitopes was significantly ($p < 0.01$) higher in the vasculitis group (67%) compared to the non-vasculitis group (10%) (Figure 2).

Relationship between epitope profiles and clinical manifestations in patients with MPO-ANCA

Table 3 shows the correlation between epitope recognition profile of MPO and clinical findings.