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Hypercomplementemia in Adult Patients With IgA Nephropathy

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IgA nephropathy (IgAN) is the most common form of chronic glomerulonephritis. Although glomerular deposition of complement components is well known, the evidence of serological complement activation in IgAN is inconclusive. We hypothesized that serum levels of complement components and regulatory proteins in patients with IgAN are correlated with its pathogenesis. In the present study we measured complement components in 50 patients with IgAN and 50 healthy volunteers. C5, C1 inhibitor, factor B, C4 binding protein, factor H, and factor I were measured with the use of single radial immunodiffusion. Mannose-binding lectin (MBL) and properdin (P) were measured by enzyme-linked immunosorbent assay (ELISA). The correlations among complements in the sera of patients with clinical gradings for IgAN (i.e., the good prognosis group, relatively good prognosis group, relatively poor prognosis group, and poor prognosis group) were evaluated. CH50, C4, factor B, P, factor I, and factor H were significantly higher in IgAN patients than in healthy controls. There were significant correlations between C5 and C4 binding protein, between C3 and C5, or between C4 and factor B in patients with IgAN. In the poor prognosis group, C4 binding protein was significantly higher than in the other groups of IgAN patients. hypercomplementemia occurs in IgAN and is associated with an increase in complement regulatory protein (CRP). C4 binding protein analyses can be used to predict disease prognosis. J. Clin. Lab. Anal. 21:77–84, 2007.

Key words: complement; complement regulatory protein; IgA nephropathy; C4 binding protein; factor H

INTRODUCTION

IgA nephropathy (IgAN) is the most common form of chronic glomerulonephritis and is characterized by mesangial deposition of IgA (mainly the IgA1 isotype) and C3. Many studies have revealed glomerular depositions of complement components in patients with IgAN. Immunohistological findings have shown deposits of C3 and properdin (P) in glomerular mesangial areas, and the absence of C1q (1–3). It is known that complement activation via alternative pathways mediates the development of IgAN by depositions of C3 and P. The deposition of mannose-binding lectin (MBL) and MBL-associated serine protease in glomerular mesangial areas has been demonstrated (4–6), and evidence of lectin pathway activation has been found histologically in patients with IgAN (7–12). IgAN develops into progressive renal failure in one-third of patients (13,14). Thus, it is important to clarify the pathogenesis, understand the pathologic condition, and prevent the progression of IgAN.

The serological complement status in IgAN is not well understood. The fact that recurrent IgA deposits in normal transplanted kidneys have been observed in the glomerular mesangial areas of recipients indicates that the basic abnormality of this condition lies within the IgA immune system rather than in the kidney (15). Human IgA1 is a glycoprotein that carries O-linked

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oligosaccharide (O-glycan) side chains in its hinge region (16,17). In patients of IgAN the abnormal O-glycosylation in its IgA1 hinge region (18–21) and the abnormalities were recognized by MBL and activates following lectin pathway (4,6,8). These changes may also lead to self-aggregation of IgA1 (22) and formation of circulating IgA-containing immune complex (IC) (23). IgG- IC, IgA-IC, and IgA1 are also present in the sera of most patients with IgAN (24–30), and the mesangial IgA deposits are composed of IC derived from circulating IC (31). Activation of the alternative pathway by IgA is supported by both in vitro and in vivo observations (9–11). In particular, activation of the alternative pathway by IgA depends on the presence of four-chain molecules F(ab′)2 fragments. Sera from individuals with mutations in the MBL gene showed significantly less activation of C4 by IgA and mannan than sera from controls (12). Thus, the fluid phase activation of complement may be mediated by both alternative and lectin pathways in IgAN. Taken together, these findings suggest that serum levels of complement components in patients with IgAN may reflect complement activities and disease progression.

The goal of the present study was to clarify variations in serum levels of complement components and regulatory proteins (CRPs), and investigate the role of serological complements in IgAN.

MATERIALS AND METHODS

IgAN Patients

Fifty patients (25 men and 25 women) with primary IgAN who had been referred to the Juntendo University Hospital were studied. The age of these patients at the time of renal biopsy ranged from 18 to 66 years (36.6 ± 12.8 years (mean $\pm$ SD)). Eight of the 50 patients were diagnosed at another hospital, and the laboratory data at the time of renal biopsy were not retained. Fifteen of the 50 patients were followed up at 9–76 months (30 ± 23 months) after the initial examination. The study was approved by the institutional review board, and informed consent was obtained from the subjects before they participated in the study. Histological diagnosis was made by standard examination of the renal biopsy specimens using light microscopy, immunofluorescence, and electron microscopy. Serum samples were obtained on the day of renal biopsy and then stored at–80 °C until analysis. Fifty healthy volunteers (30 men and 20 women) were studied as normal controls.

Clinical parameters, including the duration of the disease prior to renal biopsy, gender of the patient, proteinuria, and hematuria, were examined. A laboratory assessment that included routine urinalysis, 24-hr urine protein excretion, 24-hr creatinine clearance (Ccr), and serum levels of urea nitrogen (SUN), creatinine (s-Cr), and total protein (TP) was performed at the time of renal biopsy.

According to Japanese clinical guidelines for the prognosis of IgAN (32), the patients were divided into four groups: the good prognosis group, relatively good prognosis group, relatively poor prognosis group, and poor prognosis group. The clinical guidelines are used to predict the prognosis and select the appropriate treatment according to light-microscopy findings from renal biopsy specimens and clinical findings at the time of renal biopsy.

CRP and MBL

Rabbit antisera to human factor H (H), C4 binding protein (C4bp), P, and purified P were kindly provided by Professor Teizo Fujita of the Department of Biochemistry, Fukushima Medical University, Japan. Biotinylated anti-human P antibody was purchased from the Antibodyshop (Gentofte, Denmark). Goat antisera to human C1 inhibitor (C1INH), factor B (B), and factor I (I) were purchased from Quidel (San Diego, CA). Goat antiserum to human complement C5 was purchased from ICN Pharmaceuticals, Inc. (Aurora, OH). Mouse anti-human IgA1 antibody was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Goat anti-human IgA1 antibody was purchased from Protos Immunoresearch (Burlingame, CA). Human IgA1 was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA).

Measurements of Serum Levels of Complements, CRP, and MBL

Serum levels of C3 and C4 were measured by latex cohesive immunoassay. Serum levels of C1q and CH50 were assayed by nephelometry and Mayer's method, respectively. Serum levels of C5, C1INH, B, C4bp, H, and I were measured by single radial immunodiffusion employing the respective antisera and expressed as a percentage with respect to levels in pooled normal human serum (%NHS). MBL concentrations were measured with the use of commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (MBL-oligomer ELISA; Antibodyshop). For the ELISA developed for P, microtiter plates were coated with 1 μg/mL rabbit anti-human P antibody in carbonate buffer (pH 9.68). The plates were blocked with Block Ace (Snow Brand, Sapporo, Japan) diluted to 1:4 with phosphate-buffered saline (PBS) and incubated at 37 °C for 2 hr. After the serum samples were washed once with PBS containing 0.1% Tween 20 (PBS-Tween), they were diluted with Block Ace diluted to 1:49 with

PBS, loaded into the wells, and then incubated at 37 °C for 1 hr. After washing, the bound P was detected by the addition of 0.5 μg/mL biotinylated rabbit anti-human P antibody at room temperature for 1 hr. After washing, streptavidin-peroxidase diluted to 1:10000 was added and allowed to react at room temperature for 30 min. The plates were subsequently washed three times. The substrate (TMB Substrate Reagent Set; BD Biosciences Pharmingen, San Diego, CA) was finally added, and the absorbency was measured at 450 nm. The amount of P in the samples was calculated based on a standard curve obtained using purified P. The standard curve for P was linear between 1 and 11 ng/mL.

Measurements of Serum Levels of IgA1

IgA1 was determined by ELISA. Microtiter plates were coated with 0.25 μg/mL goat anti-human IgA1 antibody in carbonate buffer (pH 9.68). The plates were blocked with Block Ace diluted to 1:4 with PBS and incubated at 37 °C for 2 hr. After the serum samples were washed, they were diluted with Block Ace diluted to 1:49 with PBS, loaded into the wells, and then incubated at 37 °C for 1 hr. After washing, the bound IgA1 was detected by the addition of 0.25 μg/mL mouse anti-human IgA1 antibody at room temperature for 1 hr. After washing, anti-mouse IgG antibody diluted to 1:10000 was added and then allowed to react at room temperature for 30 min. The plates were subsequently washed three times, and the substrate was finally added. Absorbency was measured at 450 nm. The amount of IgA1 in the serum samples was calculated based on a standard curve obtained using human IgA1. The standard curve for IgA1 was linear between 0.15 and 2.4 μg/mL.

Statistical Analysis

Data are shown as the means ± SD. Comparisons between groups were performed with the Mann-Whitney test, and comparisons of the four classifications were performed with the Kruskal-Wallis test. Comparisons between the time of renal biopsy and after biopsy were performed with the Wilcoxon signed-ranks test. Correlations among the groups were evaluated by linear regression, and $P < 0.05$ was considered significant in all analyses.

RESULTS

Clinical and Laboratory Findings

The clinical and laboratory findings from patients with IgAN are shown in Table 1. There was a significant increase in urinary protein excretion in the poor prognosis group ($P < 0.01$), but no significant differences were shown in the levels of TP, Ccr, s-Cr, IgA, and IgA1 among the four prognosis groups.

Serum Levels of Complement Components

The serum levels of early complement components and CRPs are shown in Table 2. CH50 was significantly higher in patients with IgAN than in healthy controls ($P < 0.01$). C4, B, P, H, and I were significantly higher in patients with IgAN than in healthy controls ($P < 0.01$).

The serum levels of C4, B, P, H, and I in 15 patients with IgAN were reexamined 9–76 months (30 ± 23 months) after the initial study (Table 3). The final serum levels of C4, B, P, and I remained higher than the control levels.

Correlations Between Each Complement Component

Correlations between each complement are shown in Tables 4 and 5. There were 28 significant correlations among complement components, including those between C4 and C3 ($r = 0.723$, $P < 0.001$), C4 and C4bp ($r = 0.608$, $P < 0.001$), and C3 and C4bp ($r = 0.603$, $P < 0.001$) in patients with IgAN. There were 22

TABLE 1. Clinical and laboratory findings in patients with IgAN

Classification	TP (g/dL)	Urinary protein (g/24 hr)	Ccr (mL/min)	Cr (mg/dL)	IgA (mg/dL)	IgA1 (mg/dL)
Good prognosis (n = 6)	7.0 ± 0.9	0.3 ± 0.3	84.7 ± 25.6	0.70 ± 0.16	303 ± 83	130 ± 42
Relatively good prognosis (n = 8)	6.9 ± 0.5	0.3 ± 0.3	84.4 ± 23.1	0.74 ± 0.17	329 ± 73	149 ± 83
Relatively poor prognosis (n = 15)	6.7 ± 0.5	0.8 ± 0.7	75.6 ± 20.1	0.80 ± 0.26	317 ± 83	100 ± 50
Poor prognosis (n = 13)	6.4 ± 0.8	1.9 ± 1.5*	70.1 ± 33.4	1.02 ± 0.41	323 ± 73	96 ± 55
All patients (n = 42)	6.7 ± 0.7	1.2 ± 1.7	76.9 ± 25.9	0.84 ± 0.31	319 ± 76	112 ± 60

*$P < 0.05$ vs. others.

TABLE 2. Serum levels of complement components in patients with IgAN (n = 50)[†]

	CH50 (U/mL)	C1q (mg/dL)	C4 (mg/dL)	C3 (mg/dL)	C5 (%)	B (%)	P (μg/mL)	MBL (mg/mL)	C1INH (%)	C4bp (%)	H (%)	I (%)
IgAN	44.0±8.1*	13.4±2.8	28±11*	101±26	122±28	114±32*	32.6±27.0*	1.8±1.8	104±29	88±19	118±41*	124±35*
Controls	33.5±5.4	12.6±1.7	21±5	106±17	112±17	95±18	21.0±24.0	2.1±1.8	99±21	83±17	98±27	98±16

[†]% indicates expressed as a percentage of pooled normal human serum.
*$P<0.01$ vs. controls.

TABLE 3. Change of serum levels of complement components in patients with IgAN (n = 15)*

	C4 (mg/dL)	B (%)	P (μg/mL)	H (%)	I (%)
IgAN 1st	29 ± 11	127±38	40.8±33.4	137±42	126±31
IgAN 2nd	27±10[a]	117±28[a]	46.1±12.6[a]	99±25	117±34[a]
Controls	21±5	95±18	21.0±24.0	98±27	98±16

*% indicates expressed as a percentage of pooled normal human serum.
[a]Serum levels of complement components remain higher than the control levels.

TABLE 4. Correlation coefficient (r) between each complement component and regulatory proteins in patients with IgAN[a]

	C1q	C4	C3	C5	B	P	C1INH	C4bp	H
C1q	–	–	–	–	–	–	–	–	–
C4	0.253	–	–	–	–	–	–	–	–
C3	0.243	0.723*	–	–	–	–	–	–	–
C5	0.284**	0.311*	0.479**	–	–	–	–	–	–
B	0.259	0.485**	0.416**	0.496*	–	–	–	–	–
P	−0.033	−0.017	0.035	0.201	−0.009	–	–	–	–
C1INH	0.099	0.419**	0.173	0.524*	0.474*	0.195	–	–	–
C4bp	0.201	0.608*	0.603*	0.481**	0.423*	0120	0.353**	–	–
H	0.227	0.549*	0.434*	0.537*	0.502*	0.259	0.547*	0.483*	–
I	0.308**	0.082	0.280*	0.542*	0.440*	−0.013	0.283*	0.354*	0.421**

[a]There was no significant correlation in normal controls.
*$P<0.05$.
**$P<0.05$.

TABLE 5. Correlation coefficient (r) between each complement component and regulatory proteins in normal controls

	C1q	C4	C3	C5	B	P	C1INH	C4bp	H
C1q	–	–	–	–	–	–	–	–	–
C4	0.090	–	–	–	–	–	–	–	–
C3	−0.139	*0.606	–	–	–	–	–	–	–
C5	0.228	*0.451	0.273	–	–	–	–	–	–
B	−0.116	0.125	0.195	*0.331	–	–	–	–	–
P	0.016	−0.111	−0.090	0.055	0.026	–	–	–	–
C1INH	0.162	0.052	−0.132	*0.625	*0.413	0.210	–	–	–
C4bp	*0.458	*0.504	*0.647	0.240	*0.293	−0.072	0.257	–	–
H	0.062	*0.465	*0.417	*0.429	*0.464	−0.184	*0.285	*0.303	–
I	0.250	*0.404	*0.478	*0.335	*0.410	*0.374	*0.472	*0.382	0.194

*$P<0.05$.

significant correlations among complement components, e.g., between C3 and C4bp ($r = 0.647$, $P<0.001$), between C5 and C1INH ($r = 0.625$, $P<0.001$), and between C4 and C3 ($r = 0.606$, $P<0.001$) in normal controls. Nine significant correlations were observed in IgAN patients that were not observed in normal

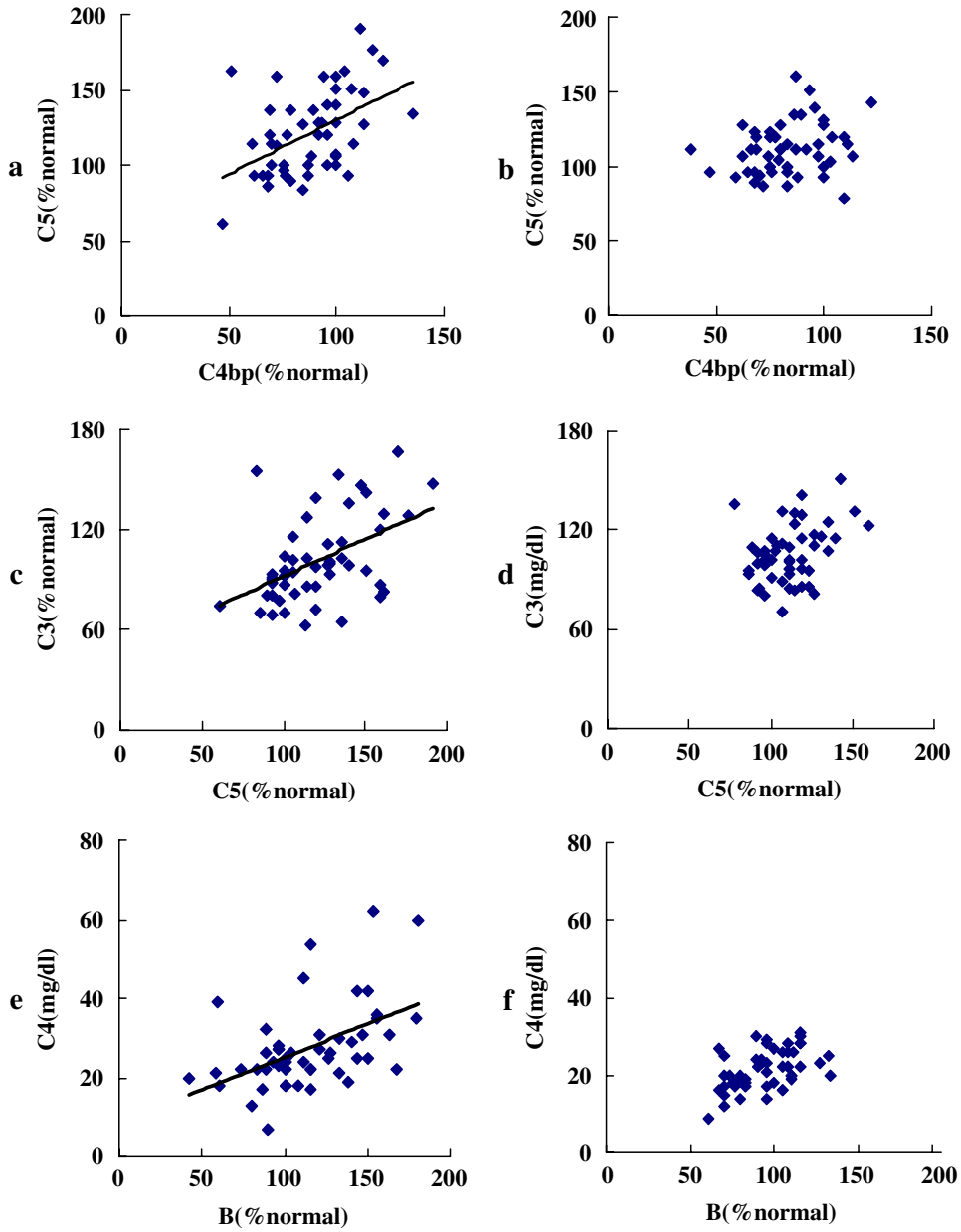

Fig. 1. Relationships between C5 and C4bp, C3 and C5, and C4 and C1INH in (**a**, **c**, and **e**) IgAN patients and (**b**, **d**, and **f**) normal controls.

controls. Figure 1 shows the correlation coefficients ($P<0.001$) between C5 and C4bp ($r = 0.481$, $P<0.001$), C3 and C5 ($r = 0.479$, $P<0.001$), and C4 and B ($r = 0.485$, $P<0.001$) in both groups.

Complement Components and CRPs With Prognosis Classification of IgAN

Complement components and CRPs (except for C4bp) showed no significant differences by prognosis classification. Although the C4bp level was not significantly higher in patients ($88\% \pm 19\%$) than in normal controls, only the poor prognosis group presented a significantly higher level of C4bp ($P<0.05$) compared to the other three groups (Fig. 2).

DISCUSSION

Histological staining in IgAN demonstrates an association between the complement system and glomerular damage. The glomerular depositions of C3, C4, MBL, and other complements indicate the occurrence of complement activation in situ (1–6). However, serum levels of C3, C4, and CH50 are generally within the

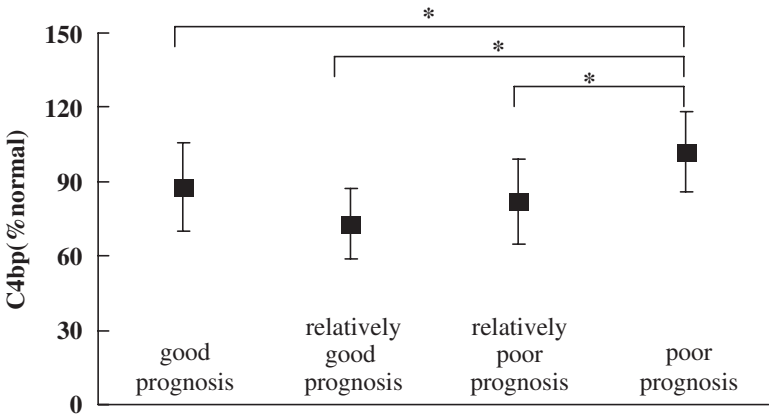

Fig. 2. Serum levels of C4bp in four prognosis groups of IgAN patients. Data are shown as the mean ± SD; $^{*}P<0.05$.

normal range, it was once considered that serum levels of complement did not reflect complement activity. Therefore, we compared serum levels of complement components between healthy controls and patients, and investigated the relationship between the clinical condition and the complement system.

In this study, adult patients with IgAN showed hypercomplementemia, and CH50, C4, B, P, H, and I titers were significantly higher in IgAN patients than in normal controls. In the clinical and laboratory findings from IgAN patients, TP and Ccr tended to decrease with the progression of IgAN. Since the levels of serum TP and urinary protein were normal or low in IgAN patients, it appears that excretion of complement components into the urine does not affect the serum levels of such components. Generally, patients with acute glomerulonephritis, membranoproliferative glomerulonephritis, and lupus nephritis tend to have hypocomplementemia. Serum levels of complements reflect excessive complement consumption. Hypercomplementemia may indicate that some factors influence the overproduction of complement components in IgAN. Most patients with IgAN show a chronic clinical course, and the production of complement may be stimulated as a form of chronic inflammation, such as in the case of chronic tuberculosis infection (33). On the other hand, regulatory proteins such as H, I, C1INH, and C4bp, which inhibit C5 convertase formation, also enhance production, and the serum levels of C3 and C4 may be within the normal range. At the step of 13 convertase formation, B, P, H, and I increased. Since these molecules stabilize and neutralize C3 convertase, it is possible that these regulatory proteins control abnormal activation of complement. In a time-course analysis, the serum levels of C4, B, P and I were remained higher in IgAN patients than in normal controls. These results indicate that hypercomplementemia is not transient in IgAN, and activation of the complement system continues for several years. It has been postulated that complement activation is affected by "tick-over" without inflammation (34–37), and in the present study many significant correlations were seen between each complement component and CRP in both IgAN patients and normal controls. For example, nine significant correlations (e.g., between C5 and C4bp, C3 and C5, and C4 and B) were observed only in IgAN patients. This indicates that overproduction of complement may occur in IgAN.

In this study, C4bp levels were significantly higher in the poor prognosis group than in the other three groups, but C4bp showed no significant difference between IgAN patients and normal controls. Miyazaki et al. (38) reported that the serum levels of C4bp were higher in patients with IgAN than in normal controls, and the frequency of glomerular C4bp deposition increased in parallel with the progression of glomerular histological changes. Although no significant correlation was found between the serum levels of C4bp and the extent or distribution of tissue deposits of C4bp, there was a significant positive correlation between the serum levels of C4bp and the extent of histological changes (38). The difference between their results and ours may stem from differences in histological classification. In the present study we used Japanese clinical guidelines established in 2003 for the prognosis of IgAN, along with additional clinical data (32); however, no such guidelines were available at the time of Miyazaki et al.'s (38) study. Since C4bp binds with protein S, C4bp may accelerate hypercoagulation in renal tissue (39). Therefore, we think that increased serum levels of C4bp and C4bp deposition in glomeruli may be used to predict aggravation of the pathological condition. It is necessary to increase the number of cases and undertake not only serological but also histological analyses. Since our study focused on adult patients with IgAN, these results do not extend to children.

In summary, we found that hypercomplementemia occurs in the pathogenesis of IgAN and is controlled by an increase of CRPs. The results suggest that the complement system may contribute to the pathogenesis of IgAN, and in particular, higher levels of serum C4bp may indicate the severity of histological damage.

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