Antigen and Epitope Specificity of Anti–Glomerular Basement Membrane Antibodies in Patients with Goodpasture Disease with or without Anti-Neutrophil Cytoplasmic Antibodies

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Goodpasture disease (GP) is defined by the presence of anti–glomerular basement membrane (anti-GBM) antibodies and rapidly progressive glomerulonephritis. Besides anti-GBM, many patients with GP produce anti-neutrophil cytoplasmic antibodies (ANCA). For elucidation of the pathophysiologic significance of ANCA in this setting, epitope and antigen specificity of the anti-GBM antibodies and antigen specificity of ANCA were studied. Bovine testis α(IV)NC1 (tNC1); recombinant human α1, α3, α4, and α5(IV)NC1 (rα1 through rα5); and three chimeric proteins that contain previously defined epitope regions designated E_A, E_B, and S2 were used to examine the anti-GBM antibodies by ELISA in 205 Chinese patients with GP with or without ANCA. In the 205 anti-GBM antibody–positive sera, 63 (30.7%) were also ANCA positive (61 myeloperoxidase-ANCA and six proteinase 3–ANCA, four being triple positive). All 205 sera recognized tNC1 and rα3(IV)NC1. In the double-positive group, 54.0, 66.7, 71.4% of the sera could recognize rα1, rα4, and rα5, respectively, compared with 49.3, 60.6, and 55.6% for patients with anti-GBM antibodies alone. The levels of the antibodies to rα3, tNC1, and the α3/α1 ratio were lower in the double-positive group than that in patients with anti-GBM antibody alone ($P < 0.05$). Most of the sera could recognize the epitope regions E_A, E_B, and S2, but the absorbance values to E_A, E_B, and S2 were lower in double-positive group ($P < 0.05$). Double-positive patients had a broader spectrum of anti-GBM antibodies and lower levels of antibodies against α3(IV)NC1 compared with that of patients with anti-GBM antibodies alone.

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Anti–glomerular basement membrane (anti-GBM) disease, also called Goodpasture disease (GP), is a rare autoimmune disorder that is characterized by the production of autoantibodies directed to the GBM, rapidly progressive glomerulonephritis, and a high risk for alveolar hemorrhage. The central role of anti-GBM antibodies in the pathogenesis of GP has been demonstrated by their ability to transfer the disease to monkeys and by the recurrence of disease in human kidney allografts (1,2). The target autoantigen has been identified as the noncollagen domain 1 of the α3 chain of type IV collagen [α3(IV)NC1] (3). Type IV collagen of human basement membranes consists of six different α chains named α1 through α6. Most basement membranes contain only α1 and α2, whereas adult GBM contains mainly α3, α4, and α5. Sera from patients with GP also have autoantibodies against other GBM components, in particular the NC1 domain of other α chains (4). In rare cases, only antibodies that recognize other α chains of collagen IV are found and no antibodies that react with α3(IV)NC1 (5).

Extensive efforts have been focused on identifying the epitopes of the anti-GBM antibodies. It has been clearly shown that anti-GBM antibodies react with conformational epitopes of α3(IV)NC1, which limit the application of linear synthetic peptides for mapping strategies. Then chimeric molecules of human α3(IV)NC1 and α1(IV)NC1 expressed in a mammalian cell line were used for epitope mapping (6–9). Hellmark *et al.* (8) identified nine critical amino acid residues in the amino-terminal part of the α3(IV)NC1 sequence (positions 17, 18, 19, 21, 24, 27, 28, 31, and 57) and produced a recombinant construct named S2 that expresses these substitutions in the α1(IV) background (8). In other studies, two regions that harbor conformational anti-GBM epitopes had been defined at residues 17 to 31 and 127 to 141 of the α3(IV)NC1 domain, which were named as E_A and E_B, respectively (7). Anti-neutrophil cytoplasmic antibodies (ANCA), targeting myeloperoxidase (MPO) or proteinase 3 (PR3), are associated primarily with systemic vasculitis. However, it has been reported that up to one third of patients with GP also produce ANCA, usually p-ANCA, that is specific for MPO (10–14).

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Neither the mechanism of the coexistence of the two kinds of autoantibodies nor its clinical significance is clear. Animal studies have shown that the presence of MPO-ANCA increases the toxicity of anti-GBM antibodies (15). Contrasting to this, some clinical studies have found that ANCA-positive patients with GP have a better prognosis than ANCA-negative patients (13,16). However, other studies have indicated that double-positive patients have similar outcomes to patients with anti-GBM antibody alone (11,12,17).

In vitro biochemical characteristics such as specificity, concentration, and avidity of anti-GBM antibodies may have clinical importance (13). The aim of this study was to compare the antigen specificity and epitope recognition of the anti-GBM antibodies in sera from patients with or without ANCA in a large cohort of Chinese patients with GP.

Materials and Methods

Sera

Sera from 205 patients with GP were collected from a referential diagnostic center in the Institute of Nephrology, Peking University from 1996 to 2005 and were preserved at −20°C until use. GP was diagnosed in these patients by the presence of circulating anti-GBM antibodies and glomerulonephritis. Anti-GBM antibodies were detected by ELISA using bovine α(IV)NC1 as solid-phase ligands. ANCA were screened by indirect immunofluorescence assay and ELISA for anti-MPO antibodies and anti-PR3 antibodies as described previously (18). In brief, standard indirect immunofluorescence assay was performed according to the manufacturer's instructions (Euroimmun, Lübeck, Germany). Highly purified PR3 and MPO were used as solid-phase ligands in antigen-specific ELISA. The antigens were diluted to 2 μg/ml with coating buffer (50 mM sodium bicarbonate [pH 9.6]); antigen-free wells (with coating buffer only) were used to exclude nonspecific binding. The volumes of this step and subsequent steps were 100 μl; all incubations were carried out at 37°C for 1 h, and plates were washed three times with PBS that contained 0.1% Tween 20 (PBST) between stages. Test serum samples were diluted 1:50 with PBST, and both antigen-coated wells and antigen-free wells were coated in duplicate; every plate contained positive, negative, and blank (PBST) controls. The binding was detected with horseradish peroxidase–conjugated goat anti-human IgG (Life Technologies BRL, Grand Island, NY; 1:5000 in PBST). The horseradish peroxidase substrate o-phenylenediamine was used at 0.4 mg/ml in citrate phosphate buffer (pH 5.0). The reaction was stopped by 2 M H_2SO_4, and the results were recorded as the net absorbance at 490 nm (average value of antigen wells minus average value of antigen-free wells) and were expressed as a percentage of the known positive controls. Samples were considered positive when they exceeded the mean plus 3 SD from 100 normal blood donors.

Antigens

Preparation of Bovine Testis α(IV) NC1. Bovine testis basement membrane was prepared as described previously (8) with minor modifications. In brief, the bovine testes were decapsulated, ground, and extracted by 3% Triton X-100 with protease inhibitors (5 mM benzamidine, 10 mM EDTA, 4 mM NEM, and 1 mM PMSF) at 4°C overnight. It was then resuspended in DNAse (100 KU/ml), in 1 M NaCl for 2 h at 4°C, and extracted by 6 M guanidine-HCl overnight at 4°C. After extraction, the testis basement membrane was digested by bacterial collagenase (Worthington Biochemical Corp., Freehold, NJ) for 20 h at 37°C. The supernatant was applied to a DEAE-Sephacel column (Pharmacia, Uppsala, Sweden). NC1 domains, which did not bind to the column, were collected, concentrated, and dialyzed against 0.01 mol/L PBS.

Preparation of Recombinant Human α(IV) NC1 and Chimeric Proteins. The recombinant proteins were produced as described previously (8,19). In brief, cDNA from the NC1 domain of human type IV collagen α1, α3, α4, and α5 were ligated to a type X collagen triple-helix leader sequence and subcloned into the pcDNA3 vector. The constructs were then stably transfected into a human embryonic kidney cell line (HEK 293), and recombinant proteins were harvested and purified from the medium and designated rα1, rα3, rα4, and rα5. Chimeric constructs that contained various combinations of sequences from α1(IV) and α3(IV) were produced by extension PCR technique. Construct T195 consists entirely of α1(IV) NC1 domain with 45 amino acids of α3(IV) NC1 that contain the Hudson E_A site. T194 consists entirely of α1(IV) with 37 amino acids of α3(IV) NC1 that contain the Hudson E_B site (19). Construct S2, which contains a specific epitope of α3(IV) NC1, was constructed in the α1(IV) background by changing each of nine amino acid residues into the corresponding amino acids in α1(IV) (8).

ELISA

Polystyrene microtiter plates (Nunc Immunoplate, Roskilde, Denmark) were coated with 100 μl of antigen in coating buffer (50 mM sodium carbonate [pH 9.6]) overnight at room temperature. All of the antigens were coated at 0.5 μg/ml. As a control for nonspecific binding, 0.5 μg/ml BSA was coated on plates in coating buffer mentioned previously. The plates were then washed three times. A total of 100 μl of human sera, diluted 1:100 in PBS that contained 0.2% BSA, was added to each well. The plates were incubated at room temperature for 1 h; after washing, alkaline phosphatase–conjugated goat anti-human IgG (Fc specific; Sigma, St. Louis, MO) diluted 1:20,000 was added. Incubation resumed for 1 h. P-nitrophenyl phosphate (1 mg/ml; Sigma) in substrate buffer (1 M diethanolamine and 0.5 mM $MgCl_2$ [pH 9.8]) was used as substrate, and color development was measured spectrophotometrically at 405 nm. All assays were run in duplicate, and when standard errors >10% were found, samples were reanalyzed. When the reactivity to the antigens was <0.05 absorbance units above the reactivity to BSA, the sample was regarded as nonspecific. All of the nonspecific samples were excluded from further analysis. Plasma from 20 healthy blood donors was used to build up a cutoff value as the means + 2 SD.

Statistical Analyses

Statistical differences between groups were evaluated by the *t* test or nonparametric test, depending on whether the data were in normal distribution and equal variance. The positive percentages to each antigen between groups were evaluated by χ^2 test.

Results

Among the 205 anti-GBM antibody–positive sera, 63 were also ANCA positive (30.7%). Fifty-seven of the 63 recognized MPO, two recognized PR3, and four recognized both MPO and PR3.

Demographic Data of Patient with GP with and without ANCA

The patients in the double-positive group were significantly older than those with anti-GBM antibody alone (median 64 *versus* 34.5 yr; $P < 0.05$; Table 1). In both groups, there was a male dominance, but it was significantly less profound in the double-positive group (Table 1). Each group exhibited only one

Table 1. Demographic data of patients with GP with and without ANCA[a]

Parameter	ANCA-Positive Patients (n = 63)	ANCA-Negative Patients (n = 142)	P
Age (yr; median)	64.0	34.5	<0.05
Male/female ratio	39/24	108/30	<0.05

[a]ANCA, anti-neutrophil cytoplasmic antibodies; GP, Goodpasture disease.

clear incidence peak, between 60 and 70 yr of age in the double-positive group and between 20 and 30 yr of age in the single-positive group (Figure 1).

Extrarenal Clinical Features of Patients

Fifty-two of the 63 double-positive patients had clinical data. Thirty-nine (75%) of 52 cases had pulmonary involvement, and 23 (44.2%) of 52 cases had hemoptysis. Thirty-one (59.6%) of 52 cases had alveolar infiltration and interstitial changes on chest x-ray and computer tomography scan, 11 (35.5%) of which were diagnosed as pulmonary fibrosis, five (16.1%) which had nodules, and one (3.2%) of which had cavitations. Of the 52 patients, nonspecific symptoms such as fever and malaise were common.

Seventy-five of the 142 ANCA-negative patients with GP had clinical data. Forty-nine (65%) of 75 cases had pulmonary involvements, and 41 (55%) of 75 cases had hemoptysis. Forty-one (55%) of 75 cases had alveolar shadowing or infiltration on chest x-ray and computer tomography scan. The pulmonary features did not show any significant differences between the two groups.

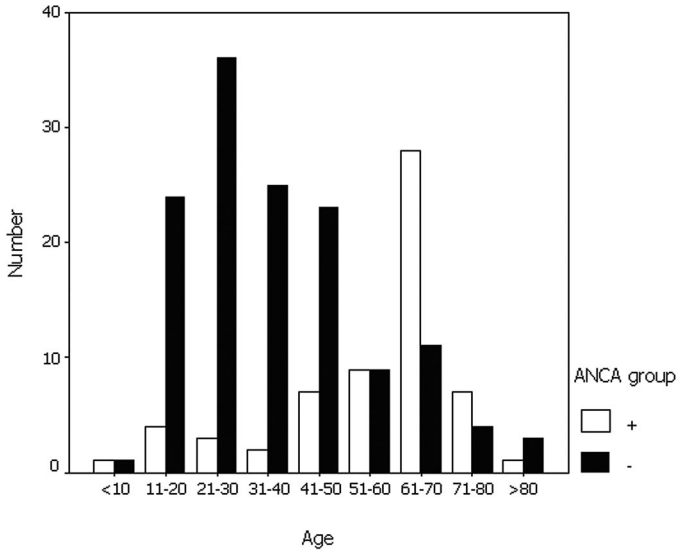

Figure 1. Distribution of age of patients with Goodpasture disease with and without anti-neutrophil cytoplasmic antibodies (ANCA).

Histopathologic Characteristics

Renal biopsies were performed in 60 patients, 12 of whom were double positive. Fifty (83.3%) of 60 had crescent formation in >50% of the glomeruli, and 41 of (68.3%) 60 had crescent formation in >85% of the glomeruli. Eight had crescents in <50% of the glomeruli. The rest had mild mesangial lesions.

Direct immunofluorescence examination was performed in 53 patients. Forty-eight of (90.6%) 53 had linear or fine granular IgG and/or C3 deposition along glomerular capillary wall. Five (9.4%) were with trace IgG and/or C3 deposition, and their absorbance values of antibody against α3(IV)NC1 were 0.388, 1.082, 1.937, 2.762, and 2.793, respectively. The histopathologic characteristics were comparable between patients with and without ANCA (Table 2).

Antigen Specificity

To compare the levels of antibodies against various antigens, we tested all of the sera using tα(IV)NC1, rα1, rα3, rα4, rα5, and BSA. All 205 sera were positive to tNC1 and rα3. Most sera (76.1%) could also recognize at least one of the three other NC1 domains rα1, rα4, or rα5. In the double-positive group, 54.0% of the sera could recognize rα1, 66.7% rα4, and 71.4% rα5. The corresponding figures in the single-positive group were 49.3, 60.6, and 55.6%; the difference regarding rα5 was statistically significant (Table 3). The higher degree of positivity to other NC1 domains was not a consequence of overall higher levels of anti-GBM antibodies. The levels of the antibodies to α3 and tNC1 were lower in double-positive group than that in patients with anti-GBM antibody alone ($P < 0.05$), whereas the levels of the antibodies to α5(IV)NC1 were higher in the double-positive group than that in patients with anti-GBM antibody alone ($P < 0.05$; Table 4). Another way to analyze the antigen diversity is to compare the ratio between absorbance values that were obtained from rα3 and rα1 ELISA. Also these calculations showed a significant difference between ANCA-positive and ANCA- negative patients with GP (Table 4). This was not simply caused by the age differences between the groups, because rα3, rα1, and the ratio rα3/rα1 did not show any significant correlation with age (data not shown).

Epitope Recognition

We also studied the epitopes of the antibodies by three chimeric proteins: T195, T194, and S2. T195 and T194 contain E_A and E_B epitopes, respectively. Almost all sera were positive against all three chimeric proteins. Only two sera failed to recognize S2, whereas eight and 18 sera did not react with E_A and E_B, respectively (Table 3). The patients who exhibited negative results were evenly distributed between the ANCA-positive and ANCA-negative groups. However, by comparing the absorbance values between the two groups, the levels of the antibodies to E_A, E_B, and S2 were significantly lower in the double-positive group than those in patients with anti-GBM antibody alone ($P < 0.05$; Table 4).

Discussion

The current series represent one of the largest collections of anti-GBM–positive sera ever reported. Previous studies indi-

Table 2. Histopathologic characteristics of patients with GP with and without ANCA

Histopathologic Characteristics	ANCA-Positive Patients	ANCA-Negative Patients	*P*
Crescents >50%	11/12	39/48	0.386
Crescents <50%	1/12	7/48	0.569
Mild mesangial lesions	0/12	2/48	0.472
Linear or fine granular IgG and/or C3 deposition	12/12	36/41	0.204

Table 3. Prevalence of anti-GBM antibodies specific to various antigens[a]

Antigen and Epitope	ANCA-Positive Patients (%; $n = 63$)	ANCA-Negative Patients (%; $n = 138$)	*P*
tNC1	100	100	
α1(IV)NC1	54.0	49.3	0.537
α3(IV)NC1	100	100	—
α4(IV)NC1	66.7	60.6	0.405
α5(IV)NC1	71.4	55.6[b]	0.033
E_A	95.2	96.5	0.672
E_B	87.3	93.0	0.187
S2	100	98.6	0.344

[a]GBM, glomerular basement membrane.
[b]$P < 0.05$.

cated that the age distribution of patients with GP seems to be bimodal, with one peak among young adults and another peak between 60 and 70, and that a significant proportion of patients with GP also have ANCA (10,12,13,16,17,20,21). This study confirms both of these observations and brings them together; as shown in Figure 1, the bimodality seems to stem from two distinct subgroups, one ANCA negative with a peak incidence age between 20 and 30 and one double-positive group with an incidence peak between 60 and 70. In this study, men were predominant in the young group with anti-GBM antibodies alone, whereas the gender distribution was more even in the double-positive group; this finding is consistent with reports from white populations (10,12,13,16). Another finding that is in accordance with results from white populations is the preponderance for MPO-ANCA among double-positive patients; only six patients were PR3-ANCA positive, and four of these six were triple positive. In this study, the majority of patients had typical linear or fine granular IgG and/or C3 deposition along the glomerular capillary wall in renal histopathology; however, five patients with GP showed only trace IgG and/or C3 deposition in immunofluorescence assay. This might be due to late diagnosis; the glomeruli were severely damaged and the deposited IgG or C3 might be reabsorbed.

It has been suggested that immunochemical characteristics of autoantibodies, such as subclass, titer, affinity, and epitopes, are associated with clinical and pathologic manifestations of autoimmune diseases (22–25). Recent studies from our groups have revealed that this seems to be the case also for anti-GBM antibodies from patients with GP (13,26). In this study, we tested the antigen spectrum of anti-GBM antibodies by recombinant human α1, 3, 4, 5(IV)NC1. We did not produce recombinant α2(IV)NC1 because the anti-α2(IV)NC1 antibody is rare (4). We found the prevalence of autoantibodies to α5(IV)NC1 to be significantly higher in the double-positive group. The prevalence of antibodies to α1 and α4(IV)NC1 also tended to be higher in the double-positive group, but the differences were NS. A higher tendency to react with the α1 chain was also seen when $\alpha3/\alpha1$ ratio was compared. The ratio was significantly lower for double-positive patients; similar results could also be demonstrated for the $\alpha4/\alpha1$ and the $\alpha5/\alpha1$ ratios (data not shown). These results are consistent with the notion that double-positive patients have a broader spectrum of their antibodies to type IV collagen. In addition to the broader spectrum, the double-positive patients exhibited lower levels of anti-GBM antibodies, both when the NC1 hexamer was used and when recombinant human α3(IV)NC1 was used. Some previous studies reported that the levels of anti-GBM antibodies were lower in double-positive patients (14,16), whereas Hellmark *et al.* (10) did not detect any difference between the two groups when using α3(IV)NC1 monomer. In this study, the double-positive group had a lower absorbance value to the NC1 hexamer and recombinant human α3(IV)NC1, and the $\alpha3/\alpha1$ ratio was much lower in the double-positive group, which indicated that the anti-GBM antibodies in the double-positive group had a lower specificity to α3(IV)NC1.

We found no major difference in epitope specificity between single-positive and double-positive patients. Most patients recognized all three recombinant proteins that harbored the S2, E_A, and E_B epitopes. That all 63 double-positive sera and 140 of the 142 sera from patients with anti-GBM antibody alone could recognize the S2 epitope underscores the critical importance of the nine amino acids that constitute the difference between S2 and the native α1(IV)NC1 sequence. The absorbance values for the three epitopes were significantly lower in the double-positive group. Although it was indicated that ANCA and anti-GBM could act synergistically in an experimental study (15), clinical studies had reported various responses to therapy (11–13,16,17). This study could not determine whether double positivity could affect the prognosis of patients with GP because the outcome data were limited.

It is unclear why both sets of the autoantibodies coexist, but obviously they could not be explained by chance. Some reports show that anti-GBM antibodies appear a few months after the

Table 4. Median absorbance values of the anti-GBM antibodies to various antigens in patients with and without ANCA

Parameter	ANCA-Positive Patients (Median [1st and 3rd Quartiles])	ANCA-Negative Patients (Median [1st and 3rd Quartiles])	*P*
tNC1	1.188 (0.412 to 2.741)	2.801 (1.294 to 3.180)[a]	0.000
α1(IV)NC1	0.306 (0.222 to 0.497)	0.294 (0.194 to 0.642)	0.951
α3(IV)NC1	1.419 (0.469 to 2.902)	2.751 (1.509 to 2.984)[a]	0.000
α4(IV)NC1	0.335 (0.244 to 0.526)	0.317 (0.205 to 1.109)	0.992
α5(IV)NC1	0.229 (0.170 to 0.365)	0.198 (0.144 to 0.299)[a]	0.034
E_A	0.837 (0.387 to 2.309)	1.951 (0.621 to 2.812)[a]	0.007
E_B	0.857 (0.363 to 2.339)	1.484 (0.537 to 2.650)[a]	0.024
S2	0.992 (0.170 to 0.365)	2.429 (0.832 to 2.992)[a]	0.001
α3/α 1	3.465 (1.584 to 6.280)	5.815 (2.605 to 10.531)[a]	0.018

[a] $P < 0.05$.

first detection of ANCA (10,27). It was presumed that ANCA-associated conditions might be the initial and underlying disease (10,16); a possible approach was that ANCA-associated damage to GBM might uncover the "hidden antigens," α3(IV)NC1 or other components, from the GBM, inducing the formation of antibodies to GBM, but some reports described the opposite sequence of events (28,29), which is hard to explain by this hypothesis. No cross-reaction of anti-GBM antibodies and ANCA has been found, but whether such cross-reactivity appears on the T cell level has not been investigated. A recent study suggested that GP might be triggered by molecular mimicry (30), and it is possible that the coexistence of anti-GBM antibody and ANCA could be explained by minimal primary sequence homology that might initiate cross-reactive T cell responses. That the double-positive patients had a broader spectrum of their autoantibodies is consistent with such a speculation.

An association had been shown between GP and HLA (31). HLA-DRB1*1501 alleles increased susceptibility; HLA-DRB1*07 and DRB1*01 were negatively associated with GP. The difference in HLA types between Chinese and white patients with GP and the difference in HLA types between patients with or without ANCA need further studies.

Conclusion

Our investigation showed that in a large cohort, Chinese patients with GP seem to fall into two distinct groups, one consisting primarily of young men with high levels of specific anti-GBM antibodies that do not have ANCA and one older, double-positive group exhibiting lower levels of anti-GBM antibodies with broader reactivity.

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Disclosures

None.

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