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 α1, α3, α4, and α5(IV)NC1 (α1 through α5); and three chimeric proteins that contain previously defined epitope regions designated EA, EP, 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 ro3(IV)NC1. In the double-positive group, 54.0, 66.7, 71.4% of the sera could recognize ro1, ro4, and ro5, respectively, compared with 49.3, 60.6, and 55.6% for patients with anti-GBM antibodies alone. The levels of the antibodies to ro3, 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 EA, EP, and S2, but the absorbance values to EA, EP, 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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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 αI(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, Lubeck, 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 H₂SO₄, 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 αI(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). NCI domains, which did not bind to the column, were collected, concentrated, and dialyzed against 0.01 mol/L PBS.

Preparation of Recombinant Human αI(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 α1, α3, α4, and α5. 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 Eₐ site. T194 consists entirely of α1(IV) with 37 amino acids of α3(IV) NC1 that contain the Hudson Eₐ 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) 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 χ² 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
and without ANCA.

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ANCA-Positive Patients (n = 63)</th>
<th>ANCA-Negative Patients (n = 142)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr; median)</td>
<td>64.0</td>
<td>34.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>39/24</td>
<td>108/30</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*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 shadowing or infiltration 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 involvement, 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.

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, rα3, 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 rα3 and tNC1 were lower in 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 E₄ and E₅ 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 E₄ and E₅ 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 E₄, E₅, 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-
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 (10,12,13,16,17). 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 patients were 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 patients 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 immunochromometric 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 not significant. A higher tendency to react with the α1 chain was also seen when α3/α1 ratio was compared. The ratio was significantly lower for double-positive patients; similar results could also be demonstrated for the α4/α1 and the α5/α1 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 α3/α1 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 2. Histopathologic characteristics of patients with GP with and without ANCA

<table>
<thead>
<tr>
<th>Histopathologic Characteristics</th>
<th>ANCA-Positive Patients</th>
<th>ANCA-Negative Patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crescents &gt;50%</td>
<td>11/12</td>
<td>39/48</td>
<td>0.386</td>
</tr>
<tr>
<td>Crescents &lt;50%</td>
<td>1/12</td>
<td>7/48</td>
<td>0.569</td>
</tr>
<tr>
<td>Mild mesangial lesions</td>
<td>0/12</td>
<td>2/48</td>
<td>0.472</td>
</tr>
<tr>
<td>Linear or fine granular IgG and/or C3 deposition</td>
<td>12/12</td>
<td>36/41</td>
<td>0.204</td>
</tr>
</tbody>
</table>

### Table 3. Prevalence of anti-GBM antibodies specific to various antigens

<table>
<thead>
<tr>
<th>Antigen and Epitope</th>
<th>ANCA-Positive Patients (%)</th>
<th>ANCA-Negative Patients (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>tNC1</td>
<td>100</td>
<td>100</td>
<td>0.537</td>
</tr>
<tr>
<td>α1(IV)NC1</td>
<td>54.0</td>
<td>49.3</td>
<td>0.672</td>
</tr>
<tr>
<td>α3(IV)NC1</td>
<td>100</td>
<td>100</td>
<td>0.405</td>
</tr>
<tr>
<td>α4(IV)NC1</td>
<td>66.7</td>
<td>60.6</td>
<td>0.405</td>
</tr>
<tr>
<td>α5(IV)NC1</td>
<td>71.4</td>
<td>55.6</td>
<td>0.405</td>
</tr>
<tr>
<td>E_A</td>
<td>95.2</td>
<td>96.5</td>
<td>0.405</td>
</tr>
<tr>
<td>E_B</td>
<td>87.3</td>
<td>93.0</td>
<td>0.405</td>
</tr>
<tr>
<td>S2</td>
<td>100</td>
<td>98.6</td>
<td>0.405</td>
</tr>
</tbody>
</table>

*GBM, glomerular basement membrane.

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.

Acknowledgments
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Disclosures
None.

References

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

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

*aP < 0.05.


