Association of Epitope Spreading of Antiglomerular Basement Membrane Antibodies and Kidney Injury

Jun-liang Chen, Shui-yi Hu, Xiao-yu Jia, Juan Zhao, Rui Yang, Zhao Cui, and Ming-hui Zhao

Summary

Background and objectives Antiglomerular basement membrane autoantibodies are pathogenic in antiglomerular basement membrane disease with two major epitopes, E\textsubscript{A} and E\textsubscript{B}, on a\textsubscript{3} chain of type IV collagen. This study investigated the epitope spectrum of antiglomerular basement membrane autoantibodies, aiming to identify the association between epitope specificity and kidney injury.

Design, setting, participants, & measurements All 108 patients with antiglomerular basement membrane disease and complete clinical data were divided into three groups according to renal dysfunction: mild group (n=20) with serum creatinine $\leq 1.5$ mg/dl; moderate group (n=22) with serum creatinine=1.5–6.8 mg/dl; severe group (n=66) with serum creatinine$\geq 6.8$ mg/dl. Epitope spectrums of antibodies were determined by ELISA, and their associations with kidney damage were analyzed. Sequential serum samples in 40 patients were examined during disease courses.

Results E\textsubscript{A} and E\textsubscript{B} were recognized in 79.6% and 72.2% of patients, respectively. E\textsubscript{A} and E\textsubscript{B} reactions were the lowest in the mild group and higher in the moderate group (E\textsubscript{A}: 35.0% versus 81.8%, $P=0.002$; E\textsubscript{B}: 15.0% versus 68.2%, $P=0.001$). They were the highest in the severe group (E\textsubscript{A}: 92.4%, $P=0.01$; E\textsubscript{B}: 90.9%, $P=0.02$). Close association was observed between renal injury and E\textsubscript{A} and E\textsubscript{B} reactions. Multivariate Cox regression analysis showed that E\textsubscript{B} reaction was an independent risk factor for renal failure (hazard ratio =6.91, $P=0.02$). The recognition for non-E\textsubscript{AB} remained low among groups. No augmentation of epitope spectrum was shown in serial serum samples.

Conclusions Intramolecular epitope spreading might occur before the onset of human antiglomerular basement membrane disease. The autoimmunity to E\textsubscript{A} and E\textsubscript{B}, especially E\textsubscript{B}, was crucial for kidney dysfunction.

Introduction

Antiglomerular basement membrane (anti-GBM) disease, also termed Goodpasture’s disease, is a rare autoimmune disorder characterized by rapidly progressive glomerulonephritis and high risk of pulmonary hemorrhage (1). Anti-GBM autoantibody has been shown as a pathogenic factor to disease initiation (2), with the major target antigen located on the noncollagenous domain of a\textsubscript{3} chain of type IV collagen [\alpha3(IV)NC1] on GBM (3–5). Two conformational epitopes have been discovered: residues 17–31 are named E\textsubscript{A}, and residues 127–141 are named E\textsubscript{B} (6–9).

However, the role of epitope specificity and its variation are less clear in the initiation and progression of human anti-GBM disease.

Epitope spreading is defined as the development of immune responses to epitopes distinct from and noncrossexsitive with the original epitope, which initiates the immune response (10). Epitope spreading within the same molecule (intramolecular) or to the other molecules (intermolecular) has been documented in many autoimmune disorders (11–13). The spread of autoimmunity amplifies the deleterious process of tissue injury, and the consequence is vice versa (14). Both intra- and intermolecular epitope spreading were shown in animal models of anti-GBM disease (15,16).

We recently provided evidence that intermolecular epitope spreading may occur in human anti-GBM disease (17).

In the current study, epitope specificity of anti-GBM antibodies was determined using recombinant E\textsubscript{A}, E\textsubscript{B}, and non-E\textsubscript{AB} [the region of a\textsubscript{3}(IV)NC1 apart from E\textsubscript{A} and E\textsubscript{B}] in a large cohort of patients with different kidney injury and sequential serum samples. Clinical and pathologic features were also investigated, aiming to find the association between epitope specificity and severity of kidney injury and the possible intramolecular epitope spreading in disease initiation.

Materials and Methods

Sera and Patients

Sera from 108 patients with anti-GBM disease, diagnosed in Peking University First Hospital during 1997–2008, were collected on diagnosis before immunosuppressive treatment or plasmapheresis. Serial serum samples collected during disease courses were available in 40 patients. All the sera were positive...
for anti-GBM autoantibodies by ELISA using purified bovine α1(IV)NC1 and recombinant human α3(IV)NC1 as solid-phase antigens. All the sera were negative for anti-neutrophil cytoplasmic antibody by indirect immunofluorescence using ethanol-fixed human neutrophils and antigen-specific ELISA against purified myeloperoxidase and proteinase 3. Sera from 50 healthy blood donors were used as normal controls. All the sera were stored at −20°C until use.

Clinical and pathologic data were collected from medical records at the time of presentation and during follow-up visits. Renal biopsies were performed in 82 patients with linear deposition of IgG with or without C3 along GBM by immunofluorescence. Crescentic glomerulonephritis was defined as a large crescent (>50%) formation involving in over 50% of glomeruli. Informed consent was obtained for each sampling of tissue and blood. The research was in compliance with the Declaration of Helsinki and approved by the ethics committee of our hospital.

Preparation of Recombinant Human Eα, Eβ, and Non-EAB

Recombinant proteins were produced as described earlier (6,18). Briefly, cDNA from the NC1 domain of human type IV collagen α1 and α3 was ligated to a type X collagen triple helix leader sequence and subcloned into pcDNA3 vector, respectively. The constructs were then stably transfected into a human embryonic kidney cell line (HEK 293). Recombinant proteins were harvested and purified from the medium by affinity chromatography, and they were designated as recombinant α1 and α3. Chimeric constructs containing different combinations of sequences from α1(IV) and α3(IV) were produced by the extension PCR technique. Eα consisted entirely of α1(IV)NC1 domain, with 45 amino acids of α3(IV)NC1 containing the Hudson Eα site (8). Eβ consisted entirely of α1(IV)NC1, with 37 amino acids of α3(IV)NC1 containing the Hudson Eβ site (8). Non-EAB consisted entirely of α3(IV)NC1, with the region of Eα and Eβ substituted by α1(IV)NC1.

Detection for Antibodies against Eα, Eβ, and Non-EAB by ELISA

The recombinant human Eα, Eβ, and non-EAB were diluted 2 μg/ml with 50 mEq/L bicarbonate buffer (pH 9.6) and coated onto three-quarters of the wells of a polystyrene microtitre plate (Nunc; Roskiled, Denmark). The other one-quarter of the wells were coated with 50 mEq/L bicarbonate buffer as antigen-free wells to exclude nonspecific binding. Incubation was performed at 37°C for 60 minutes. Test sera were diluted 1:50 in PBS containing 0.1% Tween-20 (PBST) and added to both antigen-coated and -free wells at 37°C for 30 minutes. Then, alkaline phosphatase-conjugated goat anti-human IgG (Fc-specific; Sigma, St. Louis, MO) diluted 1:4000 was added at 37°C for 30 minutes. P-nitrophenyl phosphate (100 mg/dl; Sigma, St. Louis) in substrate buffer (105 g/L diethanolamine, 4.8 mg/dl MgCl₂, pH 9.8) was used as substrate, and color development was measured spectrophotometrically at 405 nm (Bio-Rad, Tokyo, Japan). The plates were washed three times between steps, and the volume of each well was 100 μl. Each plate contained positive, negative, and blank (PBST) controls. Sera from a patient with predetermined high titers of autoantibodies against Eα, Eβ, and non-EAB were used as positive controls. When standard errors over 10% were found, samples were re-examined. Absorbance values from anticyc bicarbonate ELISA were subtracted from the results of anti-Eα, Eβ, and non-EAB ELISAs. Sera from 50 normal individuals diluted 1:50 were used to build up the cutoff values using mean + 2 SD.

Statistical Analyses

Differences of quantitative parameters were assessed using t tests or one-way ANOVAs. Differences of qualitative data were compared using chi-squared tests. Pearson or Spearman rank correlation was performed to analyze the relationship between the levels of antibodies against epitopes and the quantitative clinical parameters. Kaplan-Meier curves were used to analyze renal survival and patient survival. Univariate survival analyses were performed using log-rank tests. Multivariate survival analyses were performed using Cox regression models. Covariates were selected using the variables that showed a prognostic role in the previous univariate survival analysis. Results were expressed as hazard ratio (HR) with 95% confidence intervals (95% CIs). A P value < 0.05 was considered significant. All statistical analyses were performed using PASW Statistics 18.0 (SPSS Inc., Chicago, IL).

Results

Demographic and Clinical Data

Among the 108 patients, 75 patients were male, and 33 patients were female. The mean age was 38.4 ± 17.3 years (15–89 years), and 40 (37.0%) patients had hemoptysis. The mean concentration of serum creatinine (Scr) on diagnosis was 8.2 ± 5.2 mg/dl. The mean level of circulating anti-GBM autoantibodies was 68.1 ± 39.8 U/ml. Sixty-seven (81.7%) patients presented with crescentic glomerulonephritis. Fifty (61.0%) patients had crescents in more than 85% of the glomeruli.

The patients were divided into three groups according to the Scr on diagnosis. Group A (mild kidney dysfunction) included 20 patients with Scr < 1.5 mg/dl; group B (moderate kidney dysfunction) included 22 patients with Scr = 1.5–6.8 mg/dl, and group C (severe kidney dysfunction) included 66 patients with Scr ≥ 6.8 mg/dl.

The demographic, clinical, and pathologic data of patients in the three groups are shown in Table 1. Patients in group C had a significantly higher proportion of oliguria/anuria (P < 0.001) and gross hematuria (P < 0.001), higher Scr on diagnosis (P < 0.001), higher level of anti-GBM autoantibodies (P < 0.001), more crescents in glomeruli (P < 0.001), and worse renal survival (P < 0.001) than patients in groups A and B as expected.

Epitope Specificity of Anti-GBM Autoantibodies in Patients with Different Kidney Functions

The cutoff values of each epitope-specific ELISA were 0.043 for Eα, 0.074 for Eβ, and 0.034 for non-EAB.

All sera from the 108 patients could recognize α3(IV) NC1, among which 86 (79.6%), 78 (72.2%), and 12 (11.1%) sera recognized Eα, Eβ, and non-EAB, respectively (Table 2). In group A (mild kidney dysfunction), the frequencies of sera recognizing Eα and Eβ were 35.0% (7/20) and 15.0% (3/20), respectively. In group B (moderate
To group C, the recognition of EA and EB was greater across the severity of impaired kidney function from group A to C (92.4% (61/66); P=0.001) and 90.9% (60/66; P=0.002) than patients not recognizing EA. Patients recognizing EB presented with higher levels of anti-EA antibodies but also, a significantly higher proportion of oliguria/anuria (50.0% versus 13.3%, P<0.001) and a higher percentage of crescents in glomeruli (83.4±25.8% versus 53.2±40.2%, P=0.002) than patients with negative anti-EB antibodies (Table 3).

The prognostic significance of EA and EB reaction was determined by Kaplan–Meier curves and log-rank tests for univariate survival analyses to assess renal and patient outcomes. We found that the recognition for both EA (HR=4.10, 95% CI=1.77–9.50, P=0.001) and EB (HR=4.63, 95% CI=2.20–9.71, P<0.001) (Figure 2) and the reaction to both EA and EB (HR=3.74, 95% CI=1.96–7.16, P<0.001) were predictors for renal failure during follow-up visits. Furthermore, multivariate Cox regression analyses showed that the recognition for EA (HR=6.91, 95% CI=1.38–34.65, P=0.02) was an independent risk factor for renal failure (Table 4). The recognition for neither EA nor EB was a predictor for patient death (P>0.05).

The recognition for non-EAB had no significant association with the clinical or pathologic data of patients (P>0.05).

### Association between Epitope Specificity and Clinical Manifestations

Patients recognizing EA presented with higher levels of anti-GBM antibodies (72.7±40.2 versus 51.3±34.4 U/ml, P=0.03), higher Scr on diagnosis (9.3±4.8 versus 4.1±4.8 mg/dl, P<0.001), and higher frequencies of crescentic glomerulonephritis (88.2% versus 50.0%, P=0.003) than patients not recognizing EA. Patients recognizing EB presented with not only the same clinical features as the patients with anti-EA antibodies but also, a significantly higher proportion of oliguria/anuria (50.0% versus 13.3%, P<0.001) and a higher percentage of crescents in glomeruli (83.4±25.8% versus 53.2±40.2%, P=0.002) than patients with negative anti-EB antibodies (Table 3).

### Antibody Levels of Epitopes and Their Associations with Clinical Manifestations

The levels of antibodies against EA and EB both exhibited a greater tendency from group A to C, but no significant difference was found among the groups (P>0.05) (Table 2). The levels of antibodies against non-EAB remained low in each group (P>0.05). None of the clinical manifestations were revealed to have correlations with the levels of antibodies against EA, EB, or non-EAB (P>0.05).

### Epitope Spectrum of Anti-GBM Antibodies in Disease Courses with Serial Serum Samples

Anti-GBM autoantibodies against EA, EB, and non-EAB were detected in 40 patients (7 patients from group A, 14 patients from group B, and 19 patients from group C) with sequential serum samples during disease courses with...
treatments and follow-up. There were 33 (82.5%), 31 (77.5%), and 5 (12.5%) sera recognizing EA, EB, and non-EAB, respectively. No difference in the recognition of EA, EB, or non-EAB was shown between the 40 patients and the other 68 patients ($P > 0.05$).

Epitope specificity of anti-GBM autoantibodies was detected in these sequential serum samples. The frequencies of sera recognizing EA, EB, and non-EAB were all slightly decreased with time. No augmentation of epitope spectrum was observed on anti-GBM antibodies during disease courses (Figure 3).

### Table 2. The frequencies and levels of antiglomerular basement membrane antibodies against each epitope in patients with different renal functions

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Group A (mild renal dysfunction; $n=20$)</th>
<th>Group B (moderate renal dysfunction; $n=22$)</th>
<th>Group C (severe renal dysfunction; $n=66$)</th>
<th>$P$ (group A versus group B versus group C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA</td>
<td>Frequency 35.0% ($7/20$)$^a$</td>
<td>81.8% ($18/22$)$^b$</td>
<td>92.4% ($61/66$)$^c$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Absorbance values 0.3±0.3</td>
<td>0.6±0.4</td>
<td>0.6±0.4</td>
<td>0.09</td>
</tr>
<tr>
<td>EB</td>
<td>Frequency 15.0% ($3/20$)$^d$</td>
<td>68.2% ($15/22$)$^e$</td>
<td>90.9% ($60/66$)$^f$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Absorbance values 0.5±0.4</td>
<td>0.5±0.4</td>
<td>0.7±0.4</td>
<td>0.17</td>
</tr>
<tr>
<td>Non-EAB</td>
<td>Frequency 15.0% ($3/20$)</td>
<td>4.5% ($1/22$)</td>
<td>12.1% ($8/66$)</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>Absorbance values 0.1±0.0</td>
<td>0.1</td>
<td>0.1±0.0</td>
<td>0.13</td>
</tr>
</tbody>
</table>

GBM, glomerular basement membrane.

$^a$Comparison between groups A and B ($P=0.002$).

$^b$Comparison between groups B and C ($P=0.31$).

$^c$Comparison between groups A and C ($P<0.001$).

$^d$Comparison between groups A and B ($P=0.001$).

$^e$Comparison between groups B and C ($P=0.02$).

$^f$Comparison between groups A and C ($P<0.001$).

Figure 1. | The frequencies of sera against EA, EB, and non-EAB compared among patients with different renal functions. Patients with serum creatinine $\leq 1.5$ mg/dl were defined as group A (mild renal dysfunction); patients with serum creatinine $=1.5–6.8$ mg/dl were defined as group B (moderate renal dysfunction), and patients with serum creatinine $>6.8$ mg/dl were defined as group C (severe renal dysfunction). The frequencies of sera against EA and EB exhibited an increasing tendency from group A to group C. *$P<0.05$; **$P<0.01$.

**Discussion**

In the current study, it was shown that both EA and EB were major epitopes for anti-GBM antibodies with high frequency of recognition; for EA, it was 79.6%, and for EB, it was 72.2%. It is consistent with previous reports [5,8,9,18–20]. Difference in epitope specificity showed up when the patients were divided into three groups according to their renal functions at presentation. Among the three groups, the recognition for EA and EB became greater gradually from patients with normal kidney function (group A) to patients with moderate and severe renal...
Table 3. The association between epitope recognition and clinical manifestations of patients with antiglomerular basement membrane disease

<table>
<thead>
<tr>
<th></th>
<th>Anti-(E_A)–positive ((n=86))</th>
<th>Anti-(E_A)–negative ((n=22))</th>
<th>(P)</th>
<th>Anti-(E_B)–positive ((n=78))</th>
<th>Anti-(E_B)–negative ((n=30))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>36.4±16.1</td>
<td>46.6±19.8</td>
<td>0.01</td>
<td>35.7±14.2</td>
<td>45.7±22.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>58/28</td>
<td>17/5</td>
<td>0.37</td>
<td>53/25</td>
<td>22/8</td>
<td>0.59</td>
</tr>
<tr>
<td>Hydrocarbon exposure, % ((n))</td>
<td>10.5 (9/86)</td>
<td>18.2 (4/22)</td>
<td>0.52</td>
<td>11.5 (9/78)</td>
<td>13.3 (4/30)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Smoking, % ((n))</td>
<td>40.7 (35/86)</td>
<td>40.9 (9/22)</td>
<td>0.82</td>
<td>41.0 (32/78)</td>
<td>40.0 (12/30)</td>
<td>0.99</td>
</tr>
<tr>
<td>Prodromal infection, % ((n))</td>
<td>51.2 (44/86)</td>
<td>27.3 (6/22)</td>
<td>0.06</td>
<td>45.2 (36/78)</td>
<td>46.7 (14/30)</td>
<td>0.89</td>
</tr>
<tr>
<td>Gross hematuria, % ((n))</td>
<td>34.9 (30/86)</td>
<td>13.6 (16/22)</td>
<td>&lt;0.001</td>
<td>34.6 (27/78)</td>
<td>20.0 (6/30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oliguria/anuria, % ((n))</td>
<td>44.2 (38/86)</td>
<td>22.7 (5/22)</td>
<td>0.07</td>
<td>50.0 (39/78)</td>
<td>13.3 (4/30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>9.3±4.8</td>
<td>4.1±4.8</td>
<td>&lt;0.001</td>
<td>9.9±4.4</td>
<td>3.8±4.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Level of anti-GBM antibodies (U/ml)</td>
<td>72.7±40.2</td>
<td>51.3±34.4</td>
<td>0.03</td>
<td>77.9±40.9</td>
<td>43.9±24.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Crescentic glomerulonephritis, % ((n))</td>
<td>88.2 (60/68)</td>
<td>50.0 (7/14)</td>
<td>0.003</td>
<td>87.5 (56/64)</td>
<td>61.6 (11/18)</td>
<td>0.011</td>
</tr>
<tr>
<td>Percentage of crescent</td>
<td>81.7±25.7</td>
<td>52.9±47.1</td>
<td>0.08</td>
<td>83.4±25.8</td>
<td>53.3±40.2</td>
<td>0.002</td>
</tr>
<tr>
<td>Complement deposition, % ((n))</td>
<td>63.2 (43/68)</td>
<td>64.3 (9/14)</td>
<td>&gt;0.99</td>
<td>62.5 (40/64)</td>
<td>66.7 (12/18)</td>
<td>0.79</td>
</tr>
<tr>
<td>Renal survival at 1 yr, % ((n))</td>
<td>24.4 (21/86)</td>
<td>72.7 (16/22)</td>
<td>&lt;0.001</td>
<td>19.2 (15/78)</td>
<td>73.3 (22/30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Patient survival at 1 yr, % ((n))</td>
<td>81.4 (70/86)</td>
<td>86.4 (19/22)</td>
<td>0.27</td>
<td>82.1 (64/78)</td>
<td>83.3 (25/30)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

GBM, glomerular basement membrane.

Figure 2. Kaplan–Meier analysis of the renal outcome of patients with antiglomerular basement membrane disease compared between positive and negative anti-\(E_B\) antibodies (log-rank test).
dysfunction (groups B and C). This difference reached significance among groups and was notable for epitope EB. This finding suggests that the epitope spectrums of anti-GBM antibodies might become broader gradually as renal function gets worse. In patients with different renal functions at different stages of disease progression, our findings provide the first evidence of intramolecular epitope spreading occurring in the development of human anti-GBM disease.

Epitope spreading of B cells has been proven in the work by Bolton et al. (15) in an animal model; sera and kidney elute from rats immunized by a synthetic peptide of rat α3(IV)NC1 reveal reactivity to not only the immunizing peptide but also, epitopes of α3(IV)NC1 external to the peptide immunogen. Another study indicates that a single T cell epitope of α3(IV)NC1 is sufficient to initiate the full clinical spectrum of anti-GBM disease, and it could also elicit antibody responses to diverse native GBM proteins (21). In human anti-GBM disease, this study and our previous study (17) both provide evidence for epitope spreading occurring in disease development. Delineation of epitope spreading is essential to understanding the pathogenesis of autoimmune disease and developing potential therapies. For example, immune tolerance may be prompted through oral or nasal administration of appropriate epitopes, especially the original one, which initiates epitope spreading. Actually, it has been successful in animal experiments (22). Help from T cells is required for epitope spreading of antibody response. The emergence, persistence, and disappearance of antibodies are all regulated by T and B

<table>
<thead>
<tr>
<th>Variables</th>
<th>Renal failure</th>
<th>Patient death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>E_A</td>
<td>2.90</td>
<td>0.69–12.18</td>
</tr>
<tr>
<td>E_B</td>
<td>6.91</td>
<td>1.38–34.65</td>
</tr>
<tr>
<td>Both E_A and E_B</td>
<td>0.43</td>
<td>0.07–2.72</td>
</tr>
<tr>
<td>Non-EAB</td>
<td>1.68</td>
<td>0.82–3.44</td>
</tr>
<tr>
<td>Age (increased by 1 yr)</td>
<td>1.00</td>
<td>0.98–1.02</td>
</tr>
<tr>
<td>Sex (female)</td>
<td>1.15</td>
<td>0.65–2.03</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>1.75</td>
<td>0.87–3.63</td>
</tr>
<tr>
<td>Initial serum creatinine (mg/dl)</td>
<td></td>
<td></td>
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<tr>
<td>First tertile (≤1.5)</td>
<td>0.01</td>
<td>0.00–0.10</td>
</tr>
<tr>
<td>Second tertile (1.5–6.8)</td>
<td>0.23</td>
<td>0.10–0.54</td>
</tr>
<tr>
<td>Third tertile (≥6.8)</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Anti-GBM antibody level (U/ml) (increased by 1 U/ml)</td>
<td>1.00</td>
<td>0.99–1.01</td>
</tr>
<tr>
<td>Plasma exchange</td>
<td>0.52</td>
<td>0.30–0.89</td>
</tr>
</tbody>
</table>

CI, confidence interval; GBM, glomerular basement membrane.

Figure 3. | The epitope spectrum of antiglomerular basement membrane autoantibodies during disease courses. No augmentation of epitope spectrum was observed. m, month; w, week.
cell crosstalks (23,24). In the absence of anti-GBM antibodies, α3(IV)NC1-specific CD4+ T cell alone is sufficient to initiate glomerular injury (25). Thus, additional investigations on autoreactive T cells are needed in human anti-GBM disease.

The pathogenic role of E₄ has been identified by the predominance of Ea reaction in the total immunoreactivity of α3(IV)NC1 with high titers and affinity (9). In rats, Ea could induce florid disease similar to full-length native GBM (18). The current study showed that patients with positive anti-E₄ antibodies had more severe kidney injury, which provides more evidence for the important role of E₄ in human anti-GBM disease.

The role of E₈ epitope in the pathogenesis is still controversial. Animal experiments showed that E₈ alone could not induce the disease; a half-dose each E₈ plus E₈ induced disease identical to E₈ alone. It suggests a non-pathogenic but augmenting effect of E₈ (18). In this study, we found that patients with anti-E₈ antibodies presented with similar clinical features to patients with Ea reaction, with higher antibody levels, higher serum creatinine, and more glomerular crescents. It implies a possible nephritogenic role of E₈ reaction in human anti-GBM disease. Furthermore, by multivariate Cox regression analysis, we identified that E₈ specificity, but not E₄, was the independent prognostic factor for renal failure. These findings imply that a major pathogenic epitope may be located in E₈.

E₈ is sequestered within the α3α4α5 protomer at the interface between the α3NC1 and α4NC1 domains (9,18,26,27). The binding of antibodies to E₈ would dissociate the interactions of α3NC1 and α4NC1, increase the accessibility of autoantibodies to these monomers, and subsequently, expand the immunogenicity of GBM antigens (26). This mechanism gives an explanation for the probable pathogenic role of E₈, and furthermore, it provides a bridge linking intra- and intermolecular epitope spreading that was revealed in human anti-GBM disease. We previously documented that α4(IV)NC1 was more frequently recognized as renal dysfunction deterioration (17). During disease development, the expanding recognition for E₈ may lead to the dissociation of α3α4α5 protomer and the immunogenicity of α4NC1, hereby developing intermolecular epitope spreading to α4(IV)NC1. These findings suggest the need for additional studies about the potential clinical use of E₈-specific immunity.

After disease presentation, we did not observe additional augmentation on the epitope spectrum of anti-GBM antibodies, which suggests that epitope spreading occurs before disease onset. Very similar observations have been reported in systemic lupus erythematosus and rheumatoid arthritis (28,29), where the accrual of new types of autoantibodies gradually increased up to the time of diagnosis and then virtually stopped. A possible explanation for this finding is the effects of immunosuppressive treatments, which are commonly prescribed after diagnosis is established.

In our patient group, 11.1% of patients had antibodies against non-E₄, which is in agreement with previous studies (9,18). However, their low frequency, low level, and lack of correlation with clinical features suggest a dispensable role in the disease.

The limitation of our study is that we used patients with different degrees of renal dysfunction to represent the progression of disease. It is not the real natural history of disease and makes our findings indirect evidence for the occurrence of epitope spreading. However, because of ethical considerations, it is impossible to perform experiments in human beings, like animal models, to investigate disease courses. Thus, we enrolled as many patients as we could with different clinical phenotypes to make the findings more credible.

In summary, we provide evidence that intramolecular epitope spreading may occur before the onset of human anti-GBM disease. The recognition of E₈ and the recognition of Ea are both essential to disease initiation, especially the autoimmunity to Eb, which is associated with the severity and prognosis of kidney impairment and may play a crucial role in antigen exposure and immune expansion.

Acknowledgments

The technical support by Ying Zhang and Miao Wang was greatly appreciated.

This work was supported by Chinese 973 Project Grant 2012CB517702, National Natural Science Fund of China to the Innovation Research Group Grant 81021004, and National Natural Science Fund of China Grant 81170645.

Disclosures

None.

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**Received:** May 21, 2012  **Accepted:** September 13, 2012

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Published online ahead of print. Publication date available at www.cjasn.org.