Supplementary materials

Preparation of autoantibodies against H1-4, H1-11 and H1-12 from plasma of patients with anti-GBM disease by using microtiter plates

According to a previously described method (Radanova M, et al, Immunobiology, 2012), microtiter plates (Costar, Corning, NY) were coated with 10 µg/ml of H1-4, H1-11 or H1-12 in 0.05 mol/l bicarbonate buffer (pH 9.6) for 1 h at 37°C. After blocking with 1% BSA, the plates were incubated overnight at 4°C with test plasma diluted to 1:50 in PBS/0.5 M NaCl. After washing, the bound autoantibodies were eluted with 50 µl/well of 0.1 M glycine–HCl, pH 2.8 (1 min incubation). The glycine buffer in the wells was immediately neutralized using 10 µl of 1.5 M Tris, pH 8.8. Aliquots of the eluted autoantibodies against H1-4, H1-11 and H1-12 were pooled, respectively and were dialyzed extensively against PBS/0.75 M NaCl. The protein concentrations of the eluted autoantibodies were determined spectrophotometrically (OD280).

Complement C3 deposition assay

A C3 deposition assay was performed according to a previously described method (Roumenina LT, et al, J Biol Chem, 2011). Briefly, microtiter plates were coated with 2 µg/ml recombinant human α3(IV)NC1 or 10 µg/ml H1-4, H1-11 or H1-12 as the solid phase antigens. After blocking with 1% BSA, the wells were washed with PBS plus 0.1% Tween-20. Purified total IgG from a patient with positive anti-α3(IV)NC1 autoantibodies at 0.2 mg/ml was applied to the wells as positive control to form immune complexes. Purified total IgG from a healthy human (Sigma,

St Louis, MO, USA) at 0.2 mg/ml was applied to the wells as negative control, and 0.2 mg/ml of autoantibodies against H1-4, H1-11 or H1-12 was applied to the wells coated with respective antigens as testing samples. After washing with veronal-buffered saline $(VBST)^{Ca+Mg}$ (VBS containing 0.15 mmol/l calcium and 0.5 mmol/l magnesium with 0.1% Tween 20), 1% fresh normal human plasma diluted in VBST was added to provide complement components. The plates were then incubated for 1 h at 37°C. After washing, the plates were incubated with a rabbit polyclonal antibody against human C3c (Dako, Denmark) for 1 h at 37°C, followed by incubation with goat anti-rabbit IgG conjugated with alkaline phosphatase for 1 h at 37°C. After three washes, P-nitrophenyl phosphate (PNPP, 1 mg/ml; Sigma, St. Louis, MO, USA) was added to the substrate buffer (1 M diethanolamine and 0.5 mM $MgCl_2$ (pH 9.8)). The OD was measured at 405 nm.

Figure 1. Cross-reactivity between autoantibodies against α3(IV)NC1, and H1-4, H1-12. A) The microtiter plate was coated with H1-4 and was competed with different concentrations of H1-4 and α3(IV)NC1. B) The microtiter plate was coated with H1-12 and was competed with different concentrations of H1-12 and α3(IV)NC1.

Figure 2. C3 deposition assay. The combination of α3(IV)NC1 and anti-α3(IV)NC1 autoantibodies could fix complement, while combination of α3(IV)NC1 and normal human IgG could not fix complement. Combination of autoantibodies against linear MPO peptides and their corresponding peptides could fix complement.

Figure 3. H1-4 and H1-12 on the surface of the MPO molecule (PDB: 3F9P). Amino acid sequence of H1 is in cyan, H1-4 is in red, and H1-11 is in magenta.

Figure 1.

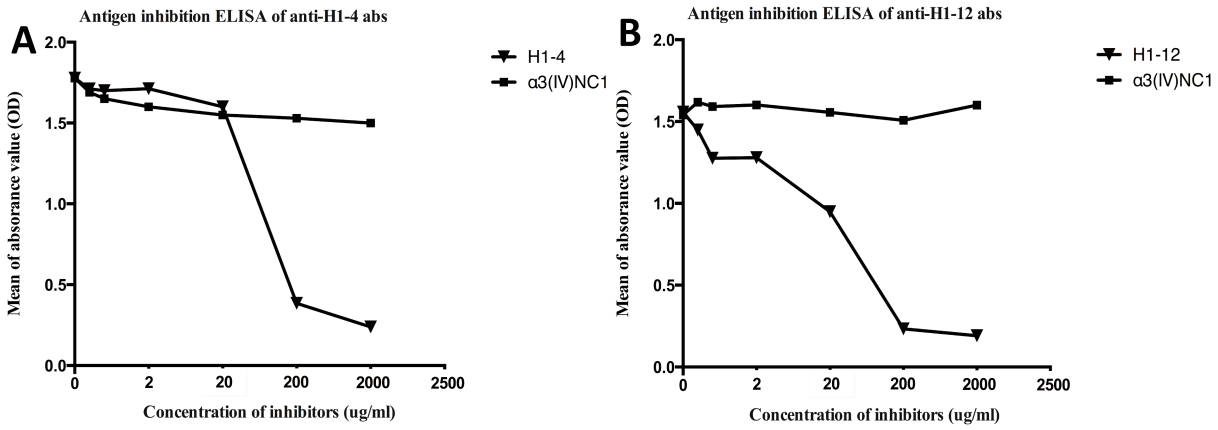
A
Antigen inhibition ELISA of anti-H1-4 abs
Mean of absorance value (OD)
2.0
1.5
1.0
0.5
0.0
0
2
20
200
2000
2500
Concentration of inhibitors (ug/ml)
H1-4
α3(IV)NC1
B
Antigen inhibition ELISA of anti-H1-12 abs
Mean of absorance value (OD)
2.0
1.5
1.0
0.5
0.0
0
2
20
200
2000
2500
Concentration of inhibitors (ug/ml)
H1-12
α3(IV)NC1

Figure 2.

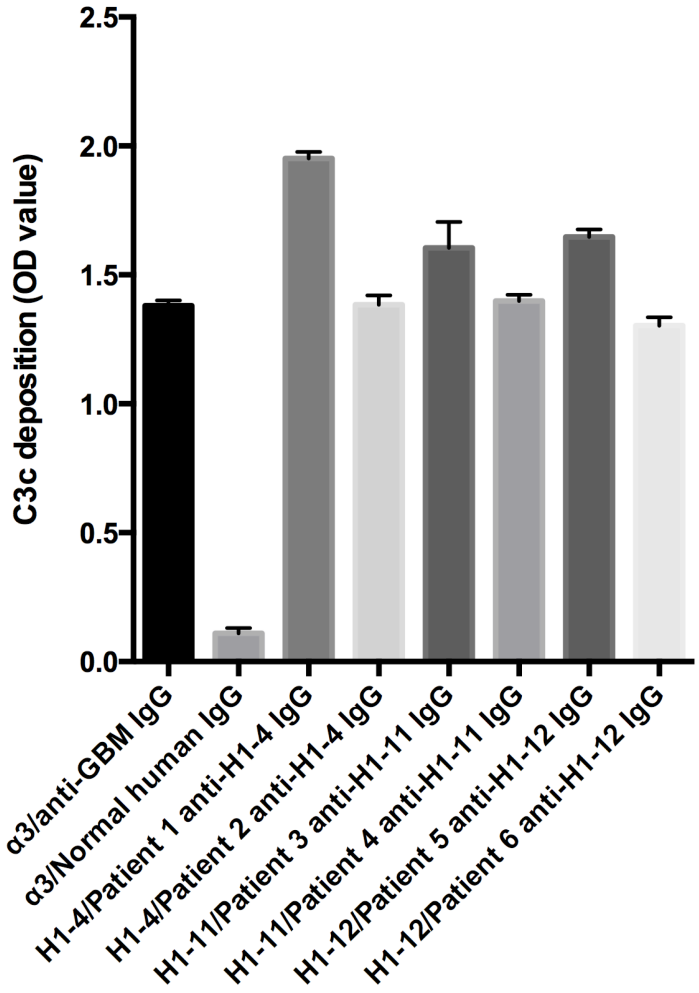
C3c deposition (OD value)
2.5
2.0
1.5
1.0
0.5
0.0
α3/anti-GBM IgG
α3/Normal human IgG
H1-4/Patient 1 anti-H1-4 IgG
H1-4/Patient 2 anti-H1-4 IgG
H1-11/Patient 3 anti-H1-11 IgG
H1-11/Patient 4 anti-H1-11 IgG
H1-12/Patient 5 anti-H1-12 IgG
H1-12/Patient 6 anti-H1-12 IgG

Figure 3