Comparison of Anti-GBM Antibodies in Sera With or Without ANCA

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Abstract. An appreciable percentage of patients with serum anti-glomerular basement membrane (anti-GBM) antibodies also have antineutrophil cytoplasmic antibodies (ANCA), against either myeloperoxidase (MPO-ANCA), or proteinase 3 (PR3-ANCA). In sera without ANCA, the anti-GBM antibodies have been shown to react mainly with the noncollagenous domain (NC1) of Type IV collagen, and especially with its $\alpha 3$ chain, $\alpha 3$(IV)NC1. In most sera, the antibodies can be partially blocked by a monoclonal antibody (Mab17) against $\alpha 3$(IV)NC1, suggesting that a limited region is recognized. Although there is evidence that some anti-GBM antibodies that coexist with ANCA react with $\alpha 3$(IV)NC1, extensive analysis of the specificity of such anti-GBM antibodies has not been reported.

In the study presented here, sera were analyzed from 332 patients tested both for anti-GBM antibodies and ANCA (MPO or PR3-ANCA) and found to have one or more positive tests. Of the 100 sera with anti-GBM antibodies, 38 also had ANCA—25 with MPO-ANCA (66%), 12 with PR3-ANCA (32%), and one with both (2%). Of the 232 sera with ANCA only, 153 had MPO-ANCA (66%), 75 had PR3-ANCA (32%), and four had both (2%). Sera was also analyzed from 259 other patients who had positive ANCA tests and were not tested for anti-GBM antibodies: 138 had MPO-ANCA (54%), and 121 had PR3-ANCA (46%). The relative frequencies of MPO or PR3-ANCA in patients with coexisting anti-GBM antibodies did not differ significantly from those in all patients with ANCA ($P = 0.35$).

Seventeen sera with anti-GBM antibodies only and 16 sera with anti-GBM antibodies plus ANCA were selected for further studies to compare the specificity of anti-GBM antibodies in sera with or without ANCA. Using enzyme-linked immunosorbent assays (ELISA), all sera in both groups were found to react with the NC1 domain (as a hexamer) of bovine Type IV collagen and with $\alpha 3$(IV)NC1 monomers. Furthermore, all but six sera also reacted with one or more of the $\alpha 1$, 2, and 4 (IV)NC1 monomers, generally with considerably lower titers. Reactivity to $\alpha 3$(IV)NC1 was partially blocked by Mab17, with comparable degrees of inhibition in both groups. Western blot analysis with the human NC1 domains revealed no differences in reactivity between the two groups. Thus, differences in antigen specificities of anti-GBM antibodies in sera with or without ANCA were not detected. The anti-GBM response in both situations is hypothesized to be driven by the same immunogen, which is probably derived from NC1 domains of endogenous Type IV collagen. (J Am Soc Nephrol 8: 376–385, 1997)

Recently developed tests for circulating anti-glomerular basement membrane (anti-GBM) antibodies and antineutrophil cytoplasmic antibodies (ANCA) provide highly specific markers for a group of conditions with overlapping features (1–3). Nearly all patients with anti-GBM antibodies, reactive with Type IV collagen, have rapidly progressive glomerulonephritis(GN), often associated with lung hemorrhage (Goodpasture's syndrome) (4–6). The glomeruli show necrotizing lesions and crescents, together with linear accumulation of immunoglobulins along the GBM (7,8). Patients with either of two types of ANCA—namely antibodies to proteinase 3 (PR3-ANCA) or myeloperoxidase (MPO-ANCA)—often have similar clinical and renal histologic findings, although the glomeruli lack appreciable immunoglobulin accumulation (pauci immune necrotizing and crescentic GN) (8,9). Furthermore, patients with ANCA frequently also have features characteristic of a group of interrelated conditions usually classified as Wegener's granulomatosis, microscopic polyangiitis (polyarteritis), or the Churg-Strauss syndrome (8,10,11).

ANCA and anti-GBM antibodies are rarely found in other conditions, including different forms of GN or vasculitis (1,3,10). Furthermore, PR3 and MPO-ANCA are rarely found together in a given patient. It is, therefore, surprising that among patients with anti-GBM antibodies, an appreciable percentage (20%–40%) also have ANCA (9,12–18). The association is either with MPO or PR3-ANCA, although in some reports, a marked preponderance of MPO-ANCA has been noted (13,15,18). The reason for the coexistence of anti-GBM

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antibodies and ANCA is unknown, but because of the rarity of these autoantibodies, it is clearly not due to chance. There is no evidence that the anti-GBM antibodies and ANCA are cross-reactive (12,14,19,20).

The autoantibodies in patients with anti-GBM nephritis or Goodpasture's syndrome have been shown to react with components of the noncollagenous domain (NC1) of Type IV collagen. The NC1 domain can be released from Type IV collagen by collagenase digestion as a hexamer, derived from two cross-linked Type IV collagen molecules. The hexamer has been used as an antigen in assays for anti-GBM antibodies and, following dissociation into dimers and monomers, to analyze reactivity of the anti-GBM antibodies with the various α(IV)NC1 domains. Several studies have shown that the anti-GBM antibodies react mainly with the α3(IV)NC1 (21–23) found in a limited group of basement membranes, including alveolar basement membranes(ABM)and GBM. The anti-α3(IV)NC1 antibodies appear to recognize a restricted group of epitopes, as indicated by the demonstration that the autoantibodies from most patients are partially blocked by monoclonal antibodies to the α3(IV)NC1 domain (22,24). In addition, all sera from 44 patients with anti-GBM antibodies were shown to inhibit partially the reactivity of antibodies to α3(IV)NC1 obtained from one patient with Goodpasture's syndrome (22). There is evidence that much of the reactivity is with epitopes confined to each end of the α3(IV)NC1 domain (25–27). In addition to reactivity with α3(IV)NC1, antibodies to other α(IV)NC1 domains have been detected, generally in lower titers (21–23); there is evidence that most such reactivity is directed at epitopes shared with α3(IV)NC1 (22,28).

Only limited information is available concerning the fine specificity of anti-GBM antibodies in patients who are also known to have ANCA. Although several studies have provided evidence that reactivity is directed against α3(IV)NC1, several findings suggest that other basement membrane components are recognized in some cases (29,30). One patient with ANCA has been described with antibodies to α1(IV)NC1 but not to α3(IV)NC1 (31). This is apparently the only study in which reactivity to purified α(IV)NC1 monomers was investigated. In addition, it is not known whether the anti-α3(IV)NC1 antibodies in sera coexisting with ANCA can be inhibited by monoclonal antibodies to α3(IV)NC1.

In the study presented here, we assessed the frequency of ANCA types and titers in sera with or without anti-GBM antibodies. We also compared the titers and specificity of anti-GBM antibodies in a group of patients with or without ANCA.

Materials and Methods

Serum Specimens

We analyzed serum samples from 332 patients found to have ANCA, anti-GBM antibodies, or both in specimens submitted to our laboratory at the Massachusetts General Hospital between 1981 and 1995. Of the 332 patients, 100 had anti-GBM antibodies, of whom 38 also had ANCA, and 232 had ANCA without anti-GBM antibodies. Since 1988, Western blot analysis with human GBM digests, as described below, has been the method used to detect anti-GBM antibodies. Specimens with positive results obtained earlier (mainly by radioimmunoassays or ELISA with GBM digests) were retested for the study presented here by Western blot analysis, using sera stored at −70°C. ANCA tests were performed by antigen-specific immunoassays for anti-PR3 and MPO antibodies, as described below; specimens submitted before 1988, when ANCA tests were established in our laboratory, were tested for the study presented here, using sera that had been stored at −70°C. Each serum sample used in this study was from a different patient and, with a few exceptions, was the first specimen collected. Most of the patients were untreated before serum collection, although a few had been on steroids or immunosuppressive treatment for several days.

The main goal of the study was to compare the specificity of anti-GBM antibodies in sera with or without coexisting ANCA. Of the 100 patients with positive tests for anti-GBM antibodies, 38 had positive ANCA tests (25 with anti-MPO antibodies, 12 with anti-PR3 antibodies, and one with both). Specimens from 33 patients were selected for further studies on the fine specificity of the anti-GBM antibodies (17 with anti-GBM antibodies only and 16 with anti-GBM plus ANCA). Aside from selecting approximately equal numbers with anti-GBM antibodies alone or with coexisting ANCA, and equal numbers with PR3 and MPO-ANCA, the 33 specimens were chosen randomly from samples with sufficient remaining serum for the additional studies described below. In all but one case, the serum studied was the first sample received. In the one case (case 22), a sample had been obtained 31 months earlierand was found to contain anti-MPO antibodies without anti-GBM antibodies.The more recent specimen, which contained both anti-MPO antibodies and anti-GBM antibodies, was used in this study. Because the 33 serum samples were selected without review of the patients records, we could not compare clinical features in patients who had anti-GBM antibodies alone with those who also had ANCA. Although clinical and pathologic findings were not reviewed in individual patients, we showed in previous studies that among patients with the clinical features of rapidly progressive GN, positive tests for PR3 or MPO-ANCA (with negative tests for anti-GBM antibodies) obtained in our laboratory had a predictive value of>99% for pauci immune necrotizing and crescentic GN (3,8,32). Furthermore, there is evidence that all patients with anti-GBM antibodies (against α (3)IV NC1) who have renal biopsies are found to have linear GBM staining for IgG and GN (14,15,33). Only 33 specimens were studied because the complexity of the additional serologic tests made a larger study impractical. As controls in these special studies, we used 20 serum samples with ANCA only and two normal sera.

In addition to analyzing the specificity of anti-GBM antibodies in the 33 sera with or without ANCA, we investigated other aspects of the association between anti-GBM antibodies and ANCA, using larger numbers of serum samples from the 332 patients with positive tests for anti-GBM antibodies, ANCA, or both, as follows. Age and sex distribution was analyzed in 249 patients for whom the relevant information was available. There were 252 specimens with sufficient amounts of sera to permit measurement of ANCA titers in sera with or without anti-GBM antibodies. The 270 specimens with positive tests for ANCA, of which 38 also had anti-GBM antibodies, were reviewed to compare the relative frequency of anti-PR3 or anti-MPO antibodies in sera with or without anti-GBM antibodies.

Some other serum specimens that were sent to the laboratory for ANCA tests only and found to be positive were not assayed for anti-GBM antibodies if the patient had no evidence of renal disease. Although these were not included in the 332 patients for whom the above issues were studied, we analyzed the relative frequency of PR-3

ANCA and MPO-ANCA in 259 patients with positive ANCA tests who were not tested for anti-GBM antibodies.

Antibodies

Three monoclonal antibodies (Mab) to α(IV)NC1 domains were used: (Mab 6) to bovine α1(IV)NC1; (Mab 17) to bovine α3(IV)NC1; and (MabA7) to human α5(IV)NC1. These antibodies have been characterized and described elsewhere (34,35). In addition, polyclonal rabbit antibodies to bovine α2(IV)NC1 and the α4(IV)NC1 domains were used (36).

Preparation of Collagenase Solubilized Human GBM

The GBM material used for Western blot analysis was prepared from human kidneys obtained at autopsy or by nephrectomy. Glomeruli were isolated by differential sieving, according to the method of Edgington (37) as modified by Gang (38). The glomeruli were washed 2–3 times in phosphate-buffered saline (PBS) and twice in distilled water, and then sonicated by pulsation using a micro tip at 40% duty cycle. The insoluble basement membrane material was dialyzed against distilled water and lyophilized. Ten mg of GBM was suspended in 2 mL of 0.1 M Tris-HCl, pH 7.5, 5 mM $CaCl_2$ and digested according to the method of Wieslander (4) with chromatographically purified collagenase Type VII from *Clostridium histolyticum* (Sigma, St. Louis, MO) (1000 units/mg GBM). The mixture was sonicated for 30 min in a water bath ultra-sonicator and incubated at 37°C in a shaking water bath for 48 h. The digestion was stopped by adding ethylenediamine-tetraacetic acid (EDTA) to a concentration of 1 mM. The particulate solution was centrifuged at 100,000 g for 30 min at 4°C. The clear supernatant was recovered and assayed for protein concentration by the Lowry method (39) and used for Western blot analysis (see below).

Preparation of Bovine NC1 Hexamers and α(IV)NC1 Monomers

NC1 hexamers and α(IV)NC1 monomers were prepared from bovine kidneys as described previously (22). Briefly, kidney cortex was homogenized and extracted overnight with 6 M guanidine-buffer containing protease inhibitors. Nonextracted material was further digested using bacterial collagenase. The solubilized NC1 hexamers were purified from the digest using ion exchange and gel filtration chromatography. Some of the NC1 hexamer preparation was further used for the purification of different α(IV)NC1 monomers. The NC1 hexamer was denatured in 6 M guanidine-buffer, and a pool of single α(IV)NC1 monomers was isolated using gel filtration. Four populations, α1(IV)NC1, α2(IV)NC1, α3(IV)NC1, and α4(IV)NC1, were separated using reversed-phase high-performance liquid chromatography (40). The purity of the α(IV)NC1 monomers was analyzed by immunoblot analysis using the monoclonal and polyclonal antibodies to the α1–5(IV)NC1.

Preparation of PR3 and MPO

PR3 was prepared as previously described (41). In brief, normal human granulocytes were obtained from buffy coat preparations of blood from normal donors by Ficoll-isopaque separation to remove mononuclear cells, followed by hypotonic lysis of the red blood cells. The granulocyte-enriched preparation was then suspended in 0.2 M acetate buffer and sonicated. After low- and high-speed centrifugation, the supernatant was brought to neutral pH. PR3 was affinity-purified from the resulting preparation by use of a monoclonal antibody affinity column, as previously described (41). MPO was purchased from CalBiochem (San Diego, CA).

ELISA

Wells in polystyrene microtiter plates (NUNC Immunoplate, Roskilde, Denmark) were coated with 100 μl of antigen (see below) or bovine serum albumin (BSA) (control for nonspecific reactivity) diluted in 50 mM Tris-HCl, pH 7.4, and 6 M guanidine-HCl. The plates were incubated overnight at room temperature and then washed three times with 0.15 M NaCl, 0.05% (v/v) Tween 20. 100 μl of serum diluted in PBS containing 0.2% (w/v) BSA, pH 7.3 (BSA-PBS) (1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.27 M NaCl, 2.5 mM KCl, 0.05% (w/v) NaN_3) was added to each well. The plates were incubated at room temperature for 1 h and after washing, alkaline phosphatase-conjugated goat anti-human IgG, 1/2000, (Kirkegaard and Perry Laboratories, Gaitherburg, MD) was added and incubated for 1 h. *P*-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, MO (1 mg/mL) in substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8), was used as substrate, and color development was measured using a microtiterplate reader at 405 nm after 1 h incubation.

NC1 Hexamer ELISA

Wells in microtiter plates were coated with 0.5 μg/mL of bovine NC1 hexamer as described above. A reference serum was used as positive control, and sera from healthy blood donors were used as negative controls. Serum samples from 20 patients with ANCA only were also analyzed to determine if these antibodies interfered with the assay. The serum samples were diluted 1/100, and bound human IgG was assayed as described above. Because all samples were diluted to the same extent (1/100), the higher values (>2.0) may represent underestimates of the true titers. There was no reactivity in wells coated with BSA.

α(IV)NC1 Monomers ELISA

Wells in microtiter plates were coated with 0.2 μg/mL of the different α(IV)NC1 monomers. The serum samples were diluted 1/100, 1/500, 1/2500, and 1/12500 respectively to determine the dilution that yielded an absorbance of 0.5, corresponding to the steep part of the dilution curve of a positive, high-titered anti-GBM sample. Serum samples with very high antibody titers were analyzed further, using dilutions higher than 1/12500, to obtain an accurate dilution curve. Reference positive sera and sera from healthy blood donors were used as controls. The plates were assayed for bound human IgG as described above. The data were analyzed using analysis of variance single-factor test.

Inhibition ELISA

A competitive ELISA was used to measure the inhibition of reactivity of patients' sera to the α3(IV)NC1 monomer by the monoclonal antibody to α3(IV)NC1, Mab 17. Culture supernatants containing the monoclonal antibody, diluted to a concentration of 4 μg/mL, were premixed with the serum sample diluted so as to give an absorbance value of 0.5 in ELISA detecting antibodies to the α3(IV)NC1 as described above. The mixture was added to microtiter plates and coated with bovine α3(IV)NC1 monomer (0.2 μg/mL), and the plates were then assayed for bound human IgG as described above.

ANCA Assay

ELISA for quantitative measurement of anti-PR3 and anti-MPO antibodies were performed as described (2), with minor modifications. Thirty five μl of borate-buffered saline with 5 μg per mL of protein were added to wells of vinyl microtiter plates; the protein was allowed

to adhere overnight. Control wells were coated with buffer alone. The wells were blocked with buffer containing 1% nonfat dried milk and then incubated with various dilutions of sera. Two high-titer ANCA sera, one each with anti-PR3 and anti-MPO antibodies, were arbitrarily assigned 128 units of activity, and the titer of antibodies of test sera were measured relative to these positive controls (32). Activities in test samples were determined relative to the positive controls at 1:16 dilutions and at subsequent serial 1:8 dilutions. The units were determined through multiplying the dilution of the test sample relative to the dilution of the standard at the steep part of the curve by 128. As described in a previous report, positive results were defined as 5.0 or more units for the PR3 ELISA and 2.8 or more units for the MPO ELISA (2). The data were analyzed using the *t* test.

Western Blot Analysis

Collagenase digested human GBM (100 μg/gel) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Laemmli (42) in 12% nonreducing gels and transferred to nitrocellulose paper as described by Burnett (43). After transfer, the nitrocellulose paper was blocked in 5% nonfat dried milk for 1 h, cut into 3–4-mm strips, and incubated for a minimum of 1 h in 1 mL of serum diluted 1/10 in 0.5% nonfat dried milk buffer. The strips were washed, and bound IgG was detected by sequential incubations with biotin-conjugated goat anti-human IgG and avidin-biotin-peroxidase complex (Vector Labs, Burlingame, CA) followed by development in 3-amino-9-ethylcarbazole.

Results

Prevalence and Type of ANCA in Sera With or Without Anti-GBM Antibodies

Of the 332 patients tested for anti-GBM antibodies and ANCA and found to have one or more positive tests, 100 had anti-GBM antibodies, of whom 38 also had ANCA, and 232 had ANCA only. Of the 38 sera with coexisting ANCA and anti-GBM antibodies, 25 (66%) had MPO-ANCA, 12 (32%) had PR3-ANCA, and one (2%) had both types of ANCA. These percentages did not differ from those found in the 232 patients with positive tests for ANCA only: 153 (66%) had MPO-ANCA; 75 (32%) had PR3-ANCA; and four (2%) had both MPO and PR3-ANCA. However, as noted above (Materials and Methods section), specimens from some other patients with positive ANCA tests were not also tested for anti-GBM antibodies, largely because the patients lacked overt evidence of renal disease. It is therefore possible that the 232 ANCA positive specimens with negative anti-GBM antibody tests were not representative of the total population of sera with ANCA; in particular, a bias may have existed against samples with anti-PR3 antibodies because renal disease may be less prevalent in patients with PR3-ANCA than in those with MPO-ANCA. To assess this issue, we analyzed the frequency of anti-PR3 and MPO antibodies in specimens from 259 other patients with positive ANCA tests who were not tested for anti-GBM antibodies; 138(54%) had anti-MPO and 121 (46%) anti-PR3 antibodies. Thus, the frequency of anti-MPO antibodies was somewhat higher than in the 232 specimens described above. However, a comparison of specimens having coexisting ANCA and anti-GBM antibodies with all specimens having ANCA (some of which also contained anti-GBM antibodies) revealed no statistically significant difference in the relative frequencies of PR3 and MPO-ANCA ($P = 0.35$).

ANCA Titers in 252 Patients With or Without Anti-GBM Antibodies

Sufficient amounts of sera were available from 252 patients to permit measurement of ANCA titers. Serum samples with MPO-ANCA antibodies alone ($N = 146$) had a median titer of 128 units, compared with the median titer of 147 units found in samples that also contained anti-GBM antibodies ($N = 23$); these differences were not statistically significant (*t* test, $P = 0.4$). Similarly, we found no significant difference ($P = 0.9$) in PR3-ANCA antibody titers in sera with ($N = 11$) or without ($N = 72$) anti-GBM antibodies; median titers, 62 and 57 units, respectively.

Age and Sex Distribution in Patients With Anti-GBM Antibodies With or Without ANCA

Some studies have reported that patients with anti-GBM antibodies only are younger than those with ANCA plus anti-GBM antibodies or with ANCA alone (13,15). We reviewed demographic data, which were available on 249 patients with positive tests for anti-GBM antibodies, ANCA or both. As shown in Table 1, patients with ANCA only or with ANCA plus anti-GBM antibodies were somewhat older than those with anti-GBM antibodies only. There was a slight male preponderance in patients with anti-GBM antibodies only and in patients with coexisiting anti-GBM antibodies and MPO-ANCA, but not with coexisting PR3-ANCA.

Specificity of Anti-GBM Antibodies in Sera With or Without ANCA

The 33 serum samples selected for this part of the study fell into two main groups, based on Western blot analysis for anti-GBM antibodies and ELISAs for PR3 and MPO-ANCA: Group I, 17 samples with anti-GBM antibodies only; Group II, 16 samples with both anti-GBM antibodies and ANCA (IIa: eight with MPO-ANCA, and IIb: eight with PR3-ANCA antibodies). As controls, we studied 20 samples with ANCA only (10 with PR3-ANCA and 10 MPO-ANCA antibodies) and two sera from normal individuals. We compared the anti-GBM antibodies in Groups I and II as assessed by Western blot

Table 1. Age and sex distribution of patients with anti-GBM antibodies and/or ANCA

Antibodies	No. of Patients	Median Age (yr)	Range of Ages (yr)	Male (%)
Anti-GBM only	56	44	11–85	57
Anti-GBM and MPO	25	62	16–84	64
Anti-GBM and PR3	11	61	40–74	50
Anti-MPO only	105	65	3–90	49
Anti-PR3 only	52	63	16–93	52

Note. Abbreviations used: GBM, glomerular basement membrane; ANCA, antineutrophil cytoplasmic antibodies; MPO, myeloperoxidase; PR3, proteinase 3.

analysis, by ELISA to measure reactivity with bovine NC1 hexamer or its components, and by determination of the degree to which reactivity was inhibited by a monoclonal anti-α3(IV)NC1 antibody.

Western Blot Analysis

The serum samples were analyzed using the collagenase solubilized human GBM preparation. All of the sera in Groups I and II produced staining of bands corresponding to α3(IV)NC1 monomers and dimers (Figure 1). There were no differences in the location of the bands between the two groups (Figure 1). There was no reactivity with the NC1 domains in any of the control sera (Figure 1).

ELISA for Antibodies to the NC1 Hexamer

The results of ELISA to measure reactivity of the sera with the NC1 hexamer of bovine Type IV collagen are shown in Table 2. The titers showed considerable variation and although there were more sera with values below 0.5 in Group II, about half had higher values (>2.0). One of the control samples (with ANCA only) had a value of 0.22, which was the same as the lowest value in Group II, but the specimen was negative by Western blot analysis, showed no reactivity with any of the purified α(IV)NC1 monomers (see below), and was therefore not considered to have anti-GBM antibodies. The other 19 sera with ANCA only did not exceed the values for the negative controls (0.14).

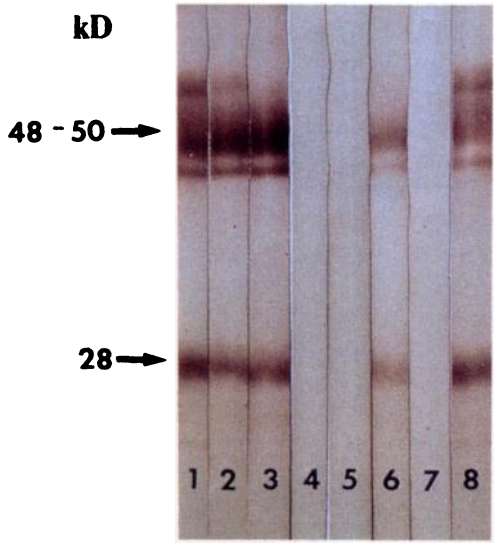

Figure 1. Western blot analysis was performed with a collagenase digest of human glomerular basement membrane (GBM). Lanes 1, 6, and 8 represent sera with anti-GBM antibodies only; lane 2, anti-GBM antibodies and proteinase 3 (PR3)-antineutrophil cytoplasmic antibodies (ANCA); lane 3, anti-GBM antibodies and myeloperoxidase (MPO)-ANCA; lane 4, PR3-ANCA only; lane 5, MPO ANCA only; and lane 7 normal serum. Reactivity (lanes 1, 2, 3, 6 and 8) is shown with a band of approximately 28 kD, corresponding to the α3(IV)NC1 monomer, and with several bands in the 48–50 kD region, corresponding to dimers containing α3(IV)NC1.

ELISA for Antibodies to α(IV)NC1 Monomers

ELISA to detect reactivity with the purified α(IV)NC1 monomers were performed in such a way as to permit comparison of titers between serum samples because the dilution required to yield a given absorbance value was determined. As shown in Table 2, all 33 sera in Groups I and II reacted with the α3(IV)NC1 monomer, and all but six also reacted with one or more of the other monomers. Except for one sample (sample 20), all sera showed the highest titers to the α3(IV)NC1. The titers of reactivity to α1, 2, and 4 (IV)NC1 monomers generally correlated with titers to the α3(IV)NC1. The median anti-α3(IV)NC1 antibody titer in sera with anti-GBM plus PR3-ANCA antibodies was lower (median 225) than in Group I, anti-GBM alone, (median 2100). However, the difference showed no statistical significance ($P = 0.2$). The median anti-α3(IV)NC1 antibody titer in sera with anti-GBM plus MPO-ANCA antibodies (median 2500) did not differ from the median titer in Group I.

None of the 22 control sera showed reactivity with any of the α(IV)NC1 monomers.

Monoclonal Antibody Inhibition of α3(IV)NC1 Reactivity

The experiments to assess blocking of anti-GBM antibodies by the monoclonal antibody to α3(IV)NC1 (Mab17) are summarized in Table 2. Four sera could not be evaluated because the titers of antibodies to the α3(IV)NC1 monomer were too low. Of the remaining sera, all showed some degree of inhibition, ranging from 17% to 77% in Group I, from 22% to 64% in Group IIa, and from 44% to 71% in Group IIb. A previous study showed that anti-GBM antibodies, in sera without ANCA, were not inhibited by a monoclonal antibody to α1(IV)NC1 (22).

Discussion

In the study presented here, we examined several issues related to the coexistence of anti-GBM antibodies and ANCA. Among serum samples submitted to the Massachusetts General Hospital laboratory from 1981 to 1995 and tested both for anti-GBM antibodies and ANCA, we selected 332 with positive tests based on current tests–immunoassays for PR3 and MPO-ANCA and Western blot analysis for anti-GBM antibodies. Among these 332 samples, 100 were positive for anti-GBM antibodies, of which 38% also had ANCA–a percentage similar to that found in several previous reports (9,12–18).

Among the 38 sera with both anti-GBM antibodies and ANCA, 66% had MPO-ANCA, 32% had PR3-ANCA, and 2% had both. Although these precentages were the same as in the above-mentioned 232 sera with ANCA only, in a larger group that included an additional 259 specimens not tested for anti-GBM antibodies, the frequency of anti-MPO antibodies was slightly but not significantly lower. In some studies, a marked preponderance of MPO-ANCA has been found in association with anti-GBM antibodies (13,15,18), although in other studies appreciable, but somewhat lower percentages were associated with PR3-ANCA (9,12,19). The present results support the

Table 2. Comparison of anti-GBM antibodies in sera with or without ANCA

Group	No.[a]	NC1 hexamer[b]	α1(IV) NC1[c]	α2(IV) NC1[c]	α3(IV) NC1[c]	α4(IV) NC1[c]	Inhibition by Mab 17[d] (%)
I: anti-GBM only	1	0.48	0	0	60	0	—
	2	0.52	0	30	170	20	21
	3	0.54	0	0	180	0	23
	4	1.30	0	0	250	250	17
	5	0.67	50	80	250	80	64
	6	2.03	120	10	1250	100	46
	7	2.28	20	80	1900	90	23
	8	2.38	200	10	1900	100	53
	9	2.43	50	0	2100	300	49
	10	2.63	120	0	2300	190	77
	11	2.55	200	0	4000	190	48
	12	3.00	1500	450	6000	1700	44
	13	2.38	190	70	7000	450	64
	14	2.40	110	30	7200	300	45
	15	2.46	1600	710	12000	1700	43
	16	2.48	380	210	12000	450	38
	17	1.29	500	280	50000	2100	66
IIa: anti-GBM and anti-MPO	18	0.22	0	0	20	0	—
	19	0.22	0	0	20	0	—
	20	0.31	0	30	40	80	22
	21	2.48	0	0	1800	420	52
	22	2.07	220	70	3200	250	35
	23	2.41	90	30	10000	310	64
	24	2.48	900	180	13000	1100	32
	25	2.51	700	170	15000	900	42
IIb: anti-GBM and anti-PR3	26	0.27	0	0	60	20	44
	27	0.39	0	0	60	0	—
	28	0.87	0	0	100	0	71
	29	0.88	0	0	150	20	56
	30	1.10	0	0	300	50	53
	31	1.99	0	0	400	20	66
	32	2.05	490	330	1400	360	60
	33	2.48	30	30	3000	1000	60

Note. Abbreviations used: GBM, glomerular basement membrane; ANCA, antineutrophil cytoplasmic antibodies; NC1, noncollagenous domain; Mab, monoclonal antibody; MPO, myeloperoxidase; PR3, proteinase 3; ELISA, enzyme-linked immunosorbent assay.

[a] The samples in each group are arranged in sequences that correspond with increasing anti-α3(IV)NC1 titers.

[b] Reactivity against bovine NC1 hexamer in ELISA. Values represent absorbance at 405 nm. A value of 0.20 or above was considered positive.

[c] Reactivity against bovine purified α(IV)NC1 monomers in ELISA. Values represent the dilution required for each serum to yield an absorbance of 0.5 at 405 nm.

[d] Percentage inhibition of α3(IV)NC1 reactivity by the monoclonal antibody Mab 17.

conclusion that the association of anti-GBM antibodies with ANCA is, at most, slightly biased toward MPO-ANCA.

We also measured serum titers of ANCA and anti-GBM antibodies in the various groups. Some previous studies have reported that anti-GBM antibody titers were lower in patients with ANCA than in those without (13–15). In the study presented here, however, titers of anti-GBM antibodies, as measured by ELISA, to the α3(IV)NC1 monomer, showed overlapping values in sera with or without ANCA, and although the levels tended to be lower in those with PR3-ANCA than in sera with anti-GBM antibodies alone, the differences were not statistically significant. With respect to ANCA, Weber *et al.* have reported that anti-MPO antibody titers were lower in serum samples that also contained anti-GBM antibodies than in sera with anti-MPO antibodies alone (18). In contrast, we found no significant difference in ANCA titers (either MPO-ANCA or PR3-ANCA), in serum samples with or without anti-GBM antibodies. Aside from the intrinsic intensity of the autoantibody responses, several factors can account for differences in titers, including variations in the time the sera were

collected in the course of disease, effects of treatment in some cases, and, with respect to reports from different laboratories, differences in techniques used. Nevertheless, our results show that the titers of coexisting ANCA and anti-GBM antibodies are usually not trivial and indeed are often quite high.

Certain findings suggest that patients with coexisting ANCA and anti-GBM antibodies have features more characteristic of ANCA-associated conditions than of anti-GBM disease. In support of this possibility is evidence indicating that patients with anti-GBM antibodies only are younger (13,15) than those with ANCA and anti-GBM antibodies, or those with ANCA alone. Our results tend to support this interpretation, but further studies with larger numbers of patients are needed to substantiate this conclusion. Furthermore, in some previous studies a considerable male sex preponderance has been found among patients with anti-GBM antibodies (44), although how many of the reported patients had coexisting ANCA is unknown. In the present study there were slightly more males than females among patients with anti-GBM antibodies alone or with coexisting MPO-ANCA, but not with coexisting PR3-ANCA.

It has been suggested that patients with ANCA and anti-GBM antibodies paradoxically have a better prognosis than those with anti-GBM antibodies alone (13), although the evidence for this is not compelling. Our study was not designed to answer this question, but in view of its practical implications, further investigation is desirable.

In the major part of the study presented here, we compared the specificity of anti-GBM antibodies in sera that also contain ANCA with anti-GBM antibodies that occur alone. Our findings confirm those of earlier studies on the specificity of anti-GBM antibodies in patients with Goodpasture's syndrome or anti-GBM nephritis (21–23) and provide new and compelling evidence that the same specificities are present in patients who also have ANCA. We found that the anti-GBM antibodies in both groups reacted mainly with the NC1 domain of the $\alpha 3$ chains of Type IV collagen, with lesser reactivity toward the $\alpha 1$, 2, and 4 (IV)NC1 domains. Furthermore, the antibodies in both groups were inhibited to roughly comparable degrees by a monoclonal antibody to $\alpha 3$(IV)NC1. Western blot analysis performed with a collagenase-solubilized human GBM preparation showed no difference in the antigens recognized by the two types of sera. Thus, based on the parameters investigated, we were unable to detect any differences in specificities of anti-GBM antibodies in sera with or without ANCA.

Previous studies on anti-GBM antibodies associated with ANCA depended largely on Western blot analysis or on immunoassays using crude collagenase digests (9,12–14,18,19). Some reports concluded that the antibodies reacted predominantly with $\alpha 3$(IV)NC1 (9,12). However, some authors (29,30) have described reactivity with unidentified GBM antigens. Furthermore, in one report, a patient with anti-MPO antibodies was found to have serum antibodies to the $\alpha 1$(IV)NC1 but not to the $\alpha 3$(IV)NC1 (31). A renal biopsy obtained from this patient failed to reveal accumulation of IgG along the GBM, suggesting the anti-$\alpha 1$(IV)NC1 antibodies themselves were not pathogenic. We found no other previous reports indicating that purified α(IV)NC1 monomers were used to assess anti-GBM antibodies in patients with ANCA. The study presented here, which is based on a large number of patients and which used several methods to assess specificity, provides substantial evidence that anti-GBM antibodies associated with ANCA are almost always reactive with $\alpha 3$(IV)NC1, frequently with lesser reactivity with other α(IV)NC1 chains.

The most tantalizing question raised by the finding of coexistence of anti-GBM antibodies with either MPO or PR3-ANCA is: what could account for the stimulation of such rare and disease-specific autoantibodies? Available evidence indicates the antibodies are not cross-reactive (12,14,19,20), which would hardly be expected in view of the differences of the three antigens involved. The finding that the age distribution (and possibly prognosis) among patients with both ANCA and anti-GBM antibodies is similar to that in patients with ANCA only provides some support, albeit meager, for the hypothesis that an ANCA-associated condition is the initial and underlying disease. We and others (8,12,13) have postulated that ANCA-associated glomerular or pulmonary damage releases, exposes, or possibly alters basement membrane material, rendering it autoimmunogenic. Evidence consistent with this idea is provided by reports describing the development of anti-GBM antibodies in a few patients with long-standing membranous GN, a glomerular disease, which, like ANCA-associated GN, is characterized by severe (although quite different) alterations of the GBM (45,46). The specificity of the anti-GBM antibodies in the patients with membranous GN has not been fully characterized. Nevertheless, several findings indicate they are pathogenic—and, therefore, probably directed at $\alpha 3$(IV)NC1. Thus, in some cases, their appearance coincided with the development of rapidly progressive renal failure and was associated with extensive crescent formation (45,46), and in one case, transfer of antibodies eluted from glomeruli to monkeys produced glomerular injury (45). Additional support for the notion that GBM components can stimulate anti-GBM antibodies comes from a report by Almquist *et al.*, who described a patient with recurrence of anti-GBM antibodies shortly after receiving a renal transplant from his identical twin brother (47). Because serum anti-GBM antibodies had been undetectable for more than a year before the transplant, the observations indicate that the introduction of the isologous renal tissue, without immunosuppressive treatment, stimulated their reappearance. Furthermore, several studies have shown that injections of basement membrane preparations in experimental animals can induce autoantibodies similar to those in the human disease (48–50). Of particular interest, immunization of Wistar-Kyoto rats with Type IV NC1 hexamers induces anti-GBM antibodies directed mainly at $\alpha 3$(IV)NC1 (Brunmark, unpublished).

Although the hypothesis that endogenous basement membrane components induce anti-GBM antibodies in patients with ANCA is plausible, there is no direct evidence to support it. Nearly all patients found to have coexisting ANCA and anti-GBM antibodies have this combination in the first serum sample collected. Nevertheless, we have seen two patients who had positive MPO-ANCA tests before anti-GBM antibodies were detected, and in one of these (referred to above), the

anti-MPO antibodies were discovered 31 months before anti-GBM antibodies were found. Conversely, however, two reports describe the opposite sequence of events (17,51). Further studies, performed with sensitive and reliable assays, are needed to determine how often one type of the autoantibodies precedes the other.

There are few clues to the initiating events in either anti-GBM or ANCA-associated disease, although there is anecdotal evidence of preceding viral infection or inhalation of hydrocarbons in some patients with anti-GBM disease (52), and patients with ANCA often have a prodromal syndrome suggestive of viral infection. In addition, a few patients have developed ANCA after propylthiouracil or hydralazine treatment, (53,54). A microbial agent could conceivably trigger the autoimmune responses, either by causing tissue damage with release of immunogenic material or by "molecular mimicry," in which a sequence or region in a microbial protein stimulates antibodies that cross-react with host proteins. To account for the development of antibodies against α3(IV)NC1, as well as either PR3 or MPO, molecular mimicry would have to involve similar regions of three totally unrelated proteins, which argues against this explanation. Nevertheless, because peptides with minimal primary sequence identity can initiate cross-reactive T cell responses, this possibility cannot be entirely excluded (55).

Genetic factors are clearly important in the anti-GBM antibody response. Thus, an association has been shown between anti-GBM disease and HLA-DR2 (33,56), but it is not known whether this association is restricted to patients with anti-GBM antibodies only or whether it is also found in those patients with coexisting ANCA.

In conclusion, the results of this study indicate that the fine specificity of anti-GBM antibodies in patients with or without ANCA is the same. In particular, in both cases, the antibodies appear to react mainly with limited regions of the α3(IV) chain in the NC1 domain. We believe these findings can be best explained by assuming that the autoantibody response to the GBM, in patients with or without ANCA, is driven by the NC1 domain of endogenous Type IV collagen.

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