CHARACTERIZATION OF TARGET ANTIGENS IN GOODPASTURE’S SYNDROME

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Summary

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by immunoblotting to nitrocellulose was performed with enzyme-digested human glomerular basement membrane (GBM) under non-reducing conditions. Sera from patients with Goodpasture’s syndrome, various other immunological diseases and healthy blood donors were incubated with the nitrocellulose sheets and bound IgG was detected by immunoperoxidase staining. High titred antibodies to GBM (anti-GBM Ab) occurring in the sera of three patients with Goodpasture’s syndrome identified components with the molecular weights of 23,500, 24,500, 41,000 and 46,000 in a collagenase-digest of human GBM as target antigens. A fourth Goodpasture’s serum with a low titre of anti-GBM Ab only reacted with the peptide of 24,500 daltons (d). The antigens were sensitive to reduction with mercaptoethanol. No reactivities with anti-GBM Ab positive sera were obtained when pepsin- or trypsin-digested GBM was used as an antigen source. We conclude that anti-GBM Ab may recognize antigens of at least four different molecular weights in a collagenase digest of human GBM and that the antigens are sensitive to reduction.

Introduction

The clinical association of pulmonary haemorrhage, rapidly progressive glomerulonephritis and antibodies to glomerular and alveolar basement membrane has been defined as Goodpasture’s syndrome. The antibodies to glomerular basement membrane (anti-GBM Ab) are assumed to be of pathogenic significance. The anti-GBM Ab can be demonstrated by various methods, indirect immunofluorescence being the common standard technique. However, radioimmunoassay and enzyme-linked immunosorbent assays (ELISA) may be more sensitive [1].

Several constituents of the glomerular basement membrane have been defined
during the last few years. The major component of human basement membranes is type IV collagen. Other distinct basement membrane macromolecules, i.e. laminin, heparan sulphate proteoglycans, entactin and 7S collagen have also been isolated [2]. The target antigen of the anti-GBM Ab, however, has not yet been entirely characterized. It was the aim of our investigation to further characterize the antigens involved in Goodpasture’s syndrome.

Materials and methods

Antigen preparation A soluble antigen of human GBM was prepared by collagenase digestion as previously described [1]. In addition pepsin and trypsin digestions were performed in a similar way.

Demonstration of anti-GBM Ab Circulating anti-GBM Ab in patients’ sera were demonstrated by indirect immunofluorescence on normal human kidney sections and by an enzyme-linked immunosorbent assay (ELISA) [1]. In brief, Dynatec polyvinylchloride microtitre plates were coated with 100 µl collagenase-solubilized GBM (100 µg/ml) in carbonate buffered saline (CBS), pH 9.6. One hundred microlitres of a dilution of sera in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) was added. Bound IgG was then detected using alkaline phosphatase (AP)-labelled rabbit anti-human IgG and enzymatic activity was measured using p-nitrophenyl phosphate (p-NPP) as substrate. The absorbances were read at 405nm after 30 minutes.

Patients Serum samples were obtained from four patients with florid Goodpasture’s syndrome. All patients had the triad of haemoptysis, rapidly progressive, crescentic glomerulonephritis with linear deposits of IgG along the GBM. Anti-GBM Ab were demonstrable in the sera by indirect immunofluorescence and by ELISA techniques. In addition, one serum obtained from a Goodpasture’s syndrome patient in clinical remission and sera from patients with systemic lupus erythematosus (10), autoimmune chronic active hepatitis (10), various forms of glomerulonephritis (16), including crescentic glomerulonephritis with granular immunofluorescence pattern (5), epimembranous glomerulonephritis (4), IgA nephritis (4), mesangioproliferative glomerulonephritis (1), exudative glomerulonephritis (2), and sera from healthy blood donors were investigated.

Electrophoresis Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under non-reducing conditions essentially as described by Laemmli [3]. In brief, 100 µl of solubilized GBM (2 mg/ml) was run into 5–20% gradient SDS-PAGE. Gels were stained with Coomassie blue (Merck, Darmstadt, FRG) unless not used for further experiments. The separated components were blotted to nitrocellulose membranes (Bio-Rad) as described by Towbin et al [4]. The sheets were then incubated with bovine serum albumin (1%) in PBS at 37°C for one hour. Thereafter, one millilitre of serum dilutions (1:10) in PBST were overlaid onto the nitrocellulose sheets overnight at 4°C
with gentle mixing and bound human IgG antibodies were detected by incubation with a dilution (1:1000) of horseradish peroxidase conjugated F(ab)2-pieces of rabbit anti-human IgG-Fc (Jackson Immunoresearch Lab, Avondale, PA) for two hours at room temperature. Peroxidase activity was detected using 4-chloro-1-naphthol as substrate.

*Modifications* One high titred Goodpasture’s syndrome serum was bound to protein A Sepharose 4B according to the guidelines of the suppliers (Pharmacia GmbH, Freiburg, FRG). Unbound material, as well as eluted IgG, was investigated as described above. In addition, SDS-PAGE of solubilized GBM was also performed after reduction with 0.1% 2-mercaptoethanol.

**Results**

SDS-PAGE demonstrated that the collagenase digest of human GBM was composed of several components which differed in molecular weight (Figure 1).

![Figure 1. SDS-PAGE (5–20%) of a collagenase digest of human GBM (2mg/ml) under non-reducing conditions, demonstrating that sufficient separation of GBM components was achieved.](image-url)
The intensity of the separated components varied between different preparations of GBM. After blotting to nitrocellulose membranes and immunoperoxidase staining reactivities on nitrocellulose sheets were obtained only by the four Goodpasture's syndrome sera when collagenase solubilized GBM was used as antigen source. The control sera and the serum of one Goodpasture's syndrome patient in clinical remission were negative. In addition, no reactivities with

![Image](image-url)

Figure 2. Positive immunoperoxidase staining of GBM components blotted to nitrocellulose membranes after incubation with anti-GBM Ab positive sera. Collagenase solubilized human GBM was separated by SDS-PAGE under non-reducing conditions and electrophoretically transferred to nitrocellulose paper. After incubation with anti-GBM Ab positive and control sera bound IgG was visualized by incubation with horseradish peroxidase conjugated monospecific antibodies and 4-chloro-1-naphthol as enzyme substrate. Results obtained with sera from four patients with Goodpasture's syndrome (GP 1-4), one Goodpasture's syndrome serum after adsorption and elution from staphylococcal protein A Sepharose 4B (GP 2 eluate) and one normal human serum (NHS) are documented.
positive sera were obtained when pepsin- or trypsin-digested GBM were used, neither in the immunoblot investigations nor in the ELISA. The four Goodpasture's syndrome sera recognized different components of the collagenase digested GBM as target antigens. Three high titred sera identified proteins of the molecular weights 23,500, 24,500, 41,000 and 46,000. The fourth serum with an anti-GBM antibody titre of 1:4 (indirect immunofluorescence) or 1:512 (ELISA) did react only with the peptide of 24,500 daltons (Figure 2). In addition, the IgG fraction eluted from protein A Sepharose 4B reacted in a similar fashion as the original serum (Figure 2). SDS-PAGE performed after reduction of the GBM antigens by 2-mercaptoethanol demonstrated that the four immuno-reactive components were no longer detectable. In addition, no reactivity was obtained with anti-GBM Ab positive sera, when the reduced antigen was used in blotting experiments or for coating in the ELISA.

Discussion

Anti-GBM Ab are demonstrable in Goodpasture's syndrome, some forms of rapidly progressive glomerulonephritis and rarely in idiopathic pulmonary haemosiderosis. The autoantibodies are thought to be of immunopathogenic relevance. Controversy exists concerning the nature of the target antigens of the anti-GBM antibodies.

McIntosh and Griswold [5] concluded that the antigens were collagenous because the antibodies were shown to be unreactive with collagenase-treated kidneys. Foidart et al [6] demonstrated anti-type IV pro-collagen antibodies in the sera of 15 of 19 patients with Goodpasture's syndrome. Mahieu et al [7] prepared a soluble GBM antigen by autoclaving, affinity chromatography, and preparative SDS-PAGE. They identified an antigen with a molecular weight of 70,000 daltons as a Goodpasture's syndrome antigen. Evidence was given that hydroxylsine galactose glucose blocked the reaction between the GBM antigen and the anti-GBM Ab, suggesting some reactivity with the more collagenous structures in some sera. However, no confirmation of this finding has appeared.

Other investigators suggested that the target antigens may be of non-collagenous origin. Holdsworth et al [8] identified the major antigenetic components in the molecular weight range of 27,000, 54,000 and 150,000 daltons when a collagenase-digestion of GBM was separated by column chromatography. Hunt et al [9] isolated three antigenic fractions from collagenase-solubilized GBM utilizing affinity columns loaded with human anti-GBM Ab. Competitive inhibition of an anti-GBM RIA was found with the fractions of 22,000–25,000, 43,000–45,000 and 65,000–70,000 daltons. More recently, Wieslander et al [10] separated a collagenase digest of GBM by column chromatography and identified components of 26,000 and 48,000 daltons as immunoreactive. SDS-PAGE (12.5% gels) followed by immunoblotting demonstrated three immunoreactive components, the third one with a molecular weight between 26,000 and 48,000 daltons.

Our investigations confirmed that the Goodpasture's syndrome target antigens may be of non-collagenous origin. No reactivity was obtained when pepsin or trypsin-digested GBM were used as antigen preparations. Moreover, the molecular
weight range of the Goodpasture's syndrome target antigens identified by SDS-PAGE and immunoblot were similar to those described in the literature. However, four immunoreactive components were identified by three Goodpasture’s syndrome sera with the apparent molecular weights of 23,500, 24,500, 41,000 and 46,000 daltons. Similar antigens were recognized by the IgG fraction of the second Goodpasture’s syndrome serum (GP2) which contained the IgG subclasses G1, G2 and G4. The fourth Goodpasture’s syndrome serum only recognized the component with the molecular weight of 24,500 daltons as target antigen. This may be due to a low titre, a low avidity or a heterogeneity of the anti-GBM Ab. The recognition of four target antigens by three Goodpasture’s syndrome sera in our investigations may be due to a better separation achieved by gradient gel electrophoresis compared to gels of a constant polyacrylamide concentration, used by other investigators. In gradient PAGE all components may migrate to their pore limit and essentially stop. Moreover, the non unit charge of unsaturated lipo- and glycoproteins will not affect their apparent molecular weights. The target antigens proved to be sensitive to reduction, demonstrating that the antigens are proteins and contain intrachain disulfide bonds that are essential for antigen activity. We conclude that anti-GBM antibodies may recognize at least antigens of four different molecular weights in a collagenase digest of human GBM and that these antigens are sensitive to reduction. Further investigations should clarify whether the antibodies recognize the same epitope on different molecules or whether different antigens may be recognized by heterogenous antibodies.

References

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