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Restricted specificity of the autoantibody response in Goodpasture's syndrome demonstrated by two-dimensional Western blotting

C. J. DERRY, M. J. DUNN,* A. J. REES & C. D. PUSEY *Renal Unit, Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, and *Department of Cardiothoracic Surgery, National Heart and Lung Institute, London, England*

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SUMMARY

The autoantigen in Goodpasture's syndrome is known to be contained within the non-collagenous (NC1) domain of type IV collagen. We have examined the specificity of autoantibodies to glomerular basement membrane (GBM) using the technique of 2-D electrophoresis followed by Western blotting. Protein stains of 2-D gels of collagenase-digested human GBM revealed extensive charge and size heterogeneity. Major components were of mol. wt 24–30 kD and 43–56 kD, corresponding to monomeric and dimeric subunits of NC1. Western blotting of 2-D gels with IgG from patients with anti-GBM disease demonstrated that the most antigenic components migrated as cationic 28-kD monomers (pI 10) and similarly charged dimers, although other components were recognized less strongly. The mobility of the strongly antigenic polypeptides was different to that of the known α1 and α2 chains of type IV collagen. Autoantibodies from all 20 patients studied showed the same pattern of reactivity, regardless of their clinical features (in particular, the presence or absence of pulmonary haemorrhage) or HLA type. A monoclonal antibody (P1) to human GBM bound in a similar pattern, particularly recognizing the cationic components. 2-D gels of affinity-purified GBM from a P1 column showed enrichment of the 28-kD monomers, which were recognized by human autoantibodies on Western blotting. These results demonstrate that the autoimmune response in Goodpasture's syndrome is of restricted specificity, and support the suggestion that the major autoantigenic determinant is present on the novel α3 chain of type IV collagen.

Keywords Autoimmunity glomerular basement membrane Goodpasture's syndrome 2-D electrophoresis

INTRODUCTION

Goodpasture's syndrome is defined clinically as rapidly progressive glomerulonephritis in association with pulmonary haemorrhage. Although various immunopathological mechanisms can be responsible for this clinical picture, the term Goodpasture's syndrome is usually reserved for cases in which autoantibodies to the glomerular basement membrane (GBM) can be detected. The pathogenicity of these autoantibodies has been demonstrated by transfer experiments, in which monkeys developed nephritis after receiving serum or kidney eluates from patients with the disease (Lerner, Glassock & Dixon, 1967). The development of quantitative solid-phase immunoassays for circulating anti-GBM antibodies has been of value in diagnosis and monitoring of treatment (Bowman & Lockwood, 1985). Reduction of antibody concentration using immunosuppressive drugs and plasma exchange is associated with clinical improvement, except in patients with advanced glomerular damage (Pusey, Lockwood & Peters, 1982). Although this approach has greatly improved the prognosis of Goodpasture's syndrome, treatment remains non-specific and has numerous side-effects. It is therefore important to elucidate the immune mechanisms involved at a molecular level with a view to designing more specific therapy.

The Goodpasture antigen (GA) has been localized to the non-collagenous (NC1) region of type IV collagen, a major component of the GBM (Wieslander *et al.*, 1984). Western blotting studies of collagenase-solubilized GBM suggest that autoantibodies from patients with Goodpasture's syndrome, and a monoclonal antibody (P1) produced to human GBM, recognize the same components, which correspond to monomeric and dimeric subunits of the NC1 domain (Pusey *et al.*, 1987). Our aim was to investigate autoimmunity to the GA in more detail using the technique of 2-D electrophoresis. We set out to determine: (i) the specificity of the autoantibody response in relation to the clinical characteristics and HLA type of the patients studied; (ii) the binding characteristics of autoanti-

Correspondence: C. J. Derry, Renal Unit, Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK.

bodies in comparison with monoclonal P1; (iii) the relationship of these results to current knowledge of the biochemistry of GBM.

PATIENTS AND METHODS

Antibodies

Human antibodies. Twenty patients with a diagnosis of anti-GBM disease, 10 male and 10 female, aged 17–76 years (mean age 43), were studied (Table 1). Controls were two patients with Wegener's granulomatosis, two with systemic lupus erythematosus, one with Henoch-Schonlein purpura, one with idiopathic focal necrotizing glomerulonephritis and two normal individuals. Plasma was obtained from all patients and disease controls at the time of their first plasma exchange. Serum was obtained from the two normal controls. IgG was purified from all samples using a protein A column (Hjelm, Hjelm & Sjoquist, 1972). The concentration of the eluted IgG, as measured by OD at 280 nm, was 2 mg/ml in all preparations. Anti-GBM antibody activity was tested in a radioimmunoassay (RIA), using collagenase-digested human GBM as the substrate (Bowman & Lockwood, 1985).

Monoclonal P1. The murine hybridoma producing P1, a monoclonal antibody to human GBM, was cultured using standard techniques (Pressey *et al.*, 1983). The activity of P1 was tested by indirect immunofluorescence on normal human kidney sections and by RIA. Antibody was purified from culture supernatant by sodium sulphate precipitation.

Antigens

Preparation of GBM. Normal human kidneys were obtained at less than 24 h post-mortem and stored at −70°C until use. Glomeruli were extracted from the cortex by differential sieving, and soluble GBM was prepared by sonication, lyophilization and collagenase digestion (Bowman & Lockwood, 1985).

Table 1. Characteristics of patients with anti-GBM disease

Patient	Age	Sex	Smoker	LH	DR2	DR4
1	17	M	+	+	−	+
2	54	M	−	−	+	−
3	21	F	+	+	+	+
4	25	F	+	+	+	−
5	76	F	+	+	−	−
6	31	M	+	−	+	+
7	60	F	−	−	+	−
8	21	M	+	+	+	+
9	35	F	−	−	+	−
10	19	M	+	+	−	+
11	48	M	+	+	+	−
12	57	M	−	−	+	−
13	30	M	−	−	−	+
14	53	F	−	−	+	−
15	54	F	−	−	+	−
16	66	M	−	−	−	+
17	20	F	−	+	+	+
18	48	F	−	−	+	−
19	62	F	−	−	−	+
20	51	M	−	−	+	−

LH, lung haemorrhage.

Affinity purification of the GA. Monoclonal P1 was bound to a cyanogen bromide-activated Sepharose 4B column (Pharmacia Fine Chemicals, Milton Keynes, UK), and used to affinity purify GBM under non-dissociating conditions. Collagenase-digested GBM (10 mg) was allowed to recirculate for 2 h, before washing unbound material through the column with phosphate-buffered saline (PBS). Bound material (500 μg) was eluted with 0·2 M glycine HCl, pH 2·5, and neutralized with 1·5 M Tris, pH 8·7.

Two-dimensional electrophoresis

Flatbed isoelectric focusing. This was performed by a modification of the method of Dunn *et al.* (1985). Four per cent polyacrylamide gels (0·5 mm), containing 8 M urea, 0·5% w/v CHAPS and 3% w/v amphyolytes were set on GELBOND PAG, a polyacrylamide gel support film (FMC Bioproducts, Rockland, ME). Prepared gels were placed on the flatbed apparatus (Pharmacia FBE 3000), cooled to 15°C and prefocused at 650 V at a maximum of 15 W and 15 mA for 1 h, to give a pH gradient of 3–10. An isoelectric focusing (IEF) calibration kit (Pharmacia), containing 11 proteins covering this pI range, was used to determine the isoelectric point of focused components. Desalted lyophilized GBM samples were dissolved in a denaturing solution (9 M urea and 3% CHAPS), and 10–12 samples were loaded onto the gel and focused at 750 V overnight, to give a final reading of 7000 Vh corresponding to equilibrium. First dimension IEF strips were stored at −70°C.

SDS-polyacrylamide gel electrophoresis. First dimension strips were equilibrated in Laemmli buffer (Laemmli, 1970) for 10 min, before loading onto a 12·5% gel. Second dimension gels were run in the Bio-Rad Protean I system at a constant voltage of 40 V overnight. Molecular weights were determined using a low molecular weight calibration kit (Pharmacia). 2-D gels were either silver stained (Morrissey, 1981) or Western blotted.

Western blotting. Separated proteins were transferred onto 0·45 μm nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany) at 0·5 mA for 3·5 h in a Hoefer transfer electrophoresis unit TE50 (Towbin, Staehelin & Gordon, 1979). Sheets were subsequently stained with the temporary protein dye Ponceau Red (Sigma Chemical Co., Poole, UK) to determine the pattern and relative amounts of transferred protein. After blocking in PBS/1% v/v Tween 20, the nitrocellulose was blotted with IgG from patients with anti-GBM disease (diluted in PBS/1% Tween to the concentration that gave 50% binding of a control positive serum in the RIA), or IgG from controls, or monoclonal P1. The secondary antibodies, goat anti-human IgG or sheep anti-mouse IgG conjugated to biotin (Sigma), were recognized by a tertiary complex of Biotin/Streptavidin linked to horseradish peroxidase (Dakopatts, High Wycombe, UK), 2 μl in 5 ml PBS/1% Tween. Blots were developed using the substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma) 0·6 mg/ml in PBS/1% Tween with 0·03% hydrogen peroxide.

PD Quest densitometry. Six of the Western blots were developed using the glucose oxidase DAB nickel method (Shu, Ju & Fan, 1988), which results in a blue product (as opposed to brown), and thus is better suited for scanning with a red laser densitometer. The blots were digitized using a Molecular Dynamics (Sunnyvale, CA) 300 A laser densitometer at a resolution of 176 × 176 μm with a dynamic range of 0–3·5 OD. Quantitative analysis of the digitized images of the blots was carried out using the PD Quest software package (Protein and DNA Imageware Inc., Huntington Station, NY) (Krauss,

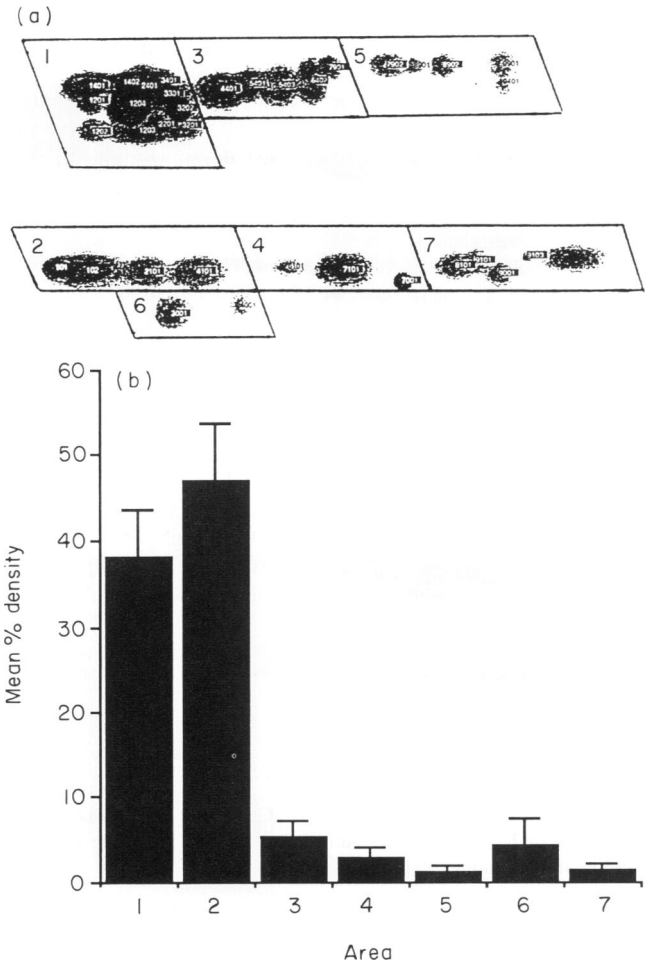

Fig. 1. (a) Composite PD QUEST scan of a Western blot of 2-D separated human GBM, indicating those areas chosen for comparison of autoantibody binding by densitometry. (b) Histogram showing mean percentage density (+s.e.m.) of autoantibody binding from six patients for each of seven areas on a Western blot of 2-D separated human GBM.

Collins & Blose, 1990), based on the QUEST system of Garrels (1989), and running on a Sparcstation 1 microcomputer (Sun Microsystems, Bagshot, UK). After background subtraction and spot detection, the patterns of the six individual blots were matched to form a composite 2-D spot image (Fig. 1a). Seven areas were then chosen corresponding to defined groups of spots, previously suggested to contain different α(IV) chains (Butkowski *et al.*, 1990). The densitometry values obtained for each of these areas were expressed as a percentage of the total integrated density for each blot, and the standard error of the mean calculated (Fig. 1b). For statistical analysis the Kruskal–Wallis test was used to test the hypothesis that there was no difference, at a 5% significance level, between the binding pattern of autoantibodies from different patients.

RESULTS

GBM composition

Silver stains of collagenase-digested GBM separated by 2-D electrophoresis revealed a number of components of approximate mol. wt 24–30 kD and 43–56 kD, spreading across a pI range of 3–10, and corresponding to the monomeric and dimeric

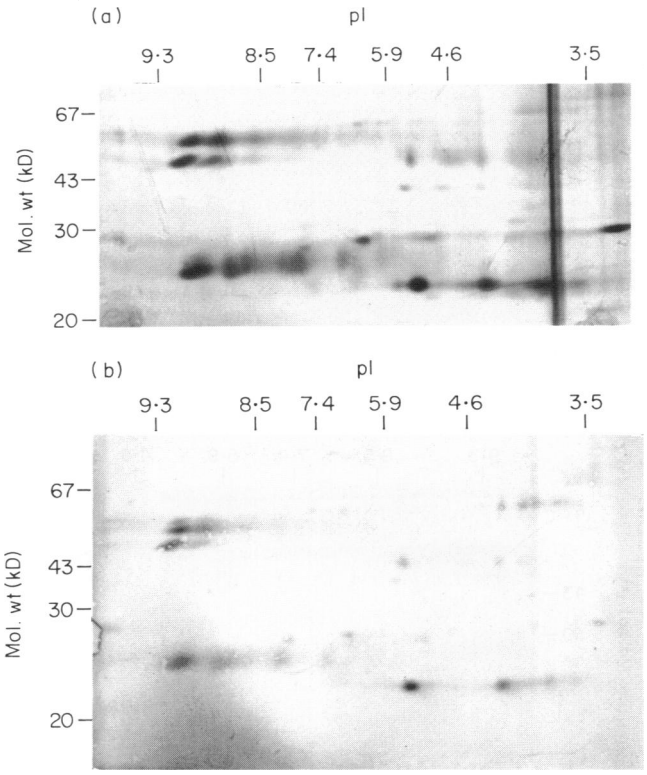

Fig. 2. Components of GBM separated by 2-D electrophoresis stained with silver (a), and with Ponceau Red after transfer to nitrocellulose (b). Note similarity in patterns demonstrating efficiency of transfer.

subunits of the NC1 domain of type IV collagen (Fig. 2). Heterogeneity of charge distribution was observed with all components, particularly the 28-kD monomers which separated across a pI range of 4·5–10. Proteins transferred to nitrocellulose were stained with Ponceau Red, to demonstrate the efficiency of transfer (Fig. 2). Despite the low sensitivity of this stain, its use allowed a qualitative estimate of the amount of protein present, which reflected that seen in silver-stained gels. The very cationic molecules were present at markedly lower concentrations than other NC1 constituents.

Western blotting of GBM

On Western blotting of 2-D separated GBM, human anti-GBM antibodies bound predominantly to the cationic components present at low concentrations. However, some binding was observed to other components (Fig. 3). All 20 patients studied showed the same pattern of autoantibody reactivity. It was possible to examine more closely six of the blots by computerized densitometry. Statistical analysis (Kruskal–Wallis test) of these results confirmed that the autoantibody response was the same in different patients (Fig. 1). The monoclonal antibody P1 showed a similar, but simplified, pattern of reactivity (Fig. 3). There was strong binding to the most cationic components, but reduced or absent reactivity to the 24-kD and 26-kD components.

On all Western blots using IgG from patients with anti-GBM disease, approximately equivalent antibody activity was achieved by blotting at a dilution that gave 50% binding of the positive control in a RIA. Differences in binding pattern related to the concentration of anti-GBM antibodies were thus elimi-

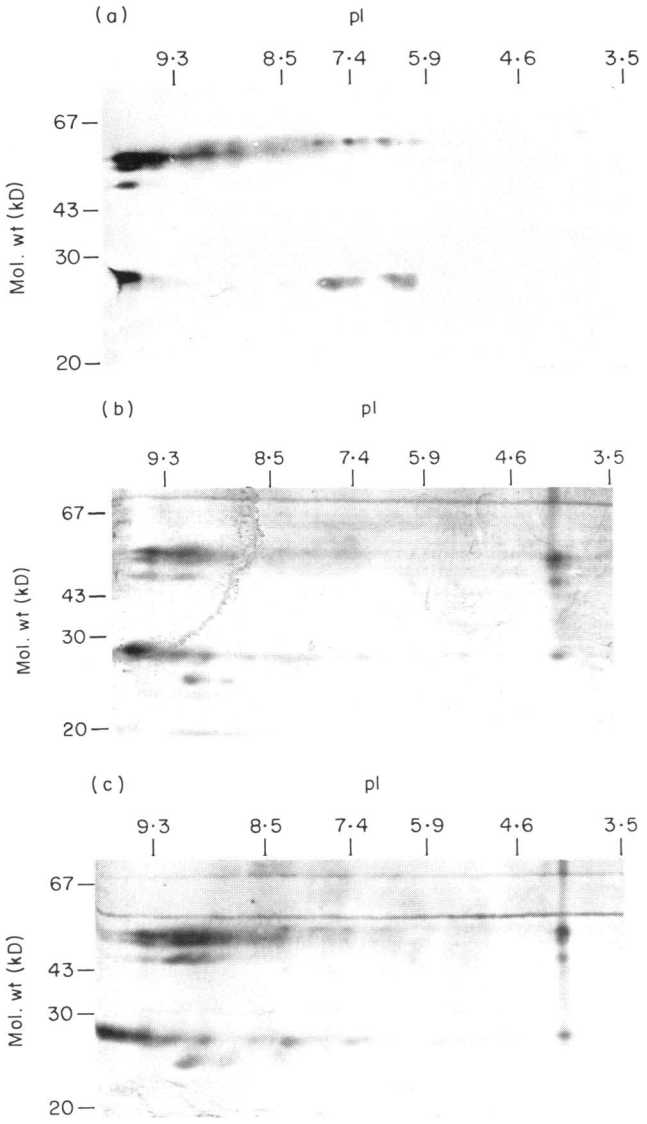

Fig. 3. Western blotting of 2-D separated GBM with monoclonal P1 (a), and IgG from two patients with anti-GBM disease: case 7 (b), and case 10 (c). See Table 1 for details of patients. Note strong binding to the most cationic components.

nated. To illustrate this point, IgG from three patients was blotted at two different concentrations. At the higher concentration there was detectable binding to more components than at the lower concentration, at which only the more cationic components were detected (Fig. 4). The 30-kD components present on these blots were not found in all preparations of GBM, and could be related to collagenase digestion at an alternative cleavage site on the α3(IV) chain (Gunwar, Noelken & Hudson, 1991).

IgG from control patients was blotted at a dilution of 1/24, corresponding to the dilution most commonly used with IgG from patients with anti-GBM disease. At this dilution no binding to GBM was observed.

Western blotting of affinity-purified GA

The GA was purified on a P1 affinity column and the resulting material analysed by 2-D electrophoresis. Silver stains of bound

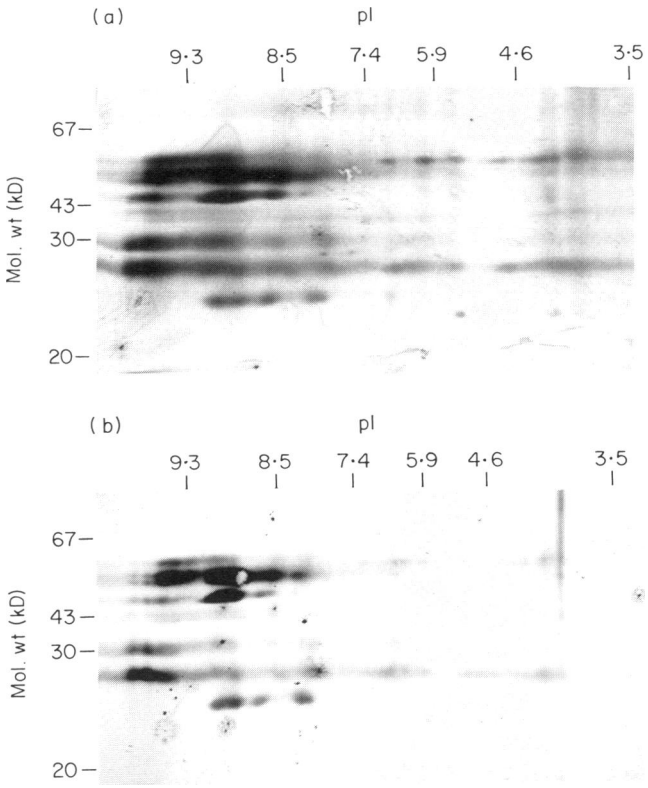

Fig. 4. Western blotting of 2-D separated GBM with IgG from case 5 at a dilution of 1/50 (a) and at a dilution of 1/1000 (b). Note stronger binding in the anionic region in Fig. 4a.

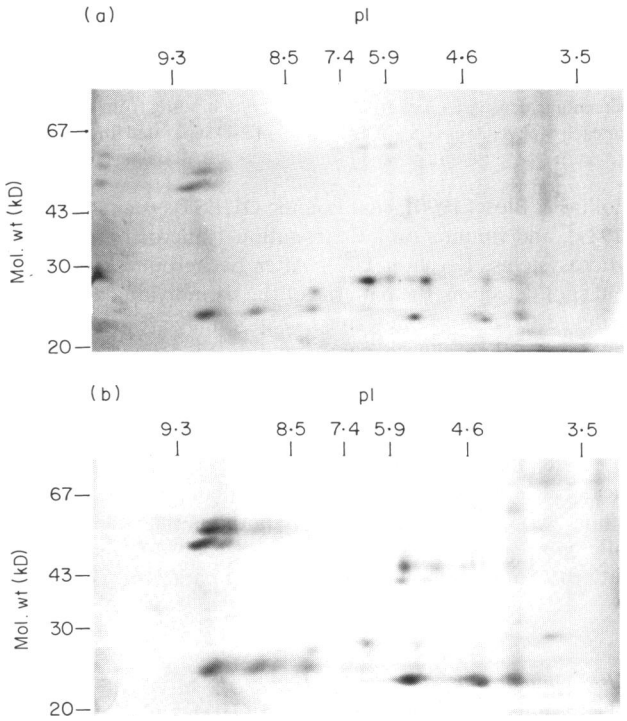

Fig. 5. Silver stains of 2-D separated GBM which had bound (a), or not bound (b) to a P1 affinity column. Note the enrichment of 28-kD monomers, and dimers of the same pI, in the bound material.

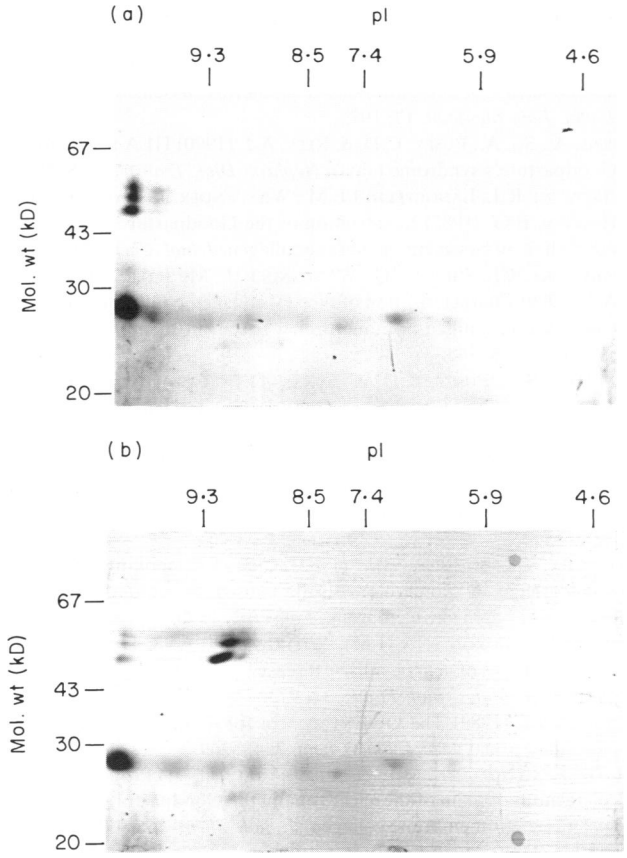

Fig. 6. Western blotting of 2-D separated GBM which had bound (a), or not bound (b) to a P1 affinity column, using IgG from case 5. Note the enrichment of very cationic antigenic components in the affinity-purified material.

material, in comparison with unbound material, showed an enrichment of 28-kD monomers and of dimers migrating to the same pI (Fig. 5). This was particularly noticeable in the cationic region. In general, there was a reduction of dimeric sized components in the bound material as compared with the unbound. Western blotting with anti-GBM antibodies demonstrated the presence of antigenic molecules in both bound and unbound material. This could be due, in part, to the limited capacity of the column, or to sequestration of antigenic sites within certain dimers in non-dissociating conditions. However, autoantibody reactivity with the bound material directly reflected the pattern seen in the equivalent silver-stained gels, and was strongest to the very cationic components (Fig. 6).

DISCUSSION

It is now well established that the GA is localized to the non-collagenous domain of type IV collagen (Wieslander, Kataja & Hudson, 1987; Butkowski *et al.*, 1987). Type IV collagen is a major structural component of GBM, and consists of a network of collagen molecules which provide a scaffold for the attachment of other BM components (Timpl *et al.*, 1981; Hudson *et al.*, 1989). Each type IV collagen molecule is made up of a triple helix of α chains, two of which, α1 and α2, have been well characterized at protein and DNA levels (Saus *et al.*, 1988; Hostikka & Tryggvason, 1988). Type IV collagen chains are unusual in that the NC1 region is not cleaved after translation, and therefore the molecules can interact not only at the N terminal 7S region but also at the C-terminal NC1 region. The hexameric structure resulting from association of adjacent triple helices via the NC1 domain is released intact by collagenase digestion. The hexamer is dissociated on SDS–PAGE into its constituent subunits, which are of mol. wt 24–30 kD and 43–56 kD, corresponding to monomers and dimers (Langeveld *et al.*, 1988). The known α1 and α2 chains are of mol. wt 26 kD and 24 kD respectively. It is now clear that there are several novel α(IV) chains (Butkowski *et al.*, 1990), of which two, α3 and α4, have a molecular weight of approximately 28 kD. The α3 chain has been isolated and sequenced from bovine (Morrison, Germino & Reeders, 1991) and human (Turner *et al.*, in press) basement membranes.

On 2-D electrophoresis, using non-equilibrium tube gels (NEPHGE) in the first dimension, it was found that the predominant 28-kD monomers migrated to different isoelectric points. These have been referred to as $M28^{+}$ (pI 7) and $M28^{+++}$ (pI 9) (Kleppel, Michael & Fish, 1986; Butkowski *et al.*, 1990); $M28^{+++}$ is thought to be the α3 (IV) chain. Our study differs from previous work in that flat-bed IEF was used in the first dimension, allowing molecules to migrate to their true pI under the conditions used. This allows more complete separation of GBM components, as illustrated in Fig. 2. Charge heterogeneity of the different monomers and corresponding dimers is extensive, and may relate to the presence of charge isoforms, or indeed to molecules of a different origin but similar molecular weight. In particular, the 28-kD monomers cover a wide range of charge, extending to a pI of approximately 10.

Western blotting of 2-D separated (NEPHGE in the first dimension) human GBM has been reported before, using sera from a small number of patients (Yoshioka, Kleppel & Fish, 1985). We agree with these authors that the 28-kD monomers and corresponding dimers, especially the more cationic species, appear to be the most antigenic components. The highly cationic bands are a minor constituent of GBM and are only weakly stained by silver, but are strongly stained on blotting with patients' IgG. However, Yoshioka *et al.* (1985) considered that there were up to three separate patterns of reactivity produced by anti-GBM antibodies, and that each of these consisted of responses to several antigens. The present study, using autoantibodies matched for concentration, and a monoclonal antibody to GBM, demonstrates that the response is highly restricted. It seems possible that the reported differences in binding pattern could be related to the concentrations of the antibodies used, rather than to their specificity. Our results provide support for this possibility, since the use of diluted IgG from three patients demonstrated that reactivity with the less cationic components became weak or undetectable while the cationic components were still easily visualized.

Anti-GBM antibodies from all 20 patients bound in a similar pattern. This was regardless of the clinical features, in particular the presence or absence of pulmonary haemorrhage and the severity of renal disease (Table 1). This finding adds support to our previous suggestion that the autoantibodies are of the same specificity whether or not there is lung involvement—which may be determined by environmental factors such as smoking (Donaghy & Rees, 1983). Preliminary results from 2-D Western blotting of alveolar basement membrane (Derry & Pusey, 1990) also demonstrate similar binding of IgG from all patients

studied. Anti-GBM disease shows strong associations with MHC genes (Rees *et al.*, 1984), in particular the haplotypes DR2 DQw6 and DR4 DQw7 (Burns *et al.*, 1990). It is of interest that there was no difference in binding of patients' antibodies in relation to their HLA type. One possible explanation is that the MHC molecules concerned can present the same autoantigenic peptide, due to similarities in their peptide binding domains.

The use of monoclonal P1, previously shown to bind in the same pattern as human autoantibodies by immunohistology (Cashman, Pusey & Evans, 1988) and Western blotting (Pusey *et al.*, 1987), allowed us to demonstrate that the same major antigenic determinant was present on different components of GBM recognized by human antibodies. The monoclonal bound to both of the major 28-kD monomers, although more strongly to the highly cationic polypeptide. There was less binding to other components recognized weakly by patients' IgG, suggesting a minor difference in specificity or avidity. Affinity purification of the GA, using a P1 column, showed enrichment for the autoantigenic 28-kD monomers. Less dimeric material bound to the column, despite possessing antigenicity on 2-D blots, suggesting that the antigenic sites in certain dimers may be sequestered in non-dissociating conditions but revealed on SDS–PAGE.

Our results support the suggestion that one or more novel chains of type IV collagen are involved in Goodpasture's disease, and in particular that the α3 chain of GBM bears the major autoantigenic epitope (Turner *et al.*, in press). The role of other novel α(IV) chains is unclear, but it is of interest that in Alport's syndrome (a form of hereditary nephritis), there is abnormal binding of anti-GBM autoantibodies and monoclonal P1 to the GBM (Savage *et al.*, 1986). The α5(IV) chain gene maps to the same region on the X chromosome that is associated with X-linked Alport's syndrome (Hostikka *et al.*, 1990) and mutations in the COL4A5 gene, encoding this chain, have been reported in kindreds with Alport's syndrome (Barker *et al.*, 1990). The association of Alport's syndrome with the α5 chain is apparently at odds with the abnormal binding of autoantibodies. This paradox may be explained by the hypothesis that the α3 and α5 chains are associated in a separate type IV collagen network found only in certain specialized basement membranes.

Precise characterization of molecules bearing the GA should be of value in the understanding of both Alport's and Goodpasture's syndrome. This study suggests that there is a single major antigenic determinant, which is present on the highly cationic form of the 28-kD molecule, thought to be the α3(IV) chain. Further investigation of the immune response to these molecules should allow the development of specific forms of immunotherapy for Goodpasture's syndrome.

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