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Characterization of the human Goodpasture antigen

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SUMMARY

The glomerular basement membrane antigen involved in Goodpasture syndrome was purified from human kidneys. The antigen was solubilized by collagenase digestion and purified by ion exchange chromatography, gel filtration and reversed phase HPLC. The monomer proteins (M1, M2*, and M3) were immunochemically compared with the corresponding bovine monomers and appeared to be identical. The Goodpasture reactivity was localized to the same monomer (M2*) as in bovine material. It could also be shown that eight out of nine patients with Goodpasture syndrome had circulating antibodies reacting with a crude collagenase digest of human glomerular basement membrane that could be inhibited by the active monomer peptide. The ninth patient had, besides antibodies to this peptide, antibodies to the 7S domain of type IV collagen. Further immunochemical studies indicate that all patients sera recognize the same site(s) on the monomer protein. Thus the major antigenic determinant(s) of Goodpasture syndrome resides in monomer M2* which is a constituent of the globular domain of collagen IV.

Keywords glomerular basement membrane Goodpasture syndrome globular domain collagen IV

INTRODUCTION

Goodpasture syndrome (GP) is a rare autoimmune disease representing approximately 5% of all glomerulonephritis patients (Wilson & Dixon, 1981). It can be described as rapidly progressive glomerulonephritis with or without attendant lung haemorrhage (Wilson & Dixon, 1981). A diagnostic finding is the presence of anti-basement membrane antibodies that are found both in a linear pattern along the glomerular basement membrane (GBM) (Wilson & Dixon, 1974; Fish *et al.*, 1984) and circulating in serum (Wilson, Marquardt & Dixon, 1974; Mahieu, Lambert & Miescher, 1974; Wieslander, Bygren & Heinegård, 1981). In most patients antibodies are also found along the tubular basement membrane and in some cases along the lung basement membrane (Wilson & Dixon, 1981). Collagen as well as noncollagen glycoproteins of the GBM have been proposed to contain the antigenic sites(s) responsible for immunization (McIntosh & Griswold, 1971; Mahieu, Lambert & Maghuin-Rogister, 1974; Marquardt, Wilson & Dixon, 1973; Foidart *et al.*, 1980; Hunt, McDonald & McGiven, 1982). Recently, several studies have shown that the antigen is present in collagenase resistant domains of the GBM with molecular weights around $M_r = 25{,}000$ daltons and $M_r = 50{,}000$ daltons (Holdsworth, Golbus & Wilson, 1979; Wieslander, Bygren & Heinegård, 1983; Fish *et al.*, 1984; Yoshioka, Kleppel & Fish, 1985). These domains were isolated (Wieslander,

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Bygren & Heinegård, 1984b) and shown to be noncollagenous proteins with a monomer-dimer relationship. It was subsequently shown that the peptides were derived from the noncollagenous domain of collagen IV (Wieslander *et al.*, 1984a), i.e., the globular domain. Further studies of the collagenase resistant peptides from bovine GBM showed that they could be separated into active and inactive monomers and dimers (with respect to reactivity with Goodpasture antibodies) by reversed phase HPLC (Butkowski *et al.*, 1985) and that the epitope is sequestered inside a hexameric structure (Wieslander *et al.*, 1985b). Antibodies to the active monomer (M2*) react with collagen IV (Butkowski *et al.*, 1985), further supporting the localization of the Goodpasture antigen to collagen IV.

The following study was undertaken to determine whether the human monomer preparation could also be subdivided into several peptides, which monomer contained the major reactivity with Goodpasture sera, and whether all GP patients recognize the same site(s) on this antigen.

MATERIALS AND METHODS

Isolation of the Goodpasture antigen. Glomeruli were isolated from human cadaver kidneys by sieving procedures as already described, in the presence of protease inhibitors (Wieslander *et al.*, 1984b). Human GBM was then extracted with 6 M guanidine HCl (GuHCl), 0·05 M Tris-HCl, pH 7·5 with protease inhibitors for 16 h at 37°C. The insoluble material was suspended in 0·05 M HEPES buffer, 0·01 M $CaCl_2$ with protease inhibitors except EDTA. Collagenase (Worthington CLSPA), 0·1% by weight, was added and the material digested for 20 h at 37°C followed by dialysis against 8 M urea, 0·05 M Tris-HCl, pH 7·5. The insoluble material was removed by centrifugation and the soluble proteins fractionated on a column of DEAE-cellulose (2·5 × 40 cm) equilibrated in 8 M urea, 0·05 M Tris-HCl, pH 7·5 as described (Wieslander *et al.*, 1984b). The unbound fraction was concentrated on a YM10 filter (Amicon) to 10 ml, dialysed against 6 M GuHCl, 0·05 M Tris-HCl, pH 7·5 for 24 h and the peptides separated on a column of Sephacryl S-200 (2·5 × 100 cm, Pharmacia Fine Chemicals) as described (Wieslander *et al.*, 1984b). The monomer and dimer pools were concentrated on a YM10 filter to 10 ml and trifluoroacetic acid (TFA) added to 0·5%. The pools were then separately fractionated by reversed phase HPLC as described (Butkowski *et al.*, 1985) on a C18 column (4·6 × 25 mm, Vydac). A 30 min gradient from 32–39% acetonitrile in 0·1% TFA was used to elute the peptides.

Immunochemical techniques. Goodpasture antigen was assayed using inhibition ELISA as already described (Wieslander *et al.*, 1984b). Antigen (crude collagenase digest of GBM after DEAE cellulose or purified monomer from HPLC) was coated on polystyrene microtitre plates (NUNC Immunoplate) in 6 M GuHCl, 0·05 M Tris-HCl, pH 7·5. Samples were diluted in 0·05 M sodium phosphate, pH 7·5, 0·15 M NaCl, 0·05% Tween 20 with 0·2% bovine serum albumin. For studies of the site specificity of GP sera, the first incubation with sera was for 2 h, the plates were washed and a second incubation with sera was done for 1 h. Protein blotting was done by the method of Burnette (1981) as described by Wieslander *et al.* (1984b). Antisera against the bovine monomers M1, M2*, M3 (Butkowski *et al.*, 1985) were raised in rabbits by standard procedures. Prestained protein molecular weight standards were obtained from Bethesda Research Laboratories.

Electrophoretic analysis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli (1970) on 6–22% gels. Molecular weight markers for SDS-PAGE were obtained from Sigma Chemical Company.

RESULTS

Isolation of the Goodpasture antigen. The antigen was solubilized from human GBM by collagenase, separated from noncollagenous proteins by DEAE cellulose chromatography, separated into monomer (30 K) and dimer (50 K) protein pools after dissociation in 6 M GuHCl,

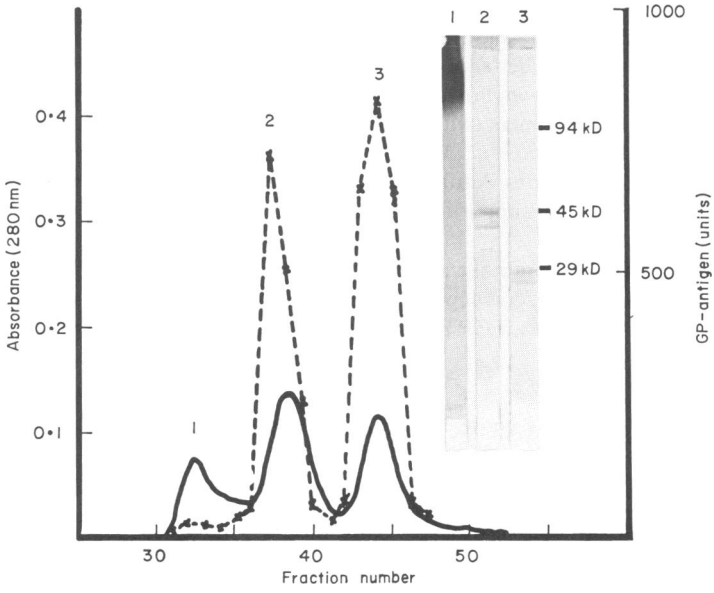

Fig. 1. Separation of human monomer proteins from dimer proteins by gel filtration in 6 M GuHCl, 0·05 M Tris-HCl, pH 7·5 on a column of Sephacryl S-200. The amount of Goodpasture antigen was measured by inhibition ELISA and expressed in arbitary units (– – – –). Fractions from peaks 1, 2 and 3 were analysed by SDS-PAGE and are shown as inserts. Molecular weight markers are represented in kilodaltons on the right hand side of the insert. (——) Absorbance.

0·05 M Tris-HCl, pH 7·5 and gel filtration chromatography on a column of Sephacryl S-200 eluted in 6 M GuHCl, 0·05 M Tris-HCl, pH 7·5 (Fig. 1).

The monomer and dimer constituents separate from each other as previously shown and are contained in pools III and II, respectively. Pool I contains mainly the 7S domain of collagen IV. SDS-PAGE (Fig. 1, insert) reveals the presence of three monomer polypeptides and two dimer polypeptides. The Goodpasture serum reacts well with both monomer and dimer fractions.

The monomer and dimer constituents were next fractionated by HPLC using a reversed phase column, as described for the separation of bovine constituents (Wieslander *et al.*, 1984b). In Fig. 2, the monomer separation can be seen. The major protein peak elutes between 35–36% acetonitrile followed by several smaller peaks. SDS-PAGE analysis of the indicated pools show that the two faster migrating peptides elute in the major peak (fractions 1 and 2) while the slower migrating peptides elute from fractions 3 to 8. No difference could be seen in mobility of the proteins in fractions 3 or 8. Protein blotting on nitrocellulose paper followed by reaction with a Goodpasture serum shows that fractions 4 to 8 stain well with the GP antibodies. This indicates that fraction 3 contains a protein with the same mobility but that does not react with GP antibodies. The immunoblotting results also show that the major peptide in fraction 1 and 2 is not reactive with the GP antibodies. The proteins in the dimer pool from S-200 were also separated by reversed phase HPLC (Fig. 3). A major peak eluted between 36 and 37% acetonitrile followed by several smaller peaks. Analysis by SDS-PAGE showed that fraction one contained residual monomers, fractions 2 and 3 contained a major doublet with a minor band migrating slightly faster. Fractions 4–7 migrated to almost identical positions as the major doublet although a slightly slower migration is observed. Protein blotting showed that the GP antibodies stained mostly in fractions 5 and 6 with a low amount of staining in fractions 4 and 7 which also indicates that the major dimer doublet, as well as the major monomer doublet, is unreactive with GP antibodies.

Comparison with bovine products. The HPLC isolated human monomers were immunochemically compared with the bovine monomers M1, M2*, and M3 (Butkowski *et al.*, 1985). The bovine M2* was shown to react with the Goodpasture antibodies and both M1 and M2* are derived from

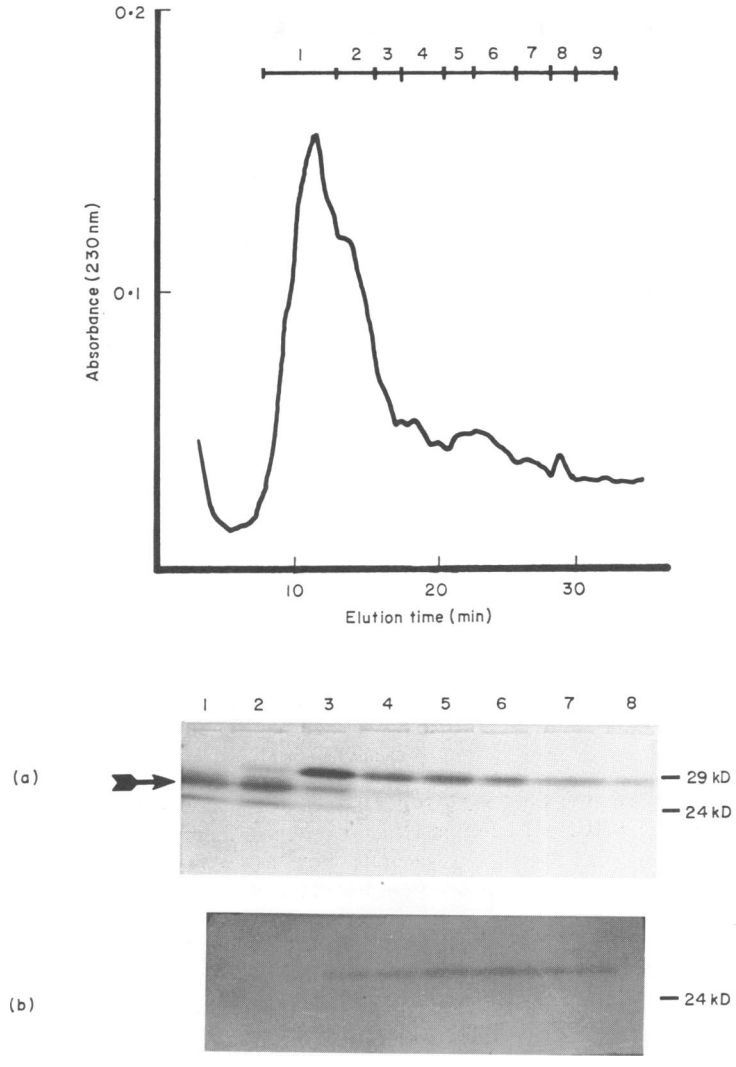

Fig. 2. Separation of monomer proteins by reversed phase HPLC. The peptides were eluted with a 30 min gradient from 32–39% acetonitrile in 0·1% TFA. Fractions from the reversed phase HPLC run were pooled as indicated. (a) The indicated pools were analysed by SDS-PAGE and silver stained. One sample of each fraction was also analysed by protein blotting and stained following reaction with a Goodpasture serum. Molecular weight markers, represented in kilodaltons, for SDS-PAGE and western blots are given on the right hand side of Panels (a) and (b), respectively. The arrow on the left hand side of Panel (a) designates the major monomer polypeptide.

collagen IV (Butkowski *et al.*, 1985). Inhibition ELISA was performed on the HPLC fractions (Fig. 4, upper half) and showed that fractions 1 to 3 contain the M1 antigen, fractions 3 and 4 the M3 antigen and fractions 5 to 7 the M2* antigen. This was confirmed by immunoblotting (Fig. 4, lower half) were 1 and 3 stained with anti-M1 antibodies, 3 with anti-M3 antibodies and 5 and 7 with the anti-M2* antibodies. The Goodpasture antigen ELISA is mostly reactive with fractions 5–8 (data not shown). This shows the cross-reactivity of bovine and human antigens and also that the antiserum to M2* recognizes the same peptide as the Goodpasture antibody (Figs 2 and 4). Furthermore, this shows that the human monomer pool is composed of the same monomers as the bovine.

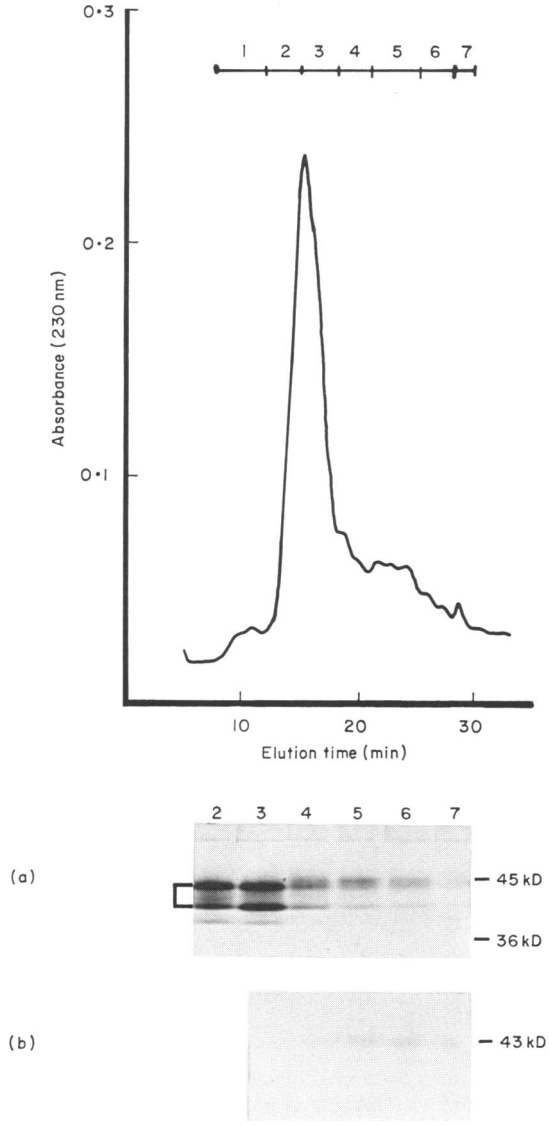

Fig. 3. Separation of dimer proteins by reversed phase HPLC. The conditions were as indicated in Fig. 2. The indicated pools were analysed by SDS-PAGE. (a) One sample was stained for protein by silver and (b) another for reactivity with Goodpasture antibodies after protein blotting. The bracket on the left side of Panel (a) specifies the major dimer doublet. Molecular weight markers are as described in Fig. 2.

Specificity of Goodpasture antibodies. The HPLC purified human monomer containing the GP epitope (fractions 6–8) was then studied to determine if this peptide contained the major antigen in Goodpasture syndrome and if different GP sera react with the same site(s) on this peptide.

The coating mixture for inhibition ELISA consisted of a crude collagenase digest of human GBM that had been passed through the DEAE-cellulose column. This enriched fraction gave a higher binding of Goodpasture antibodies than the unfractionated material and was therefore used. The binding of antibodies from nine GP patients to this crude protein mixture was studied in the presence of different amounts of the pure monomer (HPLC fractions 6–8). It could be shown (Fig. 5) that antibody binding of eight patients could be inhibited by 70–90% by this peptide. The ninth

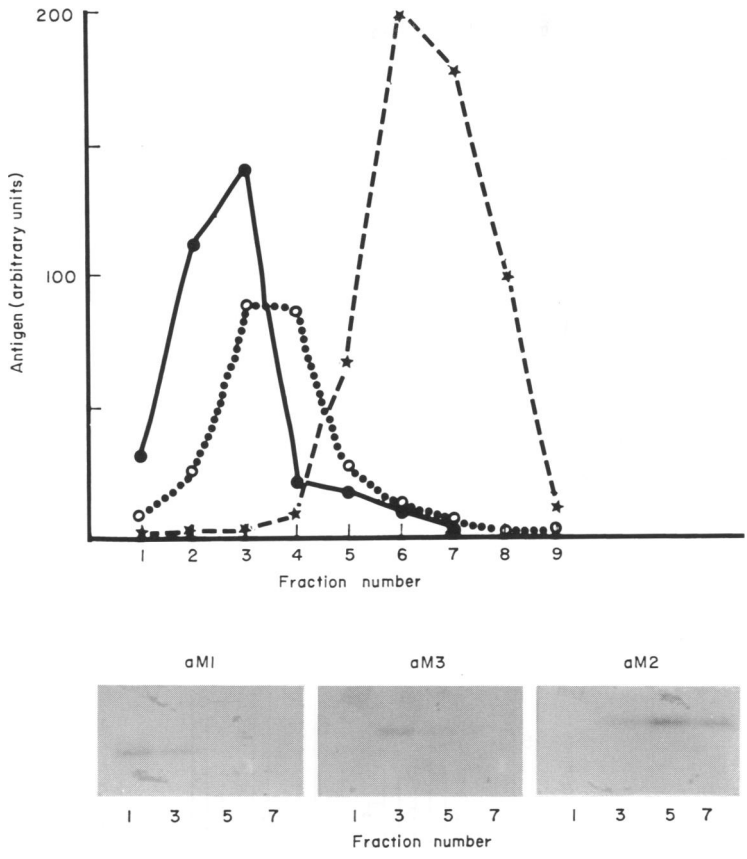

Fig. 4. Comparison of human with bovine monomers by immunochemical techniques. The individual pools 1 to 9 were analysed by inhibition ELISA for the content of M1, M2*, M3 utilizing antisera to M1, M2*, M3 (upper half). Monomer fractions 1, 3, 5 and 7 (Fig. 2) were also separated by SDS-PAGE, transferred to nitrocellulose papers and stained with antibodies to the bovine monomer M1, M2* and M3 (lower half).

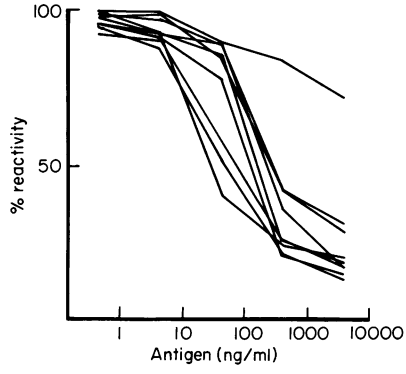

Fig. 5. Reactivity of Goodpasture sera with the isolated monomer peptide. ELISA plates were coated with a crude digest of human GBM after passage through a DEAE-cellulose column. Sera from nine Goodpasture patients were incubated with the isolated monomer peptide (Fig. 2, fractions 6–8) diluted to the indicated protein concentration.

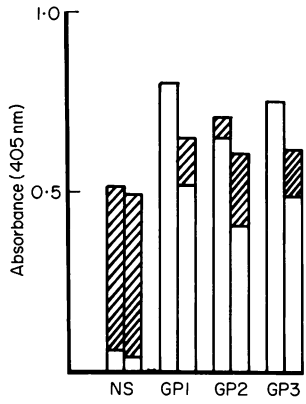

Fig. 6. Site specificity of Goodpasture antibodies. The microtitre plate was coated with the isolated human monomer (fraction 6–8). The first incubation was with one normal serum (NS, open bars) and three Goodpasture sera (GP) in two dilutions (1/10 and 1/100, open bars) and the second incubation with one Goodpasture serum diluted 1/10 (hatched bars).

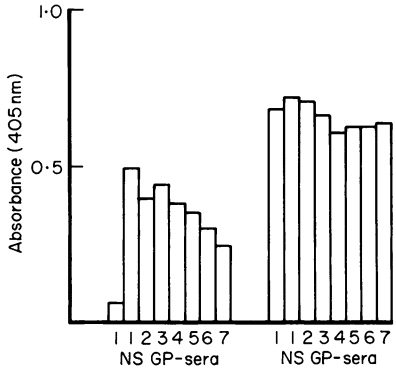

Fig. 7. Site specificity of Goodpasture antibodies. The ELISA plates were coated with the human monomer. The first incubation was with a normal serum (left panel) and with a Goodpasture serum (right panel) both diluted 1/20 and followed in both cases by a second incubation with one normal and seven Goodpasture sera all diluted 1/20.

patient could only be inhibited by 30%. This patient had antibodies to the 7S domain besides the Goodpasture antigen (Wieslander & Heinegård, 1985a).

Next the human monomer (HPLC fractions 6–8) was coated to microtitre plates in an attempt to study if the patients reacted with the same site(s) on this protein. Four sera (one normal and three GP), in two different dilutions, were incubated with the monomer, the plate was washed and one GP serum was then incubated in all the wells. The results are shown in Fig. 6. The normal serum gave little binding while all the GP sera bound to the monomer. The second incubation with one GP serum increased the absorbance, where the normal serum was used in the first incubation and where diluted (1/100) GP sera were used, but not where a 1/10 dilution of GP sera were used. This indicated that the first incubation with antisera had blocked the binding site for the second incubation. The experiment was also done in the reverse order, i.e. one serum for the first incubation and seven sera for the second incubation. In fig. 7 (the left part) a first incubation with a normal serum was followed by a second incubation with seven sera (of which one is a normal serum). All the GP sera have the capacity to increase the binding of antibody to the antigen. When the first incubation was with a GP serum (Fig. 7, right part), on the other hand, none of the GP sera could

give a higher binding than the normal serum when used in the second incubation, supporting the idea that the GP sera all react with the same site(s) on the human monomer peptide.

DISCUSSION

The rationale for isolating the basement membrane antigens involved in Goodpasture syndrome from human kidneys were (a) a lack of cross-reactivity between human and bovine material which could give reactivity with one peptide only, even though others in the human system are reactive, and (b) isolation of the human antigen makes it possible to study if this protein carries the major epitope involved in Goodpasture syndrome and subsequently, if the patients antibodies recognize the same site(s) on this protein.

The Goodpasture antigen was isolated from human GBM in order to obtain a high yield by methods already described (Wieslander *et al.*, 1984b; Butkowski *et al.*, 1985). The insoluble GBM was solubilized by collagenase, the noncollagenous proteins removed by ion exchange chromatography, monomer peptides separated from dimer peptides by gel filtration in 6 M GuHCl, and finally, the individual peptides were isolated by reversed phase HPLC. One of the monomer peptides reacted with both the Goodpasture sera and with antiserum against the bovine M2* peptide, while the major monomer peptide did not react with Goodpasture sera but reacted with antiserum to the bovine peptide M1. We concluded that the bovine and human collagenase-resistant peptides are the same and that studies on the bovine system did not neglect the identification of any major antigen for Goodpasture antibodies. Moreover, the monomer polypeptide contains the major antibody reactivity for eight out of nine patients with Goodpasture syndrome. Only one patient's serum contained additional antibodies that also reacted with the 7S domain of type IV collagen (Wieslander & Heinegård, 1985a).

Double incubation with the autoantibodies against the purified monomer, i.e. first incubation with one serum and then a second incubation with another serum, could show if the patients have antibodies to different sites on this peptide. If the patients have differing specificity one should expect that the binding of antibodies would increase with a double incubation while if their antibodies recognize the same site there would be no increase in binding. In no instance did any increase in binding occur, supporting the conclusion that the patients sera react with the same site(s). Possibly there is only one site since Pressey *et al.* (1983) have produced a monoclonal antibody that can block binding of Goodpasture antibodies to the antigen. In these double incubation and blocking experiments, one must keep in mind that the binding of one antibody may block the binding of a second antibody, not only by reacting with the same site, but also by reacting with a nearby site. Thereby it can interfere with the binding of the second antibody by steric hindrance, or by changing the conformation of the antigen once bound.

The monomer peptide (M2*), which contains the Goodpasture epitope, appears to be an integral part of the globular domain of collagen IV based on several lines of evidence obtained from studies of the bovine antigen. After its release from collagen IV by bacterial collagenase, the globular domain exits in the form of a hexamer under nondenaturing conditions (Wieslander *et al.*, 1985b). Treatment of the hexamer with the denaturants, GuHCl or SDS, dissociates it into monomeric and dimeric (disulphide crosslinked) subunits. Both the monomer and dimer constituents exhibit similar denaturation-renaturation curves and all reassociate to form a hexamer even after extensive unfolding in 6 M GuHCl (Butkowski *et al.*, 1985; Wieslander *et al.*, 1985b). Concomitant with the dissociation of the hexamer, is the increased reactivity of the Goodpasture epitope which can be reversed upon reassociation of the subunits (Wieslander *et al.*, 1985b).

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