Ann. rheum. Dis. (1971), **30**, 631

Chemical and immunological characterization of a basement membrane-like glycoprotein in the rat

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Two glycoproteins have been isolated from mammalian glomerular basement membrane. One is collagen-like rich in hydroxyproline, hydroxylysine, and carbohydrate; the other lacks these two amino acids (Kefalides, 1966). The chemical composition of basement membranes have been studied extensively (Chow and Drummond, 1969; Kefalides, 1968; Kefalides, 1969; Kefalides and Winzler, 1966; Misra and Kalant, 1966; Spiro, 1967a, b). A glucosyl galactosyl disaccharide linked glycosidically to hydroxylysine and a heteropolysaccharide containing hexosamine, mannose, galactose, fucose, and sialic acid have been isolated from glomerular basement membrane (GBM) (Dische, Pappas, Grauer, and Dische, 1965; Spiro, 1967a, b).

The role of anti-GBM antibodies in renal disease is well recognized (Lerner, Glassock, and Dixon, 1967; McPhaul and Dixon, 1970). The isolation of GBM fragments in the urine of man (McPhaul and Dixon, 1969) and experimental animals (Lerner and Dixon, 1968) and the increase of these with immunological injury (Hawkins and Cochrane, 1968) has been well documented. Glomerulonephritis has been produced with homologous and autologous solubilized GBM (Steblay, 1962), GBM glycoproteins (Dinh and Brassard, 1967; Shibata, Miyakawa, Naruse, Nagasawa, and Takuma, 1969), and urinary GBM fragments (Pollak, Galzutis, and Rezaian, 1968).

Dinh and Brassard (1967) showed that the major urinary protein (MUP) in rats produced glomerulonephritis after serial immunization. The purpose of this study was to isolate, purify and characterize this protein and to compare it with GBM glycoproteins.

Material and methods

(1) *Isolation and purification of urinary glycoprotein*
Pooled urine obtained from 200 g. Sprague-Dawley rats was filtered through Whatman No. 1 paper and centrifuged at 1,500 r.p.m. for 30 min. and the supernate was concentrated by pressure dialysis (Diaflo Amicon Labs., Mass.) (Pollak and others, 1968) and dialysed extensively against 0·05 M phosphate buffer, pH 8. DEAE cellulose ion exchange chromatography was performed according to the method of Sober and Peterson (1958). All fractions were concentrated and tested for the presence of serum and GBM glycoproteins by Ouchterlony double diffusion in agar gel (Ouchterlony, 1958). Preparation of antisera is described below. Sephadex G200 gel column chromatography was performed on the major fraction from DEAE. Immunodiffusion studies were performed on the fractions as above. The major peak from G200 Sephadex purified major urinary glycoprotein (MUPpg) was dialysed extensively against distilled water and lyophilized, and this purified protein was used for chemical and immunological studies. Gel chromatography on Sephadex G200 column of the same size was performed on rat albumin (Pentex Labs., Ill.) and rat IgG (Pentex Labs.), and on GBM prepared and solubilized as described below.

(2) *Preparation of glomerular components*
Glomeruli and glomerular basement membrane were isolated from kidneys from Sprague-Dawley rats by the method of Krakower and Greenspon (1951). One portion of isolated glomeruli was sonicated and lyophilized; the remainder was used for GBM preparation, solubilization, immunization, and isolation of the two GBM glycoproteins. The collagenous component of GBM was isolated by TCA extraction and the non-collagenous component by 8 M urea extraction and Sephadex G200 chromatography as described by Kefalides (1966). GBM was solubilized with highly purified Pronase (Sigma) at 18°C. for 6 hrs and the supernate after centrifugation at 14,000 r.p.m., dialysed against distilled water, lyophilized, and purified on Sephadex G200 column (Kefalides, 1969).

(3) *Preparation of antisera*
Rabbit antisera to whole rat serum was obtained from Immunologic Inc., Glen Ellyn, Illinois. Rabbit antisera to

[1,3] Supported by G. R. S. Grant, United States Public Health Service No. 1-501-FR 05354, University of California, Los Angeles School of Medicine, and the Westwood Hills Junior Foundation.
[4,5] Supported by United States Public Health Service Training Grant HD0060.
Accepted for publication May 11, 1971.
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rat IgG was obtained from Immunologic, Inc., and rabbit antisera to rat β_1A-β_1C was prepared by serial immunization with Zymosan absorbed rat β_1A-β_1C (Müller-Eberhard, Nilsson, and Aronsson, 1960). Antiserum was prepared in rabbits to 8 M urea solubilized rat GBM by serial immunization. Antiserum to purified rat major urinary glycoprotein (MUPpg) was prepared in a similar manner. Antiserum to rat urine was prepared by serial immunization of rabbits with lyophilized urine. The antisera were absorbed with normal rat serum. All these antisera were tested for the presence of antiglomerular antibody by direct immunofluorescent technique using FITC-labelled antisera and the double-layer technique using unlabelled antisera and FITC goat anti-rabbit IgG (Immunologic, Inc.) (Coons, 1956; Coons and Kaplan, 1950) on normal rat glomeruli. The methods for preparation of tissue and microscopy have already been described (McIntosh, Kaufman, Kulvinskas, and Grossman, 1970). The antisera were also tested for the absence of antibody to serum proteins by Ouchterlony immunodiffusion using normal rat serum. Normal rat serum and rat albumin (Pentex, Inc.) were used as controls.

(4) ***Absorbtion studies***

Antiserum to MUPpg. was absorbed with:

(*a*) Collagen-like GBM glycoprotein prepared by TCA extraction as described above;

(*b*) Sonicated glomeruli;

(*c*) Non-collagen-like glycoprotein prepared by 8 M urine extraction and gel chromatography as described above;

(*d*) MUPpg;

(*e*) Lyophilized rat urine;

(*f*) Acid-soluble tail tendon collagen isolated by the method of Watson, Rothbard, and Vanamee (1954) and with a combination of collagen and non-collagenous component GBM glycoprotein.

After repeated absorption with at least one part of the absorbent to three parts of the anti-MUPpg, the antiserum was used for immunofluorescent staining of 4 micron sections of frozen rat kidney cortex by both the direct and double layer immunofluorescent techniques.

(5) *Blocking studies*

Using labelled and unlabelled antisera to both 8 M urea solubilized GBM and MUPpg, blocking or inhibition studies were performed by immunofluorescent methods (Coons and Kaplan, 1950).

(6) *Chemical studies*

Amino acid analysis was performed on lyophilized MUPpg after hydrolysis in 6 N HCl at 110°C. for 21 hrs on a Beckman amino acid analyser by the method of Moore and Stein (Spackman, Stein, and Moore, 1958), hexose analysis by the orcinol method of Weimer and Moshin (1953), and glucose and galactose analyses were performed on the hydrolysates by enzymatic methods in 2 N HCl for 2 hrs at 100°C. (glucostat and galactostat reagents, Worthington Biochemical Corp., Freehold, New Jersey). Carbohydrates were identified by paper chromatography after hydrolysis in 2 N HCl for 2 hrs at 100°C. for neutral sugars. Neutral sugars were separated by Dowex 50H$^+$. Aliquots were spotted on Whatman No. 1 paper and descending chromatography was performed (Boas, 1953; Partridge, 1946).

(7) *Experimental production of renal disease*

Rat MUPpg was administered to five 150 g. Sprague-Dawley rats as a single dose of 40 mg. in complete Freund's adjuvant (Hyland Labs., Calif.). Five control animals received complete Freund's adjuvant in PBS. 24-hr quantitative urinary protein was determined 14 days after the injection. The animals were killed on day 14. Four micron sections of formalin fixed tissue were stained with haematoxylin and eosin and periodic acid-Schiff (PAS) reagent for light microscopy. Immunofluorescent studies were performed on frozen sections using FITC-labelled rabbit antisera to rat IgG and β_1C.

Results

Concentrate dialysed urine was eluted from a DEAE cellulose column as a large single fraction and several components (Fig. 1) were recovered, all of which showed a single identical precipitin band when concentrated and tested by immunodiffusion in agar gel using rabbit antisera to both solubilized basement membrane and to concentrated urine. These antisera contained no antibody to rat serum by immunodiffusion; anti-rat GBM antibody was demonstrated by immunofluorescence.

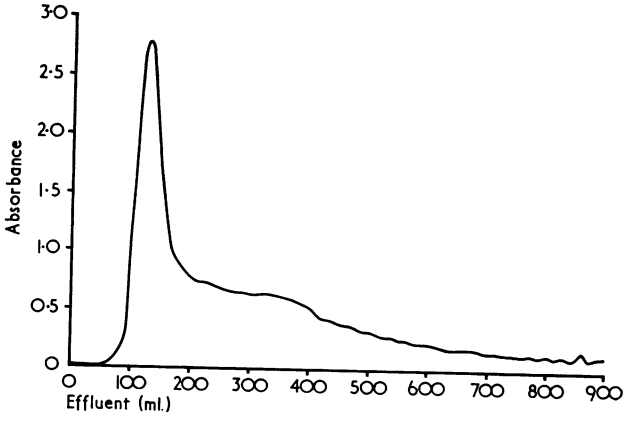

FIG. 1. *Elution of concentrated rat urine on DEAE cellulose column.*

The elution of the concentrated large fraction from DEAE cellulose on Sephadex G200 is shown in Fig. 2. The second peak, the major fraction, when concentrated, showed no serum proteins by immunodiffusion using rabbit antisera to normal rat serum but showed a single precipitin band to anti-rat GBM and absorbed anti-rat urine.

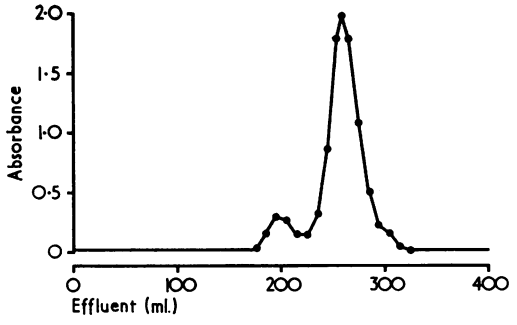

FIG. 2 *Elution of major fraction from DEAE on Sephadex column.*

Rat MUPpg was eluted from Sephadex G200 column in the area of rat albumin. Rat IgG, the non-collagenous component of GBM, the first fraction of the supernate after high-speed centrifugation of pronase-digested GBM, are eluted earlier than MUPpg from a Sephadex G200 column of the same size (Fig. 3).

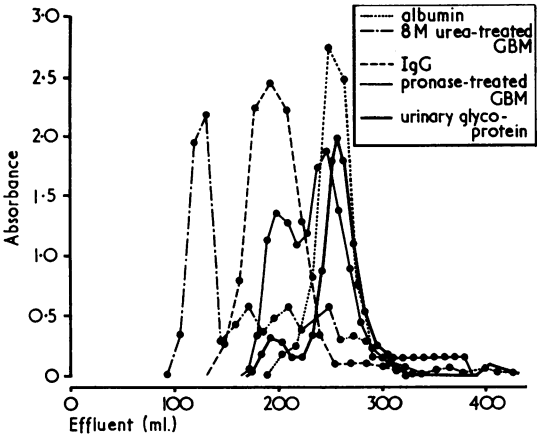

FIG. 3 *Comparison of elution of rat MUPpg with rat IgG, rat albumin, and the collagen-like and non-collagenous component of GBM.*

Antisera to rat MUPpg showed no antibody activity to rat serum proteins, but linear glomerular basement membrane fluorescence was observed by staining normal rat kidney, using both direct and double-layer techniques.

The results of absorption studies are shown in Table I, and those of blocking studies in Table II.

Table I *Immunofluorescence with rabbit antisera to urinary glycoprotein on normal rat kidney* (absorption studies)*

Antisera treatment	*Immuno-fluorescence*
None	4+
Absorbed with sonicated glomeruli	Negative
Absorbed with GBM	Negative
Absorbed with TCA extract of GBM	2+
Absorbed with lyophilized urine	Negative
Absorbed with urinary glycoprotein	Negative
Absorbed with 8M urea extract of GBM	±
Absorbed with collagen	2+
Absorbed with collagen + 8M urea extract	Negative

*Graded by intensity: 0 to 4+
± = equivocal

Table II *Blocking studies by immunofluorescence methods**

FITC rabbit anti-rat GBM	4+
FITC rabbit anti-rat urinary glycoprotein (UP)	4+
Rabbit anti-rat GBM + FITC rabbit anti-rat UP	1+
Rabbit anti-rat UP + FITC rabbit anti-rat GBM	2+

*Graded by intensity: 0 to 4+

Representative immunohistological studies on glomeruli are shown in Fig. 4 (overleaf). These immunofluorescent studies suggest that MUPpg is immunologically more similar to the non-collagenous glycoprotein, although gel filtration studies suggest it to be of smaller molecular size than both.

The amino acid and carbohydrate composition of MUPpg is compared with that of GBM and GBM glycoproteins isolated by different methods by various investigators in Table III (overleaf). The amino acid composition of MUPpg most closely resembles the non-collagen-like glycoprotein of GBM. The glycine, hydroxyproline, and hydroxylysine composition is particularly striking. Sugar chromatography revealed the presence of mannose, galactose, glucose, and fucose (Fig. 5, overleaf).

Intramuscular administration of MUPpg to rats was associated with proteinuria of 50–110 mg./24 hrs in three out of five animals. At 14 days, morphological studies revealed focal mild basement membrane thickening and focal moderate mesangial cell proliferation. Immunohistological studies showed a linear pattern of deposition of host IgG and β_1C on the capillary walls—the hallmark of anti-GBM autoimmune disease. No changes were observed in control animals.

Discussion

The chemistry of glomerular basement membrane,

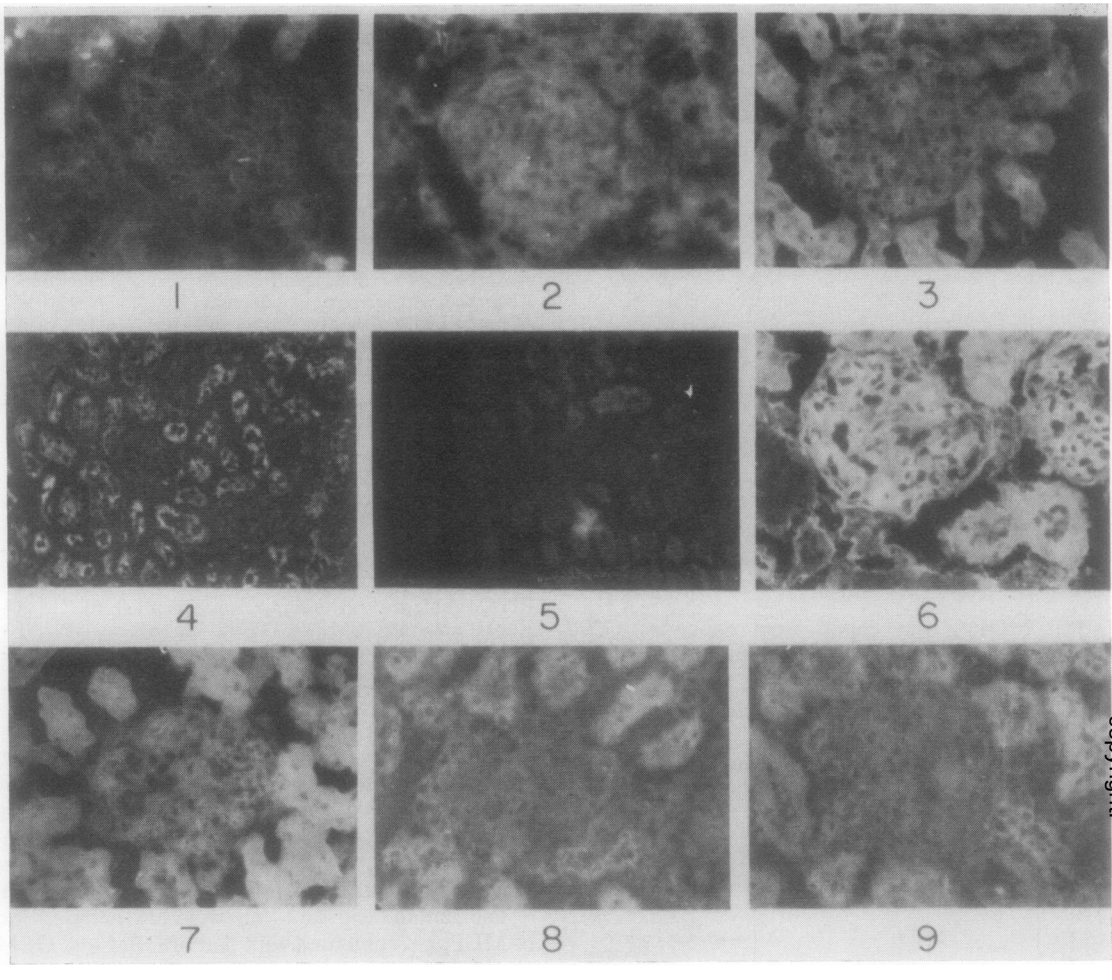

FIG. 4 *Results of absorption studies. Staining of normal rat kidney with antisera to MUP after absorption with*:

(1) *Urinary glycoprotein.*
(2) *TCA extract of GBM.*
(3) 8 *M urea of extract GBM.*
(4) *GBM.*
(5) *Sonicated glomeruli.*
(6) *No absorption.*
(7) *Collagen.*
(8) *Collagen* + 8 *M urea extract of GBM.*
(9) *Lyophilized urine.*

The antisera is absorbed with 1, 4, 5, 8, *and* 9, *but not with* 2, 6, 7, *and almost completely with* 3 *and* 8.

GBM glycoproteins, and GBM urinary fragments has been receiving considerable attention, particularly since the role of anti-GBM antibodies in the pathogenesis of renal disease in man and experimental animals has been emphasized, and GBM fragments shown to be capable of producing autoimmune nephritis have been isolated (Chow and Drummond, 1969; Dische and others, 1965; Hawkins and Cochrane, 1968; Intorp and Milgrom, 1968; Kefalides, 1966). Although quantitative studies show an increase in GBM fragments in glomerular injury, and biochemical studies on diseased glomerular basement membrane have revealed alterations (Chow and Drummond, 1969; Misra and Berman, 1968), extensive chemical studies have not been performed on urinary GBM in health and disease.

There have been variations in the chemical analyses of GBM and GBM glycoproteins reported by various investigators. However, despite these discrepancies, it appears that MUPpg is chemically most similar to the non-collagen-like component in GBM. Immunological studies lend some support to this. These immunological and biochemical studies suggest that MUPpg may represent a fragment of GBM which consists primarily of a product of the non-collagen-like glycoprotein alone or a fragment of this component only. The partial blocking by inhibition studies and the incomplete absorption

Table III *Comparison of chemical composition of MUP (pg.) with GBM and GBM glycoproteins isolated by different methods by various investigators*

*Amino acid**	*Non-collagen*		*Urinary** glycoprotein (MUPpg)*	*Collagen-like component*		*GBM*	*GBM glycoprotein*
	(1)	(2)		(3)	(4)	(5)	(6)
Aspartic	8·3	8·2	10·98	5·9	5·1	7·3	6·14
Threonine	6·0	5·7	7·37	2·1	2·1	4·0	3·07
Serine	8·7	7·3	8·25	3·9	4·1	4·9	5·11
Glutamic	13·2	11·4	10·53	8·6	7·5	11·0	11·15
Proline	8·3	6·8	4·02	7·1	6·0	7·0	8·59
Glycine	13·5	12·0	12·8	31·0	33·2	21·0	37·76
Alanine	7·6	8·8	8·74	5·6	3·3	6·7	3·99
Valine	3·8	5·1	6·67	2·7	2·6	4·7	3·37
½ Cystine	2·9	2·9	2·9	1·0	0·62	1·6	4·50
Methionine	0·9	0·8	0·61	0·2	0·75	0·9	—
Isoleucine	4·3	3·5	4·31	1·8	2·96	2·8	3·37
Leucine	8·9	8·1	8·25	4·6	5·0	6·7	6·44
Tyrosine	1·6	2·1	1·50	0·5	0·3	1·8	tr
Phenylalanine	4·2	3·3	4·11	2·1	2·5	3·3	2·86
Lysine	1·0	4·0	2·62	1·3	0·4	2·8	1·33
Histidine	1·2	1·9	1·83	0·6	0·8	1·4	0·51
Arginine	5·4	6·0	4·5	2·9	2·9	4·9	2·04
Hydroxyproline	tr	0	tr	15·8	16·1	5·3	tr
Hydroxylysine	tr	tr	tr	4·3	4·2	3·2	—
Hexose	5·7 per cent.	6 per cent.	15·1 per cent.		19 per cent.	6 per cent.	12·45 per cent.

(1) Non-collagenous component prepared by 8 M urea extraction at 38–40°C. and gel filtration chromatography (10, 11, 12)
(2) Residue after TCA extraction (10)
(3) TCA extract (10)
(4) Prepared by Pronase digestion and gel chromatography (11)
(5) (12)
(6) Trypsin
* Residues per 100 residues
** This study

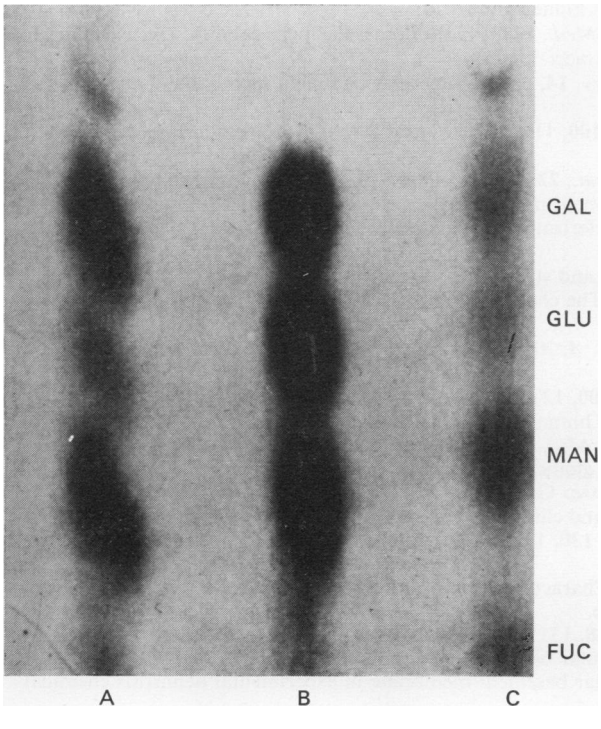

FIG. 5 *Sugar chromatography of MUPpg. B = standard; A and C = MUPpg.*

suggest that the former hypothesis may be true, although the purity of the materials used in these studies may be an important factor. Multiple precipitin bands are observed by immunodiffusion when solubilized GBM is tested against rabbit anti 8 M urea solubilized GBM (Kefalides and Winzler, 1966). However, MUPpg gives only a single precipitin line. Since glomerular basement membrane (the lamina densa) permeability may be a major factor in the passage of protein molecules into the urine (Graham and Karnofsky, 1966) and the deposition of immune complexes in various areas of capillary loops, it has been suggested that alteration in the molecular composition of GBM may be related to these abnormalities. The simple methodology for the isolation and chemical characterization of MUPpg may provide an easy method of studying molecular changes in immunological and non-immunological renal disease states.

Summary

A glycoprotein similar in amino acid composition but richer in hexose than the non-collagen-like component of glomerular basement membrane was isolated from rat urine by DEAE cellulose ion-exchange and Sephadex G200 chromatography. This protein appeared to be of smaller molecular size than either of the glomerular basement membrane glycoproteins. Immunodiffusion and immuno-fluorescence studies suggested that this protein was either a fragment of the non-collagen-like glomerular basement membrane component or was a glomerular basement membrane fragment containing this component predominantly. It is suggested that this protein may be used to provide an easy method of studying chemical alterations in basement membrane in glomerular diseases which lead to proteinuria.

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