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Glomerular Basement Membrane Antigens of Masugi Nephritis

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Summary. Glomerular basement membrane (GBM) isolated from rat, dog and human kidneys were treated with enzymes to obtain soluble membrane preparations. Gel filtration on calibrated columns of soluble membrane preparations produced several macromolecules which were identified as glycoproteins and glycopeptides, depending on their molecular sizes. A sialoglycoprotein of 50,000 Daltons is shown to be the nephrotoxigenic antigen of the GBM. Lipids were shown to be a non-essential component of the nephrotoxigenic antigen. Collagen and several other glycopeptides were found to be non-nephrotoxigenic antigens of the GBM. Use of pronase or combinations of certain enzymes (for example, collagenase and pronase, pepsin and trypsin) to obtain the soluble components of the membrane led to an alteration of the nephrotoxigenic antigen molecule.

INTRODUCTION

The pathogenesis of antikidney serum (AKS) nephritis has been studied extensively since its description by Masugi (1933). Subsequently, several workers have shown that the antigen(s) capable of raising nephrotoxic serum in heterologous species were located in the glomeruli (Solomon, Gardella, Fanger, Dethier and Ferrebee, 1949; Krakower and Greenspon, 1951). It was also shown that the nephrotoxic sera were not strictly species specific. Instead, they showed immunologic cross reactivity between heterologous GBM obtained from different species (Steblay and Lepper, 1961a, b; Krakower and Greenspon, 1969). Such sera are not completely organ specific, as cross-reactivity and localization in various organs of the nephrotoxic sera have now been demonstrated (Steblay, 1962; Nagasawa and Shibata, 1969). Until very recently, little was known about the number and chemical nature of various antigens of the GBM (Misra and Berman, 1969a). Based on the evidence gained from the cross-reactivity, absorptive capacity and biologic activity of heterologous antibodies raised against basement membrane containing tissue, Krakower and his associates held the view that there were multiple antigens in the GBM (Goodman, Greenspon and Krakower, 1955; Krakower and Greenspon, 1969) which were involved in the pathogenetic processes. In the past, several attempts to isolate, purify and characterize these antigens by chemical, physical and immunological means have not met with success (Misra, 1971a; Misra and Berman, 1969a; Steblay, 1962) due to virtual insolubility of GBM in common physiologic solvents. Recent studies (Misra, 1971a; Misra and Berman, 1969a) have shown that GBM has a complex molecular structure and consists of glycoproteins, collagen and glycolipoproteins which are known to be insoluble by usual procedures.

Use of enzymes, strong chemical agents (acids, alkalis and reducing reagents) and organic solvents to solubilize GBM have invariably led to contamination and/or alterations in the molecular structure of membrane components and production of complex molecules consisting of parts of more than one molecular species of GBM components. Earlier, Misra (1964) had shown that the GBM was a rich source of nephrotoxigenic and non-nephrotoxigenic antigens and that the chemical nature of a nephrotoxigenic antigen of rat GBM was a sialoglycoprotein. This study has since been extended and confirmed by several investigators (Huang and Kalant, 1968; Misra, 1971a, b; Rothbard and Watson, 1969; Shibata, Miyakawa, Naruse, Nagasawa and Takuma, 1969; Thoenes and Hammer, 1967; Skoza and Mohos, 1969). The present report describes isolation and purification of soluble GBM components from rat, dog and human kidneys and characterization of their chemical nature and antigenic behaviour. Our aim is to describe further the nephrotoxigenic antigens of GBM and alterations induced in their biological activities by commonly used proteolytic enzymes.

EXPERIMENTAL METHODS

Preparation of the GBM

A two step procedure was used to obtain highly pure GBM preparations from human, mongrel dog and Sprague–Dawley rat kidneys. In the first step, glomeruli were isolated from cortical homogenates by the magnetic iron oxide method (Misra and Berman, 1966) or graded nylon sieve technique (Misra, 1972). The homogeneity of glomerular preparation ranged between 95 and 98 per cent, using the criteria described by Misra and Berman (1966, 1968). In the second step, uniform suspensions of glomeruli in 8 per cent NaCl solution were subjected to controlled ultrasonication until 98 per cent of the glomeruli lost their cellular complement and became 'glomerular ghosts.' The material was washed five times with distilled water, using centrifugation at 1000 ***g***. These were dialysed against 20 volumes of distilled water for 16 hours at 4° with two changes of water in a mechanical dialyser. Dialysed material was lyophilized and labelled as 'GBM preparation.'

Preparation of GBM antigens

(a) Delipidized GBM: 10 mg of rat GBM were suspended in 10 ml distilled water in a 250 ml Soxhlet flask. To the suspension 40 ml chloroform–methanol (2:1 v/v) were added and extracted for 16 hours at 40°. The suspension was transferred into stoppered centrifuge tubes and centrifuged at 1000 ***g*** for 15 minutes. The chloroform-methanol layer was removed carefully. Ten millilitres (×2) aliquots of cold ether (reagent grade) were added to the tubes containing the deposits, stirred to mix well, centrifuged as before, and the ether layer was removed using Pasteur pipettes. Deposits (lipid-free GBM) were stored in a desiccator over silica gel at 4° till used.

(b) Solubilization of GBM by enzymatic digestion: 10–20 mg portions of GBM were treated with highly purified collagenase, pepsin, trypsin, pepsin followed by trypsin in sequence, papain, pronase and collagenase followed by pronase. Various conditions for enzymic digestion were followed as reported earlier (Misra, 1964; Misra and Kalant, 1966). The sources of enzymes used were as follows: collagenase (Sigma, U.S.A.) purified by the method of Keller and Mandl (1963) and purchased from Worthington; crystalline pepsin, 2× (Sigma, U.S.A.); crystalline trypsin, 2× (Sigma, U.S.A.); pronase, B grade free of nucleases (Calbiochem, U.S.A.); and papain, 2× crystallized (Sigma, U.S.A.).

These enzymes were tested for their specific and nonspecific activities against known substrates (Table 1). The K_{av} of enzymes from calibrated gel columns used were also ascertained (Fig. 1).

(c) Separation and fractionation of soluble GBM components: the reaction mixtures were centrifuged at 1000 *g* for 10 minutes. The supernatant was carefully transferred to a dialysis bag and dialysed in a mechanical dialyser against 10–20 volumes (×4 changes) with distilled water at 4°. The dialysed materials were lyophilized and stored at −20°. The deposits were resuspended in 5 ml distilled water and dialysed as above. These were then lyophilized and stored at −20°. The dialysates were flash evaporated to dryness, extracted with 0·5 per cent HCl in absolute alcohol and redried by means of a flash evaporator and stored at 4° in a desiccator.

The Sephadex G-75 columns (20 × 1 cm) were equilibrated with 0·15 M NaCl at 24° and calibrated using protein markers. Void volume was ascertained using blue Dextran (Fig. 1). One millilitre fractions of the eluates of glycoproteins and glycopeptides were collected and the optical density was read at 278 nm. A representative graph of such fractions obtained from rat GBM is presented in Fig. 2. Ninhydrin and anthrone reactions were carried out on each fraction.

Preparation of antiserum

Antiserum was produced in New Zealand rabbits weighing 2·5 kg against intact or delipidized GBM, or against basement membranes treated with collagenase, trypsin, pronase or papain, or combinations of collagenase and pronase or pepsin and trypsin. One to 2 mg of dry material was suspended by homogenization in 1 ml of Freund's complate adjuvant (Difco, U.S.A.). The first injection was 0·75 to 1 ml of the uniform suspension injected into footpads of each rabbit. Three weeks later a second dose by subcutaneous injection and 2 weeks later a third injection by the same route was administered. Blood was collected by cardiac puncture after an interval of 1 week. Blood was collected every other month following a booster injection of 0·5 ml of antigen preparation. The blood was allowed to clot at room temperature. The serum was separated by centrifugation (1000 *g*) and stored without preservatives in sterile ampules or serum tubes at −20° till used. All antisera were incubated at 56° for 30 minutes prior to use. The antiserum from each rabbit was tested separately.

Immunological studies

(i) Precipitation reaction. Three types of tests were used: (a) interfacial Ring test (Maurer, 1971); (b) modified Preer's test (1956) using 100 µl capillary tubes; and (c) Ouchterlony's plates (1962). Ring tests were used to screen the immune sera to ascertain the presence or absence of specific antibodies in the serum. Preer's test and/or Ouchterlony's double diffusion gel plates were employed using undiluted and diluted antisera to ascertain the number and specificity of antigen–antibody reactions.

The antigens for the above tests were prepared as follows: soluble antigens were dissolved in 0·15 M NaCl solution. The insoluble antigens were homogenized in 0·15 M NaCl solution using 0·5 mm uniform diameter glass beads in a mortar and pestle of agate. Homogenates were centrifuged at 1000 *g* for 30 minutes. Clear supernates were used. The concentrations of homologous and heterologous antigens ranged between 1 and 2 mg/ml.

(ii) Localization of antibodies in the kidneys by immunofluorescence.

An indirect technique was used (Mellors, 1959). Fresh normal rat kidneys were quick

frozen using 'Cryokwik' in a Cryostat (IEC) at −20°. Sections 4–6 μm thick were placed on clean fluoroslides (Aloe). These sections were then treated with various immune sera mentioned above. Binding of rabbit serum in the kidneys was located with fluorescein labelled goat or sheep antiserum to rabbit γ-globulin (Fig. 3). Immunofluorescent staining was controlled by the application of unconjugated sheep anti-rabbit γ-globulin serum followed after washing by the application of the conjugated sheep anti-rabbit γ-globulin serum (Fig. 4). The sections were examined microscopically for fluorescence, using an HBO200 ultraviolet light source (Bausch and Lomb).

Biological tests

The nephrotoxicity of the immune serum was determined by its ability to produce proteinuria (>100 mg/24 hours) in Sprague-Dawley rats weighing about 200 g and having a normal proteinuria of 10±5 mg/24 hours. Ten normal rats were injected intravenously with 0·5 to 1 ml of each antiserum which contained approximately 0·5 mg of precipitating antibodies. Twenty-four-hour urine protein content was determined on the first, third, fifth and the tenth day after the injection. A consistent rise in protein excretion in urine or a protein level of 100 mg/24 hours or more were considered as the evidence of glomerulonephritis.

Light microscopy of the kidneys

Haematoxylin and Eosin and/or periodic acid–Schiff (PAS)-stained sections of kidneys from immune serum injected rats were examined under light microscope. Evidence of mild cellular (endothelial and mesangial) proliferation and basement membrane thickening were graded as positive reactions.

Chemical analyses of antigen preparations

Proteins and peptides. Total nitrogen was measured by a micro-Kjeldahl procedure using the digestion mixture described by Campbell and Hanna (1937) and titration of absorbed ammonia in 10 ml of 2 per cent boric acid with the mixed indicator of Ma and Zuazaga (1942). Recoveries of nitrogen from known standards ranged between 99 to 101 per cent of the expected values. For amino acid analyses, samples were hydrolysed in sealed pyrex tubes under nitrogen with constant boiling 6 N HCl and heated for 22 hours at 105°. Alpha-amino nitrogen was measured by the method of Jacobs (1959). Amino acids were measured by column chromatography using single column at 60° (Hamilton, 1963). Proteins were measured by the procedure of Lowry, Rosenbrough, Farr and Randall (1951) with Folin and Ciocalteu reagent (1927) with bovine serum albumin and gelatin as standards.

Carbohydrates and sugars: Total hexoses were measured by the anthrone and cysteine sulphuric acid methods (Dische, 1955) without prior hydrolysis. Neutral hexoses were partitioned by the procedure described by Misra and Berman (1968). Fucose was measured by the procedure of Dische and Shettles (1948). Hexosamines were measured as described by Boas without the column purification procedure (Misra and Kalant, 1966). Glucosamine and galactosamine were separated by the method reported earlier (Misra and Berman, 1968). Total sialic acid was determined by the procedure of Warren (1959).

Lipids: Total lipids were extracted from the GBM preparations using chloroform–methanol (2:1 v/v) or ethanol–diethyl ether (3:1 v/v) mixture at 24° and 40° respectively. Multiple extractions over a period of 24 hours were also carried out in some cases. Aliquots of extracted GBM were subjected to 0·2 N NaOH at reflux temperature for 16 hours to

check for release of long chain fatty acids. All extracted GBM were free of 'total' lipids. Lipid–phosphorus and cholesterol were measured by our published procedures (Misra and Berman, 1972).

RESULTS

Isolated GBM showed tinctorial properties and morphological appearance similar to GBM in histological sections of kidney (Misra, 1971a; Misra and Berman, 1966; Misra and Kalant, 1966) and had the chemical composition presented in Table 2. Data indicate that collagen, glycoproteins and glycolipoproteins are the constituent macromolecules of the rat, dog and human GBM preparations.

TABLE 1

SPECIFICITY OF ENZYMES USED TO OBTAIN SOLUBLE COMPONENTS OF GBM

Activity	Substrates	Enzymes				
		Collagenase	Pepsin	Trypsin	Pronase	Papain
Collagenolytic	Collagen	+	0	0	+	0
Proteolytic	Haemoglobin	0	+	+	+	+
	Casein	0	+	+	+	+
	Azocoll	±	+	+	+	+
α-Glycosidase	Glycogen	0	0	0	+	±
	Starch	0	0	0	±	±
Ketosidase	Neuraminlactose	0	±	0	+	+
	Mucin	0	±	0	+	±

For conditions of enzyme reactions see Misra and Kalant (1966).
+ = Positive; 0 = Negative; ± = doubtful.

Our studies on the specificity of enzymes have shown that pronase and papain have a broad spectrum of enzymic activity. Pronase catalysed proteolytic, collagenolytic, glycosidase and ketosidase activities while papain showed proteolytic and ketosidase activities (Table 1). Collagenase and trypsin were found to be specific in their activities.

EXPERIMENTS WITH NORMAL RAT GBM

Analyses of two isolated fractions (Fig. 2) of rat GBM are presented in Tables 3 and 4. Major differences between the two components, on the basis of chemical measurements, are presence of sialic acids and a high hexosamine content in the one, labelled as sialoglycoprotein, and less hexosamine and no detectable amounts of sialic acids in the other, marked as glycopeptide (Table 3). Total amounts and relative proportions of aminoacids and sugars were also found to be quite different (Table 4). Glycine, aspartic acid and alanine accounted for large portions of the total amino acids in both of these fractions. Molar ratios of glucose: galactose: mannose were 1·0:3·1:2·6 in the sialoglycoprotein and 1·5:1·0:0·5 in the glycopeptide fractions. Glucosamine and galactosamine were present as equimolar ratios in both these groups. Sialoglycoprotein had an approximate mol. wt of 50,000 Daltons whereas the glycopeptide averaged only 10,000 Daltons (Table 3 and Fig. 1).

The immunological behaviour of intact GBM, lipid-free GBM and GBM treated with several proteolytic enzymes and isolated glycoprotein components are presented in Table 5.

TABLE 2

CHEMICAL COMPOSITION OF NORMAL RAT, DOG AND HUMAN GBM (mg% DRY WEIGHT)

Components	Rat	Dog	Human
Protein	86·0	88·0	90·0
Collagen*	51·2	48·0	52·0
Non-collagen	34·8	40·0	38·0
Carbohydrates	9·6	9·5	8·1
Hexoses	5·8	6·3	5·6
Glucose	1·6	2·2	1·8
Galactose	3·0	2·1	2·9
Mannose	1·2	2·0	0·9
Fucose	0·4	0·7	0·6
Hexosamines	2·6	1·4	2·0
Sialic acids	0·8	1·1	0·6
Lipids	4·4	2·5	2·0
Phospholipids	2·7	1·5	1·4
Cholesterol	1·7	1·0	0·6

* Based on hydroxyproline measurement.

Lipid free GBM and trypsin treated GBM preparations behaved very like the intact GBM immunologically and biologically. Treatment of GBM preparations with multiple enzymes or pronase altered the immunologic behaviour of the membrane drastically, suggesting modification or denaturation of antigenic determinants which compromised its ability to raise immune sera in a heterologous species. It is possible that such enzyme treatments led to degradation of GBM antigens into low molecular weight haptens.

The sialoglycoprotein is shown not only to be able to produce immune sera which

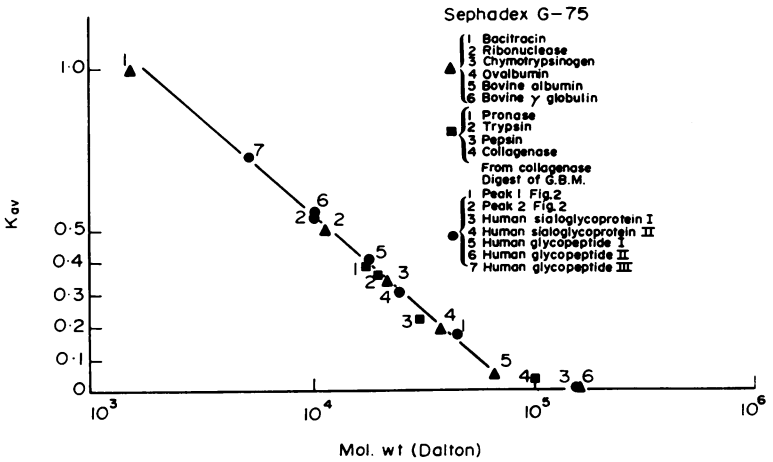

FIG. 1. Selectivity curve for the calibrated Sephadex G-75 Column. See text for details.

behaved quite like the immune sera raised against intact GBM preparations, but also like the anti-GBM antibody produced proteinuric renal disease when injected intravenously in normal rats (Table 5). The other glycopeptide fraction could also produce immune sera in rabbits, as was evidenced from the *in vitro* immunological tests but lacked the attributes of anti-GBM antibodies in producing renal disease with proteinuria in normal rats (Table 5).

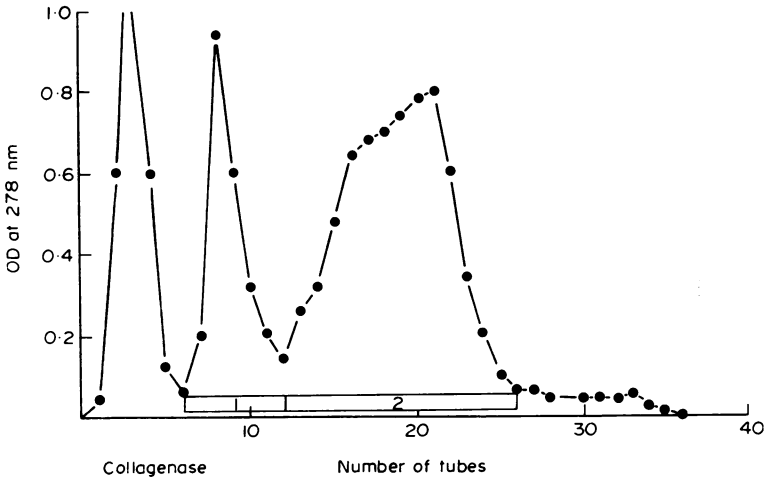

FIG. 2. Elution profile of a typical fractionation of collagenase digested rat GBM.

TABLE 3

COMPOSITION OF ANTIGENIC GLYCOPROTEINS OF NORMAL RAT GBM* (mg% DRY WEIGHT)

Constituents	Sialoglycoproteins	Glycopeptide
Proteins	85·0	78·8
Carbohydrates	15·2	13·5
Hexoses	5·4	10·0
Aminosugars	7·0	3·5
Sialic acids	2·8	0
Approx. mol. wt $\times 10^4$ Daltons	5	1

* See Table 4 for amino acid and sugar compositions and Table 5 for immunological and biological behaviour of the antigens.

Control experiments using normal rabbit serum and immune sera preabsorbed with homologous antigens were performed. The results were negative. The dialysates obtained from enzyme-treated GBM preparations failed to produce immune sera in the rabbits and hence were not studied further.

EXPERIMENTS WITH NORMAL HUMAN GBM

The chemical composition of five fractions obtained from normal human GBM are presented in Table 6. The two macromolecular components are sialoglycoprotein in nature while the other three components are devoid of sialic acids. None of these macromolecules contained hydroxyproline and/or hydroxylysine. The sialoglycoprotein-1 contained all of the sugars present in the intact GBM, apart from fucose.

The immunologic and biologic behaviour of intact normal human GBM, decollagenised, trypsin-treated and lipid-free GBM preparations were quite similar (Table 7). The sialoglycoprotein-1 and 11 showed similar *in vitro* immunologic features but failed to

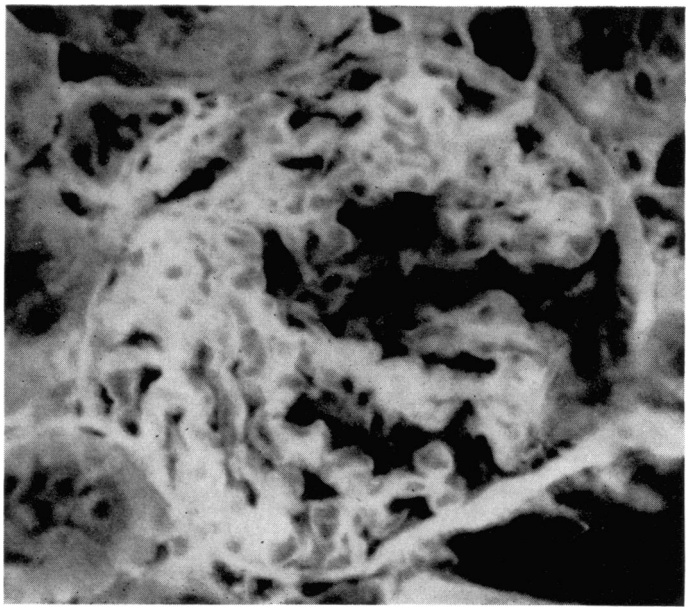

FIG. 3. Positive immunofluorescent reaction (magnification × 160).

TABLE 4

AMINO ACIDS AND SUGARS OF NEPHROTOXIGENIC SIALOGLYCOPROTEIN AND NON-NEPHROTOXIGENIC GLYCOPEPTIDE OF NORMAL RAT GBM

Composition	Sialoglycoprotein mol/5 × 10^4 Daltons	Glycopeptide mol/10^4 Daltons
Amino acids		
Aspartic acid	89	15
Glutamic acid	50	10
Serine	30	5
Glycine	130	30
Alanine	75	15
Proline	20	3
Leucine	10	4
Sugars		
Glucose	2·6	2·5
Galactose	6·0	2·0
Mannose	6·2	1·0
Aminosugars		
Glucosamine	8·0	1·0
Galactosamine	11·0	1·0
Sialic Acids	4·8	0·0

produce proteinuric renal diseases. The three glycopeptides were immunogenic, as judged from the precipitation reactions. The antiglycopeptide sera did not show disease producing activities (Table 7).

TABLE 5

IMMUNOLOGICAL AND BIOLOGICAL PROPERTIES OF RABBIT IMMUNE SERA RAISED AGAINST ANTIGENS OF NORMAL RAT GBM

Antigens	Number of sera tested	Number of positive sera			
		Immunological tests		Biological tests	
		Precipitation	Fluorescent	Proteinuria (>100 mg/24 hr)	Histological
Intact GBM	65	65	65	65	65
Delipidized GBM	8	8	5	8	8
Decollagenized GBM	12	12	12	12	11
Trypsin-treated GBM	3	3	3	3	2
Collagenase and pronase-treated GBM	2	0	0	0	0
Pepsin and trypsin-treated GBM	4	0	0	0	0
Pronase-treated GBM	3	0	1	0	0
Sialoglycoprotein of GBM	2	2	2	2	2
Glycopeptide of GBM	2	1	2	0	0

EXPERIMENTS WITH NORMAL DOG GBM

The chemical compositions and the approximate molecular weights of various fractions isolated following extensive digestion of canine GBM with collagenase, trypsin, pronase and papain are presented in Table 8. All of these fractions are glycoprotein or glycopeptide in nature. The various enzymes used did not produce macromolecules of similar chemical composition, instead a marked degree of heterogeneity was observed (Table 8). The immunological and biological behaviour of immune sera raised in rabbits against these isolated components and assayed in normal rats and rat kidneys are presented in Table 9.

Data indicate that large molecular weight glycoproteins with complex structures are potent nephrotoxigenic antigens, while the small molecular weight fractions were either less potent as nephrotoxigenic antigens or may belong to the group of so-called 'common antigens' of Krakower and Greenspon (1969). Results (Table 9) confirmed earlier observations by Misra (1971b) and others (Huang and Kalant, 1968; Skoza and Mohos, 1969), that pronase does in fact alter the molecular structure of nephrotoxigenic antigens and/or their antigenic determinants.

TABLE 6

COMPOSITION OF ANTIGENIC GLYCOPROTEINS OF NORMAL HUMAN GBM* (% DRY WEIGHT)

Constituents	Siagloglycoproteins		Glycopeptides		
	I	II	I	II	III
Proteins	88·35	72·52	70·10	84·81	88·87
Carbohydrates	9·30	27·12	28·60	15·00	8·80
Hexoses	4·95	20·20	16·10	14·20	8·79
Aminosugars	3·58	5·37	12·50	1·00	0
Sialic acids	0·77	1·55	0	0	0
Approx. mol. wt × 10^4 Daltons	16·0	2·8	2·0	1·0	0·5

* See Table 7 for immunological and biological behaviour of the isolated antigens.

TABLE 7

IMMUNOLOGICAL AND BIOLOGICAL PROPERTIES OF RABBIT IMMUNE SERA RAISED AGAINST ANTIGENS OF NORMAL HUMAN GBM

Antigens	Number of sera tested	Number of positive sera			
		Immunological tests		Biological tests	
		Precipitation	Fluorescent	Proteinuria (>100 mg/24 hr)	Histological
Intact GBM	10	10	10	10	8
Decollagenized GBM	3	3	3	3	3
Trypsin-treated GBM	2	2	2	2	2
Delipidized GBM	1	1	1	1	1
Sialoglycoprotein					
I	2	2	2	0	1
II	2	1	2	0	0
Glycopeptide					
I	2	2	NT	0	0
II	2	2	NT	0	0
III	2	2	NT	0	0

NT, not tested.

TABLE 8

COMPOSITION OF ANTIGENIC GLYCOPROTEIN AND GLYCOPEPTIDES OF NORMAL CANINE GBM

Glycoproteins from	Approx. mol. wt Daltons	Chemistry		
		Molar ratio: hexose = 1		
		Nitrogen	Hexosamines	Sialic acids
Collagenase-treated GBM				
No. 1	$>5\times10^4$	1·0	1·0	0·2
No. 2	$<4\times10^4$	3·0	0·7	0·0
Trypsin-treated GBM				
No. 1	$>5\times10^4$	1·0	1·0	0·1
No. 2	5×10^4	2·0	1·0	0·01
No. 3	$<4\times10^4$	5·0	1·5	0
Pronase-treated GBM				
No. 1	$<4\times10^4$	2	0	0
No. 2	2×10^4	3	0	0
No. 3	1×10^4	6	0	0
Papain-treated GBM				
No. 1	$>5\times10^4$	5	1	0·5
No. 2	$<4\times10^4$	3	1	0

DISCUSSION

Within the last few years, it has become clear from the chemicophysical and immunological studies on the isolated GBM components that the nephrotoxigenic antigens of the membrane belong to a class of glycoproteins. We (Misra, 1971a, b) and others (Krakower and Greenspon, 1969; Huang and Kalant, 1968; Rothbard and Watson, 1969; Skoza and Mohos, 1969; Shibata, Naruse, Nagasawa, Takuma and Miyakawa, 1967 and Shibata, Miyakawa, Naruse, Nagasawa and Takuma, 1969) have repeatedly shown that successful removal of collagen and lipids (Tables 5 and 7) from the GBM by enzymatic and chemical

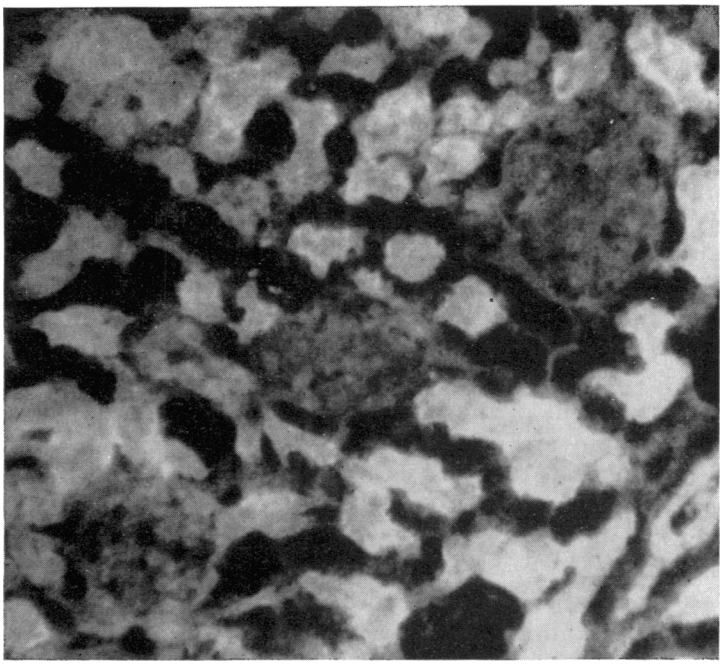

FIG. 4. Negative immunofluorescent reaction (magnification ×80).

TABLE 9

IMMUNOLOGICAL AND BIOLOGICAL PROPERTIES OF RABBIT IMMUNE SERA RAISED AGAINST ANTIGENS OF NORMAL DOG GBM

Antigens	Number of sera tested	Number of positive sera			
		Immunological tests		Biological tests	
		Precipitation	Fluorescent	Proteinuria (>100 mg/24 hr)	Histological
Intact GBM	5	5	5	5	5
Glycoproteins of collagenase-treated GBM					
No. 1	4	4	4	4	4
No. 2	2	2	2	0	NT
Glycoproteins of trypsin-treated GBM					
No. 1	3	3	3	3	2
No. 2	2	2	2	2	2
No. 3	2	2	2	0	0
Glycoproteins of pronase-treated GBM					
No. 1	2	0	0	0	NT
No. 2	2	0	0	0	NT
No. 3	2	0	0	0	NT
Glycoproteins of papain-treated GBM					
No. 1	1	1	NT	1	NT
No. 2	1	1	NT	0	NT

0 = Negative; NT, not tested; see Table 8 for glycoprotein numbers.

means did not alter its ability to raise nephrotoxic sera in a heterologous species. Krakower and Greenspon (1958) had earlier used exhaustive pyridine and ether extractions of isolated dog glomeruli and did not observe any change in the ability of the glomeruli to induce nephrotoxic serum in heterologous species. It can be said with reasonable certainty that lipids *per se* do not play an important role in the immunogenic make-up of the nephrotoxigenic antigens of GBM. Past studies (Cruickshank and Hill, 1953; Millazzo, 1957) had suggested that the reticulin or collagen were the nephrotoxigenic antigens of the GBM. Rothbard and Watson (1961) using heterologous anticollagen antibody and rats were able to show that although anticollagen antibodies localize in the kidneys, they failed to induce proteinuric renal disease in rats. Anticollagen antibodies were later shown to localize in human kidney also (Rothbard and Watson, 1967). Data presented in this paper (Tables 5 and 7) and those very recently reported (Huang and Kalant, 1968; Rothbard and Watson, 1969; Shibata *et al.*, 1967, 1969) strongly suggest that GBM collagen is not the nephrotoxigenic antigen of the membrane. Earlier Misra (1964) from his results obtained from chemicophysical and immunological studies on rat GBM and Krakower and Greenspon (1969) from their data obtained from the studies on cross-reactivity, absorptive capacity and biological activity had arrived at similar conclusions that the nephrotoxigenic antigen of the GBM was a part of 'non-collagenous' glycoprotein component of the membrane.

We have now shown (Table 5) that one species of large mol. wt glycoprotein of rat GBM containing sialic acid and aminosugars is able to raise nephrotoxic serum in rabbits which when injected in normal rats produced proteinuria, localized in their kidneys (Fig. 3) and induced typical histological appearance of Masugi nephritis in the kidneys. We have also shown that another species of small mol. wt glycopeptide devoid of sialic acids and aminosugars can raise antisera that localized in the kidneys but fails to produce proteinuria or morphological changes in the kidneys typical of Masugi nephritis. Huang and Kalant in 1968 reported isolation of six antigenic macromolecules from collagenase digests of rat GBM. Antibodies against these molecules localized in kidneys, but only those glycoproteins of very high molecular weight containing heterosaccharide units were able to raise disease-producing antibodies. They had further shown one of the disease-producing glycoproteins to contain 10·7 per cent carbohydrate, no hydroxyproline and hydroxylysine and glycine accounting for 95 per cent of the amino acid residues. Shibata *et al.* (1967, 1969) have reported isolation of glycoproteins from rat renal cortex and GBM which contained no hydroxyproline or hydroxylysine and glycine accounted for 37·8 per cent of the amino acid residues. These authors (1969) also reported that total hexoses accounted for 11–12 per cent of the glycoprotein molecule and over 50 per cent of the sugar residues were present as glucose.

Since proteolytic enzymes like pronase, pepsin, trypsin are extensively used to isolate glycopeptides of membrane preparations (see review by Misra, 1971a) we have recently examined the antigenic nature of GBM fragments obtained from dog GBM treated with collagenase, trypsin, pronase and papain (Misra, 1971b). Collagenase, trypsin and papain digestion of GBM produced large molecular weight (>50,000 Dalton) components consisting of proteins and heterosaccharides with sialic acids (Table 8). Antisera directed against these glycoproteins behaved quite like those raised against intact dog GBM immunologically and biologically (Table 9). Immune sera raised against small molecular weight (<40,000 Daltons) glycopeptides contained precipitins and GBM localizing antibodies but no disease-producing antibodies. Pronase digestion of the GBM led mainly to

small mol. wt (Tables 8 and 9) glycopeptides, which failed to produce demonstrable antibodies. We have extended our earlier studies on the isolated glycopeptides obtained from collagenase digests of normal and diseased human GBM preparations (Misra and Berman, 1969b). The macromolecules of normal human GBM containing proteins, sialic acids, amino sugars and neutral sugars (Table 6) were antigenic in rabbits leading to precipitins capable of localizing in the normal rat kidney (Table 7). However, the immune sera failed to induce proteinuric renal diseases in rats on intravenous injections. The results suggest a partial immunological identity of the GBM of the human and rat kidneys. Interspecies cross-reactivity of GBM has been observed by other investigators also (Steblay and Lepper, 1961a, b).

Collagenase or trypsin treatments of the GBM, to obtain soluble components, did not alter the nephrotoxigenic antigenicity of the membrane components. On the other hand, use of several other enzymes like pepsin, pronase and combinations of enzymes, such as pepsin and trypsin, or collagenase and pronase altered the molecular structures of the nephrotoxigenic glycoproteins of GBM, and reduced their capacity to produce nephrotoxic antibodies in the heterologous species. We believe that indiscriminate use of enzymes with a broad spectrum of activities to obtain immunologically potent and biologically active molecules from GBM may lead to undesirable consequences. We have also observed that removal of large portions of carbohydrates including sialic acids by exposing GBM to mild alkalies (0·1 NaOH at 24° for 48 hours) or treatment with neuraminidase did not affect the nephrotoxigenicity of the membrane (Misra, 1964, 1971a). We maintain that the essential antigenic structure for eliciting potent nephrotoxic antibodies is either closely associated with the peptide portion or with the protein–polysaccharide region of the antigen molecules (Misra, 1971a).

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