VARIATIONS IN THE SIALIC ACID CONCENTRATION OF GLOMERULAR BASEMENT MEMBRANE PREPARATIONS OBTAINED BY ULTRASONIC TREATMENT

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INTRODUCTION

Several authors (10, 15, 6, 1, 20) have suggested that sialic acid is a component of the glomerular basement membrane (BM). Investigations by Spiro (20) have shown that BM preparations obtained by ultrasonic treatment of whole glomeruli contain two distinct carbohydrate moieties, in approximately equal proportions: one is a disaccharide containing glucose and galactose, believed to be intrinsically associated with collagen, and the other is a heteropolysaccharide consisting of galactose, mannose, hexosamines, sialic acid, and fucose. We have presented histochemical evidence that sialic acid is present in the glomerulus and that it is located not in the basement membrane proper but in the cell membranes lining the basement membrane (18, 14). Preceding us, Rambourg and Leblond (17) demonstrated a carbohydrate-rich cell coat, acidic in nature, at the surface of glomerular cells, and recently Gronowski et al. (3) and Jones (4) published results similar to ours.

The present study was undertaken in an attempt to demonstrate that basement membrane preparations obtained by ultrasonic treatment, a customary procedure for biochemical analysis, are contaminated by cell membranes and that the sialic acid content of such preparations increases with the rising centrifugal force at which they are collected.

MATERIALS AND METHODS

Glomerular Membrane Preparations

Glomeruli were collected according to the method of Krakower and Greenspon (8), using beef kidneys obtained frozen from a local abattoir. The kidney tissue was dissected so as to obtain the cortex. This cortex was fragmented by a tissue press, and the resulting preparation was strained through 150-mesh stainless steel sieves using chilled 0.15 M NaCl. This suspension was washed with 0.15 M NaCl and centrifuged until the supernatant remained clear. It was then sedimented by gravity at least five times, and each time the upper, nonglomerular portion of the sediment was discarded. The purity of such preparations was ascertained by light microscope examination. The preparations were used only when tubular and other contaminants constituted less than 5% of the total. However, in critical experiments, preparations with less than 1% contaminants were used.

The glomerular preparations were suspended in cold 1.5 M NaCl, capable of solubilizing the DNA proteins, and sonicated for 5 min, several minutes of cooling time being allowed after each 1-min burst. A Branson Sonifier, Model S125 (Branson Ultrasonic Corp., Stamford, Connecticut) 20 kc/sec at the No. 6 power setting was used for sonic disruption. Repeated trials showed that such sonication was sufficient to disrupt the glomeruli completely, so that only translucent, refractile plates remained. After this preparation was kept at 4°C overnight, it was centrifuged at 5,000 rpm (3,200 *g*). The sediment was washed at least five times with 1.5 M NaCl and collected at 5,000 rpm until the supernatant remained clear. This treatment was followed by five washings in cold distilled water, and the sediment was collected at 15,000 rpm (28,700 *g*). The final glomerular membrane preparation (GMP) was used for the low-speed centrifugation studies.

The GMP was evenly suspended and divided into three equal portions. The portions were centrifuged for 30 min at 500 rpm, 1,500 rpm, and 3,000 rpm (42 *g*, 383 *g*, 1200 *g*), respectively. The supernatants were transferred to three other tubes and their particulate content was collected at 15,000 rpm. These six experimental samples were weighed and used for biochemical and electron microscope studies.

Biochemical Studies

Sialic acid was determined by the thiobarbituric acid assay of Warren (22) with *N*-acetyl neuraminic acid as the standard. The sialic acid was first liberated from the preparation by hydrolysis with 0.1 N H_2SO_4 at 80°C for 60 min.

The hydrolysates, as prepared for the sialic acid test, were further hydrolyzed at 100°C for 40 hr. This "graded hydrolysis," as described by Spiro (21), represented partial but substantial hydrolysis for glucose and was used to determine the relative glucose content by applying glucose oxidase.

Total neutral sugars were detected by the anthrone procedure of Morris (15) with glucose as the standard.

Electron Microscopic Studies

Samples of the six centrifugal sediments were suspended with a short burst of sonication in 4% neutral formaldehyde. After overnight fixation, they were washed in distilled water and collected by centrifugation at 15,000 rpm. These sediments were resuspended with colloidal iron in 25% acetic acid for 20 min as prepared by the method of Muller (16). This was followed by three washings in 30% acetic acid, and each time the sediment was collected at 15,000 rpm. This sediment was then cut into minute blocks, dehydrated, and embedded in Epon 812. Ultrathin sections were studied in an Elmiskop 1A. For augmenting the recognition of the basement membrane and cell membrane structures, the sections were counterstained with uranyl acetate (13). On randomly selected electron micrographs of the 500 and 3,000 rpm sediments, the surface areas of the BM and of the contaminants were compared by the aid of a planimeter.

RESULTS

Distribution of Sialic Acid and Glucose in the Various Centrifugal Sediments of GMP's

The sialic acid content of the GMP was 240 mg/100 g wet weight. This corresponds approximately to 1.2% sialic acid by dry weight. Table I shows the amount of sialic acid present in the three sediments obtained at 500, 1,500, and 3,000 rpm and in the particulates collected from their corresponding supernatants. As indicated, the sialic acid content in the sediment increases with rising centrifugal force. At 500 rpm, the sediment comprised 49% of the sialic acid present in the original GMP. In the 3,000 rpm sediment it reached 82%.

Analysis of the standard GMP for total neutral sugars obtained by the anthrone method gave an average value of 5.1% of the dry weight of the membrane expressed as glucose equivalents. This value is in close approximation with values reported recently (20). Analysis of the same material by the glucose oxidase (glucostat) reaction gave an average value of 2.3% of the dry weight.

In contrast to the sialic acid content, the relative glucose content of GMP changed very little with increasing centrifugal force.

Estimation of Sialic Acid in the Various Centrifugal Sediments of GMP by Electron Microscopy

On the basis of the assumption that the deposition of colloidal iron is in association with sialic acid, we have shown (18) by histochemical methods that the presence of sialic acid in the glomerulus can be demonstrated at the light microscope level by Spicer and Warren's method (19), and at the electron microscope level by Gasic's method (2). In such glomerular preparations (Fig. 1) the colloidal iron is localized at the cell membrane, but not in the BM proper. Furthermore, this localization could easily be prevented by treating the sections beforehand with neuraminidase, an enzyme capable of selectively removing the sialic acid. This indicated that the localizations of iron and sialic acid are interrelated.

TABLE I

Distribution of Sialic Acid in the Supernatants and Sediments of Sonicated Beef Glomeruli Obtained at Various Centrifugal Forces

	Weight			Weight ratio	Sialic acid content			Sialic acid ratio
Centrifugal force	Supernatant	Sediment	Total	Supernatant/ Sediment	Supernatant	Sediment	Total	Supernatant/ Sediment
	mg	*mg*	*mg*		*μg*	*μg*	*μg*	
42 *g* (500 rpm)	58.2 (52.7%)	52.2 (47.3%)	110.4	1.11	122.80 (50.8%)	119.03 (49.2%)	241.83	1.03
383 *g* (1500 rpm)	46.8 (45.1%)	56.9 (54.9%)	103.7	0.82	95.60 (38.5%)	152.87 (61.5%)	248.47	0.63
1200 *g* (3000 rpm)	33.3 (34.1%)	64.3 (65.9%)	97.6	0.51	41.62 (18.0%)	189.01 (82.0%)	230.63	0.22

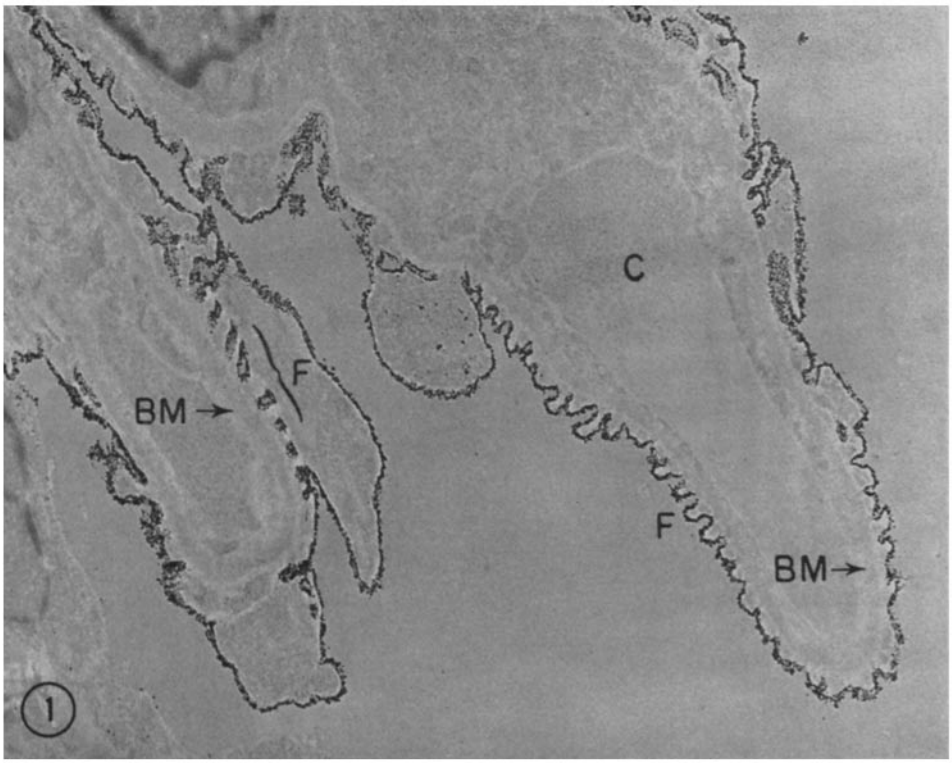

FIGURE 1 Electron micrograph of a mouse glomerular loop stained with colloidal iron. No counterstain. Observe the heavy metallic deposits on the epithelial cell membrane including the foot processes (*F*), and the absence of these deposits in the *BM*. *C*, lumen of capillary loop. × 16,000.

The same colloidal iron technique was used on the sediments of the GMP obtained at different centrifugal forces and examined by electron microscopy. It was demonstrated that the preparations were considerably contaminated with cell membrane marked with colloidal iron deposits, and that the contamination was greatest at the highest centrifugal force. The 500 rpm sediment predominantly contained BM fragments. Many of these BM fragments were completely free of cell membranes, while others showed attached, iron-positive cell membrane fragments or foot processes. The corresponding supernatant was composed of cell membranes and less than 10% BM fragments (Fig. 2). Iron-positive particles were absent in neuraminidase-treated, control preparations. Quantitative comparison of the surface areas of the BM and of the contaminants on randomly selected electron micrographs of the 500 and 3,000 rpm sediments indicated 16.5% and 36% contaminants, respectively.

DISCUSSION

Because sialic acid–containing heteropolysaccharides are known constituents of cell membranes (24), the possibility presented itself that the sialic acid, presumably present in the glomerular basement membrane, may actually be present in the cell membranes contaminating the ultrasonic BM preparations. Sonic fragmentation of the glomerulus followed by differential centrifugation, as used by most investigators for the procurement of BM preparations, cannot achieve complete separation of BM and cell membrane.

Electron microscope studies of the different sediments, with the colloidal iron technique as a method for the localization of sialic acid, and consequently for the identification of cell membrane material, indicated that even the 500 rpm sediment contained substantial amounts of cell membrane usually attached to the BM. Furthermore, there was a considerable increase of free cell mem-

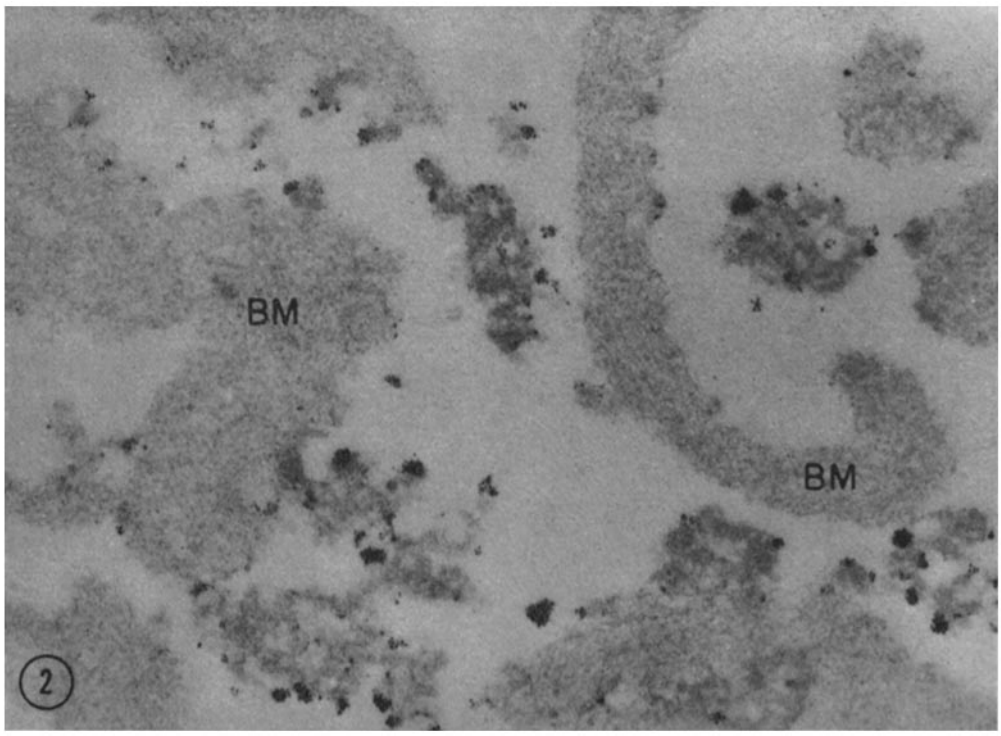

FIGURE 2 3000 rpm centrifugal BM sediments stained with colloidal iron and poststained with uranyl acetate. Note substantial iron-tagged cell membrane contamination. × 65,000.

brane fragments in the pellets obtained at a higher centrifugal force. In accord with these findings, the sialic acid concentration of the sediments increased with rising centrifugal force. In contrast, the glucose concentration did not show any significant change. The large amount of contaminants could explain why the collagen content of reported BM preparations varies from 50 to 70% (10, 12, 9, 6, 11). While this contamination parallels the sialic acid content, it has no appreciable effect on glucose concentration believed to be intrinsically associated with collagen. These findings support our contention that sialic acid is not a component of the glomerular BM, but rather one of the cell membrane.

A similar linear relationship between cell membrane contamination of human BM collagen and centrifugal force was found recently by Westberg and Michael (23) using hydroxyproline and phospholipids as the parameters.

In Table II, we compiled the published glomerular sialic acid concentrations and our results in an effort to find further support for the "issue of contamination." The values show considerable variation. Some of this variation may be due to deviations from the method adopted. Most of the publications refer to the method by Krakower and Greenspon (8) for the harvesting of glomeruli and for the preparation of BM, yet it is not at all clear whether 20 min of sonic treatment and collection at 1,500 rpm were uniformly adhered to. Nor are frequency and energy output of the sonic instrument uniformly stated. In addition to deviations from the original technique, we have found age and species differences in glomerular sialic acid concentration, which may further obscure interpretation. There is 30% more sialic acid in young beef glomeruli than in glomeruli obtained from older animals. The high values obtained by Kefalides could be explained by his method of 2.5 hr of acid hydrolysis (5), instead of 1 hr, customarily used for the sialic acid assay of Warren (22). In spite of the discrepancies, Table II seems to support a direct relationship between sialic acid content and centrifugal force: with similar ultrasonic treatment and centrifugal force as Spiro, our sialic

TABLE II

Comparison of Sialic Acid Content of Glomeruli and Glomerular Membrane Preparation (GMP) Obtained by Different Investigators

Item	Authors	Species tested	Methods of separation: Whole glomeruli	Methods of separation: GMP — Removal of cells	Methods of separation: GMP — Collection of BM	Dry weight
						g SA*/100 g
1	Lazarow & Speidel (10)	H	D.C.	0.05 N NaOH 3–8 days	—	0.00
2	Lange & Markowitz (9)	H	D.C.	U.S. *20′*	1,*500* rpm *5–10′*	0.55
3	Misra & Berman (12)	H	Magnetic Fe_2O_3	—	—	1.23–1.37
4	"	H	Magnetic Fe_2O_3	U.S. 8–12′	200 *g* 10′	0.27–0.68
5	Kefalides & Winzler (5)	C	D.C.	U.S. *20′*	*1,500* rpm *5–10′*	2.10
6	Kefalides (6)	C	D.C.	U.S. *20′*	*1,500* rpm *5–10′*	2.00
7	" (7)	H	D.C.	U.S. *20′*	*1,500* rpm *5–10′*	1.50
8	Spiro (20)	B	D.C.	U.S. 5′	3,000 rpm 10′	1.14–1.24
9	Lidsky et al. (11)	B	D.C.	1000 rpm grinder	3,000 rpm (810 *g*)	0.99
10	Mohos & Skoza (14)	B	D.C.	—	—	0.90–1.20
11	"	B	D.C.	U.S. 5′	3,000 rpm 10′ (1,200 *g*)	1.00–1.40‡

* SA = Sialic acid. H, human, C, canine, B, beef, D.C., differential centrifugation, U.S., ultrasonic treatment. Italicized numbers represent the procedure of Krakower & Greenspon (8). It is the methodology referred to by the respective authors. Wet/Dry ratio of whole glomeruli = 20, and that of GMP = 5, and indicates the approximate water content of these preparations.

‡ Based on more than 100 determinations.

acid values for the *GMP* are in the same range, ~ 1.20%. In contrast, Misra and Berman, using more extensive ultrasonic disruption and a much lower centrifugal force (200 *g*), obtained much lower sialic acid values (0.27–0.68). Nevertheless, sialic acid content of the *whole glomerulus* is, in both instances, approximately the same. Whether the low values of GMP, as reported by Misra and Berman, can further be reduced is not known. As stated elsewhere (14), one can not disregard the possibility that the basement membrane proper may contain sialic acid in a local concentration insufficient for histochemical demonstration. But then, the sialic acid would be a minor component, considering the high local concentration of sialic acid on the plasma membrane of the epithelial cells.

SUMMARY

The possibility of contamination of ultrasonically prepared glomerular basement membranes by cell membranes collected at different centrifugal forces was investigated by means of sialic acid determination in combination with electron microscopy. Such preparations showed that the sialic acid titer and the degree of cell membrane contamination increased with a rising centrifugal force.

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