Assessment of the charge selectivity of glomerular basement membrane using Ficoll sulfate

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Bolton, Glen R., William M. Deen, and Barbara S. Daniels. Assessment of the charge selectivity of glomerular basement membrane using Ficoll sulfate. Am. J. Physiol. 274 (Renal Physiol. 43): F889–F896, 1998.—The extent to which the glomerular basement membrane (GBM) contributes to the charge selectivity of the glomerular capillary wall has been controversial. To reexamine this issue, the size and charge selectivity of filters made from isolated rat GBM were assessed, using polydisperse Ficoll and Ficoll sulfate as test macromolecules. Ficoll sulfate, a novel tracer with spherical shape synthesized for this purpose, exhibited little or no binding to serum albumin, thereby avoiding a major difficulty that has been reported with dextran sulfate. The sieving coefficients of Ficoll sulfate were not different from those of Ficoll at physiological ionic strength, although the values for Ficoll sulfate were depressed at low ionic strength. These results confirm that the GBM possesses fixed negative charges but suggest that its charge density is insufficient to confer significant charge selectivity under physiological conditions, where electrostatic interactions are relatively well screened. The sieving coefficients of Ficoll sulfate and Ficoll were elevated significantly and by similar amounts when bovine serum albumin (BSA) was present in the retenate at 4 g/dl. This could be explained as the combined effect of two nonspecific physical factors, namely, the reduction in filtration velocity due to the osmotic pressure of BSA and the effect on macromolecular partitioning of repulsive solute-solute interactions. The view that BSA does not affect the intrinsic properties of the GBM is supported also by the absence of an effect on the hydraulic permeability of isolated GBM. The sieving coefficient of BSA was roughly half that of Ficoll or Ficoll sulfate of similar Stokes-Einstein radius. Given the finding of negligible charge selectivity, this difference may be attributed to the nonspherical shape of albumin. The results suggest that, to the extent that isolated GBM is similar to GBM in vivo, the charge selectivity of the glomerular capillary wall must be due to the endothelial and/or epithelial cell layers.

The information that can be gained from measurements of sieving coefficients, either in vivo or in vitro, is very dependent on the properties of the test molecules employed. With proteins, it is not possible to vary molecular size and/or charge while maintaining the same molecular structure. Moreover, the reabsorption of proteins by the renal tubules complicates the interpretation of their urinary clearances. To avoid tubular reabsorption and to allow isolation of charge and size effects, many studies have used dextran and dextran sulfate. However, dextran and its derivatives do not resemble ideal, rigid spheres, and the interpretation of data obtained using dextran sulfate is complicated by its binding to plasma proteins. Ficoll, a cross-linked copolymer of sucrose and epichlorohydrin, has the biological inertness of dextran and, in addition, is rigid and spherical. It has been used recently in fractional clearance studies in both laboratory animals and humans. This suggests that Ficoll sulfate, which has not been used previously in physiological studies, might be an excellent probe for examining glomerular charge selectivity. In the present study, Ficoll sulfate was synthesized, and its permeation across isolated GBM was compared with that of neutral Ficoll to examine the size and charge selectivity of this part of the glomerular capillary wall.

METHODS

Synthesis and labeling of Ficoll sulfate. Ficoll sulfate was synthesized by reacting Ficoll 70 (Pharmacia, Piscatway, NJ) with chlorosulfonic acid in the presence of pyridine, as described by Ricketts (26) for the preparation of dextran sulfate. The extent of sulfation was controlled by varying the amount of Ficoll added to the chlorosulfonic acid-pyridine complex. Two Ficoll sulfate preparations were employed, one with 9% sulfur by weight and the other with 16–17% sulfur. The sulfur assays were performed by Galbraith Laboratories (Knoxville, TN). Gel chromatography revealed that the molecular size distribution of the Ficoll sulfate was of similar
breath to that of Ficoll 70 but that sulfation resulted in a modest increase in the average molecular radius.

Ficoll sulfate was labeled using 6-[4,6-dichlorotiazin-2-yl)-amino]fluorescein (DTAF) (Sigma, St. Louis, MO), following De Belder and Granath (9). Unreacted DTAF was removed by elution through 10-ml disposable desalting columns (Econo-Pac 10DG; Bio-Rad, Hercules, CA) with 1 M NaCl. The labeled Ficoll sulfate was concentrated in a 200-ml ultrafiltration cell (model 8200; Amicon, Danvers, MA) with a 5-kDa-molecular-mass cutoff membrane (PLCC, Amicon) prior to freeze drying. The degree of fluorescein substitution was estimated by comparing the Ficoll sulfate fluorescence with a fluorescein standard curve, as determined with a fluorimeter (model RF-551; Shimadzu, Columbia, MD). The preparation with 9% sulfur had one fluorescein group per 18 ± 4 Ficoll sulfate molecules. Ficoll sulfate with 16% sulfur had ~400 times less fluorescence and was more difficult to detect. In this case, it appears that almost all of the hydroxyl groups that are normally substituted by DTAF were occupied by sulfate.

Ion exchange chromatography showed that the Ficoll sulfate was uniformly anionic. When labeled Ficoll sulfate was injected into a 5-ml disposable gel column containing cationic tertiary amine high trap Q (Pharmacia), none of the sample eluted in 0.02 M Tris buffer at pH 7.4, whereas it eluted promptly when 1.0 M NaCl was added to the buffer. In contrast, almost all fluorescein Ficoll eluted at the low ionic strength. The fact that some "neutral" Ficoll eluted at the high ionic strength (15% of that recovered) may be attributed to the small amount of negative charge conferred by the label (−1 charge/fluorescein).

It is worth noting that Ficoll sulfate tends to be more highly charged than bovine serum albumin (BSA). For a Stokes-Einstein radius (r) of 36 Å (similar to BSA) and a sulfur content of 9%, the molecular weight of Ficoll sulfate is ~38,000, indicating that there are ~100 sulfur groups. Thus the Ficoll sulfate charge is −100, compared with a BSA charge under physiological conditions of about −20.

Ficoll sulfate-BSA binding. Prior to the studies with isolated GBM, the binding of Ficoll sulfate and other test molecules to BSA was assessed using two methods. The first involved interactions with immobilized BSA. Beads of 4% agarose with 15 mg of BSA insolubilized per milliliter of gel (Sigma) were packed in a column (16/20 C, Pharmacia). Five fluorescently labeled polymers were tested: Ficoll, Ficoll sulfate with 9% sulfur, Ficoll sulfate with 17% sulfur, dextran sulfate with 7% sulfur, and dextran sulfate with 15% sulfur. The synthesis of the dextran sulfates (from dextran 40, Sigma) was similar to that for the Ficoll sulfates, and the labeling procedure was the same for all of the polymers. The tracers were injected and eluted first with phosphate-buffered saline (PBS) at pH 7.4. After the peak was detected, the ionic strength of the buffer was increased (PBS with 1.0 M NaCl instead of 0.12 M). It was anticipated that the high ionic strength would suppress the electrostatic interactions and cause any macromolecules bound to the immobilized BSA to desorb. The percentage of the injected macromolecule that was bound was estimated by comparing the peak areas at the physiological and high ionic strengths. To determine whether the gel or the linker arm used to immobilize BSA contributed to the binding, control experiments were performed using a column packed with agarose-glycine (Sigma).

The results are shown in Fig. 1. For both Ficoll sulfate and dextran sulfate, the amount of binding to immobilized BSA increased greatly as the sulfur content was increased. The structure of the sulfated polysaccharide (linear coil for dextran sulfate, cross-linked sphere for Ficoll sulfate) was of only secondary importance. There was little evidence for binding of neutral Ficoll to BSA or for binding of the sulfated polymers to the control (glycine) beads. These results suggest that electrostatic forces are responsible for the binding of the sulfated polymers to BSA and that the charge density of the test molecule is more important than its shape or rigidity.

The second method used to evaluate binding to BSA was to compare sieving curves measured with cellulose ester ultrafiltration membranes, with or without BSA present in the retentate. Approximately 1 mg/ml of polydisperse Ficoll, Ficoll sulfate with 9% sulfur, or dextran sulfate with 18% sulfur (similar to commercial dextran sulfates) was added to PBS or to PBS containing 0.4 g/dl BSA, and the pH was adjusted to 7.4. (A subphysiological concentration of BSA was used to minimize osmotic effects and yield filtration rates similar to those for protein-free solutions.) The solutions were ultrafiltered across a 30-kDa-molecular-mass cutoff cellulose ester membrane (PLCC, Amicon) in a 3-ml stirred cell (model 3, Amicon). The cell was pressurized to 100 mmHg, using nitrogen, and the filtrate was collected for 15 min. Samples of the filtrate, retentate, and initial solutions were chromatographed on a 10/20 C column packed with Superdex 200 (Pharmacia). The void volume was determined from the elution of fluorescently labeled 2,000-kDa dextran (FITC-dextran, Sigma). The eluent was 0.05 M ammonium acetate with 0.15 M KCl at pH 7. The column was calibrated with fluorescently labeled Ficoll standards with Stokes-Einstein radii of 29.7, 37.7, 46.4, and 58.7 Å (16). Sieving curves were constructed by plotting the ratio of filtrate to retentate concentration (θ) as a function of molecular radius.

It was found that the sieving curves for Ficoll were not affected by BSA. Those for Ficoll sulfate showed a slight reduction of θ for the largest molecules when BSA was present, possibly due to BSA binding. In contrast, the sieving curves for dextran sulfate were depressed significantly by BSA for all molecular radii, consistent with the formation of dextran sulfate-BSA complexes in the ultrafiltration cell. More revealing was the effect of BSA on the chromatographic elution profiles. As shown in Fig. 2, the dextran sulfate samples with BSA yielded a new peak that eluted early, which is evidence for large complexes. No such secondary peak was observed with Ficoll or Ficoll sulfate. It was concluded that
filtration cell was evaluated as

\[ k_c = 2.35 \mu^{1/6} D^{1/2} \]  

where \( k_c \) is in cm/s, \( \mu \) is the viscosity of water in centipoise, and \( D \) is the solute diffusivity in cm²/s [12]. For the results reported here with isolated GBM, the true sieving coefficients were calculated to be ~30% lower than the apparent values.

The hydraulic permeability (\( L_p \)) of the GBM layer was calculated as

\[ L_p = \frac{v}{\Delta P - \sigma_{\text{BSA}} \Delta \Pi_{\text{BSA}}} \]  

where \( \Delta P \) is the applied pressure and \( \sigma_{\text{BSA}} \) and \( \Delta \Pi_{\text{BSA}} \) are the reflection coefficient and osmotic pressure difference, respectively, for BSA. The reflection coefficient was estimated as \( \sigma_{\text{BSA}} = 1 - \Theta_{\text{BSA}} \) and \( \Delta \Pi_{\text{BSA}} \) was calculated from the concentrations of BSA at the upstream surface and in the filtrate, using the correlation of Vilker et al. [29].

In the first series of experiments with GBM, the Ficoll or Ficoll sulfate was added to Krebs buffer, with or without 4 g/dl BSA. A single collection period was employed with each membrane. These data were analyzed in an unpaired manner to determine the effects of tracer charge and BSA. In a second series of experiments, there were two collection periods, allowing a paired comparison of Ficoll and Ficoll sulfate in each membrane. These data were analyzed in an unpaired manner.
Results

Ultrafiltration across synthetic membranes. One possible interpretation of the results with isolated GBM is that the difference in charge between Ficoll and Ficoll sulfate was insufficient to yield a difference in sieving coefficients. Accordingly, ultrafiltration across anionic track-etched membranes was used to test whether the tracers could be distinguished. As shown in Fig. 4, at a high ionic strength, the Ficoll and Ficoll sulfate sieving curves were essentially identical, but, at a low ionic strength, the sieving coefficients for Ficoll sulfate were significantly lower than those for Ficoll. The results at low ionic strength confirm that, when electrostatic interactions are not screened by a high salt concentration, the filtration of Ficoll sulfate is affected by the membrane charge. The results at the high ionic strength reinforce the assertion that the two tracers differ only in their charge characteristics.

Ultrafiltration across GBM at low ionic strength. The absence of any difference between the sieving coefficients of Ficoll and Ficoll sulfate in GBM at physiological ionic strength could mean that there was little or no fixed membrane charge or it could indicate that the electrostatic interactions were completely screened under these conditions. To test for functional membrane charges, paired comparisons of Ficoll and Ficoll sulfate sieving were made at low ionic strength. As shown by a representative experiment in Fig. 5A, at low ionic strength, the sieving coefficients for Ficoll sulfate were well below those for Ficoll. This is the behavior expected for a negatively charged membrane. However, as shown by another representative experiment in Fig. 5B, there was little or no difference between Ficoll sulfate and Ficoll at physiological ionic strength. An analysis of seven paired experiments at the low ionic strength indicated that the differences in the sieving coefficients of Ficoll sulfate and Ficoll were statistically significant (P < 0.05) for 20 ≤ r₅ ≤ 48 Å. The three paired experiments at physiological ionic strength merely reinforced the findings shown in Fig. 4, namely, that there was no significant charge discrimination under these conditions. These experiments confirm that GBM has fixed negative charges, although not at
high enough concentration for them to be functionally effective at physiological ionic strength.

The filtration data in Table 1 show a slight tendency for $L_p$ to be reduced at low ionic strength, although the difference was not statistically significant.

DISCUSSION

The main finding of this study is that there were no significant differences between the sieving curves of Ficoll sulfate and Ficoll in isolated GBM at physiological ionic strength (Fig. 3). This absence of charge selectivity is generally consistent with previous results obtained with various GBM preparations. Bray and Robinson (5) found only a slight reduction in the sieving of dextran sulfate relative to neutral dextran. Likewise, Bertolatus and Klinzman (3) found only a small difference in the filtration rates of native (anionic) or cationized BSA. Moreover, they showed that neutralization of carboxyl groups by methylation, which should have abolished much of the GBM charge, had only a slight effect on the sieving of BSA. Daniels (7) found that treating the GBM with heparatinase to remove heparan sulfate proteoglycan, adding protamine to neutralize GBM polyanions, or reducing the experimental pH to the isoelectric point of the GBM or BSA had little or no effect on the sieving coefficient of BSA. Robinson and Walton (27) also found that the sieving of BSA across isolated GBM was the same at pH 7.4 as at pH 5.7, the isoelectric point of GBM. Taken together, these results suggest that it is unlikely that the GBM makes an important contribution to the charge selectivity exhibited by the glomerular capillary wall in vivo.

The conclusion that the GBM makes little or no contribution to normal glomerular charge selectivity is based, of course, on the assumption that isolated GBM is not functionally different from that in vivo. The possibility that GBM is altered during the isolation process has been examined previously using a variety of methods. Immunofluorescent microscopy of consoli-

Fig. 4. Sieving curves for Ficoll and Ficoll sulfate in track-etched membranes at low (A) or high (B) ionic strength. Values are shown as means ± SE, with $n = 3$ for each case.

Fig. 5. Representative sieving curves for Ficoll and Ficoll sulfate in GBM at low (A) and high (B) ionic strength. Each plot shows results of paired experiments using same membrane.
dated GBM filters prepared as in the present study demonstrated the presence of laminin, type IV collagen, and the core protein of heparan sulfate proteoglycan (8), the main components of GBM. The sulfated side chains of GBM proteoglycans are also present in GBM isolated using the present methodology (7). The permeability of the GBM filters was not changed when a milder detergent, Triton X-100, which has been shown to preserve heparan sulfate proteoglycan, was used to lyse glomerular cells (7). That isolated GBM is relatively intact is suggested also by electron microscopy studies; the spatial distribution of cationic ferritin has been found to be similar to that in vivo (18).

Functional confirmation of the presence of negatively charged groups in isolated GBM was provided by the present results at low ionic strength, in which the sieving coefficients for Ficoll sulfate were found to be substantially lower than those for Ficoll (Fig. 5). Reductions in ionic strength amplify electrostatic interactions by increasing the distance in an electrolyte solution over which fixed charges are “felt”; the relevant length scale is termed the Debye length. A comparison of the results at the two ionic strengths indicates that fixed negative charges are indeed present in the GBM but at too low a concentration to confer appreciable charge selectivity under physiological conditions. This conclusion was reached previously by Zamparo and Comper (30) on the basis of studies using model anionic polysaccharide matrices. Experimental estimates of GBM charge density have been reported using titration (5) and an isotopic ion exchange technique (6).

The conclusion that the GBM is unlikely to contribute significantly to normal glomerular charge selectivity contradicts inferences made from numerous histological studies employing electron-dense tracers (19). Such studies have advanced the understanding of structure-function correlations, but they are qualitative and reflect transient phenomena rather than steady-state conditions. Distinguishing between tracer particles that are strongly bound to GBM and those that are freely mobile is one of several difficulties in using electron micrographs to reach quantitative conclusions about the sieving characteristics of molecules at steady state. As emphasized above, the present results do not dispute the existence of fixed negative charges in the GBM, as inferred using electron microscopy and other methods; they suggest only that the charge density is insufficient to be functionally important.

The sieving coefficient of BSA in isolated GBM was found to be roughly half that of comparably sized Ficoll or Ficoll sulfate. Of interest is that such differences in F between BSA and uncharged test molecules of similar size are sometimes taken as evidence of charge selectivity. The fact that the sieving coefficients of Ficoll and Ficoll sulfate did not differ (at physiological ionic strength) indicates that some other factor was responsible for the relatively low sieving coefficient of BSA, such as its nonspherical shape.

If the GBM makes little or no contribution, then glomerular charge selectivity must be conferred by the endothelial or epithelial cell layers. In support of a role for the cells, Daniels (7) found that removal of heparan sulfate proteoglycans increased the permeability of intact glomeruli (studied in a filtration cell) to BSA but not the permeability of GBM. Likewise, protamine increased the permeability of layers of whole glomeruli but not those of GBM. It has been hypothesized that the negative charges on the epithelial cells and slit diaphragms help maintain spaces between foot processes for the passage of filtrate, because neutralization of these charges with protamine or cationic ferritin causes foot processes to broaden and occlude filtration channels (17, 20). Negative charges on the slit diaphragms could also be responsible for the charge selectivity observed in vivo, in that these fibrous structures are in close proximity to molecules passing between the foot processes. An alternative hypothesis is that the passage of anionic tracers across the glomerular capillary wall is selectively reduced by cellular uptake (28). Whether cellular uptake is physiologically important seems uncertain, in that electron microscopy studies have not shown cationic or anionic ferritin (18, 25) bound to or within glomerular epithelial cells.

The sieving curves of both Ficoll sulfate and Ficoll were significantly higher when BSA was present than when it was not (Fig. 3). This can be explained as the combined result of two physical factors: the reduced filtration rate of water with BSA and the effect of BSA on the partitioning of other molecules within the membrane. Neither factor is specific to BSA or GBM; rather, these phenomena occur generally for macromolecular solutes in any porous or fibrous membrane. The theory for the second (partitioning) effect has been developed only for macromolecules of uniform size. Accordingly, the calculations described below focus on the sieving behavior of a Ficoll with \( r_s = 36 \) \( \text{Å} \), the same Stokes-Einstein radius as BSA. The sieving coefficient of this size of Ficoll was 73% higher, on average, when BSA was present.

The effect of the filtration rate on the sieving coefficient, which is due to the increased importance of diffusion at lower filtration velocities, is a well-known phenomenon in ultrafiltration. Decreasing the filtration rate enhances the tendency of diffusion to equilibrate the filtrate and retentate and thereby increases the sieving coefficient. The inverse relationship predicted between \( \Phi \) and filtration rate for a membrane of thickness \( \delta \) is described by

\[
\Theta = \frac{\Phi K_c}{1 - (1 - \Phi K_c) \exp (-Pe)}
\]

\[
Pe = \frac{(\Phi K_c)v\delta}{(\Phi K_c)D_c}\]

where \( Pe \) is the membrane Peclet number, \( \Phi \) is the partition coefficient, \( K_c \) is the convective hindrance factor, and \( K_d \) is the diffusive hindrance factor (12). The osmotic pressure of BSA caused a reduction in \( v \) (Table 1). To calculate the corresponding change in \( Pe \), it was necessary to estimate the values of \( \Phi K_c \) and \( \Phi K_d \). Both of these products are expected to decrease rapidly with
The corresponding increase in sieving of the 36-A˚ Ficoll was predicted to increase by 106%. Given the uncertainties in applying the idealized theory to the GBM, this prediction is in remarkably good agreement with the observed increase of 73%. Thus it appears that there is no need to postulate an effect of BSA on the GBM itself. It seems unlikely that this conclusion would be altered if a more structurally realistic theory were available to describe solute-solute interactions in the GBM. Supporting the view that BSA does not affect the intrinsic properties of the GBM is the observation that there was no change in the hydraulic permeability (Table 1). We conclude that, although BSA binds to GBM (1), any structural changes in the membrane that might result are too small to affect its permeability properties.

In filters prepared by consolidating many glomerular skeletons, as done here, a small amount of the filtrate may pass around the GBM rather than through it. This was suggested by the tendency of the Ficoll sulfate and Ficoll sieving coefficients to asymptotically approach a small but nonzero value at large molecular radii and was evidenced in a previous study by the passage of small amounts of 2,000-kDa dextran (7). Such a shunt would lead to overestimates of the true sieving coefficients for the GBM, with the percentage error increasing with molecular size. To minimize the effect of such artifacts, the calculations described above used Ficoll sieving data only for \( r_s \leq 50 \text{ Å} \). To examine this source of error, the calculations were performed also by first subtracting the sieving coefficient of the 80-A˚ Ficoll (an upper bound for the magnitude of the shunt) from the entire curve. When the calculations were done in this manner, the predicted increase in the Ficoll sieving coefficient due to the presence of BSA was again in excellent agreement with that measured.

In conclusion, we found that there was no significant difference between the sieving coefficients of Ficoll sulfate and Ficoll in isolated GBM, suggesting that GBM is unlikely to contribute significantly to glomerular charge selectivity in vivo. This supports the hypothesis that the glomerular cells and not the GBM are responsible for glomerular charge selectivity. The sieving coefficients of Ficoll sulfate and Ficoll were elevated similarly in the presence of BSA, which could be explained as the combined effect of nonspecific physical interactions, the sieving coefficient for a 36-A˚ Ficoll was predicted to increase by 106%. Given the uncertainties in applying the idealized theory to the GBM, this prediction is in remarkably good agreement with the observed increase of 73%. Thus it appears that there is no need to postulate an effect of BSA on the GBM itself. It seems unlikely that this conclusion would be altered if a more structurally realistic theory were available to describe solute-solute interactions in the GBM. Supporting the view that BSA does not affect the intrinsic properties of the GBM is the observation that there was no change in the hydraulic permeability (Table 1). We conclude that, although BSA binds to GBM (1), any structural changes in the membrane that might result are too small to affect its permeability properties.

In conclusion, we found that there was no significant difference between the sieving coefficients of Ficoll sulfate and Ficoll in isolated GBM, suggesting that GBM is unlikely to contribute significantly to glomerular charge selectivity in vivo. This supports the hypothesis that the glomerular cells and not the GBM are responsible for glomerular charge selectivity. The sieving coefficients of Ficoll sulfate and Ficoll were elevated similarly in the presence of BSA, which could be explained as the combined effect of nonspecific physical interactions.
factors. The more novel of these, which seems not to have been recognized previously in physiology, is the ability of finite concentrations of one macromolecule (e.g., BSA) to augment the transmembrane passage of a second, tracer macromolecule (e.g., Ficoll), via intermolecular repulsions. The theory for this effect needs further development, in that it is presently restricted to molecules of the same size. A more complete theory, as well as additional data, is needed to determine whether this phenomenon is important for the interpretation of sieving data in vivo. Finally, this study represents the first use of Ficoll sulfate to probe the charge selectivity of a biological tissue. The minimal binding of this tracer to serum albumin, together with its more rigid and spherical shape, make it an attractive alternative to dextran sulfate for use in future physiological studies.

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