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LABORATORY INVESTIGATION

Glomerular permeability and polyanion in Adriamycin nephrosis in the rat

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Glomerular permeability and polyanion in Adriamycin nephrosis in the rat. Alterations in glomerular permeability were studied in Adriamycin-induced proteinuria in rats by measuring fractional clearances (C/GFR) of uncharged labeled dextrans of varying molecular radii (a_e) and of anionic, native, and cationic horseradish peroxidases (HRP) in experimental and control animals. Experimental animals were studied between days 14 and 55 after a single intravenous dose of Adriamycin (doxorubicin), 7.5 mg/kg. Mean proteinuria in the experimental animals was 98 mg/24 hr. Glomerular morphology showed few changes except for epithelial cell swelling, vacuolization, and foot process obliteration, and a significant reduction of glomerular colloidal iron staining. Polyethyleneimine staining revealed a similar distribution of anionic sites in the laminae rarae interna and externa in proteinuric rats as compared with controls. Inulin clearances revealed reduction in GFR and RPF of 20 and 15%, respectively. Dextran C/GFR values showed in experimental animals a size defect for molecules with an a_e exceeding 40 Å, with a four- to fivefold increase over the values found in control animals for dextrans with a_e of 58 and 60 Å. The peroxidase clearances showed a slight increase in C/GFR of anionic HRP in experimental animals, as could be expected on the basis of the sieving defect, whereas the C/GFR values for native and cationic HRP were virtually unchanged, indicating an intact functional charge barrier in the proteinuric animals. The results indicate that proteinuria in this model, which morphologically resembles aminonucleoside nephrosis, is due to a sieving defect, the charge barrier being functionally intact. The results further suggest, at least in this model, that a reduction in colloidal iron reactive glomerular polyanion is not associated with a defect in the charge selective barrier function.

Perméabilité glomérulaire et polyanions dans la néphrose par Adriamycine chez le rat. Les altérations de la perméabilité glomérulaire ont été édutiées chez le rat au cours de la protéinurie induite par l'adriamycine en mesurant les clearances fractionnelles (C/GFR) de dextrans neutres marqués de différents rayons moléculaires (a_e), et de peroxydases de raifort anioniques, neutres, et cationiques (HRP) chez des animaux expérimentaux et contrôles. Les animaux expérimentaux étaient étudiés entre les jours 14 et 55 après une injection unique intra-veineuse d'Adriamycine (doxorubicine), 7.5 mg/kg. La protéinurie moyenne chez les animaux expérimentaux était de 98 mg/24 hr. La morphologie glomérulaire indiquait peu de modifications excepté un gonflement des cellules épithéliales, une vacuolisation et une fusion des pédicelles, et une réduction significative de la coloration glomérulaire par le fer colloïdal. La coloration au polyéthylèneimine a révélé une distribution identique des sites anioniques dans les lamina rarae interna et externa de rats protéinuriques par rapport aux contrôles. Les clearances de l'inuline ont montré une réduction de GFR et RPF de 20 et 15%, respectivement. Les valeurs de C/GFR du dextran ont indiqué chez les animaux expérimentaux un trouble portant sur la taille pour les molécules dont a_e dépasse 40 Å, avec une augmentation de quatre- à cinqfois au-dessus des valeurs trouvées chez les animaux contrôles pour des dextrans d'a_e de 58 et 60 Å. Les clearances des peroxydases ont indiqué une augmentation modérée de C/GRF pour HRP anionique chez les animaux expérimentaux, comme cela était attendu sur la base d'un trouble de la filtration, tandis que les valeurs de C/GFR pour les HRP natives et cationiques étaient virtuellement inchangées, indiquant une barrière fonctionnelle de charge intacte chez les animaux protéinuriques. Ces résultats indiquent que la protéinurie dans ce modèle qui ressemble morphologiquement à la néphrose des aminonucléosides, est dûe à un trouble de la filtration, la barrière de charges restant fonctionnellement intacte. Ces résultats suggèrent en outre, au moins dans ce modèle, que une réduction des polyanions glomérulaires réagissant avec le fer colloïdal n'est pas associée à une anomalie de la barrière sélective aux charges.

Ultrastructural and functional studies using macromolecular tracers have shown that the glomerular capillary wall in the rat constitutes a size and charge selective barrier to the filtration of circulating macromolecules [1–5]. For any given size the charge selective filter restricts negatively charged molecules more than neutral ones, whereas transport of cationic molecules is facilitated [3–7]. These charge selective properties are due to the presence of fixed negatively charged elements within the structures that constitute the glomerular capillary wall. Glomerular fixed anionic sites have been visualized by light and electron microscopy by investigators using cationic probes such as colloidal iron (CI) [8, 9], Alcian Blue [8, 9], lysozyme [10], ruthenium red [11], and polyethyleneimine (PEI) [12]. Biochemical studies have demonstrated sialic acid rich glycoproteins [13] and sulphated glycosaminoglycans [14] in isolated glomerular basement membranes. The precise functional rule of these different polyanionic constituents present in the glomerular capillary wall is still uncertain.

The present study was designed to investigate the relationship between glomerular permselectivity and the glomerular polyanion in Adriamycin-induced proteinuria, a model that has been shown to be associated with relatively unaltered GFR and with a reduction in colloidal iron reactive glomerular polyanion [15].

Methods

Model. Adriamycin® was administered in a single intravenous dose of 7.5 mg/kg to female Wistar Furth rats (150 to 180 g, Microbiological Associates), known to be resistant to mass cell degranulation after injection of horseradish peroxidases (HRP)

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[16]. All animals received food and water ad libitum and were kept in metabolic cages. Urinary protein excretion was measured daily using the sulfosalicylic acid method [17]. The relative amount of albumin in the urinary protein was measured by SDS-polyacrylamide gel electrophoresis [18]. Total plasma proteins were measured by the Lowry technique [19]. Clearances and morphological studies were performed in control animals and proteinuric rats 14 to 55 days after the administration of Adriamycin.

Clearance studies. Experimental (N = 17) and control animals (N = 16) were divided into groups of five or six animals each. Fractional clearance of anionic (a), native (n), and cationic (c) HRP were performed in one control and one experimental group for each tracer. Clearance of uncharged, tritium-labeled dextrans was performed in the control and proteinuric animals which also received nHRP [20]. Native HRP (Sigma Chemical Co., St. Louis, Missouri; grade II) was purified by gel chromatography and ion-exchange chromatography [6]. Anionic HRP was prepared by succinylation and cHRP by carbodiimide activation and hexanediamine substitution of carboxyl groups of the native enzyme [6]. The tracer enzymes were characterized for their size and isoelectric point (pI) as described previously with the following results: a HRP, a_e = 31.2 Å, pI = 3.7; nHRP, a_e = 29.6 Å, pI = 7.9; cHRP, a_e = 28.8 Å, pI = 9.5. Dextran T40 (Pharmacia, Uppsala, Sweden) was methylated with tritium-labeled iodine (New England Nuclear Corporation, Boston, Massachusetts) according to the method described by Hakomori [21].

Two polyethylene tubings (PE10, Clay-Adams, Parsippany, New Jersey) were inserted in the left jugular vein for infusion of inulin and macromolecular tracers. PE50 tubing in the right femoral artery was connected to a transducer (Statham P23-DC, Statham Medical Instruments, Puerto Rico). Arterial blood pressure was recorded with a Grass 79D polygraph (Grass Instruments Corp., Quincy, Massachusetts). Urine was collected from the left ureter. The transition time (TT) was determined by lissamine green infusion. Inulin (5 g/dl) was infused at a constant rate of 0.034 ml/min. The HRP tracers were administered as a small bolus (0.2 to 0.6 ml) followed by continuous infusion at a rate of 0.0069 to 0.026 ml/min. Tritium-labeled dextran (specific activity 32 μCi/mg; 200 to 300 μCi per animal) was added to the solution containing 5% inulin to animals also receiving nHRP. The clearance rates of inulin and macromolecular tracers were determined over a 30-min period during which time arterial blood was obtained by continuous withdrawal from the femoral artery. Urine samples were collected from the left kidney for a similar time period (corrected for TT).

At the end of the 30-min clearance period, 0.3-ml blood samples were collected from the renal vein and the aorta in heparinized syringes to calculate renal plasma flow (RPF) from the renal extraction of inulin and the GFR. The kidney was then perfused with saline and homogenized as described [20] for the determination of reabsorbed peroxidase tracers. Inulin concentration in urine and plasma samples was determined by the anthrone colorimetric assay [22]. HRP concentration in urine, plasma, and kidney homogenate was measured by enzymatic activity [23].

GFR and RPF were then calculated from the inulin concentration obtained in the urine, peripheral arterial, and renal venous plasma samples. The clearance (C) of each peroxidase tracer was calculated as the ratio of the amount excreted in the urine plus the reabsorbed fraction determined in the kidney homogenate over the time-averaged concentration in the peripheral artery. Fractional clearance was defined as C/GFR. The clearance rate for graded dextrans was calculated after separation by gel chromatography of plasma and urine, using a 2.2 by 54 cm column of an acrylamide agarose mixture (Ultrogel AcA 34 and 44, LKB, Sweden). Fractions of 1.7 ml were collected and isotope content was determined by standard liquid scintillation (Beckman, Model LS 250, Fullerton, California). Clearances for graded dextrans at 2 Å intervals from 16 to 60 Å were then calculated. The unpaired Student's t test was used for statistical analysis. All values given are the mean ± 1 SD.

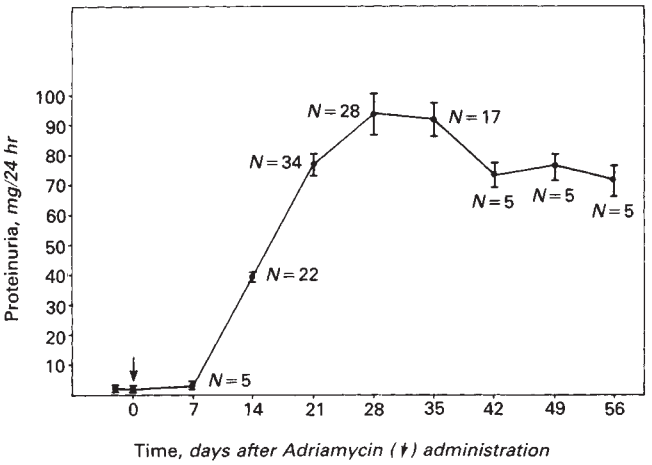

Fig. 1. *Twenty four-hour urine protein excretion following administration of Adriamycin at day 0.*

Morphology and immunohistology. Kidneys of five proteinuric and five control animals were processed for light and electron microscopy. The kidneys were fixed by arterial perfusion with 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and processed for electron microscopy as described before [20]. Tissue samples for light microscopy were postfixed in formalin 10%, embedded in paraffin, and sections were stained with Haematoxylin Eosin and PAS. For direct immunofluorescence and colloidal iron staining the renal cortex of five proteinuric and five control animals was snapfrozen in Freon at −90°C; 3-μm cryostat sections were processed for direct immunofluorescence using fluorescein isothiocyanate conjugated rabbit anti-rat IgA, IgG, IgM, C3, albumin, and fibrinogen (Cappel Laboratories, Cochranville, Pennsylvania). Colloidal iron staining on frozen sections was performed at a pH of 1.8 following the method described by Rinehart and Abul-Haj [24].

Staining of anionic sites in the laminae rarae interna and externa of the glomerular basement membrane was performed in two additional groups of five proteinuric and five control rats each, using polyethyleneimine (PEI) according to the method of Schurer et al [12]. The animals were anesthesized with Inactin as described above and placed on a heated operating table. A tracheostomy was performed and a PE10 tubing was inserted into the left jugular vein. PE50 tubing in the right femoral artery was used for continuous blood pressure registration as described above. PEI (Sigma Chemical Co., St. Louis, Missouri;

Table 1.[a]

	Body weight *g*	AP *mm Hg*	Urinary protein *mg/24 hr*	Plasma protein *g/dl*	GFR *ml/min*	RPF *ml/min*	FF
Experimental group (N = 17)	169 ± 12	125 ± 12	100 ± 35[b]	5.9 ± 0.7	0.51 ± 0.05[b]	1.63 ± 0.37[b]	0.33 ± 0.04
Control group (N = 16)	171 ± 15	130 ± 13	2 ± 1	5.9 ± 0.5	0.63 ± 0.08	1.92 ± 0.39	0.34 ± 0.04

[a] Values represent the mean ± SD.
[b] $P < 0.05$.

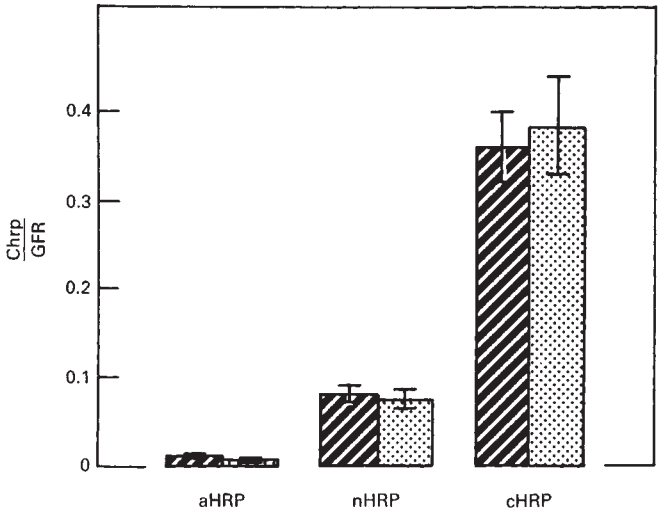

Fig. 2. *Fractional clearances of aHRP, nHRP, and cHRP in Adriamycin-injected animals (▨) as compared to control rats (▦).* Values are the mean of five animals in each group ± SD of the mean.

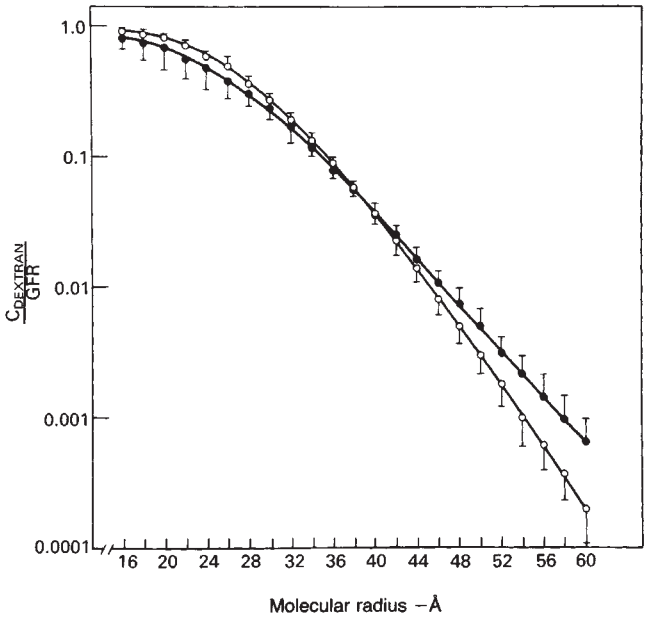

Fig. 3. *Fractional clearance of tritiated, neutral dextran as a function of effective molecular radius in six experimental (adriamycin-injected, ●) and five control animals (○).* The difference between experimental and control groups is not statistically significant for molecules 48 Å in molecular radius and smaller.

40,000 daltons) was infused through the venous catheter at a dose of 4 μg/g body weight during 2 min. Arterial blood pressure dropped somewhat during the infusion, but the diastolic pressure never fell below 100 mm Hg, and recovered as soon as the infusion was completed. After 30 min, the animals were sacrificed and the kidneys were fixed by perfusion of a mixture of 2% phosphotungstic acid and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Small pieces of tissue samples were postfixed in 1.25% glutaraldehyde and subsequently in osmium tetroxide, and processed for electron microscopy as described before [20].

Results

The pattern of urinary protein loss after administration of Adriamycin is shown in Figure 1. Three weeks after injection, proteinuria ranged from 53 to 101 mg/24 hr. After 4 weeks proteinuria reached a peak at a range from 60 to 189 mg/24 hr. Urinary protein loss remained at these levels until 5 weeks after injection; animals observed for a prolonged period of time were found to have persistent proteinuria of 60 to 90 mg/24 hr at 8 weeks (N = 5). An increase in urine production was present between days 5 and 15. Oliguria did not occur. Albumin accounted for 60 to 65% of the urinary protein.

Functional studies. Renal functional parameters, bodyweight, and protein excretion of experimental and control rats are given in Table 1. There was a decrease of GFR and RPF in the experimental animals of 20 and 15%, respectively. The filtration fraction was unchanged. Plasma protein concentration in both groups of animals was similar (5.9 g/dl).

The reabsorbed fraction of filtered HRP tracers was similar in control and in Adriamycin-treated rats and averaged for aHRP 16 and 15%, respectively; for nHRP 5.5 and 6%, respectively, and for cHRP 3 and 4%, respectively. Mean C/GFR values for HRP tracers in proteinuric and control animals are represented in Figure 2. The C/GFR of the cationic cHRP in the experimental group was 0.360 ± 0.041 as compared to 0.383 ± 0.054 in the control group. For the native nHRP, the values obtained were 0.079 ± 0.011 and 0.074 ± 0.009 in proteinuric and control rats, respectively. The differences between the means of both groups of animals for the cationic and native HRP did not attain statistical significance. For the anionic aHRP the fractional clearance in the experimental group was 0.0109 ± 0.0015 as compared to 0.0082 ± 0.0020 in control animals. This represents a 33% increase in experimental over control values, a difference which is statistically significant ($P < 0.05$).

Fractional clearance values for dextrans of graded sizes are given in Figure 3. The values obtained in experimental animals

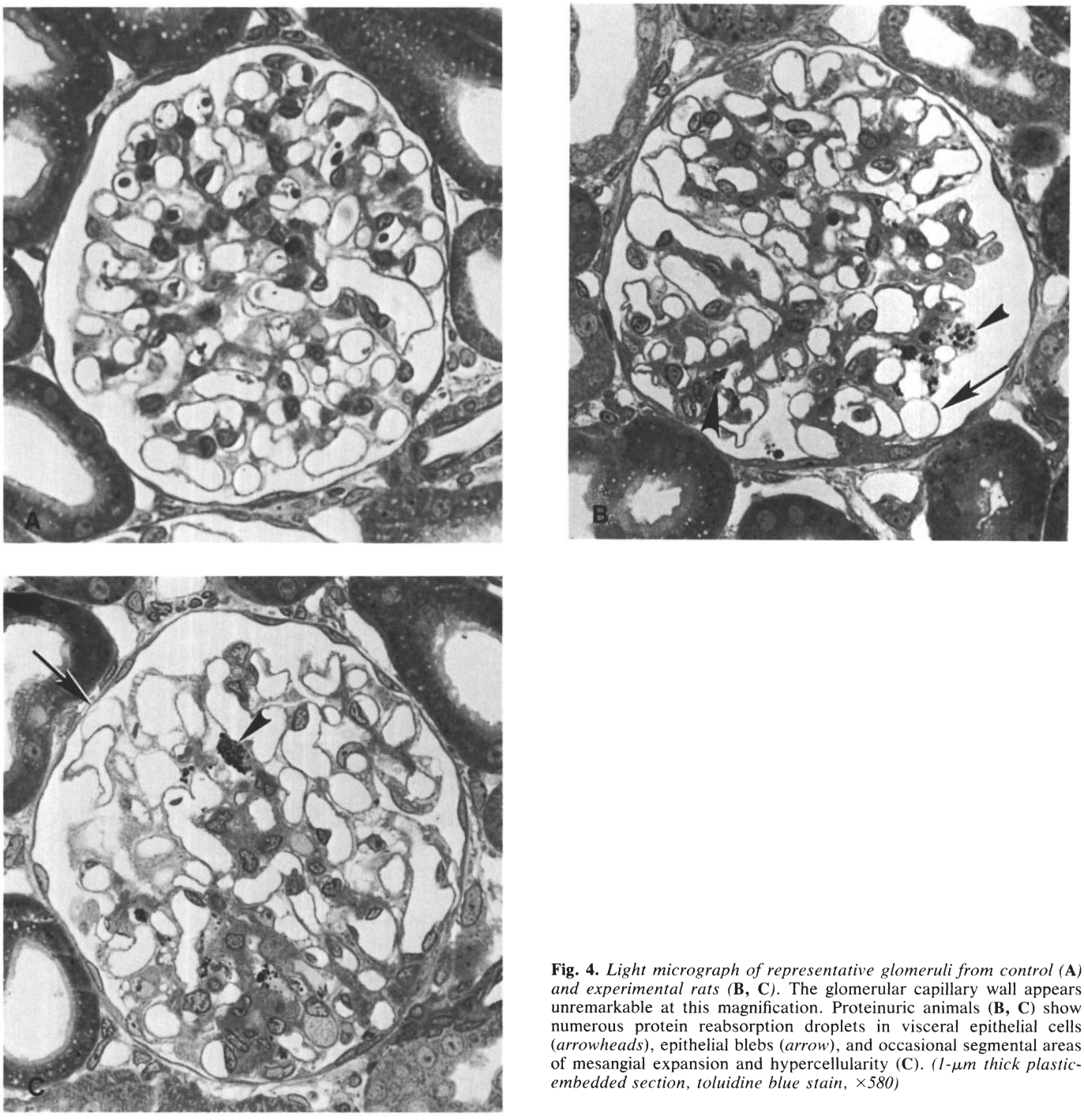

Fig. 4. *Light micrograph of representative glomeruli from control* (**A**) *and experimental rats* (**B, C**). The glomerular capillary wall appears unremarkable at this magnification. Proteinuric animals (**B, C**) show numerous protein reabsorption droplets in visceral epithelial cells (*arrowheads*), epithelial blebs (*arrow*), and occasional segmental areas of mesangial expansion and hypercellularity (**C**). *(1-μm thick plastic-embedded section, toluidine blue stain, ×580)*

were not significantly different from those in control rats for dextran up to 48 Å a_e. For larger dextran in the size range of 50 to 60 Å, proteinuric animals showed a statistically significant higher value when compared to controls ($P < 0.05$). No significant differences in GFR or in C/GFR were found between animals that were studied early and those that were studied late (2 or 3 as opposed to 7 or 8 weeks) after Adriamycin administration.

Glomerular morphology and immunohistology. By light microscopy proteinuric animals showed minimal glomerular changes consisting of focal increase in reabsorption droplets and blebbing and mild expansion of mesangial matrix. An occasional glomerulus showed segmental mesangial hypercellularity (Fig. 4). Tubules revealed mild to moderate increases in lysosomes with an occasional cast occluding the lumen of the distal segments. Immunofluorescence microscopy did not reveal glomerular deposits of immunoglobulins, complement, or fibrin; epithelial cell reabsorption droplets were found to contain immunoreactive albumin and IgG. Ultrastructural examination revealed effacement of epithelial cell foot processes, in-

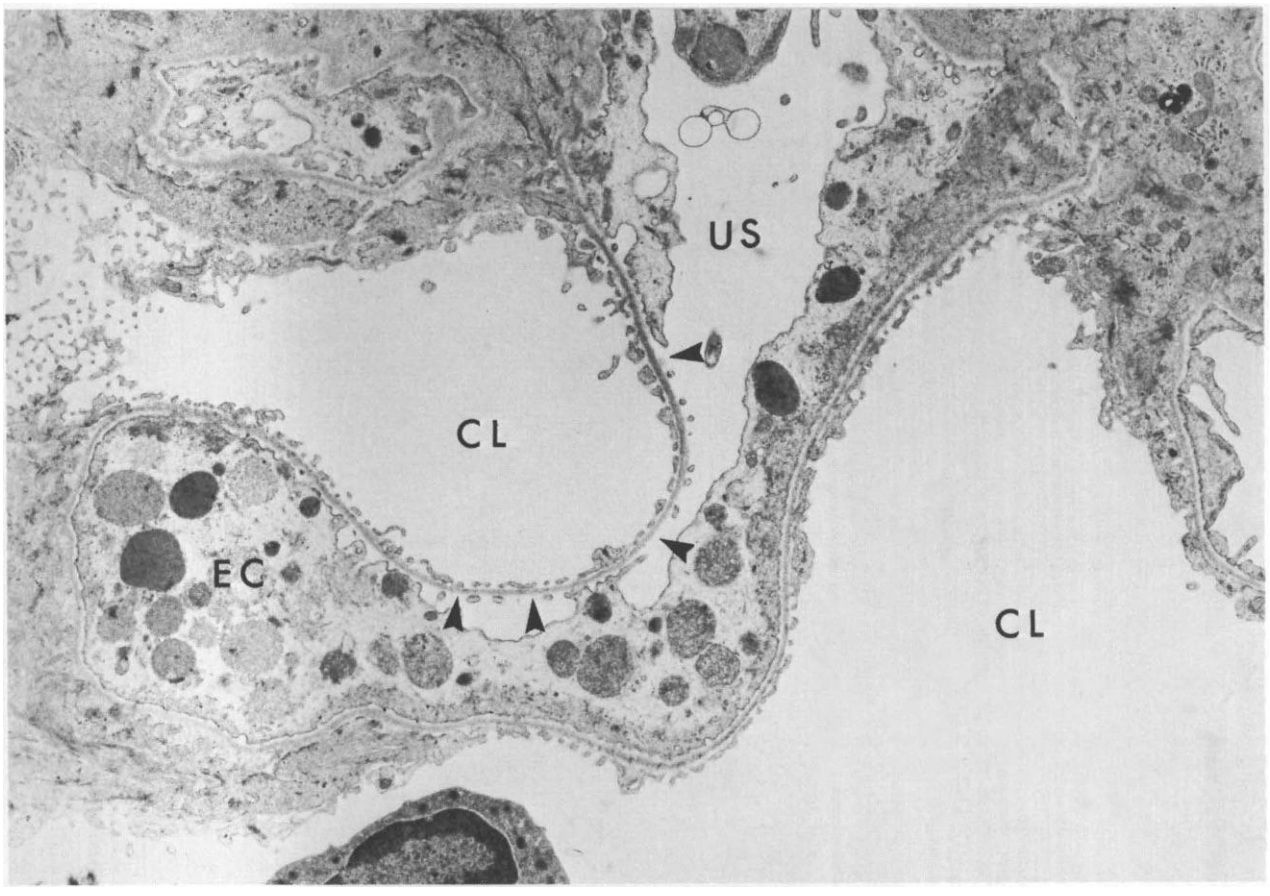

Fig. 5. *Electron micrograph of glomerular capillaries showing massive obliteration of epithelial foot processes, numerous electron dense reabsorption droplets and focal retraction of epithelial cells leaving an extensive area of the capillary wall devoid of podocytes* (arrowheads). Abbreviations are: CL, capillary lumen; EC, epithelial cell; US, urinary space. *(Uranyl nitrate and lead citrate, ×8,100)*

creased numbers of reabsorption vacuoles and lysosomes, and segmental detachment of epithelial cells from the underlying basement membrane (Fig. 5).

Colloidal iron reactive polyanions were reduced significantly in the glomeruli of proteinuric animals when compared to control rats (Fig. 6). The reduction in staining intensity was uniform and consistent in all proteinuric animals. The distribution of anionic sites in the lamina rarae interna and externa of the glomerular basement membrane as revealed by electron microscopy after PEI staining was found to be similar in proteinuric and control animals (Fig. 7).

Discussion

Our findings in this model of proteinuria indicate that the increased filtration of macromolecules to a great extent is due to a defect in the size selectivity of the glomerular capillary wall. Morphologically this model shows focal areas of the capillary wall in which the epithelial cells have become detached from the underlying basement membrane. This structural defect has been shown to be the site of increased permeability for large macromolecules in the aminonucleoside nephrosis and neuraminidase-treated kidneys [20, 25–29]. Such defects are associated with increased concentration in Bowman's space of large, uncharged dextran molecules [25], peroxidases [26], and negatively charged protein macromolecules such as catalase [27] (pI 6.5); a_e = 51 Å) and ferritin (pI 4.2 a_e = 61 Å) [20]. Both in aminonucleoside nephrosis and Adriamycin-induced proteinuria, epithelial cell detachment is rather focal and involves only a small fraction of the total glomerular filtration surface area. The limited nature of the epithelial cell defect in these models is consistent with a sieving defect most noticeable for macromolecules greatly restricted under normal conditions. Recently, Myers et al [30] described glomerular barrier function in diabetic nephropathy in humans by measuring fractional clearances of graded neutral dextrans. They showed that nonselective proteinuria in advanced diabetic nephropathy is associated with the development of large pores or defects within the glomerular capillary wall (GCW). Such a defect can be expected to increase the fractional clearance of the larger uncharged dextrans and of anionic proteins normally restricted due to charge interactions with the capillary wall. Our clearance results are consistent with such a defect as evidenced by an increase in the fractional clearance of uncharged dextrans with a molecular radius greater than 50 Å (Fig. 3) and a 33% increase in the fractional clearance value for the anionic HRP. The increase in filtration of the anionic HRP is not a result of a charge selective defect since there is not a simultaneous decrease in the fractional clearance of the cationic HRP, as would be expected had a decrease in the net fixed charge of the capillary wall occurred. A 33% increase in fractional clearance of the anionic aHRP in Adriamycin-

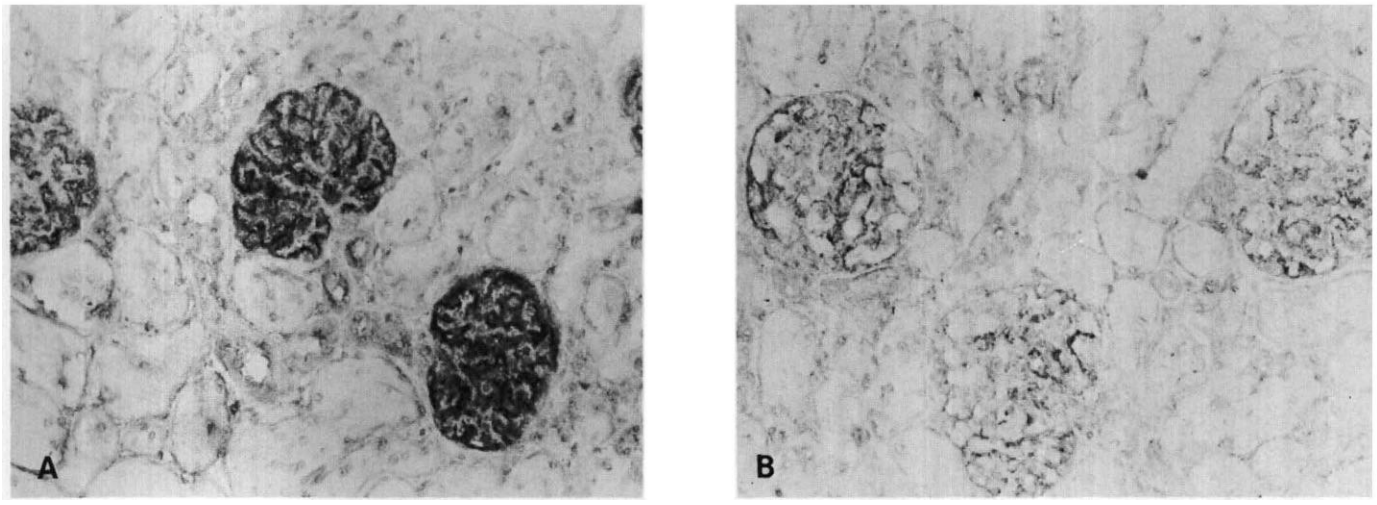

Fig. 6. *Representative glomeruli from control* (**A**) *and proteinuric* (**B**) *rats following colloidal iron staining of unfixed frozen sections.* Note uniform reduction of colloidal iron stainable polyanion in glomeruli from proteinuric animals. *(×200)*

treated animals over the value obtained in control rats (0.0109 vs. 0.0082) could be attributed to the altered sieving properties of the GCW, as evidenced also by a 30% increase in fractional clearance of a neutral dextran fraction which is restricted due to its size under normal conditions to the same extent as our anionic HRP tracer (that is, a dextran fraction with a_e~46 Å, C/GFR 0.0107 in proteinuric animals versus 0.0082 in controls (Fig. 3).

The fractional clearance values of the smaller dextrans in the proteinuric rats were somewhat lower than those in the control animals, and the curves plotted in Figure 3 show a crossing point at the values for the dextrans with an a_e between 36 and 42 Å. This finding is similar to what was found in rat aminonucleoside nephrosis [20] and proteinuric diabetic patients [30]; it is probably due to a decrease in GFR and RPF in combination with structural alterations in the GCW, resulting in a lowered hydraulic flux [31]. Since the fractional clearance values for cationic and native HRP are in the same range as the smaller dextrans, a slight decrease in C/GFR for the cationic tracer and an unaltered C/GFR of the native one are therefore to be expected in the proteinuric rats.

Renal function, as determined by GFR and RPF, was only slightly affected by Adriamycin, with a decrease of 20 and 15%, respectively. In aminonucleoside nephrosis, GFR is reduced by about 80% of the normal value [20]. Since the filtration fraction in the adriamycin nephrotic animals was unchanged, the slight changes in GFR and RPF are not likely to affect the permselectivity of the glomerular filter to macromolecules, since convective forces have not changed [1]. The most visible effect is that on smaller molecules, as discussed above; this effect was statistically not significant (Fig. 3). The present studies, therefore, indicate that in this model proteinuria is secondary to the sieving defect of the GCW, and the charge selective barrier remains unaltered. In this respect, Adriamycin-induced nephrosis differs from aminonucleoside nephrosis, a model with similar morphologic changes but with a well documented functional charge selective defect [20, 31]. This difference may be related to an additional effect of aminonucleoside on the proximal components of the GCW. One could speculate that this latter drug induces endothelial cell changes which result in a loss of anionic sites from the GBM [32].

Notwithstanding the preservation of the charge selective properties of the glomerular capillary wall in this model of proteinuria, total glomerular polyanions were found to be reduced, as evidenced by a marked and uniform reduction in colloidal iron staining. These results indicate that a reduction in total glomerular polyanion is not always associated with a defect in the charge selective barrier function of the glomerular capillary wall as has been suggested previously by other investigators [3, 5, 33]. The decrease in total glomerular polyanion as observed with the colloidal iron technique by light microscopy in a variety of human and experimental conditions associated with proteinuria is most likely the result of a decrease in total epithelial cell surface glycoproteins. This reduction in total cell surface polyanion could be the result of the effacement of epithelial cell foot processes, a constant feature associated with proteinuria of glomerular origin, and/or a reduction of the density of such glycoproteins induced by metabolic changes in the epithelial cell. Sialic acid containing glycoproteins appears to play a significant role in the morphologic integrity of the glomerular capillary wall [29]. Perfusion of the kidney with sialidase-containing solutions results in detachment of endothelial and epithelial cells from the glomerular basement membrane [28, 29]. Some of the epithelial cell changes observed in Adriamycin-treated animals, in particular effacement and detachment of the foot processes from the basement membrane, most likely result from a reduction in some critical component of the cell surface glycoproteins as a further result of drug-induced metabolic derangements. Despite the loss of glomerular polyanion in this model as evidenced by light microscopic examination of colloidal iron reacted frozen sections, the amount and distribution of anionic sites in the glomerular basement membrane by electron microscopy appear undisturbed, which agrees with our findings of intact charge-selective properties of the glomerular capillary wall.

As mentioned above, the sieving defect in this model becomes most apparent for molecules greatly restricted under normal conditions either by their size or charge characteristics.

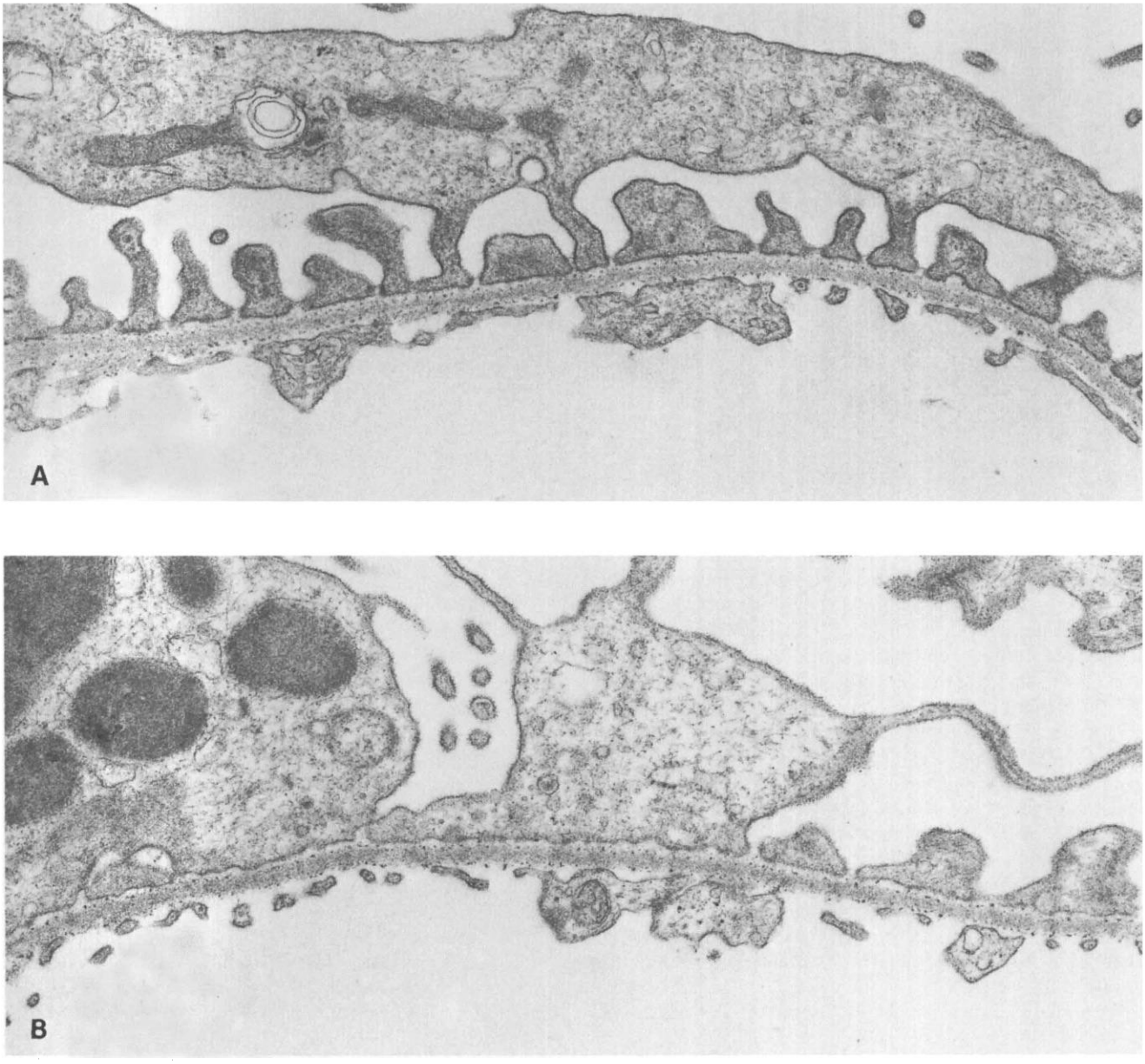

Fig. 7. *Electron micrograph from control* (**A**) *and experimental rats* (**B**) *showing a similar distribution of anionic sites within the laminae rarae interna and externa after staining with polyethyleneimine. (Uranyl nitrate, lead citrate, ×26,000)*

Whether a 25- to 50-fold increase in protein excretion as observed in this model is entirely due to this defect remains uncertain, because our neutral dextran sieving curve does not extend into the range of the sieving coefficient reported for serum albumin by others on the basis of direct micropuncture measurements [34–36].

In conclusion, in Wistar Furth rats Adriamycin induces glomerular epithelial cell damage and proteinuria, which is due to a defect in the size selective barrier function of the glomerular capillary wall. Notwithstanding a marked loss of colloidal iron reactive glomerular polyanion, the functional charge barrier is virtually intact, associated with a normal distribution of anionic sites within the glomerular basement membrane.

Finally, the chronic character of proteinuria in this new model with relatively well preserved renal function offers an opportunity to study long-term changes such as focal glomerulosclerosis and the effect of metabolic and nutritional factors on the course of proteinuria and renal damage.

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