The Renal Handling of IgG in the Aging Rat and in Experimental Kidney Disease

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Abstract. **The urinary excretion of total protein, low-MW proteins, albumin, high-MW proteins, and intact IgG was measured in male Wistar rats between the ages of 5–52 weeks, and in rats with experimentally induced glomerular or tubular proteinuria. About 25% of aging rats spontaneously developed focal glomerulosclerosis and a mild glomerular proteinuria. By age 52 weeks, total protein excretion in rats with glomerulosclerosis exceeded that of unaffected rats by a factor of seven (39.5 vs 5.4 mg/24 hr × 100 g body wt), and albumin excretion was seven times higher than IgG excretion in affected rats (21.2 vs 2.9 mg/24 hr × 100 g body wt). Rats with chromate toxicity exhibited a reversible tubular proteinuria, with low-molecular weight protein excretion reaching 16.8 mg/24 hr × 100 g body wt (75% of total protein excretion) at the time of peak toxicity. IgG excretion remained less than 0.6 mg/24 hr × 100 g body wt. Aminonucleoside induced a massive but reversible glomerular proteinuria (204 mg/24 hr × 100 g body wt), with IgG excretion reaching 11.4 mg/24 hr × 100 g body wt (6% of total protein excretion) at the time of peak toxicity. Biochemical and immunochemical studies showed that, while some intact IgG is present in normal rat urine, most IgG immunoreactivity is derived from low-molecular weight catabolic fragments of IgG which interfere with the immunoassay of intact urinary IgG. One of these fragments, probably Fc fragment, may be involved in the pathogenesis of focal segmental glomerulosclerosis.** [P.S.E.B.M. 1997, Vol 216]

The analysis of proteinuria beyond the measurement of total protein excretion is useful in the diagnosis of kidney disease (1). As glomerular disease progresses, microalbuminuria is an early and specific marker of glomerular proteinuria (2), followed by an increase in the excretion of anionic IgG (3–5), and then total IgG (6–8). Glomerular filtration theory has promoted the concept that IgG crosses the glomerular capillary wall exclusively through large pores of the shunt pathway (9, 10). The model, however, does not account for the facilitated clearance of anionic IgG over that of cationic IgG in health and disease (11, 12).

Recently, a comparative analysis of dextran and ficoll glomerular sieving data has challenged the premise that albumin and IgG traverse different populations of pores (13). Furthermore, the analysis indicates that the fraction of filtrate traversing the large pores of the shunt pathway is about 10 times smaller in normal rats than in normal humans (13). Since much research in the pathogenesis of glomerular disease has been done in rats, and since a clear understanding of IgG ultrafiltration is lacking, further studies concerning the renal handling of IgG were warranted. We therefore investigated the renal handling of albumin, proteins with molecular weights lower than albumin (low-MW), higher than albumin (high-MW), and IgG in the aging male Wistar rat, and in rats with reversible glomerular and tubular proteinuria.

Our studies provide new reference values for IgG excretion in normal and proteinuric rats. We provide new

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information regarding the MW profile of IgG-related molecules in normal rat urine, and show that the measurement of urinary IgG in the rat is invalid using heavy-chain–specific antiserum.

Material and Methods

Animals. Male Wistar rats (Rockland Laboratories, Gilbertsville, PA) were used in all studies. Rats were maintained in group cages in a room with a 12:12-hr light:dark cycle and were fed Wayne Lab Blox (24% protein, 5.5% fat) and water *ad libitum.*

Aging Rat Study. Two groups of rats were studied simultaneously over a 6-month period. The younger group was received at age 3 weeks, and studied from 5 to 29 weeks of age ($n = 20$). The older group was received at age 27 weeks and studied from 29 to 52 weeks of age ($n = 22$). At intervals of 2–5 weeks, body weight (BW) was measured and urine and tail vein blood was obtained on each rat. During urine collection periods (4 PM–8 AM), food was withheld but water was available *ad libitum.* Following urine collections, but before food was again made available, heparinized blood samples were collected.

Chromate Toxicity Study. Rats aged 14 weeks (mean BW of 206 g) were entered in the study ($n = 12$). Following a 2-day control period, a 400-mg/dl aqueous solution of research grade potassium dichromate was administered on Days 3 and 15 by subcutaneous injection at doses of 1 mg/100 g BW (Day 3) and 1.5 mg/100 g BW (Day 15). During the 29-day study, BW was measured and overnight urines were collected every 1–7 days. Blood samples were collected every 3–7 days.

Aminonucleoside Toxicity Study. Rats aged 8 weeks (mean BW of 126 g) were entered in the 34-day study ($n = 10$). Following a 2-day control period, a 500-mg/dl aqueous solution of aminonucleoside of puromycin was administered on Days 3–9 by subcutaneous injection at a dose of 1.5 mg/100 g BW. During the 34-day study, BW was measured and overnight urines were collected every 1–7 days. Blood samples were collected every 2–16 days.

Analytical Methods. A microcontinuous gradient gel electrophoresis procedure was used for separation and quantization of proteins in plasma (diluted 1:101) and urine (usually undiluted, but, in cases of heavy-proteinuria, diluted up to 1:51). As previously described (14), the acrylamide continuous gradient gels were made in 5-μl microcapillary tubes. After electrophoresis at pH 8.8, the gels were extruded from the capillary tubes, stained overnight, and scanned directly in an ultramicrodensitometer (Joyce-Loebl, Gates Head-on-Tyne, United Kingdom). Densitometer counts under each protein peak were individually quantitated. Recognition of plasma and urinary albumin permitted differentiation of proteins into low-MW, albumin, and high-MW classes. In scans shown, electrophoretic migration is from left (low-density end of gel) to right (high-density end of gel).

Urinary total protein determination was based on the Lowry method (15) in the aging rat study, and with the method of Bradford (16) in the glomerular and tubular toxicity studies. Plasma total protein was determined using a biuret method (17). Plasma and urine creatinines were determined following adsorption and elution from Lloyds reagent using the method of Hare (18).

Plasma IgG was assayed immunologically using a radial immunodiffusion (RID) assay kit available from ICN Biochemicals, Inc. The kit uses a rabbit, anti-rat IgG antiserum which is heavy-chain-specific and provides rat serum IgG standards in the range of 150–1250 mg/dl. Although the RID assay was suitable for the measurement of IgG in undiluted plasma samples, it was found to be unsuitable for the measurement of intact urinary IgG (19). This is due to the presence of low-MW fragments of IgG in urine, which interfere with the assay of intact IgG.

Urinary IgG was measured nonimmunologically using the gradient gel electrophoresis assay. We determined where urinary IgG migrates in our electrophoresis system by adding rat IgG (ICN Biochemicals, Inc.) to urine samples and comparing scans of "spiked" versus "unspiked" samples. Knowledge of IgG peak location, densitometer counts under the IgG peak, total scan densitometer counts, and total protein concentration permitted the calculation of urinary IgG concentration (19).

Biochemical and Immunochemical Studies of Normal Rat Urine. Urine was collected once a week from 37 healthy rats aging between 11 and 31 weeks. Pooled urine was dialyzed against distilled water and ultrafiltered under negative pressure using Spectopor 3 dialysis tubing (54 mm, 3.5-kDa MW cutoff) reinforced with tubegause. The protein rich retentate was lyophilized and 3.5 g of dry powder (derived from about 1700 ml of urine) was resuspended in 15 ml of Tris-HCl buffer at pH 8.0. Preparative gel filtration chromatography of urinary proteins was performed at 4°C in a 90 × 5–cm column containing regular Sephadex G-100 in the same buffer. The column was calibrated using protein standards in the Pharmacia Calibration Kit (Pharmacia Biotech, Inc., Piscataway, NJ). *Ve/Vo* values for ovalbumin (45 kDa), β-lactoglobulin (35 kDa), chymotrypsinogen (25 kDa), and cytochrome C (12.5 kDa) were 1.49, 1.61, 1.79, and 2.10, respectively. Column fractions were dialyzed against water and lyophilized to obtain dry powder. One-percent solutions (dry weight) of column fractions were assayed for IgG using the RID assay. Immunoelectrophoresis was done at pH 8.8, courtesy of Ms. Doris Skarha, formerly of Miles Laboratories, Elkhart, IN.

Statistical Methods. In the aging rat study, comparisons between means of normal and spontaneously proteinuric animals at any given age were done using the unpaired Student's *t* test. In the chromate and aminonucleoside toxicity studies, means between control periods and treatment periods were compared using the analysis of variance,

followed by secondary Sheffe t test. Results are reported as mean ± SEM.

Results

Aging Rat Study. In rats studied between 5 and 29 weeks, mean BW increased from 145 to 521 g. No significant changes ($P < 0.05$) occurred in plasma creatinine or creatinine clearance at any age relative to values measured at age 5 weeks. At age 29 weeks, plasma creatinine was 0.37 ± 0.02 mg/dl and creatinine clearance was 0.89 ± 0.05 ml/min × 100 g BW.

Figure 1 shows changes in total protein excretion and the excretions of low-MW, albumin, and high-MW proteins. Between 5 and 9 weeks, the time of sexual maturation in the male rat (20), low-MW protein excretion increased. Throughout the period of observation, the character of the proteinuria remained normal, with low-MW proteins comprising more than half of all proteins excreted. IgG was not consistently detectable as a discrete high-MW peak by gradient gel electrophoresis until age 29 weeks, when it was 0.31 ± 0.04 mg/24 hr × 100 g BW, representing only 5% of total protein excretion and 25% of high-MW protein excretion.

Among the 22 rats studied between ages 29 and 52 weeks, 5 (23%) consistently excreted more protein than the other 17. In both the "normal" (Nl; $n = 17$) and the "spontaneously proteinuric" (SP; $n = 5$) groups, BW increased from about 435 g at 29 weeks to about 570 g at 52 weeks. At any given age, there were no significant differences in body weight, plasma creatinine, or creatinine clearance between the two groups. In both groups, there was no significant change in plasma creatinine or creatinine clearance until age 52. At age 52 weeks, both Nl and SP groups had a mean creatinine clearance between 0.47 and 0.48 ml/min × 100 g BW, compared with 0.82–0.88 ml/min × 100 g BW at age 29 weeks ($P < 0.005$ in both groups).

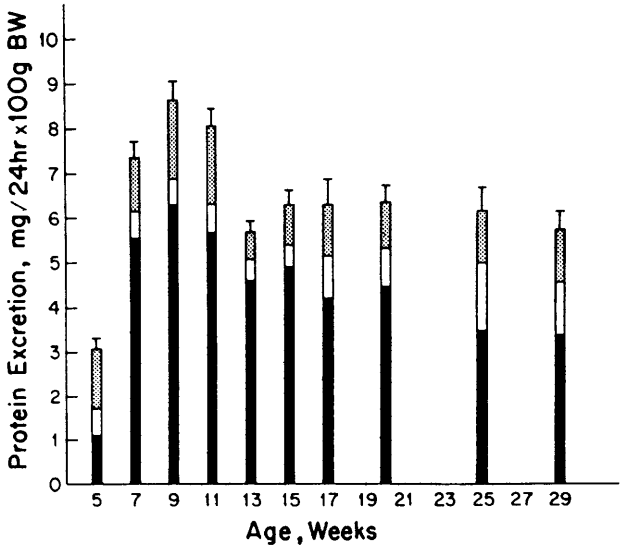

Figure 1. Time course of protein excretion in normal male Wistar rats, ages 5–29 weeks ($n = 20$). Vertical bars differentiate the excretion of low-MW (■), albumin (□), and high-MW (▨) proteins. High-MW protein includes IgG excretion. Lines above bars indicate SEM for total protein excretion.

Figure 2 presents total protein excretion and the excretions of low-MW, albumin, IgG, and high-MW (including IgG) proteins in Nl versus SP rats between the ages of 29 and 52 weeks. By age 45 weeks, the excretion of all protein classes and IgG was significantly greater in SP rats than in Nl rats ($P < 0.01$). At age 50 weeks, mean total protein excretion and albumin excretion in the SP group were maximal, exceeding corresponding values in the Nl group by factors of 7.46 and 11.18, respectively. At that time, albumin represented 57% of total protein excretion in the SP group, compared with 38% in the Nl group.

The fractional excretion (% of total protein excretion) of low-MW proteins never dropped below 49% in the Nl group. At age 41 weeks and older, it was consistently below 23% in the SP group. The fractional excretion of high-MW proteins was comparable in both groups, ranging between 16% and 24% of total protein excretion at all times. The proteinuria in the SP rats was glomerular in origin, with the principal component being albumin.

In all five SP rats, an IgG peak was recognizable by gradient gel electrophoresis at age 29 weeks and older. At age 29 weeks, mean IgG excretion was 0.35 ± 0.19 mg/24 hr × 100 g BW, and steadily increased thereafter. At 45 weeks and older, IgG excretion comprised 27%–31% of all high-MW proteins excreted, and 6%–7% of total proteins excreted by the SP rats. Within the Nl group, a discrete IgG band was not consistently recognizable by gradient gel electrophoresis until age 45 weeks. Between ages 45 and 52 weeks, IgG excretion comprised 22%–39% of all high-MW protein, and 4%–7% of total protein excretion in the Nl group. Maximal IgG excretion in both groups occurred at age 50 weeks (0.42 ± 0.11 mg/24 hr × 100 g BW in the Nl group compared with 3.50 ± 0.79 mg/24 hr × 100 g BW in the SP group; $P < 0.001$).

Figure 3 illustrates the qualitative differences in protein excretion between a Nl and an SP rat. As the SP rat aged from 29 to 52 weeks (right panel), the fractional excretion of albumin steadily increased, becoming the principal urinary protein by 45 weeks of age. The fractional excretions of high-MW proteins (all bands to left of albumin) and IgG (solid black band) also increased with age. In contrast, low-MW proteins (all bands to right of albumin) remained the predominant class of proteins excreted in the Nl rat between ages 29 and 52 weeks (left panel). These results are representative of other rats in the Nl and SP groups.

Table I compares BW, creatinine clearance, plasma albumin, plasma IgG, and urinary protein excretions in Nl versus SP rats at age 52 weeks. Note that there was no significant difference between plasma albumin in the two groups at age 52 weeks. However, plasma IgG (measured by RID assay) was significantly lower in the SP group compared with the Nl group (0.54 ± 0.08 vs 0.79 ± 0.04 g/dl; $P < 0.025$). At this time, the excretion of IgG in the SP group

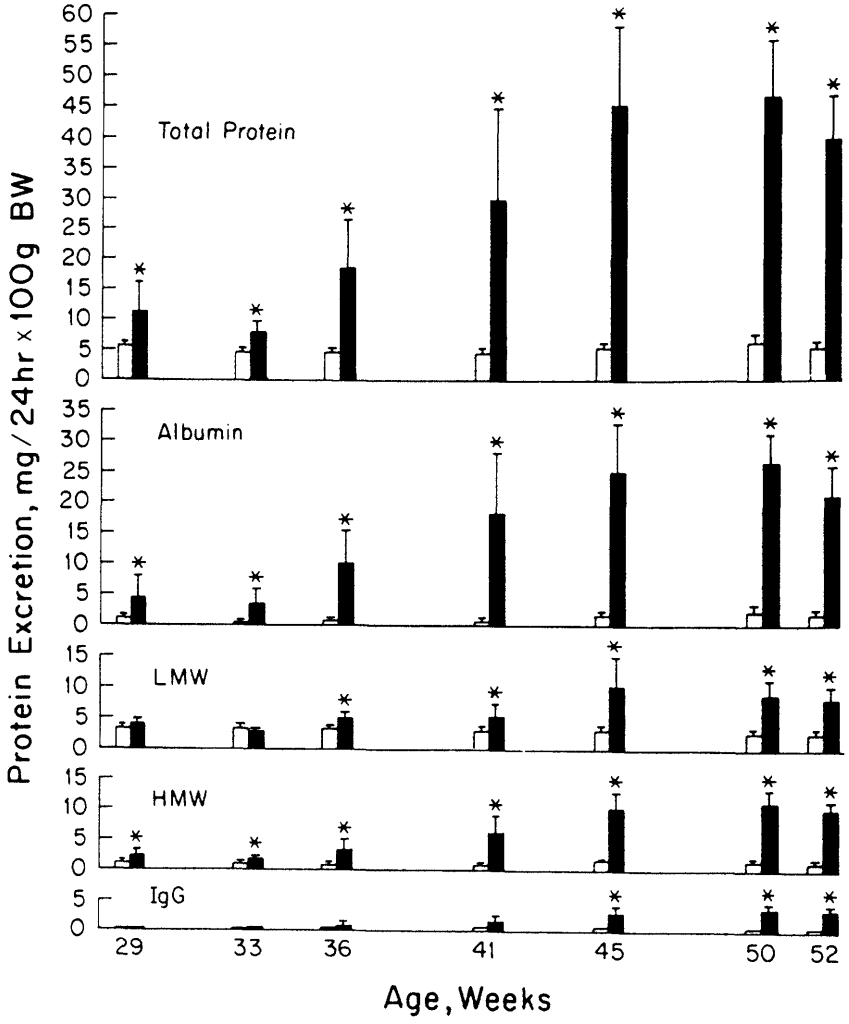

Figure 2. Protein excretion in normal (n = 17; □) and spontaneously proteinuric (n = 5; ■) rats, ages 29–52 weeks. High-MW (HMW) protein excretion includes IgG excretion. *Significant differences between normal and proteinuric group means ($P < 0.01$ in all cases except those at age 29 weeks, where $P < 0.05$).

was eight times greater than that in the Nl group (2.92 ± 0.54 vs 0.35 ± 0.04; $P < 0.001$).

Kidney histology of rats sacrificed at 52 weeks revealed focal glomerulosclerosis in all five SP rats, compared with normal glomeruli in Nl rats. Glomerular changes included increased mesangial matrix, sclerotic capillary loops, and a thickening of the basement membrane of Bowman's capsule. Other changes included dilated tubules, with and without proteinaceous casts, and interstitial infiltration by predominantly mononuclear cells. The degree of histopathology correlated with the degree of proteinuria.

Chromate Toxicity Study. During the study, rats exhibited no signs of illness. BW increased from 206 ± 6 g on Day 1 to 263 ± 7 g on Day 29. Plasma creatinine, creatinine clearance, and plasma proteins were all normal throughout the study. Plasma IgG, determined by RID assay, was significantly decreased on Day 18 relative to the control period level (0.55 ± 0.05 vs 0.84 ± 0.06 g/dl; $P < 0.025$).

Figure 4 shows changes in total protein excretion during control (Day 2), toxicity (Days 4–20) and recovery (Day 22) periods. Note that following the first administration of chromate on Day 3 (first arrow) only a modest elevation in total protein excretion was observed, with return to baseline levels by Day 8. However, following a second and higher dose of chromate on Day 15 (second arrow) a more pronounced proteinuria resulted, peaking on Day 18. On Day 17, the fractional excretions of low-MW, albumin, and high-MW proteins were 75%, 7%, and 18%, respectively, documenting the tubular proteinuria induced by potassium dichromate. At this time, urinary IgG was detectable by gradient gel electrophoresis in only 2 of the 12 rats.

Aminonucleoside Toxicity Study. Rats exhibited no signs of illness. BW increased from 126 ± 3 g on Day 1 to 264 ± 9 g on Day 34. Creatinine clearance decreased significantly on Day 17, during the time of peak proteinuria. Its level of 0.68 ± 0.06 ml/min × 100 g BW on Day 17, was significantly lower compared with the level measured on Day 7 of 0.91 ml/min × 100 g BW ($P < 0.05$).

Figure 5 shows changes in total protein excretion during control (Days 1–2), toxicity (Days 3–20) and recovery (Day 28) periods. Note that following the final injection of aminonucleoside on Day 9 (arrows), total protein excretion increased markedly, reaching 203.81 ± 23.54 mg/24 hr × 100 g BW on Day 16, or 91 times that measured on Day 2 ($P < 0.001$). Thereafter, total protein excretion returned to

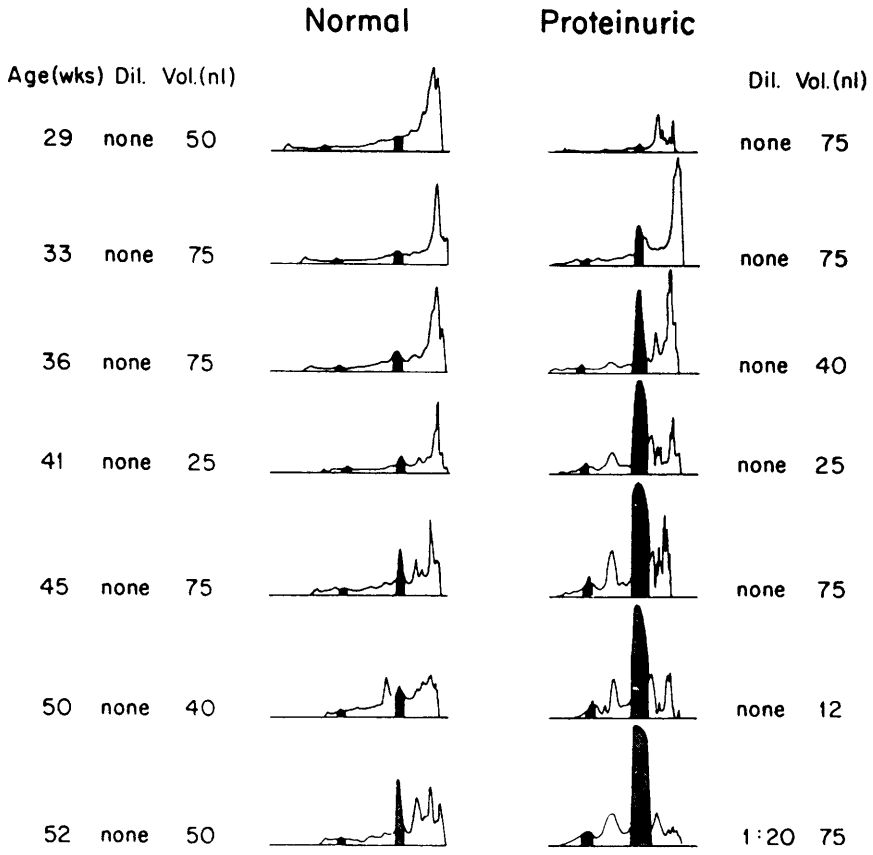

Figure 3. Gradient gel electrophoresis of urinary proteins in a normal and a spontaneously proteinuric rat between ages 29 and 52 weeks. Scans to the left are from a single rat with normal protein excretion (2.9–6.3 mg/24 hr × 100 g BW). Scans to the right are from a single rat with progressive proteinuria (5.1–32.0 mg/24 hr × 100 g BW). Stippled areas identify locations of Albumin (vertically aligned). Solid areas identify locations of IgG. Sample volume (nl) and dilutions are noted.

Table I. Comparison of Normal versus Spontaneously Proteinuric Rats at Age 52 Weeks

Variable	Normal	Proteinuric	Significance
Body weight (g)	565 ± 15	578 ± 16	
Creatinine clearance (ml/min)[a]	0.48 ± 0.02	0.47 ± 0.01	
Plasma albumin (g/dl)	3.07 ± 0.07	2.90 ± 0.15	
Plasma IgG (g/dl)	0.79 ± 0.04	0.54 ± 0.08	$P < 0.025$
Protein excretion (mg/24 hr)[a]			
Total	5.42 ± 0.72	39.54 ± 6.98	$P < 0.001$
Low-MW	2.48 ± 0.26	7.96 ± 1.97	$P < 0.001$
Albumin	1.74 ± 0.32	21.15 ± 4.98	$P < 0.001$
High-MW[b]	1.01 ± 0.13	9.69 ± 1.76	$P < 0.001$
IgG	0.35 ± 0.04	2.92 ± 0.54	$P < 0.001$

Note. N1 group, $n = 17$; SP group, $n = 5$.
[a] Clearance and protein excretion rates are per 100 g BW.
[b] High-MW protein excretion includes IgG excretion.

nearly baseline levels (8.13 ± 1.12 mg/24 hr × 100 g BW on Day 34). On Day 16, the character of the massive proteinuria induced by aminonucleoside was glomerular, with the fractional excretions of albumin, high-MW protein, and low-MW protein being 53%, 32%, and 15%, respectively.

On Day 16, IgG excretion was 11.44 ± 2.47 mg/24 hr × 100 g BW. This high level of IgG excretion (6% of total protein excretion and 18% of all high-MW protein excretion) represents the loss of approximately 18 mg IgG/rat/day during the period of peak proteinuria.

As shown in the top panel of Figure 5, the significant urinary loss of IgG which occurred was associated with a significant decline in plasma IgG. On Day 2, plasma IgG measured by RID assay was 0.99 ± 0.11 g/dl. This compared with a value of 0.33 ± 0.05 g/dl on Day 17 ($P < 0.005$). As urinary IgG losses decreased during the recovery period, plasma IgG began to recover, increasing to 0.57 ± 0.05 g/dl by Day 28.

Figure 6 illustrates plasma and urinary proteins analyzed by gradient gel electrophoresis in representative chromate and aminonucleoside treated rats at times of peak proteinuria. In the urine from the rat with tubular toxicity (left lower panel) no urinary IgG peak is recognizable. The overwhelming majority of proteins present in this urine are low-MW proteins (bands to right of albumin). The urinary albumin band is relatively small in comparison to the low-

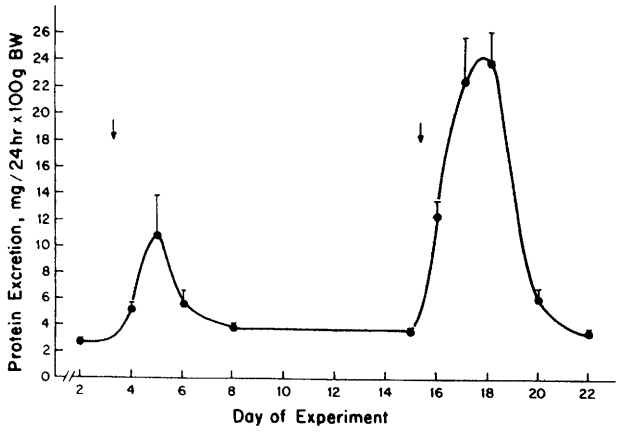

Figure 4. Total protein excretion (mean ± SEM) in male Wistar rats treated with potassium dichromate (n = 12). Arrows indicate days when the tubular toxin was administered.

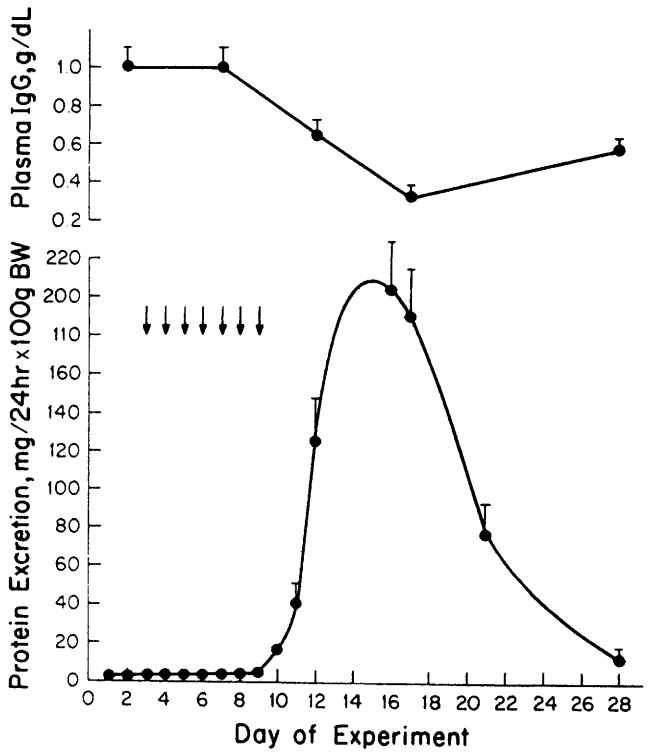

Figure 5. Total protein excretion in male Wistar rats treated with aminonucleoside of puromycin (n = 10). Arrows indicate days when the glomerular toxin was administered. In the upper panel, concurrent changes in plasma IgG concentration (mean ± SEM) are plotted.

MW protein bands present. This is contrasted by the scan of urinary proteins from the rat with glomerular toxicity (right lower panel), in which albumin is the principal urinary protein present, and urinary IgG is recognized within the high-MW protein zone (left of albumin).

Table II compares creatinine clearance, plasma albumin, plasma IgG, and urinary protein excretions in chromate- versus aminonucleoside-treated rats at the time of peak proteinuria (mean body weights of 235 and 157 g, respectively). Note that IgG excretion was 23 times higher in aminonucleoside-treated than in chromate-treated rats (11.44 vs 0.49 mg/24 hr × 100 g BW). Conversely, aminonucleoside-treated rats excreted only 1.8 times as much low-MW proteins as did chromate-treated rats at the time of peak toxicity (30.54 vs 16.76 mg/24 hr × 100 g BW). Distinct types of proteinuria are induced by these agents. Total protein and albumin excretion were 9- and 67-fold higher, respectively, in aminonucleoside-treated rats compared with chromate-treated rats.

Biochemical Studies of Normal Rat Urine. Figure 7 displays the separation of normal rat urinary proteins into MW fractions by Sephadex G-100 gel filtration chromatography. Fraction A contains urinary molecules having a MW above 80 kDa. Fractions B, C, D, and E have average MWs of 60, 40.6, 21.3, and 13 kDa, respectively. Comparisons of IgG immunoreactivity, expressed as RID assay ring diameter obtained in RID assays of standardized solutions of Fractions A–D, are depicted by the vertical bars. The smallest ring diameter was obtained in Fraction A, and the largest ring diameter was obtained in Fraction C. Fraction C was shown to be completely devoid of intact IgG by gradient gel electrophoresis. Therefore, low-MW molecules exist in normal rat urine that are immunoreactive with the rabbit, anti-rat IgG antiserum (heavy-chain-specific) used in the RID assay. Such molecules may be Fc and Fc′ fragments of IgG (21–24), which represent prerenal, catabolic fragments of IgG having molecular weights compatible with glomerular filtration and subsequent excretion. Thus, for purposes of measuring intact urinary IgG in the normal rat, the RID assay used is clearly not suitable.

To determine whether plasma IgG and urine IgG (and catabolic fragments) have different molecular charges, immunoelectrophoresis assays were conducted on Fractions A and C and rat plasma. The rabbit anti-rat IgG antiserum used was identical to the heavy-chain–specific antiserum used in the RID assay. Results are shown in Figure 8. In the lower panel, the immunoprecipitate arc farthest to the left above and below the antigen well for plasma represents plasma IgG. Thus, in this assay system IgG appears to be the most anionic species detectable in plasma. In the upper panel, IgG present in Fraction A appears to migrate slightly to the anode, signifying that intact IgG present in normal rat urine is more anionic than the form which predominates in plasma. In the central panel, the immunoprecipitate arcs formed by the IgG-related molecule(s) in Fraction C occupy similar positions relative to the arc formed by the IgG present in Fraction A. The predominant form of IgG present in normal rat urine, whether derived from the glomerular filtration of the intact molecule or its circulating catabolic fragments, is more anionic compared with the predominant form of IgG present in plasma.

Discussion

Aging Rat Study. A fundamental question posed by the study of renal disease in aging rats is why some rats within the same strain and sex develop proteinuria and focal glomerulosclerosis (23% within the first year of life in our

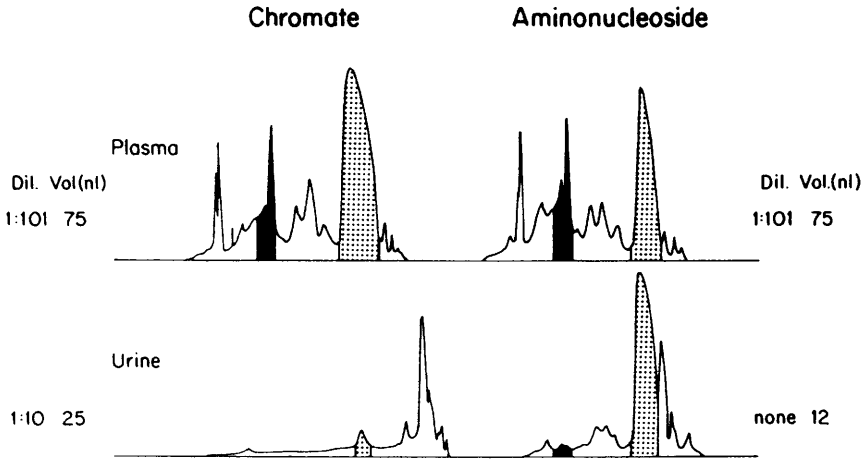

Figure 6. Gradient gel electrophoresis of plasma and urinary proteins in rats with tubular and glomerular toxicity. Scans to the left are from a chromate-treated rat with a total protein excretion of 26.4 mg/24 hr x 100 g BW. Scans to the right are from an aminonucleoside-treated rat with a total protein excretion of 255 mg/24 hr x 100 g BW. Stippled areas identify positions of albumin. Solid areas identify positions of IgG. Sample volumes (nl) and dilutions are noted.

Table II. Comparison of Rats with Tubular (Chromate) versus Glomerular (Aminonucleoside) Toxicity at Time of Peak Proteinuria

Variable	*n*	Tubular	*n*	Glomerular	Significance
Creatinine clearance (ml/min)[a]	5	0.60 ± 0.10	10	0.68 ± 0.05	
Plasma albumin (g/dl)	5	2.91 ± 0.14	7	1.78 ± 0.20	$P < 0.005$
Plasma IgG (g/dl)	12	0.55 ± 0.04	10	0.33 ± 0.05	$P < 0.005$
Protein excretion (mg/24 hr)[a]					
Total	12	22.45 ± 3.18	10	203.81 ± 23.54	$P < 0.001$
Low-MW	12	16.76 ± 2.50	10	30.56 ± 4.13	$P < 0.025$
Albumin	12	1.60 ± 0.25	10	107.70 ± 11.40	$P < 0.001$
High-MW[b]	12	4.09 ± 0.66	10	65.26 ± 11.31	$P < 0.001$
IgG	2	0.49 ± 0.07	10	11.44 ± 2.47	

Note. Values in chromate-treated rats measured on Day 17 or 18 of experiment, except for creatinine clearance, measured on Day 29 of experiment. Values in aminonucleoside-treated rats measured on Day 16 or 17 of experiment.
[a] Clearance and excretion rates are per 100 g BW.
[b] High-MW protein excretion includes IgG excretion.

study), while others do not, or take longer to develop it. Perhaps the best response is to quote the biological truth contained in Boylan's Law (25): "Some people get sick and some do not." Underscoring Boylan's Law as applied to the development of proteinuria and glomerulosclerosis in rats, a study of 1500 male Sprague-Dawley rats showed that 25% of rats had focal glomerulosclerotic changes by age 13 months (26). It has also been shown that Sprague-Dawley rats can be categorized as "high" or "low" protein excreters, and that subsequent breeding can select for rats that do, and do not, spontaneously develop nephrosis (27, 28).

Regarding the pathogenesis of focal segmental glomerulosclerosis, a circulating agent has been hypothesized for 25 years (29), but has yet to be identified (30). Early investigators recognized that patients with focal segmental glomerulosclerosis had an "anomalously high" IgG clearance, and hypothesized that the agent responsible for the disease was a low-MW IgG fragment (31). Although Hardwicke and colleagues concluded that this was not the case, it is noteworthy that they apparently only searched for IgG-related molecules with a MW greater than albumin (32), making it difficult to exclude a role for Fc fragment.

Recent work suggests that the circulating agent may be an immunoglobulin fragment with a biochemical profile

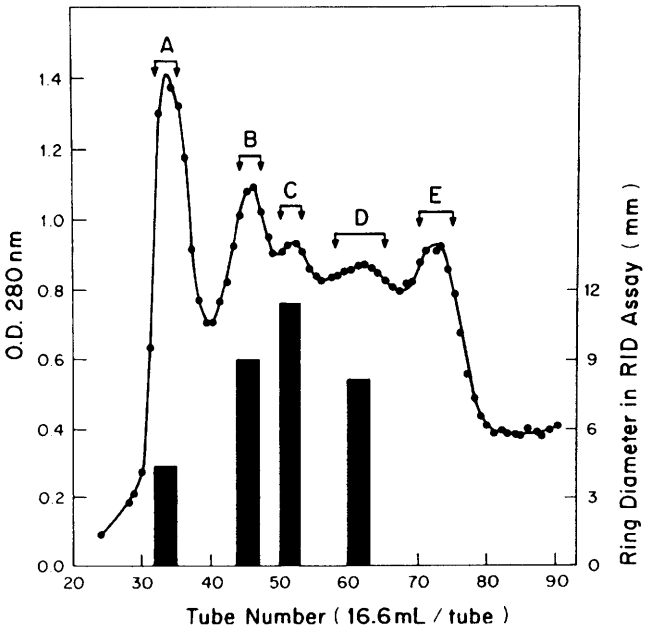

Figure 7. Gel filtration chromatography of pooled normal male Wistar rat urine. Vertical bars under the MW elution profile indicate the immunoreactivity of Fractions A–D in a radial immunodiffusion assay system for rat IgG. In the immunoassay system, a heavy-chain–specific antiserum was used.

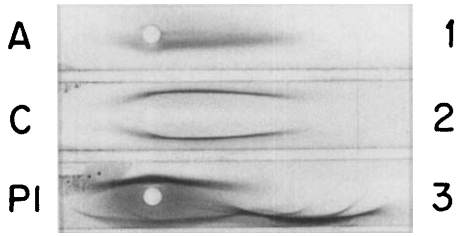

Figure 8. Immunoelectrophoresis of selected column fractions (Fig. 7) and rat plasma. Symbols to the left identify antigens added to the circular wells. Numbers to the right identify antisera added to the narrow, rectangular troughs. Well A, Fraction A; Well C, Fraction C; Well PI, pooled normal male Wistar rat plasma; Troughs 1 and 2, rabbit, anti-rat IgG (heavy-chain-specific); Trough 3, rabbit, anti-rat serum. Cathode (–) is left, anionic molecules such as albumin (pI 4.9) migrate to the right.

matching that of Fc fragment (33). We speculate that in aging rats, glomerulosclerosis occurs secondary to pathologically high levels of circulating Fc fragment. Humans, who have only trace quantities of Fc fragment in their urine, develop the disease on a more sporadic basis.

Toxicity Studies. Our chromate and aminonucleoside toxicity studies show that these nephrotoxins are useful for short-term studies of tubular and glomerular proteinuria. Both toxins produce specific and reversible changes in the renal handling of plasma proteins. Gradient gel electrophoresis is well suited for differentiating between glomerular and tubular proteinuria, and is useful for studying the serial progression of disease in individual rats.

In our aminonucleoside-treated rats, analysis of proteinuria confirms the massive glomerular proteinuria (mostly albumin) known to occur with this toxin. The large increase in IgG excretion we observed was consistent with tracer studies showing that aminonucleoside causes epithelial foot process detachment and leakage of IgG into the urinary space (34). The significant decrease in plasma IgG associated with the heavy urinary losses of IgG supports previous reports that the plasma IgG pool is not defended in nephrotic rats (35), or humans (36). Since we calculate that at the time of peak proteinuria the daily urinary loss of IgG amounted to about 20% of total plasma IgG, this is not surprising.

Why do rats with chromate toxicity recover and retain normal renal function, whereas rats who recover from acute aminonucleoside toxicity progress to recurrent proteinuria and glomerulosclerosis (37)? Chromate, while causing necrosis of the proximal convoluted tubule, does not cause tubular basement membrane disruption or chronic interstitial inflammation (38). By contrast, aminonucleoside induces a nonselective proteinuria and an acute, irreversible, and progressive interstitial inflammation (39). Whereas rats with chromate toxicity are not subject to the noxious effects of excessive macromolecular filtration, aminonucleoside-treated rats clearly are.

Current theory supports the view that increased IgG filtration leads to IgG binding to mesangial cells (40) and proximal tubular cells (41). Chemokines and potent vasoactive substances released by these cells set into motion a variety of responses leading to interstitial inflammation and fibrosis, peritubular vascular compromise, tubular atrophy, glomerulosclerotic changes and nephron obliteration (41). Furthermore, as suggested by Bohle, excessively filtered macromolecules may result in proximal tubular cell and basement membrane injury, as well as incomplete IgG catabolism by overloaded proximal tubular cells. Tubular basement membrane fragments and IgG fragments may then serve as antigens which initiate further interstitial inflammation and fibrosis, changes that correlate best with progressive renal disease (42).

Thus, nonselective proteinuria, and excessive IgG filtration in particular, is "noxious" to the kidney, initiating chronic tubulointerstitial disease processes which gradually lead to loss of glomerular function (41, 43). Clearly, excessive IgG filtration is an abnormal event which paves the way to renal disease. Diseases causing tubular proteinuria or highly selective glomerular proteinuria are more compatible with recovery and maintenance of normal renal function.

Biochemical Studies of Normal Rat Urine. In normal human urine, the ratio between Fc fragment to intact IgG is about 0.06 (23), and the immunoassay of intact urinary IgG using heavy-chain–specific antisera is considered valid (7, 44–46). However, the immunoassay of urinary IgG in the rat is nonspecific for the intact molecule when heavy-chain–specific antisera is used, and IgG is greatly overestimated by the RID assay. In agreement with earlier findings by Gardiner and colleagues (47), we suggest that a large portion of heavy-chain–specific material in normal rat urine is derived from prerenal catabolic fragments of IgG, which are readily filtered. The larger proportion of interfering IgG catabolic products in rat urine is most likely due to the shorter half-life of IgG in the rat (48).

It is therefore not surprising that, in a study measuring urinary IgG excretion in rats using the RID assay (49), the normal value of U_{IgG} *V/GFR* was expressed as <2.3 μg/ml, allowing for the excretion rate of IgG to be calculated as <5 mg/24 hr × 100 g BW. Even though expressed as an upper bound, we believe that this figure is too high and that the RID assay of urinary IgG was the source of error.

The problem with interference and overestimation by low-MW immunoreactive species present in urine which have higher immunodiffusion coefficients relative to intact IgG is well documented (50, 51). As stated by Bell and Chaplin, "The presence in urine of Fc and Fc′ fragments complicates the accurate measurement of IgG." Those authors have shown that the RID assay can be used for the measurement of intact urinary IgG if Fd-specific antisera is used, and intact IgG is used for the standard curve (52). Use of Fd-specific antiserum renders the assay specific for intact IgG because the region of the heavy chain corresponding to the Fd molecule (C-γ-1 domain) is present on intact IgG, but not on Fc fragment, or other naturally occurring catabolic products of IgG (22, 53).

Our immunoelectrophoresis finding that the IgG ex-

creted in urine is more anionic compared with the cationic form of IgG predominating in plasma underscores a problem in macromolecular filtration theory. Current theory holds that IgG is filtered by the large pores of the shunt pathway, and that these size selective pores do not discriminate on the basis of charge. In support of this, Gall *et al.* (54) have found that the ratio between anionic IgG and total IgG in normal human urine (0.07) is the same as that ratio simultaneously measured in serum (0.06). From that data it would appear that anionic and cationic IgG are equally filterable and equally reabsorbable. In other studies (55, 56), values for the ratio between anionic IgG and total IgG in urine have been inconsistent, spanning several orders of magnitude (0.003–1.03).

Supporting our conclusions, Bertolatus *et al.* found the glomerular sieving coefficient of anionic IgG to be four times that of cationic IgG (57). The authors concluded that the shunt pathway "appears to favor anionic molecules." In addition, Makker and Kanalas found that in normal rats, anionic IgG permeated the glomerular basement membrane more readily than did cationic forms (58). Clearly, there is a lack of consistency in the published data concerning the effect of charge in IgG ultrafiltration (59). The question of whether anionic IgG is filtered preferentially compared with cationic forms of the molecule deserves further study.

In summary, one-quarter of male Wistar rats develop glomerulosclerosis during the first year of life. Normal rat urine contains small amounts of intact IgG and much larger amounts of catabolic fragments of IgG. These low-MW prerenal catabolic fragments interfere with the immunoassay of urinary IgG unless specific antisera directed against the Fd portion of the heavy chain are used. For unknown reasons, anionic IgG seems to be preferentially filtered relative to cationic IgG in the rat.

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1. Peterson PA, Evrin RE, Berggard I. Differentiation of glomerular, tubular and normal proteinuria: Determinations of urinary excretion of beta-two microglobulin, albumin and total protein. J Clin Invest **48:**1169–1198, 1969.
2. Mogensen CE, Christiansen CK. Predicting diabetic nephropathy in insulin dependent patients. N Engl J Med **311:**89–93, 1984.
3. Deckert T, Feldt-Rasmussen B, Djurup R, Deckert M. Glomerular size and charge selectivity in insulin-dependent diabetes mellitus. Kidney Int **33:**100–106, 1988.
4. Gambardella S, Morano S, Cancelli A, Pietravalle P, Frontoni S, Bacci S, DiMario U, Andreani D. Urinary IgG4: An additional parameter in characterizing patients with incipient diabetic nephropathy. Diabetes Res **10:**153–157, 1989.
5. Pietravalle P, Morano S, Cristina G, De Rossi MG, Mariani G, Cotroneo P, Ghirlanda G, Clementi A, Andreani D, Di Mario U. Charge selectivity of proteinuria in type 1 diabetes explored by immunoglobulin subclass clearance. Diabetes **40:**1685–1689, 1991.
6. Friedman S, Jones HW, Golbetz HV, Lee JA, Little HL, Myers BD. Mechanisms of proteinuria in diabetic nephropathy. II. A study of the size-selective glomerular filtration barrier. Diabetes **32:**40–46, 1983.
7. Deen WM, Bridges CR, Brenner BM, Myers BD. Heteroporous model of glomerular size-selectivity: Application to normal and nephrotic humans. Am J Physiol **248:**F374–F389, 1985.
8. Tomlanorich S, Deen WM, Myers BD. Functional nature of injury in progressive diabetic glomerulopathy. Diabetes **36:**556–565, 1987.
9. Myers BD, Winetz JA, Chui F, Michaels AF. Mechanisms of proteinuria in diabetic nephropathy: A study of glomerular barrier function. Kidney Int **21:**633–641, 1982.
10. Myers BD, Okarma TB, Friedman S, Bridges C, Ross J, Asseff S, Deen WM. Mechanisms of proteinuria in human glomerulonephritis. J Clin Invest **70:**732–746, 1982.
11. Hasselbacher P. Anionic IgG appears preferentially in human urine. Clin Res **34:**597A, 1986.
12. Nakamura Y, Myers BD. Charge selectivity of proteinuria in diabetic glomerulopathy. Diabetes **37:**1202–1211, 1988.
13. Remuzzi A, Remuzzi G. Glomerular perm-selective function. Kidney Int **45:**198–402, 1994.
14. Van Liew JB, Davis FB, Davis PJ, Noble B, Bernardis LL. Calorie restriction decreases microalbuminuria associated with aging in barrier-raised Fischer 344 rats. Am J Physiol **263:**F554–F561, 1992.
15. Lowry DH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193:**265–275, 1951.
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248–254, 1976.
17. Weichgelbaum TE. An accurate and rapid method for the determination of protein in small amounts of blood, serum, and plasma. Am J Clin Pathol **7**(Tech, Sec 10):40–49, 1946.
18. Hare RS. Endogenous creatinine clearance in serum and urine. Proc Soc Exp Biol Med **74:**148–151, 1950.
19. Sabatino B. The renal handling of plasma proteins and immunoglobulin G in the aging rat and in experimental kidney disease. Ph.D. thesis, State University of New York at Buffalo, Buffalo, NY, 1993.
20. Neuhaus OW, Flory W. Age-dependent changes in the excretion of urinary proteins by the rat. Nephron **22:**570–576, 1978.
21. Turner MW, Rowe DS. A naturally occurring fragment related to the heavy chains of immunoglobulin G in normal human urine. Nature **210:**130–132, 1966.
22. Vaughan J, Jacox RF, Gray BA. Light and heavy chain components of gamma-globulins in urines of normal persons and patients with agammaglobulinemia. J Clin Invest **46:**266–279, 1967.
23. Peterson PA, Berggard I. Urinary immunoglobulin components in normal, tubular, and glomerular proteinuria: Quantities and characteristics of free light chains, IgG, IgA, and Fc gamma fragment. Eur J Clin Invest **1:**255–264, 1971.
24. Muller BR. Identification and incidence of a urinary fragment of IgG. Clin Chim Acta **92:**283–288, 1979.
25. Boylan JW. Boylan's Law. In: Strauss MB, Ed. Familiar Medical Quotations (1st ed). Boston: Little, Brown and Co., p 547, 1968.
26. Berg BN. Longevity studies in rats: II. Pathology of aging rats. In: Cotchin E, Roe FJC, Eds. Pathology of Laboratory Rats and Mice. Philadelphia: Davis Co., pp749–785, 1967.
27. Gray JE, Larsen ER, Greenberg HS. A breeding study to control chronic progressive nephrosis in rats. Lab Anim Sci **32:**609–612, 1982.
28. Goldstein RS, Tarloff JB, Hook JB. Age-related nephropathy in laboratory rats. FASEB J **2:**2241–2251, 1988.
29. Hoyer JR, Vernier RL, Najarian JS, Raij L, Simmons RL, Michael AF. Recurrence of idiopathic nephrotic syndrome after renal transplantation. Lancet **2:**343–348, 1972.
30. Lesavre P, Grunfeld JP. Idiopathic focal segmental glomerulosclerosis—New lessons from kidney transplantation. N Engl J Med **334:**914–915, 1996.
31. White HR, Mills RJ, Beetham R, Raine DN. The significance of variation in the selectivity of proteinuria. Clin Nephrol **3:**42–47, 1973.

32. Hardwicke J, White HR, Williams A. Molecular size of IgG in patients with focal glomerulosclerosis. Clin Nephrol **6:**290–294, 1976.
33. Savin VJ, Sharma R, Sharma M, McCarthy ET, Swan SK, Ellis E, Lovell H, Warady B, Gunwar S, Chonko AM, Artero M, Vincenti F. Circulating factor associated with increased glomerular permeability to albumin in recurrent focal segmental glomerulosclerosis. N Engl J Med **334:**878–883, 1996.
34. Ryan GB, Hein SJ, Karnovsky MJ. The distribution of albumin and immunoglobulin G in the glomerular capillary wall in aminonucleoside nephrosis. Pathology **10:**335–341, 1978.
35. Al-Bander HA, Martin VI, Kaysen GA. Plasma IgG pool is not defended from urinary loss in nephrotic syndrome. Am J Physiol **262:**F333–F337, 1992.
36. Ting RH, Kristal B, Myers BD. The biophysical basis of hypofiltration in nephrotic humans with membraneous nephropathy. Kidney Int **45:**390–397, 1994.
37. Anderson S, Diamond JR, Karnovsky MJ, Brenner BM. Mechanisms underlying transition from acute glomerular injury to late glomerulosclerosis in a rat model of nephrotic syndrome. J Clin Invest **82:**1757–1768, 1988.
38. Biber TUL, Mylie M, Baines AD, Gottschalk CW, Oliver JR, MacDowell MC. A study by micropuncture and microdissection of acute renal damage in rats. Am J Med **44:**664–705, 1968.
39. Diamond JR, Anderson S. Irreversible tubulointerstitial damage associated with chronic aminonucleoside nephrosis. Am J Pathol **137:**1323–1332, 1990.
40. Schlondorff D. Chemokines and renal disease. Kidney Int **51:**610–621, 1997.
41. Remuzzi G, Ruggennenti P, Benigni A. Understanding the nature of renal disease progression. Kidney Int **51:**2–15, 1997.
42. Bohle A, Wehrmann M, Bogenschutz O, Batz C, Muller CA, Muller GA. The pathogenesis of chronic renal failure in diabetic nephropathy. Pathol Res Pract **187:**251–259, 1991.
43. Ong ACM, Fine LG. Loss of glomerular function and tubulointerstitial fibrosis: Cause or effect? Kidney Int **45:**345–351, 1994.
44. Carrie BJ, Myers BD. Proteinuria and functional characteristics of the glomerular barrier in diabetic nephropathy. Kidney Int **17:**669–676, 1980.
45. Dimario U, Morano S, Mancuso M, Pietravalle P, Gambardella S, Andreani D. Urinary immunoglobulin G: A routine and highly sensitive technique based on competative binding. J Clin Lab Immunol **20:**155–158, 1986.
46. Fomsgaard A, Feldt-Rasmussen B. Micro-ELISA for the quantitation of human urinary IgG. Scand J Clin Lab Invest **47:**195–198, 1987.
47. Gardiner ME, Finley-Jones JM, Morgan EH. Catabolism and urinary excretion of albumin and transferrin before and after intravenous injection of albumin in the rat: With observations on the urinary excretion of IgG globulin. Biochem Med **8:**287–298, 1973.
48. Arend WP, Silverblatt FJ. Serum disappearance and catabolism of homologous immunoglobulin fragment in rats. Clin Exp Immunol **22:**502–513, 1975.
49. Bridges CR, Rennke HG, Deen WM, Troy JL, Brenner BM. Reversible Hexadimethrine-induced alterations in glomerular structure and permeability. J Am Soc Nephrol **1:**1095–1108, 1991.
50. Fahey JL, McKelvey EM. Quantitative determination of serum immunoglobulins in antibody agar plates. J Immunol **94:**84–90, 1965.
51. Delacroix DL, Dehennin JP, Vaerman JP. Influence of molecular size of IgA on its immunoassay by various techniques. II. Solid-phase radioimmunoassays. J Immunol Methods **48:**327–337, 1982.
52. Bell CE, Chaplin H. Application of a modified technique to study urinary light chain and whole IgG excretion in normal human subjects and patients with immune-related disorders. J Lab Clin Med **75:**636–653, 1970.
53. Beale D. Molecular fragmentation, some aspects of immunology. Devel Comp Immunol **11:**287–296, 1987.
54. Gall MA, Rossing P, Kofoed-Enevoldsen A, Nielsen FS, Parving HH. Glomerular size- and charge selectivity in type 2 (non-insulin dependent) diabetic patients with diabetic nephropathy. Diabetologica **37:**195–201, 1994.
55. DiMario U, Bacci S, Morano S, Pugliese G, Pietravalle P, Andreani D, Morabito S, Simonetti B, Pierucci A. Selective decrement of anionic immunoglobulin clearance after induced renal hemodynamic changes in diabetic patients. Am J Physiol **262:**F381–F388, 1992.
56. Deckert T, Kofoed-Enevoldsen A, Vidal P, Norgaard K, Andreasen HB, Feldt-Rasmussen B. Size and charge selectivity of glomerular filtration in type 1 (insulin-dependent) diabetic patients with and without albuminuria. Diabetologica **36:**244–251, 1993.
57. Bertolatus JA, Abuyousef M, Hunsicker LG. Glomerular sieving of high molecular weight proteins in proteinuric rats. Kidney Int **31:**1257–1266, 1987.
58. Makker SP, Kanalas JJ. Permeation of endogenous IgG with an anionic subpopulation into glomerular basement membrane in rat. Proc Soc Exp Biol Med **183:**382–391, 1986.
59. Comper WD, Glasgow EF. Charge selectivity in kidney ultrafiltration. Kidney Int **47:**1242–1251, 1995.