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Low-protein diet prevents glomerular damage in adriamycin-treated rats

GIUSEPPE REMUZZI, CARLA ZOJA, ANDREA REMUZZI, MAGDA ROSSINI, CRISTINA BATTAGLIA, MASSIMO BROGGINI, and TULLIO BERTANI

Mario Negri Institute for Pharmacological Research, Bergamo and Milano, Italy

Low-protein diet prevents glomerular damage in adriamycin-treated rats. Adriamycin (ADR) induces glomerular damage in rats with persistent proteinuria which develops 13 to 15 days after a single intravenous (i.v.) injection (5 mg/kg). Electron microscopy (EM) shows alterations of glomerular visceral epithelial cells with foot process fusions. The disease resembles minimal change nephropathy in humans. We studied the effect of two isocaloric diets with different protein content on urinary protein excretion, renal function, and glomerular morphology in rats treated with ADR. Six groups of rats were used. Group 1 received a single i.v. injection of ADR and was fed a standard diet containing 20% protein. Group 2 was fed a low-protein diet containing 6% protein starting 7 days before ADR. Group 3 was fed a low-protein diet starting the day after ADR. Group 4 served as control. Two additional groups of rats (5 and 6) were used to study the kidney distribution of ADR.

Unlike animals fed the standard diet, animals fed the low-protein diet did not develop proteinuria. The kidney distribution of ADR measured at different intervals after drug injection was not influenced by the diet. Renal function as determined by glomerular filtration rate (GFR) and renal plasma flow (RPF) was not significantly modified in nephrotic rats receiving the standard diet compared to control animals. The low-protein regimen induced a significant elevation in RPF compared to the standard diet, but had no influence on GFR.

Light and transmission EM studies showed alterations of glomerular visceral epithelial cells with fusion of foot processes in rats fed the standard diet, whereas no significant abnormalities of glomerular epithelial cells were detectable in animals receiving the low-protein diet.

We conclude that the low-protein diet prevents the development of ADR glomerulopathy. The effect of the low-protein diet does not appear to be the consequence of changes in renal blood flow (RBF) and GFR, but rather reflects preservation of the permeability properties of the filtering membrane.

Un régime pauvre en protides prévient les lésions glomérulaires chez des rats traités par l'adriamycine. L'adriamycine (ADR) induit des lésions glomérulaires chez des rats avec une protéinurie persistante qui se développe 13 à 15 jours après une injection intraveineuse (i.v.) unique (5 mg/kg). La microscopie électronique (EM) révèle des altérations des cellules épithéliales viscérales glomérulaires avec fusion des pédicelles. Cette maladie ressemble à la néphropathie à lésions glomérulaires minimes humaine. Nous avons étudié l'effet de deux régimes isocaloriques de contenu protidique différent sur l'excrétion urinaire de protéines, la fonction rénale, et la morphologie glomérulaire chez des rats traités par ADR. Six groupes de rats ont été utilisés. Le groupe 1 a reçu une injection i.v. unique de l'ADR et a été nourri avec un régime standard contenant 20% de protides. Le groupe 2 recevait un régime pauvre en protides contenant 6% de protéines commencant 7 jours avant ADR. Le groupe 3 recevait un régime pauvre en protides débutant le jour d'après ADR. Le groupe 4 servait de contrôle. Deux groupes supplémentaires de rats (5 et 6) ont été utilisés pour étudier la distribution rénale d'ADR.

A la différence des rats nourris avec le régime standard, les animaux recevant le régime pauvre en protides n'ont pas développé de protéinurie. La distribution rénale d'ADR mesurée à différents intervalles après l'injection du médicament n'était pas influencée par le régime. La fonction rénale mesurée par le débit de filtration glomérulaire (GFR) et le débit plasmatique rénal (RPF) n'était pas significativement modifiée chez les rats néphrotiques recevant le régime standard par rapport aux animaux contrôles. Le régime pauvre en protides a induit une élévation significative de RPF par rapport au régime standard mais n'a pas influencé GFR.

Des études d'EM optique et à transmission ont montré des altérations des cellules de l'épithélium viscéral glomérulaire avec fusion des pédicelles chez les rats au régime standard, alors qu'aucune anomalie significative des cellules épithéliales glomérulaires n'était détectable chez les animaux recevant le régime pauvre en protides.

Nous concluons qu'un régime pauvre en protides prévient le développement de la glomérulopahie à l'ADR. L'effet du régime pauvre en protides ne semble pas être la conséquence de changements du débit sanguin rénal (RBF) ni de GFR, mais reflète plutôt le préservation des propriétés de perméabilité de la membrane de filtration.

It has been proven that dietary manipulation has a profound influence on renal diseases in experimental animals [1–5] and humans [6]. Urinary protein excretion increases in rats when reduction of renal mass is associated with high protein intake [7]. On the other hand, protein restriction reduces glomerular injury in experimental nephrotoxic serum nephritis [2] and in the NZB × NZW mouse, an animal model of lupus erythematosus [4]. Moreover, proteinuria is reduced in the ablation model [8] when protein intake is limited. According to a recent theory, the beneficial effects of low-protein diet on the renal diseases studied must be attributed to a reduction in protein intake that limits the hemodynamic changes occurring in survival nephrons of kidney subjected to various types of experimental injury [8–10]. In fact, at variance to animals receiving a protein supplementation, in animals fed a low-protein diet glomerular capillary plasma flow rates and transcapillary hydraulic pressure gradients remain normal despite extensive renal ablation [8, 10]. It is, therefore, conceivable that hemodynamic factors are responsible for the reduced urinary protein excretion observed in this as well as in other experi-

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mental conditions in which the protein restriction has been found to be beneficial consistently.

We investigated the effect of low-protein diet in another model of renal injury that mimics a human disease closely. We have studied the model of adriamycin-induced (ADR) glomerulopathy in rats [11]. ADR given to animals as a single intravenous (i.v.) injection induces a glomerulopathy characterized by heavy and persistent proteinuria. The abnormal protein excretion begins 4 to 5 days after the ADR injection and the syndrome develops fully 13 to 15 days later. Minimal alterations at light microscopy (LM), negative immunofluorescence, and some focal fusion of foot processes can be detected in the early phase of the disease, but by 12 to 14 days profound alterations of glomerular visceral epithelial cells with extensive foot process fusions are found invariably. The clinical and morphological expression of the disease resembles minimal change nephropathy in humans.

Using this model we have evaluated the effect of dietary manipulation on protein excretion, glomerular filtration rate (GFR), renal plasma flow (RPF), and glomerular morphology. Our results show that a low-protein diet protects animals from the development of glomerular damage.

Methods

Animal studies

Sprague-Dawley CD-COBS male rats (Charles River Breeding Laboratories Inc., Wilmington, Massachusetts, USA) weighing 185 to 200 g were used. Nephrotic syndrome was induced by a single i.v. injection of ADR (Adriblastina, Farmitalia Carlo Erba, Milan, Italy), 5 mg/kg through the tail vein of non-anesthetized animals according to a method described previously [11]. Control rats received the solvent alone. Animals were divided into 6 groups. In group 1, 10 rats received a single injection of ADR and were fed a standard diet (Altromin-Rieper, Vandoies, Italy) containing 20% protein. In group 2, 10 rats were fed a low-protein diet containing 6% protein. This diet was provided for 7 days before i.v. ADR and continued throughout the experimental period. In group 3, 5 rats were fed a low-protein diet containing 6% protein for the same period, starting the day after ADR injection. In group 4, 5 rats were used as control and were fed the same diet as group 1. Two additional groups of 15 animals (groups 5 and 6) were used to study kidney distribution of ADR as described below.

Diets

Our study compared the effect of two diets differing in their protein content, 6% of weight for the low-protein diet and 20% for the standard diet, according to the nitrogen assay. Other calories were supplied by corn starch, saccharose, and soy oil (see Table 1 for details). The two diets were isocaloric (3300 kcal/kg) so that proteins provided 6 and 20% of total calories. Mineral and vitamin supplements were equal in the two diets (3.5 g sodium, 9.5 g calcium, 6.5 g phosphorus, 2.3 g potassium, 0.5 g magnesium, 15,000 U vitamin A, and 500 U vitamin D per kg). Both diets were dry (less than 4% water), had a pasty consistency, and were distributed in cups fitted with a perforated, movable lid to prevent spillage. All rats had free access to tap water.

Table 1. Composition of diets

	6% Protein	20% Protein
	g/kg	
Casein	70	220
Corn starch	660	510
Saccharose	100	100
Cellulose	40	40
Soy oil	50	50
Mineral mix	58	58
Trace elements	2	2
Vitamin mix	20	20

Renal distribution of ADR

Two groups of rats were used. Group 5 was fed the standard diet and group 6 the low-protein diet, starting 7 days before the experiment. Rats of both groups were injected i.v. with 5 mg/kg of ADR. Three animals per point of each group were killed 5, 15, 30, 120 and 360 min after ADR injection. Kidneys were removed and frozen immediately at −20°C until analysis. Tissue samples were assayed as described previously [12]. Kidney levels of ADR were quantified by high performance liquid chromatography (HPLC) (Series 3–B, Perkin Elmer, Norwalk, Connecticut, USA) with fluorescence detection, Perkin Elmer 650/10) according to a method described previously [12].

Renal clearance studies

The effect of dietary manipulation in ADR nephrosis on renal hemodynamics (GFR and RPF) was studied by inulin and p-aminohippuric acid (PAH) clearance methods. Rats were anesthetized by intraperitoneal (i.p.) injection of Inactin, 16 mg/100 g body wt, tracheotomized, and placed on a constant temperature table at 37°C. Two hundred mg PAH (Sigma Chemical Co., St. Louis, Missouri, USA), 5 g inulin (E. Merck, Darmstadt, Federal Republic of Germany), and 900 mg NaCl were dissolved in 100 ml distilled water. Thus, the final solution contained 0.2% PAH and 5% inulin. As a priming load, 2.5 ml of this solution was infused in the internal jugular vein, followed by constant infusion of the same solution via a syringe pump at 2.7 ml/hr. Before starting to measure clearances, a 60-min period of constant infusion was allowed in order to attain constant plasma levels of inulin and PAH. 100 μl blood samples were obtained from the femoral artery at the mid-point of each clearance period. Urine was collected in preweighed vessels via a large-bore polyethylene tube inserted in the bladder via a small incision at the fundus. Inulin and PAH concentrations in plasma and urine were determined by methods described previously [13, 14], modified for microliter samples. During the experiments, arterial blood pressure was monitored constantly from a polyethylene tube (PE-50) in the femoral artery, connected to a pressure transducer and a carrier amplifier (Battaglia Rangoni, Bologna, Italy). Each experiment involved three clearance periods, each lasting 40 min.

Histological studies

Renal tissue specimens were obtained from kidney biopsies. For light microscopy (LM), fragments of the cortex were fixed in Dubosq-Brazil fluid (80% alcohol, 150 ml; formol, 60 ml; acetic acid, 15 ml; picric acid, 1 g) and embedded in paraffin.

Table 2. Urinary protein excretion in ADR-treated and control rats[a]

		Day after ADR		
		14	21	28
Group	*N*	Protein excretion *mg/day*		
1	10	199 ± 61	398 ± 112	607 ± 136
2	10	6 ± 2	6 ± 2	8 ± 3
3	5	5 ± 3	6 ± 4	7 ± 4
4	5	6 ± 4	8 ± 4	8 ± 5

Abbreviations: *N*, number.
[a] Results are expressed as mean ± SD.
Groups 2, 3, 4 = $P < 0.001$ for each determination (Duncan's multiple range test) compared to Group 1.

Table 3. Urine volume of ADR-treated and control rats[a]

		Day after ADR		
		14	21	28
Group	*N*	Diuresis *ml/day*		
1	10	16 ± 4	21 ± 8	20 ± 4
2	10	15 ± 7	17 ± 6	17 ± 9
3	5	15 ± 7	18 ± 6	18 ± 6
4	5	19 ± 3	21 ± 4	19 ± 3

[a] Results are expressed as mean ± SD.
P = NS for all groups of ADR-treated animals (Duncan's multiple range test) compared to Group 4.

Table 4. Kidney levels of ADR at different times after injection in rats on a standard and low-protein diet[a]

		Minutes after injection				
		5	15	30	120	360
Group	*N*	Concentration *μg/g tissue*				
5	15	41.4 ± 1.6	31.7 ± 1.4	18.3 ± 0.6	10.1 ± 0.7	7.4 ± 0.2
6	15	41.0 ± 3.2	45.9 ± 6.8	16.7 ± 0.7	12.7 ± 2.4	6.5 ± 1.0

[a] Values are mean ± SE.

Table 5. GFR and RPF in ADR-treated and control rats[a] 28 days after ADR injection

Group	*N*	GFR *ml/min/100 g*	RPF *ml/min/100 g*	FF *%*
1	10	0.76 ± 0.24	2.14 ± 0.37	36.0 ± 13.7
2	10	0.95 ± 0.21	3.82 ± 0.75[b]	26.0 ± 9.1
4	10	0.86 ± 0.16	2.57 ± 0.43	34.0 ± 6.1

Abbreviations: GFR, glomerular filtration rate; RPF, renal plasma flow; FF, filtration fraction.
[a] Results are expressed as mean ± SD.
[b] Values of Group 2 compared with Group 1, or 4 $P < 0.01$ (Duncan's multiple range test).

Sections of 2 μ were stained with Masson's trichrome, hematoxylin and eosin, periodic acid staining (periodic acid-Schiff), and Wilder's reticulin.

For electron microscopy (EM), small pieces of the cortex were fixed with phosphate-buffered 2.5% glutaraldehyde (pH 7.2) for 6 hr, then rinsed in 0.2 M cacodylate buffer (pH 7.4). Subsequently, the samples were postfixed in 1% osmium tetroxide at 4°C for 1 hr, washed in buffer, and immersed in 0.5% Veronal uranyl acetate. Then samples were dehydrated through graded alcohol and embedded in Spurr resin. Ultrathin sections (60 to 80 nm) were cut on a Reichert OmU–2 ultramicrotome (Reichert, Vienna, Austria) and examined with a Zeiss EM 109 (Carl Zeiss, Oberkochen, Federal Republic of Germany).

Other investigations

Serum was obtained after clotting (at 37°C for 30 min) of native blood collected by intracardiac puncture from ether-anesthetized animals. Total serum proteins were measured according to the method of Lowry et al [15] with bovine serum albumin (BSA) as a standard. Serum creatinine was determined by the method of Hare [16] using a Beckman analyzer (Astra 4 model, Beckman Instruments Inc., Fullerton, California, USA). Urines were collected using metabolic cages over a 24-hr period and protein was determined by the sulfosalicylic acid method. Results were analyzed by unpaired Student's *t* test and Duncan's multiple range test.

Results

Time course of proteinuria

ADR-treated animals on the standard diet, group 1, developed the glomerular disease. Proteinuria started 5 to 7 days after the i.v. injection. All animals were heavily proteinuric (398 ± 112 mg/day) at day 21 and the abnormal protein excretion persisted throughout the experimental period (Table 2). In contrast, animals fed the low-protein diet, groups 2 and 3, developed no glomerular damage. Protein excretion was within the normal range (8 ± 5 mg/day) throughout the experimental period. This was true when the low-protein diet was started 7 days before ADR injection and when it started the day after ADR. Urine volume was not significantly different in ADR treated animals and controls (Table 3).

Pharmacological studies

These studies were designed to assess whether the protective effect of a low-protein diet was due to different tissue exposure of ADR. For this purpose we followed ADR concentrations in the kidney at different intervals after drug injection in rats fed a standard or a low-protein diet. As shown in Table 4, initial kidney distribution and the subsequent disposal of ADR in rats fed the standard diet were similar to that of rats on the low-protein diet. Thus, the difference in protein content of the diet does not influence the exposure of kidneys to ADR.

Clearance studies

In animals of group 1 and 2, GFR, as measured by inulin clearance, was the same as in the control group (Table 5). An increase in RPF, as measured by PAH clearance, was observed in ADR-treated rats on the low-protein diet, compared to controls ($P < 0.01$). The filtration fraction calculated from GFR

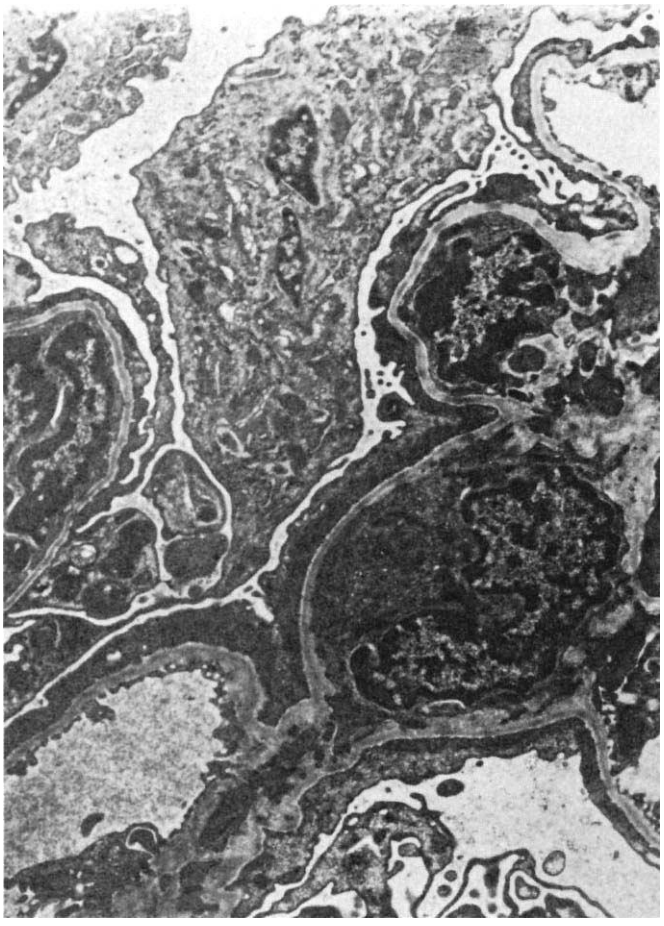

Fig. 1. *Electron micrograph of glomerular capillaries in an adriamycin-treated rat fed a standard diet.* Note severe changes of glomerular visceral epithelial cells showing widespread fusion of foot processes (×7000)..

although it was slightly higher in group 1 than in controls and slightly lower in group 2. These differences were not statistically significant.

Morphological studies

The kidneys of all animals were removed at the end of experiments for LM and EM examination. The usual epithelial cell abnormalities [11] were found in the rats fed the standard diet (group 1). At LM glomeruli displayed a marked swelling of podocytes, not associated with other relevant glomerular lesions. Some protein droplets were seen in the cytoplasm of proximal tubules and a few eosinophilic casts were present in the lumen of distal tubules. Interstitium, arteries, and arterioles were unremarkable. EM findings (Fig. 1) consisted of the typical changes to glomerular visceral epithelial cells, including fusions of foot processes, cytoplasmic blebs, protein droplets, and villous transformation.

In contrast, in both groups of animals fed the low-protein diet, no alterations in the kidney specimens were found by LM, and glomerular and tubular ultrastructure appeared normal in all specimens examined by EM (Fig. 2).

Other investigations

Serum protein and serum creatinine, measured at the end of the experimental period in the three groups of ADR-treated rats, were comparable to the values found in the control group (Table 6). Our data indicate that ADR-treated rats assigned 6 or 20% protein diets ingested similar amounts of food (20.4 ± 3.5 vs. 21.3 ± 3.8 g/day).

Discussion

In rats with severe reduction of renal mass, progressive glomerular damage develops, characterized by changes in glomerular hemodynamics and severe proteinuria, that leads ultimately to focal sclerosis [8, 17]. Low-protein feeding has been found to protect against the development of proteinuria [8, 10]. In another model of glomerular damage, "desoxycorticosterone acetate-salt" hypertension, protein restriction has been shown to limit glomerular hemodynamic changes and to relieve proteinuria [18]. Much evidence indicates that the restriction of dietary protein exerts a favorable effect in other animal models of glomerular injury [2, 4, 8], that are closer to human glomerulopathies.

We report now that dietary protein restriction protects animals from the development of glomerular damage induced by ADR. In the glomerular disease that develops in rats after a single i.v. injection of ADR [11], we found that animals fed a low-protein diet had negligible proteinuria in comparison to rats fed a standard diet. In order to determine if the protective effect of the low-protein diet in ADR nephrosis reflected a reduced delivery of the drug to the kidney, we performed two distinct experiments. First, we measured proteinuria in rats starting the low-protein regimen one day after i.v. ADR. Our preliminary data have demonstrated that the toxic effect of ADR on the kidney glomerular structures requires only a few minutes exposure to the drug when animals are fed a normal diet (unpublished observation). Since animals starting the low-protein regimen the day after ADR had exactly the same degree of protection as animals starting the diet one week before ADR injection, the diet does not appear to influence the tissue disposition of ADR but interferes directly with the mechanisms responsible for proteinuria. Moreover, evidence that the kidneys of rats fed a low-protein or standard diet are exposed to the same level of ADR is concluded by comparing direct measurements of the drug concentration in renal tissue. However, it must be pointed out that equal concentrations in a complex organ such as the kidney do not mean equal regional or subcellular concentrations of ADR in the two experimental conditions.

The mechanism by which a low-protein diet protects against proteinuria remains speculative. GFR and RPF were not significantly modified in nephrotic rats receiving a standard diet, compared to control animals. This finding is in agreement essentially with the findings of Weening and Rennke [19] that renal function, determined from GFR and RPF, was affected only slightly by ADR. In our experimental condition the low-protein regimen induced a significant elevation in RPF compared to the standard diet, but had no influence on GFR. These results indicate that the protective effect of low-protein diet cannot be attributed to a reduction in GFR. On the other hand,

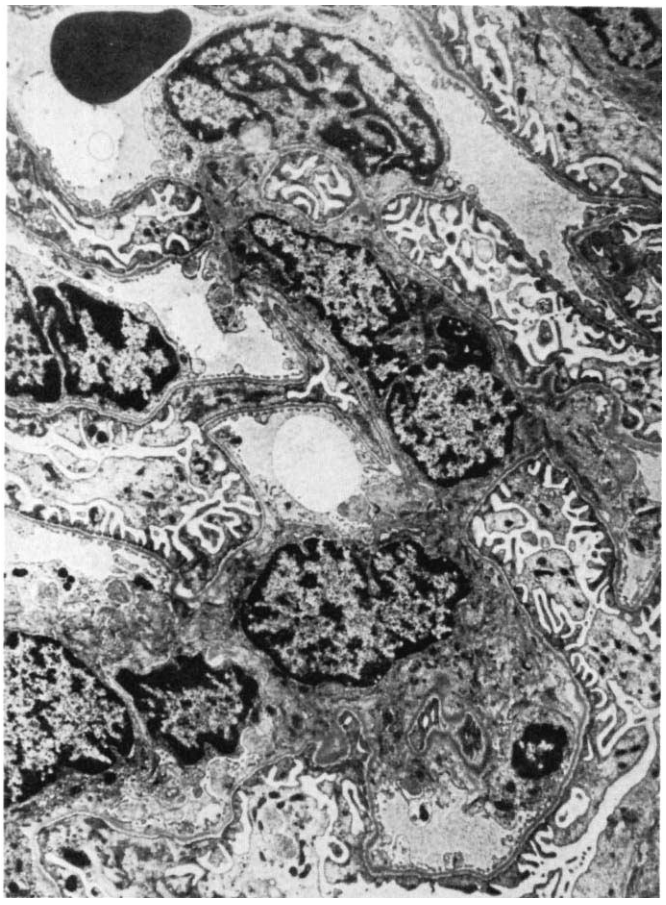

Fig. 2. *Electron micrograph of glomerular capillaries in adriamycin-treated rats fed a low-protein diet.* Note the absence of significant changes of glomerular visceral epithelial cells. In particular, note the normal arrangement of interdigitating foot processes (×4000).

Table 6. Total serum proteins and serum creatinine in control and treated rats[a]

Group	*N*	Total proteins *g/dl*	Serum creatinine *mg/dl*
1	10	6.7 ± 1	0.81 ± 0.14
2	10	6.5 ± 0.8	0.71 ± 0.07
3	5	6.4 ± 0.9	0.72 ± 0.06
4	5	7.0 ± 0.5	0.71 ± 0.06

[a] Results are expressed as mean ± SD.

the unexpected finding that low-protein diet is associated with a significant increase in RPF deserves some comment.

It has been recognized that glomerular filtration of plasma proteins is governed by the permeability properties of the glomerular membrane and depends also on the hemodynamic conditions through the convective and diffusive transport of macromolecules across the glomerular capillary wall [20]. Since dietary manipulation in our experimental conditions was associated with changes in RPF we wondered whether these changes could be responsible for the anti-proteinuric effect of protein restriction. According to the model of glomerular ultrafiltration proposed by Deen, Robertson and Brenner [21], an increase in RPF without change either in GFR and in afferent arterial plasma protein concentration (Ca) would result in a decreased protein concentration along the capillary bed. The theoretical plasma protein concentration in the efferent arteriole (Ce) can be calculated by:

$$Ce = \frac{Ca}{(1 - FF)}$$

Using the values of Ca and FF reported in Tables 5 and 6, we estimated that the low-protein regimen was associated with a decrease in Ce from 10.4 to 8.8 g/dl. We wondered whether the related difference in protein concentration along the glomerular capillary might influence the passage of protein through the membrane. To address the issue we followed the scheme used by Vehaskari et al [22], considering the general flux equation of Kedem and Katchalsky as modified for macromolecular filtration by Chang et al [23]. In any point of an idealized capillary bed, the solute flux (J_s) can be expressed as:

$$J_s = \omega R T \Delta C_s + J_v (1 - \sigma) \overline{C}_s$$

where J_v is the volume flux; ω, solute permeability parameter; R, universal gas constant; T, absolute temperature; ΔC_s, transcapillary solute concentration difference; σ, reflection coefficient; $\overline{C}_s$, logarithmic mean solute concentration at any point along idealized glomerular capillary. It derives that the solute flux is affected by the filtering membrane properties, such as permeability and reflection coefficient, by the protein concentration along the glomerular capillaries and by the volume flux. According to this equation, the flux of proteins through the glomerular membrane is decreased by a decreased protein concentration along the capillary bed. However, our data indicate a dramatic decrease in proteinuria that is unlikely to be justified by the slight decrease in the protein concentration along the glomerular capillary bed as shown by the calculated decrease in Ce. Therefore, because the observed hemodynamic changes can only offer a partial explanation for the protective effects of low-protein diet in our model, this can be explained better by a preservation in the permeability and reflection coefficient of the membrane. In this context it has been demonstrated previously that ADR exerts its toxic effect through a direct injury on the glomerular structure, probably on glomerular podocytes [11, 19].

Morphologically, our light and transmission electron microscopic studies showed that low-protein feeding prevented also the visceral epithelial cell abnormality that is a constant finding in rats treated with the same dose of ADR but fed a standard diet. These findings might have different implications. The glomerular capillary normally impedes filtration of circulating macromolecules, acting as a size- and charge-selective barrier [24]. It is conceivable that ADR induces proteinuria, altering one or both these membrane filtering properties. Relevant are the observations, in human disease as well as in experimental glomerulopathies such as puromycin aminonucleoside (PA) nephrosis, ablation of renal mass, and perfusion of polycations, that the loss of electrostatic glomerular barrier is followed by an increased transit of circulating anionic macromolecules in the urinary space [8, 25–30].

In a previous study we have shown that glomerular

sialoproteins, as evaluated by LM, were reduced markedly in ADR-treated rats [11]. This finding suggested that the loss of negative charges may play a role in the pathogenetic cascade leading to proteinuria in the ADR model. However, Weening and Rennke [19], using the fractional clearances of anionic, native and cationic horseradish peroxidase, found that the charge barrier is intact in ADR nephrosis. These data have been challenged by the recent studies of Bertolatus and Hunsicker [31] who, using the fractional clearances of ^{131}I-labeled native and cationic BSA, found a marked increase in the native BSA, suggesting a loss of the charge-dependent permselectivity. The problem of size selectivity has been studied less extensively in ADR nephrosis, as compared to the other experimental models of proteinuria. The results of Weening and Rennke [19] show an increase in the fractional clearance of dextrans with molecular radius greater than 50 Å. These findings indicate strongly that the size-selective barrier of glomerular capillary walls is defective in ADR nephrosis. All together these results prompt one to consider a defect in size-selectivity as the most likely explanation for ADR-induced urinary protein loss in animals fed a standard diet. However, the possible role of an additional defect in charge-selectivity cannot be ruled out completely on the basis of the available evidence.

We conclude that low-protein intake protects animals against ADR glomerulopathy, the protective effect of a low-protein diet does not result from a different exposure of the kidneys to ADR, and variations in GFR or blood flow do not appear to be responsible for the reduced amount of protein excreted in the urine of animals fed a low-protein diet.

Whether low-protein diet prevents glomerular damage and proteinuria, interfering with one or both properties of the filtering membrane, or whether other factors that are not yet defined contribute to the beneficial effects of the diet, cannot be established by the present study.

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Reprint requests to Dr. G. Remuzzi, Mario Negri Institute for Pharmacological Research, Via Gavazzeni, 11, 24100 Bergamo, Italy.

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