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Tubulo-interstitial lesions mediate renal damage in adriamycin glomerulopathy

TULLIO BERTANI, FRANCA CUTILLO, CARLA ZOJA, MASSIMO BROGGINI, and GIUSEPPE REMUZZI

Mario Negri Institute for Pharmacological Research, Via Gavazzeni 11, 24100 Bergamo, Italy, and Mario Negri Institute for Pharmacological Research, Via Eritrea 62, 20157 Milano, Italy

Tubulo-interstitial lesions mediate renal damage in adriamycin glomerulopathy. The present study was designed to investigate the relationship between proteinuria, focal sclerosis, and tubulo-interstitial changes in the evolution of renal damage in experimental nephrosis. We utilized an accelerated unilateral model of adriamycin (ADR) nephrosis characterized by morphological changes more severe than in the classical model. The first events in ADR-induced glomerulopathy were epithelial cell damage and proteinuria. Subsequently, tubular casts were formed at the distal level. The cast formation preceded the development of interstitial damage, which was determined by tubular obstruction and breaking of tubular basement membrane (TBM), which in turn promoted an interstitial inflammatory reaction. Despite the severity of tubulo-interstitial damage observed after a long period of heavy proteinuria, the incidence of focal segmental glomerulosclerosis (FSG) was very low. The results of the present study indicate that chronic proteinuria is not necessarily accompanied by the development of focal sclerosis. Tubulo-interstitial lesions appear to be the most important determinant for the progression of renal damage in this model.

A single injection of 5 mg/kg of adriamycin (ADR) promotes in rat a state of heavy and persistent proteinuria [1]. Proteinuria appears a few days after the injection of the drug and persists for several months. In a previous study [2], we have shown that a six–month period of high urinary protein excretion in 60% of animals induced the development of focal and segmental glomerulosclerosis (FSG). The focal and segmental changes were associated with pronounced tubulo-interstitial changes consisting of the presence of large tubular casts, tubular dilatation, interstitial fibrosis and inflammation. However, in the remaining animals FSG was not detected and tubulo-interstitial changes were rather mild. After nine months of sustained urinary protein excretion, all rats developed FSG of moderate severity, once again associated with severe tubulo-interstitial changes and with a moderate degree of renal insufficiency [2]. The findings of our previous study suggested a possible linkage between glomerulosclerosis and tubulo-interstitial changes, however the temporal sequence of events and the dynamic process leading to tubulo-interstitial changes were not fully clarified.

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The present investigation was designed to examine the relationship between proteinuria, focal sclerosis, and tubulo-interstitial changes in ADR nephrosis; and, furthermore, to establish which of the above mentioned phenomena plays the most relevant role in the evolution of renal damage in this model. To address this issue we utilized a new unilateral model of ADR nephrosis in which pathological changes, restricted to one kidney alone, were significantly more severe if compared with those observed in the classical model. Proteinuria was very pronounced, the ultrastructural changes of podocytes were dramatic, and after two weeks many proteinaceous casts were detected in the lumens of distal tubules. We defined this model as "accelerated ADR nephrosis". It offered the opportunity to evaluate in a shorter period of time the pathological changes in many aspects similar to those seen after several months of proteinuria in the classical model. The results of these studies demonstrate that in this model: a) proteinuria and FSG are unrelated phenomena; and b) tubular cast formation is the most important determinant in the progression of renal damage.

Methods

Experimental design

The most employed model of ADR nephrosis contemplates a dose of 7.5 mg/kg of the drug [3]. However, recently it has been reported that a single injection of lower doses (5 mg/kg and 3 mg/kg) promotes in rats a chronic proteinuria which persists for several months [1, 2, 4]. Pathological changes, especially with the dose of 3 mg/kg, appear to be less severe. The goal of our experiment was to have a model able to potentiate the lesions induced by a single intravenous injection of 7.5 mg/kg. This could have been achieved by increasing the dose of ADR. However, in our experience the use of 10 mg/kg of ADR leads to a high mortality rate in the animals. Therefore, our efforts were directed to find a way to increase the ADR nephrotoxicity without exposing the animals to an increased risk of general side effects.

The accelerated model of ADR nephrosis was carried out on Sprague–Dawley CD-COBS male rats (Charles River Italia S.p.A., Calco, Italy), weighing 280 to 300 g at the start of the experiment. Animals were fed a standard diet and had free access to water. Under ether anesthesia, the abdominal cavity

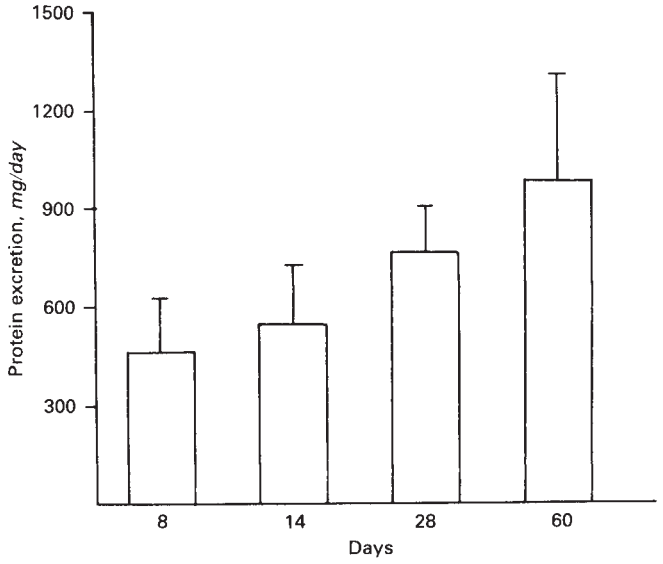

Fig. 1. *Protein excretion in rats with the accelerated model of ADR nephrosis at various intervals after a single i.v. ADR injection.* Results are expressed as means ± SD.

Table 1. Serum creatinine and GFR measured in control rats and in all groups of ADR-treated rats at the end of each experimental period[a]

*Group	*N*	Serum creatinine (mg/dl)	GFR (ml/min/100 g body wt)
1	8	0.76 ± 0.15	0.65 ± 0.27
2	10	0.75 ± 0.08	0.70 ± 0.18
3	10	0.77 ± 0.12	0.69 ± 0.22
Controls	7	0.74 ± 0.07	0.77 ± 0.05

[a] Values are means ± SD.
* Group 1: ADR-treated rats killed on day 14; Group 2: ADR-treated rats killed on day 28; Group 3: ADR-treated rats killed on day 60.

was opened through a midline incision and a clamp was placed on the left renal artery. Immediately after, ADR (Adriblastina, Farmitalia Carlo Erba, Milan, Italy) at the dose of 7.5 mg/kg was injected intravenously. The clamp was removed eight minutes later and the abdominal incision sutured, allowing rats to recover from anesthesia. Animals tolerated the surgery quite well. In selected animals a histological evaluation performed 24 or 48 hours after the drug injection did not reveal any significant tubular damage. The overall mortality during the experimental period did not exceed 25%. Animals were divided into three groups.

Group 1. Sixteen rats underwent the accelerated model of ADR glomerulopathy. Proteinuria was evaluated after 8, 14 and 16 days. On day 14, half the animals were killed and both their kidneys examined by light microscopy (LM), immunofluorescence (IF), and electron microscopy (EM). In the other 50% of rats, the unclamped kidney was removed on day 14 in order to demonstrate the unilaterality of the disease, and on day 16 the animals were killed. The clamped kidney was used for pathological studies.

Group 2. Ten rats underwent the accelerated model of ADR nephrosis. Proteinuria was evaluated after 8, 14 and 28 days. Animals were killed at day 28 and both kidneys were used for pathological studies.

Table 2. Renal distribution of ADR at different times after injection in rats with left clamped kidney[a]

Minutes	Clamped kidney[b]	Unclamped kidney[b]
2	0.73 ± 0.59	58.38 ± 27.76
5	1.26 ± 0.46	97.17 ± 9.53
10	4.14 ± 2.09	115.14 ± 25.07
15	4.51 ± 0.36	77.05 ± 6.28
30	10.50 ± 3.66	72.44 ± 11.85
60	8.90 ± 2.01	48.89 ± 13.32

[a] Values are means ± SD.
[b] μg of ADR per g of tissue.

Group 3. Thirty–two rats underwent the accelerated model of ADR glomerulopathy. Proteinuria was evaluated after 8, 14, 28 and 60 days. A bilateral renal biopsy was performed at day 14.

On day 60, all animals were killed and both kidneys removed for histological examination. An additional group (*Group 4*) was used to study kidney distribution of ADR as described below.

Renal distribution of ADR

Twenty-four rats were treated in the same way of previous groups up to the clamp removal, eight minutes after the end of ADR injection. Four animals per point were killed 2, 5, 10, 15, 30, and 60 minutes after ADR injection. Left and right kidneys were removed separately and immediately frozen at −20°C until analysis. Tissue samples were assayed as previously described [5, 6]. Kidneys were homogenized in five volumes of water. To 0.5 ml of homogenate, 50 μl of a solution (20 ml) containing ADR as internal standard and 30 μl of 33% $AgNO_3$ were added. The samples were then extracted with 8 ml of isopropanol. The organic phase was evaporated under vacuum. Dried samples were redissolved with 100 μl of mobile phase and injected into a Perkin Elmer series 3B high–performance liquid chromatograph with a fluorescence detector (Perkin Elmer 650/10, Perkin Elmer, Norwalk, Connecticut, USA) at an excitation wavelength of 475 nm and an emission wavelength of 580 nm. Separation was achieved by an isocratic solvent system of acetonitrile:water:0.1 M phosphoric acid (34:40:26) at a flow rate of 1.2 ml/min on a 25 cm "Li Chrosorb" C-18 column (E. Merck, Darmstadt, Federal Republic of Germany). The sensitivity limit was 10 ng/g of tissue.

Pathological studies

Light microscopy. Fragments of kidneys 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. Sections 3 μ thick were stained with Masson's trichrome, hematoxylin and eosin, periodic acid staining (periodic acid–Schiff), and Wilder's reticulin.

Immunopathology. Small pieces of kidney were snap–frozen in liquid nitrogen. Frozen sections of cortex and of medulla were cut at 3 μ on a cryostat (Ames Company, Miles Laboratories, Cavenago, Italy). Sections were fixed in acetone for 10 minutes and subsequently washed in phosphate–buffered saline (PBS). Then they were treated with fluorescein conjugated antisera to rat immunoglobulins, complement and albumin (N.L. Cappel Laboratories, Inc., West Chester, Pennsylvania,

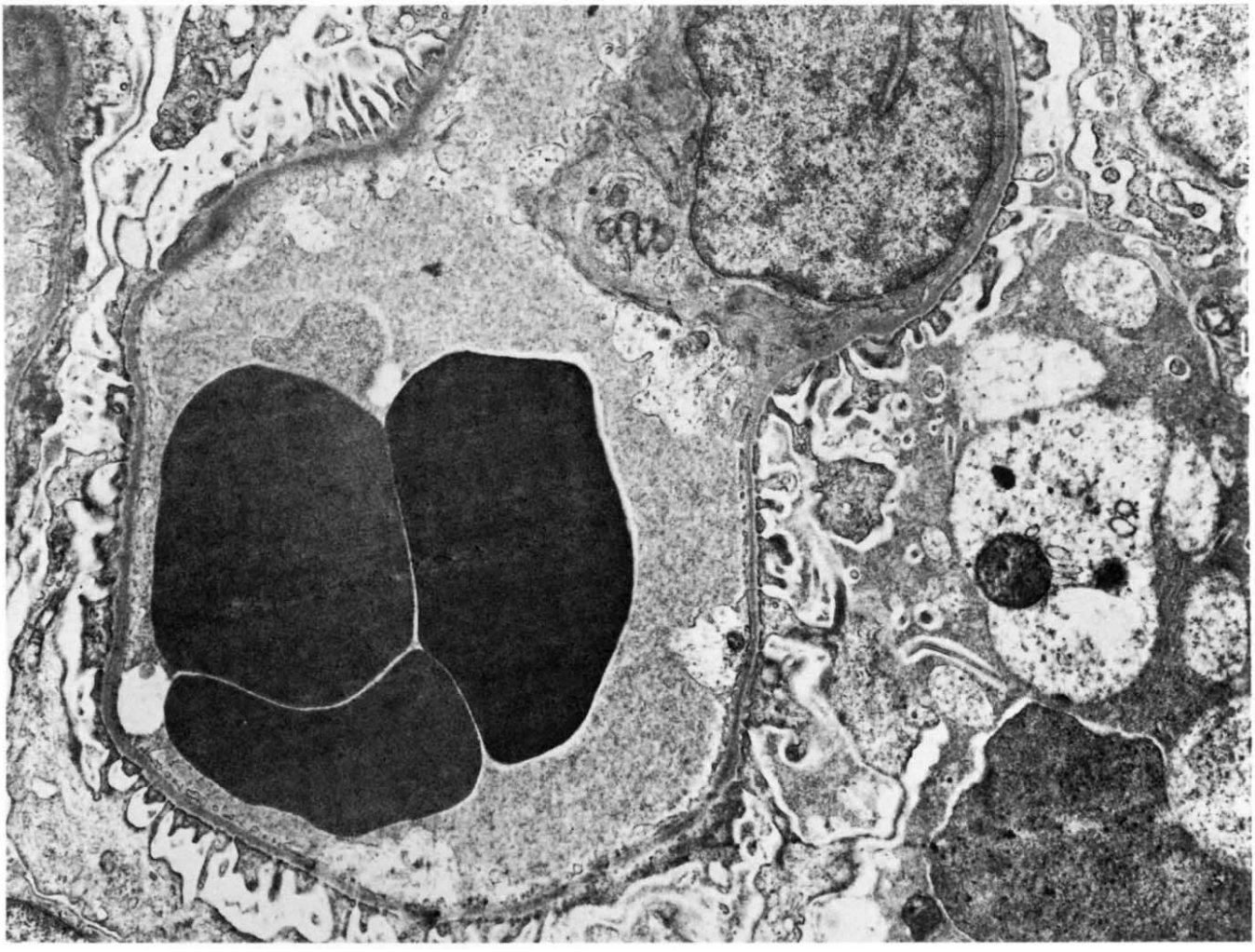

Fig. 2. *Electron micrograph of clamped kidney 14 days after ADR injection.* Note the absence of significant changes of the glomerular visceral epithelial cell (× 3,000).

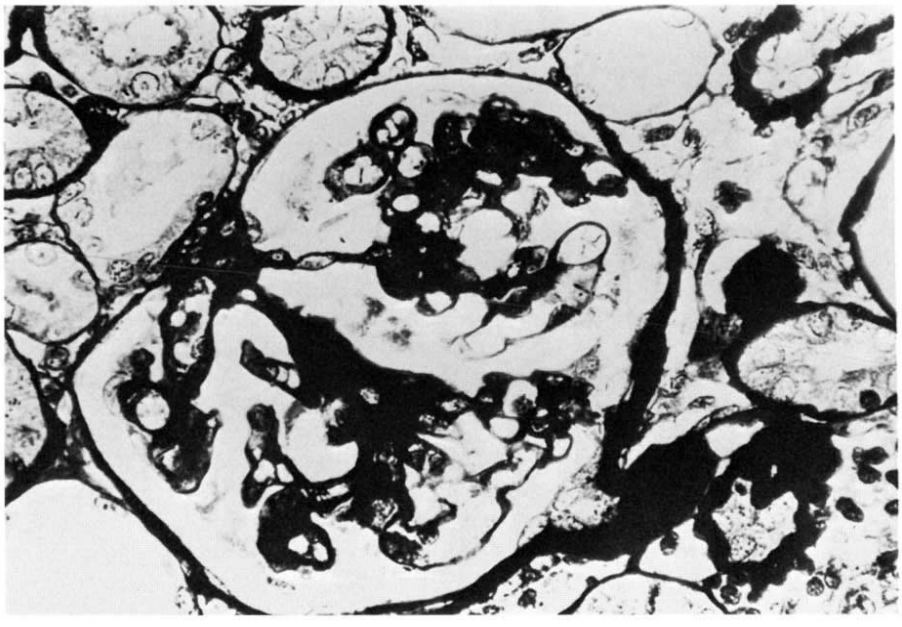

Fig. 3. *Glomerulus of unclamped kidney 60 days after ADR injection showing a small area of segmental sclerotic changes with adhesion to Bowman's capsule (Silver stain × 320).*

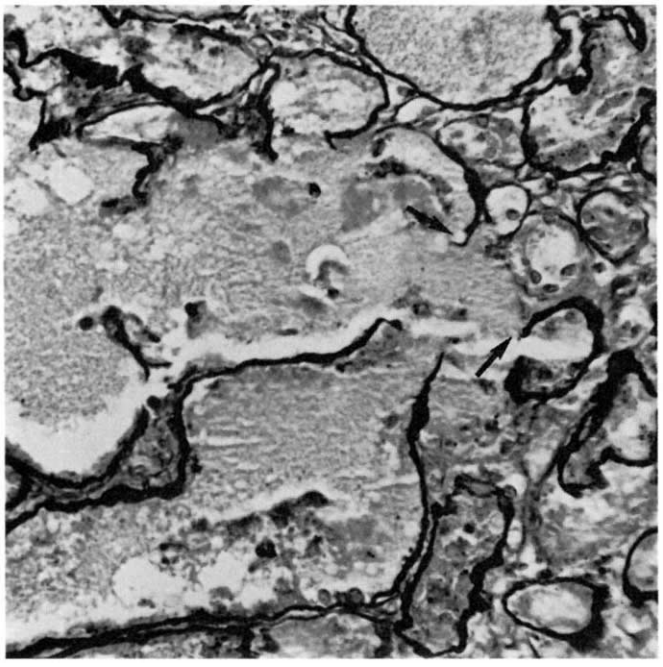

Fig. 4. *Unclamped kidney 60 days after ADR injection.* Note the presence of a large amount of eosinophilic material in the tubular lumens and some breaks of tubular basement membrane (arrows) with extravasation of the tubular content in the interstitium (Silver stain × 208).

USA). Sections were also incubated with a fluorescein conjugated antiserum to Tamm–Horsfall protein (THP). We used three different dilutions of THP 1:5, 1:10, and 1:20. Sections were extensively washed in PBS and examined in a Leitz microscope (Ernst Leitz, Wetzlar, Federal Republic of Germany) under ultraviolet light using a Ploem epifluorescence illuminator system.

Electron microscopy. Small fragments of kidney were fixed in 2.5% glutaraldehyde in 0.2 M phosphate buffer pH 7.4 for four hours at room temperature. Samples were washed in phosphate buffer and subsequently post-fixed in 1% osmium tetroxide at 4°C for one hour. After a brief wash in distilled water, they were dehydrated through ascending grades of alcohol and embedded in Epon resin. Sections were cut on an LKB V ultramicrotome (LKB Instruments, Milan, Italy). Semi-thin sections were stained with toluidine blue in borax and examined by LM. Ultrathin sections were stained with uranyl acetate and lead citrate then examined with a Zeiss EM 109 (Carl Zeiss, Oberkochen, Federal Republic of Germany).

Other investigations

Serum and urinary creatinine were determined by the method of Hare [7] using a Beckman analyzer (Astra 4 model, Beckman Instruments Inc., Fullerton, California, USA) in all groups of ADR treated animals and in seven normal rats used as controls. Urine was collected using metabolic cages over a 24-hour period and proteinuria was determined by the sulfosalicylic acid method.

Results were analyzed by unpaired Student's *t*-test and Duncan's range test.

Table 3. Semiquantitative assessment of pathological changes in the unclamped kidneys

Days	Glomerulosclerosis	Tubular casts	Tubular dilatation	Interstitial changes
14	0	+ +	+	0
28	±	+ + +	+ +	+
60	+	+ + +	+ + +	+ to + +

Results

Time course of proteinuria

All treated animals were heavily proteinuric (464 ± 161 mg/day) eight days after the ADR injection. In Group 1 the severe proteinuria, detected at day eight, fell to an almost physiological range (21 ± 5 mg/day) after the removal of the unclamped kidney, thus demonstrating that the glomerular damage was restricted to the unclamped kidney. Proteinuria was found to be further increased at days 14 (544 ± 178 mg/day), 28 (760 ± 145 mg/day) in Groups 2 and 3, and 60 (982 ± 330 mg/day) in the Group 3. Figure 1 shows the time course of proteinuria throughout the experimental period in these animals.

Renal function

In all groups of animals treated with ADR, glomerular filtration rate (GFR), measured as clearance of endogenous creatinine at the end of the experimental period, was not different from the values found in control rats (Table 1).

Distribution of ADR in the kidneys

These studies were designed to assess whether the protective effect of an eight minutes' clamping on the left kidney was due to a different exposure of the two kidneys to ADR. For this purpose, we followed ADR concentrations in unclamped and clamped kidneys at different intervals after ADR injection. As shown in Table 2, the initial kidney distribution and the subsequent disposal of ADR in clamped and unclamped kidneys were greatly different for each of the experimental points considered. Thus, the short period of clamping effectively protects the clamped kidney from ADR toxicity. The fact that at 30 and 60 minutes we found low but measurable levels of ADR also in the clamped kidney probably accounts for the modest histological changes observed 60 days after ADR injection.

Morphological studies

Clamped kidney. By LM no significant changes were detected at 14, 16, and 28 days after ADR injection. However, at day 60 animals showed minimal glomerular changes consisting of a moderate swelling of glomerular podocytes. Occasional casts were found in the lumens of distal tubules. The EM examination of clamped kidneys was in agreement with LM findings. Whereas at days 14, 16, and 28 no evident changes in the structure of podocytes could be detected (Fig. 2), at day 60 focal 'fusions' of foot processes, focal increase in reabsorption droplets, and blebbing were present. The cytoplasm of proximal tubular cells contained some protein droplets and in the lumens of distal tubules some fairly homogeneous casts were seen. IF findings were essentially negative, except for the THP staining

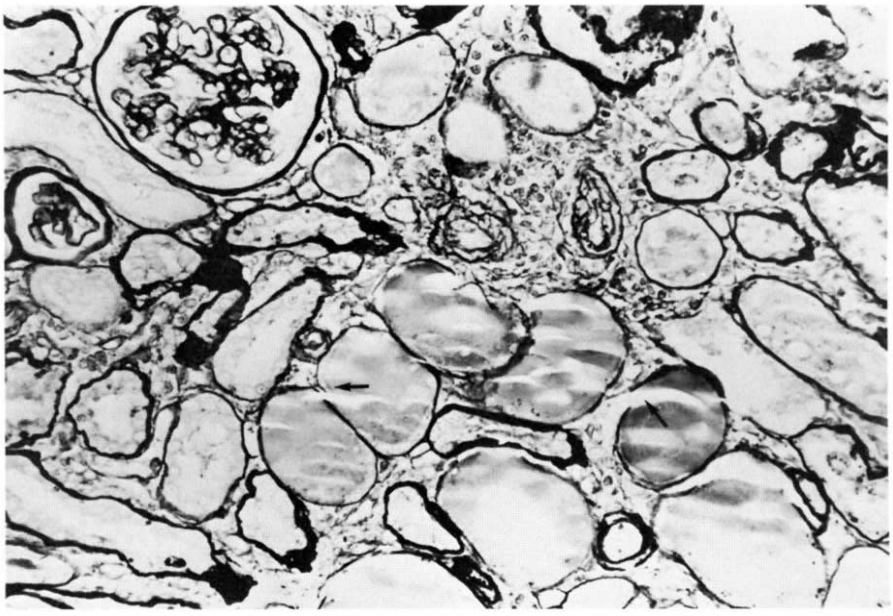

Fig. 5. *Unclamped kidney 60 days after ADR injection.* Glomerulus shows only minor sclerotic changes. Many casts are present in the tubular lumens. In some areas tubular basement membrane is thickened or broken (arrows). Tubular changes are associated with an interstitial inflammatory reaction (Silver stain × 80).

and for albumin of occasional tubular casts in those animals which at day 60 were affected by some histological changes.

Unclamped kidney. By LM at day 14, glomerular visceral epithelial cells showed a pronounced swelling of their cytoplasm with an increased number of reabsorption vacuoles and lysosomes as well as segmental detachment of epithelial cells from the basement membrane. The other glomerular structures appeared normal. However, most distal tubules were filled with a large number of eosinophilic casts. Concomitantly in the cytoplasm of many proximal tubules many protein droplets were seen, demonstrating the intense reabsorption activity of proximal tubular cells consequent to a large–scale proteinuria. At day 28 glomerular changes seen by LM were in several aspects similar to those described after 14 days. In particular, despite the severe proteinuria, areas of glomerulosclerosis were not detectable in any of the glomeruli examined. By contrast tubular casts were bigger, with a consequent marked tubular dilatation. At that time, interstitial inflammation and fibrosis were unremarkable. Sixty days after ADR injection in 5% of glomeruli limited areas of focal glomerulosclerosis were found. Compared with the previous model of nine months chronic proteinuria, in the accelerated model described in this study, FSG was significantly less pronounced as far as the percentage of affected glomeruli and the extent of glomerular sclerosis (Fig. 3). Tubular changes were impressive in most animals: the size of casts appeared to be further increased and tubular dilatation became very pronounced, leading to some discontinuities of the tubular basement membrane (TBM) (Figs. 4 and 5). Moreover interstitial inflammatory and fibrotic changes were seen in most animals, although significantly less pronounced than in the nine months model (Table 3). By IF at days 14 and 28 no significant deposits of rat immunoglobulins or C3 were detected in glomeruli; after 60 days scattered deposits of immunoglobulins and C3 were seen only in some glomeruli. The most interesting

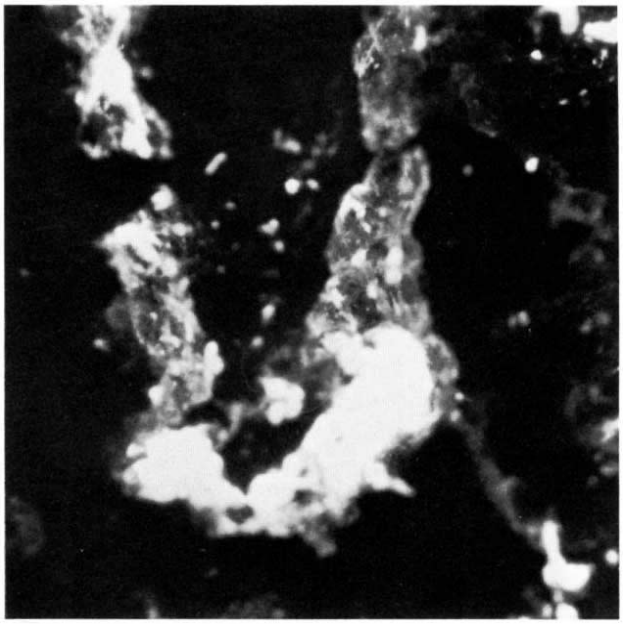

Fig. 6. *Kidney section of unclamped kidney 60 days after ADR treatment.* Note the intense staining for THP in tubular casts and in some interstitial areas (× 250).

immunopathological findings were in the tubules. All casts were strongly positive for albumin and THP. Moreover, in the animals with the most severe tubular interstitial changes, THP staining was also detected in some interstitial areas, probably as a consequence of rupture of the TBM and of extravasation of the casts in the interstitium (Fig. 6). The ultrastructural studies performed 14 days after ADR injection revealed the usual changes at the level of glomerular visceral epithelial cells:

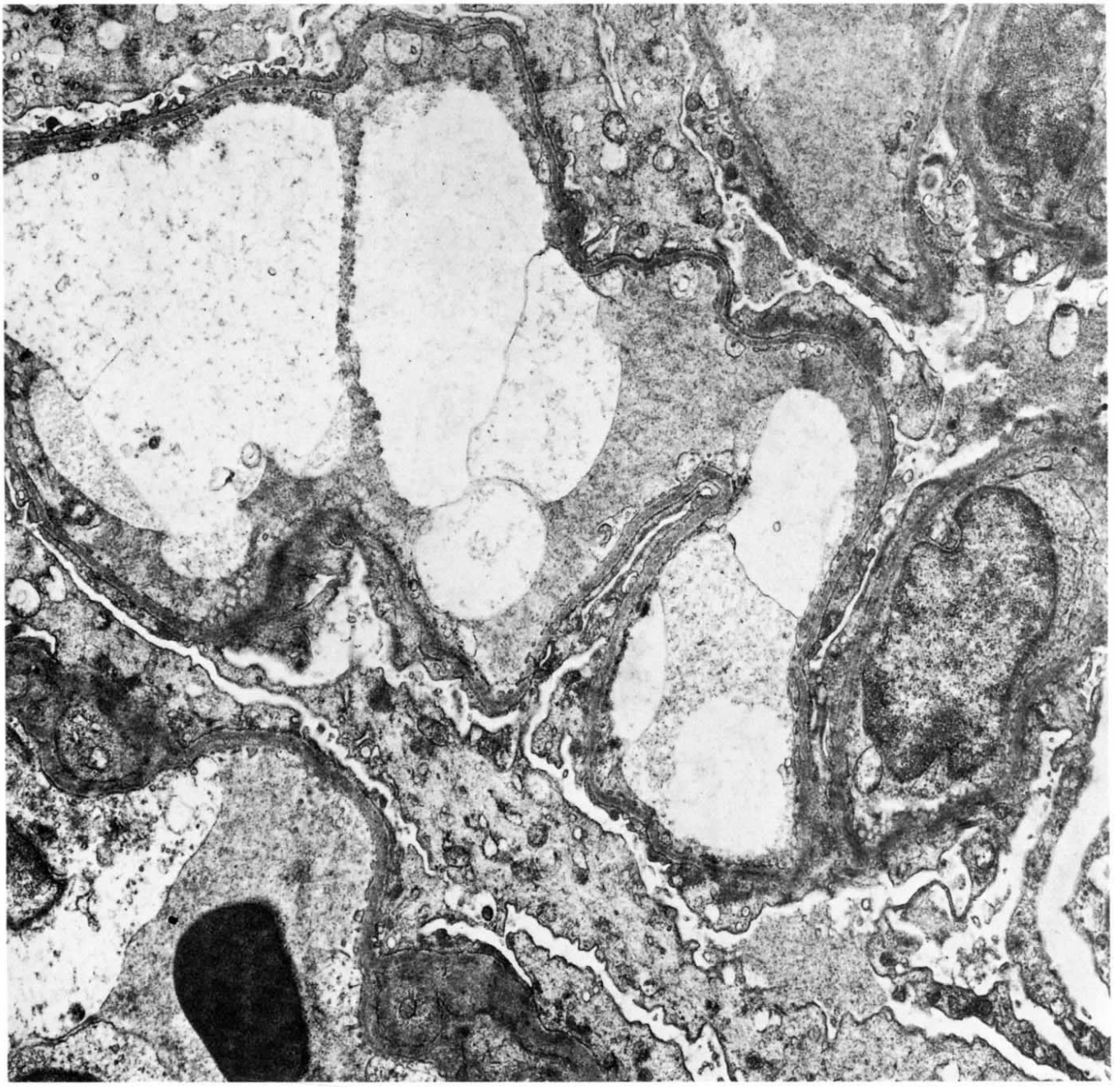

Fig. 7. *Electron micrograph of unclamped kidney 14 days after ADR injection.* Note the severe changes of glomerular podocytes with extensive 'fusion' of foot processes (× 3,000).

extensive fusion of foot processes, increase in reabsorption droplets and blebbing (Fig. 7). Proximal tubular cells contained protein reabsorption droplets, whereas in the lumens of distal tubules fairly homogeneous casts were seen. Glomerular and tubular changes worsened 28 days after ADR injection. At day 60, by the end of the experimental period, glomerular lesions became more complex. In addition to the severe changes of glomerular visceral epithelial cells, a moderate swelling of endothelial cells and an irregular expansion of the mesangial matrix were seen (Fig. 8).

Discussion

The present study describes an accelerated model of ADR nephrosis characterized by pathological changes confined to the unclamped kidney. The clamped kidney appeared normal at least until 28 days after the i.v. injection of ADR. The mechanism by which eight minutes' clamp effectively protects the clamped kidney from ADR toxicity should be attributed to the rapid disappearance of ADR from the circulation for its rapid entry into cells and tissues [8, 9]. On the basis of our pharma-

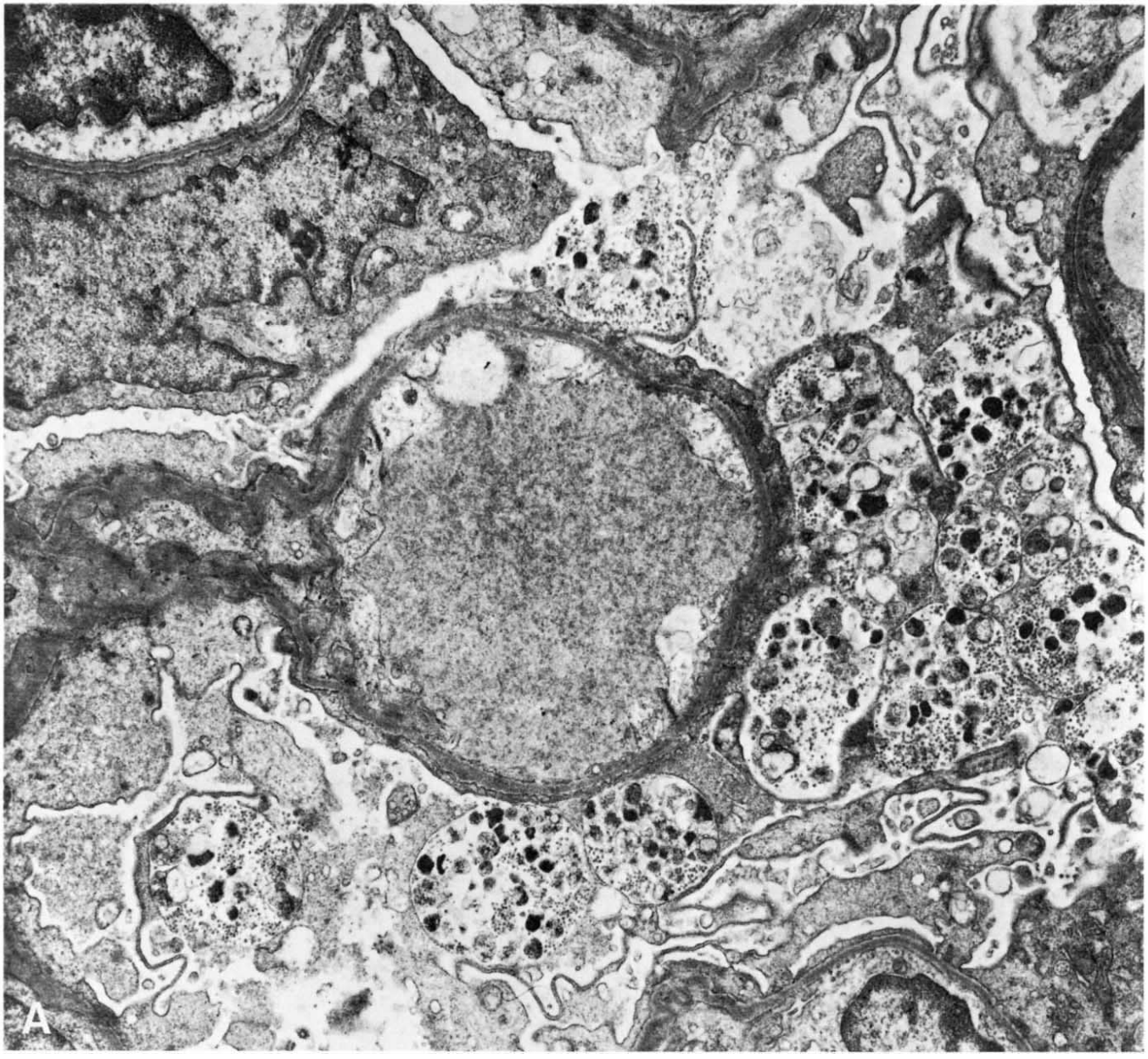

Fig. 8A and B. *Electron micrographs of unclamped kidney 60 days after ADR injection.* Note the complete obliteration of foot processes and the degenerative changes in the cytoplasm of glomerular podocytes (× 7,000).

cological studies, it is reasonable to postulate that few minutes after the i.v. injection, plasma ADR concentration is too low to induce renal damage. Conversely, as result of an increased blood flow to the unclamped kidney, the latter may be exposed to a higher amount of ADR in this model than in the classical one, and the toxic effect of the drug on the glomerular structures is potentiated. This conclusion is supported by our observation that morphological damage to the affected kidney and proteinuria were both more severe in this model than in the classical one.

Recently a model consisting of repeated injections of ADR in rats undergoing unilateral nephrectomy has also been reported [10]. Although the period of study of the pathological changes was not extended over 20 days, glomerular and tubular damage was closely similar to that seen in our model. The advantage of the model described here is that the presence of a normal kidney allows a better evaluation of the sequence of morphological changes in the affected kidney, avoiding the profound hemodynamic modification induced by unilateral nephrectomy.

Despite the similarity between the classical model and the accelerated one, in the latter, FSG appeared to develop in a lower percentage of glomeruli [1, 2]. However, in both models tubulo-interstitial damage was the prominent morphological finding associated with ADR-induced chronic proteinuria. Glomerular sclerosis and tubulo-interstitial changes have been also observed in rabbits after several months following a

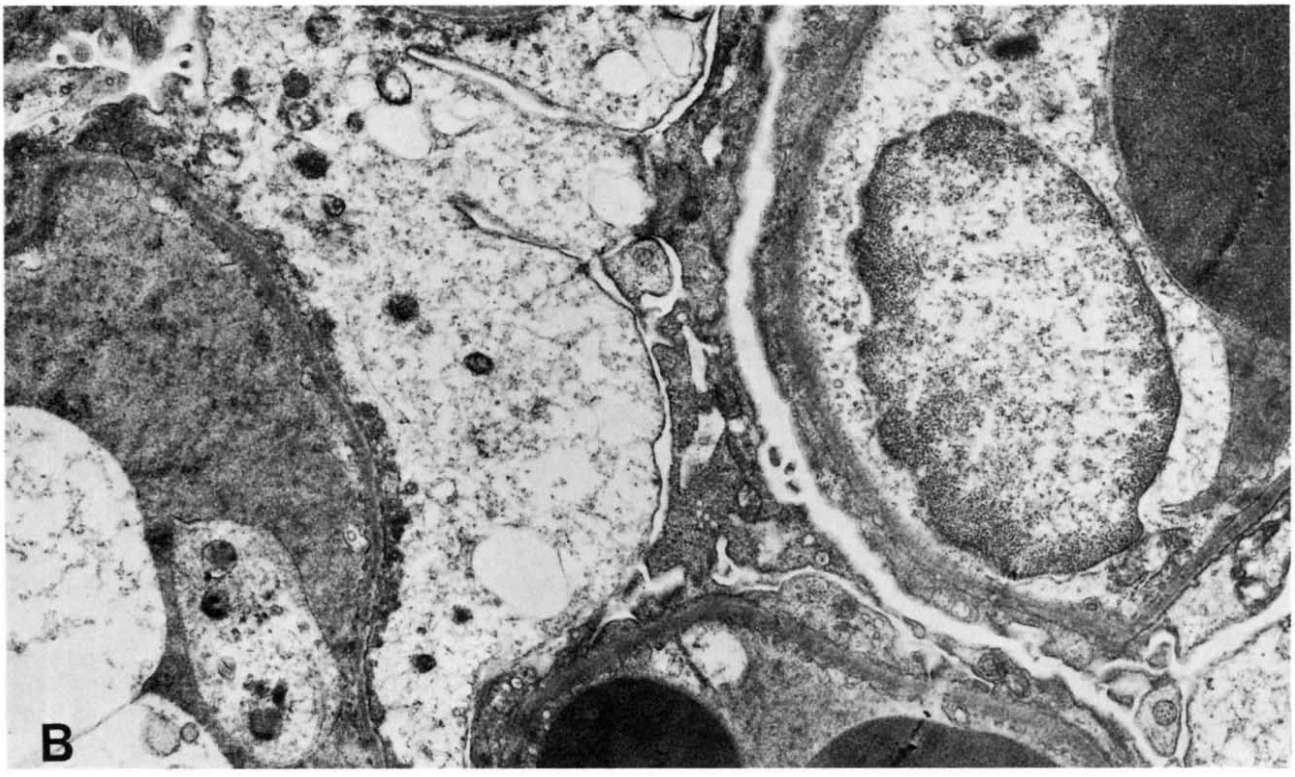

Fig. 8B.

treatment with multiple injections of ADR. However, the extent and the characters of FSG have not been described in detail [11].

After a single i.v. injection of ADR, proteinuria remains at a high level for several months [1, 2], with a gradual increase between months six and nine. According to our previous studies [1, 2] such a long period of heavy proteinuria does not produce FSG in all animals. We show with the present investigation that in the unilateral model, characterized by more severe changes, the incidence of FSG is even lower. These observations are consistent with the hypothesis that heavy proteinuria and FSG are unrelated phenomena. In this context, Grond and associates [4] have recently shown that rats made nephrotic with a lower dose of ADR than used by our group developed FSG only in a very low percentage of glomeruli in respect to animals treated with repeated injections of puromycin aminonucleoside.

In consideration of the striking similarities between the two models [3, 12, 13] it has been suggested that the normal function of the mesangium is the main reason for the low incidence of FSG in ADR nephrosis. This possibility is supported by experimental data showing alteration of mesangial function in chronic aminonucleoside, but not in ADR nephrosis [4]. As indicated by the above mentioned authors, overloading of the mesangium might trigger an excessive production of matrix substance and promote the development of FSG. Therefore, increased capillary permeability and development of FSG should be considered separate 'sequelae' of glomerular damage rather than causally related phenomena. The present study does not contribute to clarify whether 'mesangium overloading' is the main pathogenetic factor in the development of FSG; however, it adds to the available evidence [4], indicating a poor association between chronic proteinuria and the development of focal sclerosis.

As far as the pathogenesis of glomerular sclerosis, the close relationship between FSG and tubulo-interstitial changes in the nine months' model [1, 2] suggested to us a possible cause and effect relationship between the two lesions. In the present study we have documented that epithelial cell abnormalities and proteinuria are the first events in ADR-induced glomerulopathy. Subsequently, probably as a result of heavy proteinuria, tubular casts are formed at the distal level. The cast formation largely precedes the onset of FSG and the development of interstitial damage. We propose the following sequence of events: the massive entry of circulating proteins into urinary space leads to the formation in the lumen of distal tubules of casts, which progressively increase in size and cause obstruction followed by breaking of TBM. These changes might be responsible for the interstitial inflammatory reaction, which might favor the development of glomerulosclerosis [1, 2]. The intense staining for THP of some interstitial areas we have observed with immunofluorescence suggests that THP might have a role in determining interstitial damage.

Taken all together, the data obtained in both the nine months and the accelerated model of ADR nephrosis indicate that ADR exerts a direct toxicity on glomerular structure responsible for changes in glomerular permeability and proteinuria. This concept is suggested by previous studies [1, 4] in which ADR has

been employed for unilateral kidney perfusion [14]. At variance, tubulo-interstitial damage does not appear to be due to a direct toxicity of the drug, but rather a consequence of long-lasting proteinuria inducing cast formation and interstitial inflammatory reaction.

Whether the sequence of events proposed here as responsible for the progression of ADR nephropathy also applies to some proteinuric conditions in man is open to speculation. Of interest is the observation that in humans affected by reflux nephropathy, the late development of FSG is preceded by tubulo-interstitial changes. The experiments presented here, however, are not sufficient to rule out the possibility that FSG and tubulo-interstitial damage are two unrelated phenomena both consequent to proteinuria.

Acknowledgments

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

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