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Adriamycin-induced nephropathy as a model of chronic progressive glomerular disease

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Adriamycin-induced nephropathy as a model of chronic progressive glomerular disease. Serial changes in urine protein, blood chemistry, and histology of the kidney were investigated in rats for 28 weeks after injections of adriamycin (ADR). Massive proteinuria, hypoalbuminemia, and hyperlipidemia were observed at week 4 and throughout the experiment. Both BUN and serum creatinine began to increase at week 16 and reached the uremic level at week 28. Light microscopic study of the kidney demonstrated a normal appearance at week 4, vacuole formation in glomerular tuft at weeks 8 and 12, focal and segmental glomerular sclerosis at weeks 16 and 20, and extensive glomerular sclerosis with tubulointerstitial degenerations at weeks 24 and 28. Immunohistologically, IgM with a small amount of IgG and C_3 appeared in the sclerosing glomeruli from week 16. Aggregated human IgG, injected intravenously at week 24, had accumulated mainly in the glomeruli. Electron microscopy revealed degenerative changes of glomerular epithelial cells with small vacuoles in the cytoplasm at week 4. Size of vacuoles increased at the later stage. In conclusion, ADR produced chronic, progressive glomerular changes in rats, which led to terminal renal failure. The segmental glomerular sclerosis and IgM-dominant glomerular deposition in these animals are similar to pathological characteristics of focal and segmental glomerular sclerosis seen clinically.

Adriamycin (ADR) is a commonly used antineoplastic antibiotic that damages renewal systems of highly proliferative cells such as the epithelium of the intestinal tract and cell hematopoietic organs. In addition, it has nephrotoxic actions in experimental animals [1–3]. In short-term observations, heavy proteinuria associated with loss of glomerular polyanion, focal fusion of foot processes, and swelling, as well as vacuolation of epithelial cells, have been evidenced in ADR-treated rats [4, 5], in which nephrotic signs are manifest yet their renal function is preserved. In contrast, a long-term study demonstrated severe renal damage such as extensive glomerular lesions, tubular dilatation, and stromal fibrosis [6]. These observations indicate that the ADR-induced nephropathy have chronicity and a self-perpetuating property, which are characteristic features of chronic progressive renal disease in humans [7]. However, little is known of the self-perpetuating mechanism leading to terminal renal failure in chronic renal diseases. We now report details on long-term observations of rats with ADR-induced nephropathy.

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Methods

Experimental Design

The experiments were performed on adult, male Lewis rats, 8 weeks old and weighing 250 to 300 g (Charles River Breeding, Tokyo, Japan). The rats were fed a regular diet containing 0.3% NaCl and 22.0% protein. Adriamycin (2 mg/kg) was intravenously administered to 70 rats twice at a 20-day interval. This schedule was determined in preliminary experiments in which we evaluated the dosage interval to produce definite renal lesions without acute toxicity in all the rats used. Body wt, blood pressure, hematocrit, urinary protein, serum albumin, cholesterol, BUN, and serum creatinine were checked every 4 weeks after the second injection of ADR until week 28. Seven animals were sacrificed every 4 weeks for histological study. Forty rats were used for a control study in which isotonic saline was given instead of ADR, and the animals were sacrificed every 8 weeks until week 24; the final study was done at week 28.

Blood pressure measurement

Systolic blood pressure was measured in the conscious rats every 4 weeks by the tail cuff method. The animals were warmed for 10 min at 37°C before the measurement.

Analytic methods

Twenty-four-hr urine specimens were collected. Urinary protein was measured using the sulfosalycylic acid method [8]. Blood samples were obtained via percutaneous puncture of the tail artery. Serum protein was measured by the method of Lowry et al [9], and the protein fraction was analyzed electrophoretically. BUN was measured by the urease indophenol method, and serum creatinine by kinetic creatinine assay. Serum cholesterol was determined by an enzymatic method [10].

Renal histological examination

Kidneys were fixed in 10% neutral buffered formalin and embedded in paraffin for light microscopic study. Sections 2 μm

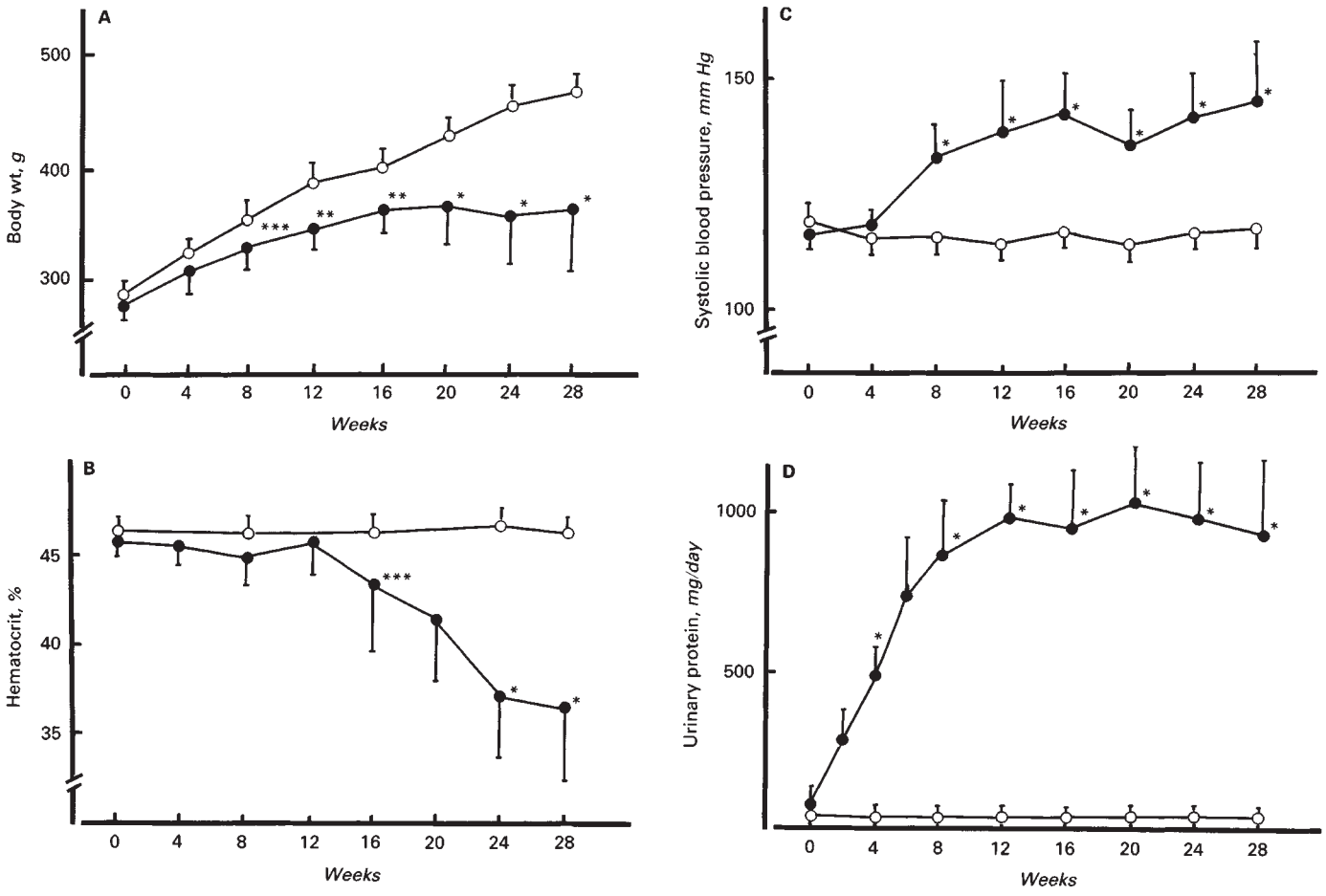

Fig. 1. *The courses of* **A** *body wt,* **B** *hematocrit,* **C** *systolic blood pressure, and* **D** urinary protein in ADR-administered rats (●—●) *and control rats* (○—○). Values are the mean of ten rats ± SD. $^{*}P < 0.001$, $^{**}P < 0.005$, $^{***}P < 0.01$ compared with the controls.

thick were stained with hematoxylin and eosin (H&E), periodic acid-Schiff reagent (PAS), or phosphotungstic acid hematoxylin. Histological evaluation was made independently by two pathologists without prior knowledge of the experimental groups. A semiquantitative score was used to evaluate the degree of glomerular sclerosis, according to the method of Raij, Azar, and Keane [11]. A minimum of 20 glomeruli (range, 20 to 60) in cortical and juxtamedullary portions was examined independently, and the severity of the lesion was graded from 0 to 4+ according to the percentage of glomerular sclerosis. Thus, a 1+ lesion represented an involvement of 25% of the glomerulus, while a 4+ lesion indicated that 100% of the glomerulus was involved. An injury score was then obtained by multiplying the degree of damage (0 to 4+) by the percentage of the glomeruli with the same degree of injury, that is, increase in mesangial material or glomerular sclerosis. The extent of the injury for each individual tissue specimen was then obtained by the addition of these scores. For example, if 5 of 20 glomeruli had a lesion of 1+ and 5 of 20 had a lesion of 3+, the final injury score for that specimen would be: $((1 \times 5/20) + (3 \times 5/20)) \times 100 = 100$.

For immunohistological studies, fresh kidney specimens were snap-frozen at −70°C in a dry ice-acetone mixture and sectioned with a cryostat microtome. Sections 4 μm thick were exposed to fluorescein isothiocyanate (FITC) conjugated anti-rat IgM, IgG, or C_3 goat serum (Cappel Lab., Cochranville, Pennsylvania, USA) for 1 hr at 37°C. The sections were examined under a fluorescence microscope (Carl Zeiss, Inc., New York, New York, USA). At the 24th week, aggregated human IgG (AHIgG, 40 mg/100 g) was injected intravenously to seven rats in both ADR-treated and control groups, and, 12 hr later, these rats were sacrificed. AHIgG was prepared by heating human γ-globulin (Midori-Juji Corp., Osaka, Japan) at 63°C for 15 min [12]. The sections were stained with FITC-conjugated anti-human IgG goat serum.

For electron microscopy, kidney specimens were fixed in 1.4% glutaraldehyde buffered with 0.1 M cacodylate, postfixed in ice-cold 1% osmium tetroxide in the same buffer for 2 hr, dehydrated in a graded series of ethanol, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a JEM-100S electron microscope (Nihon-Denshi Corp., Tokyo, Japan).

Statistical analysis

Student's *t* test was used for statistics. Data were expressed by mean ± SD.

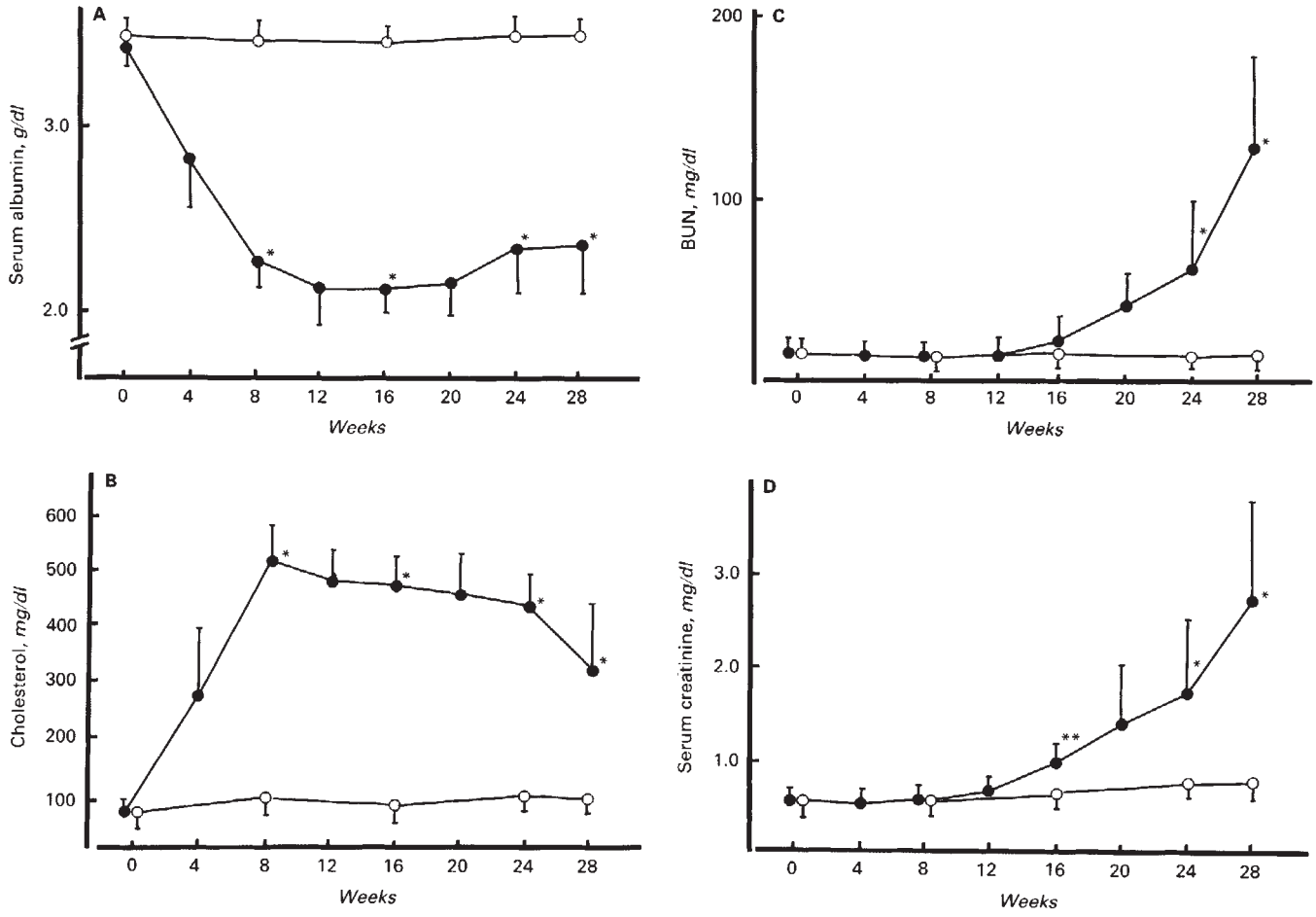

Fig. 2. *The courses of* **A** *serum albumin,* **B** *cholesterol,* **C** *BUN, and* **D** *serum creatinine in ADR-administered rats* (●—●) *and control rats* (○—○). Values are mean of ten rats ± SD. $*P < 0.001$, $**P < 0.005$ compared with controls.

Results

None of the ADR-treated rats showed acute toxicities such as severe anemia, gastrointestinal bleeding, and cachexia in an early period. Body wt was increased to a lesser extent in the ADR-treated rats than in control rats during the observation (Fig. 1A). In the ADR-treated rats, hematocrit began to decrease at week 16, and some of the rats were severely anemic by the end of the experiment (Fig. 1B). Blood pressure started to rise at week 8 and remained high thereafter (Fig. 1C). Urinary protein was positive shortly after the second ADR injection, and the amount excreted progressively increased and reached the level of 969.1 ± 122.6 mg/day at week 12 (Fig. 1D). Significant decrease of serum albumin with marked increase in cholesterol was observed at week 8 and continued throughout the experiment (Figs. 2A and B). Renal function was fairly well preserved until week 16, when both BUN and serum creatinine began to increase (Figs. 2C and D). BUN increased significantly at week 24 or later, compared with the controls, and serum creatinine also showed the same tendency at week 16 or later. Uremic manifestations such as marked azotemia, anemia, cachexia, and convulsion were observed in most of ADR-treated rats at week 28, at which time three animals died.

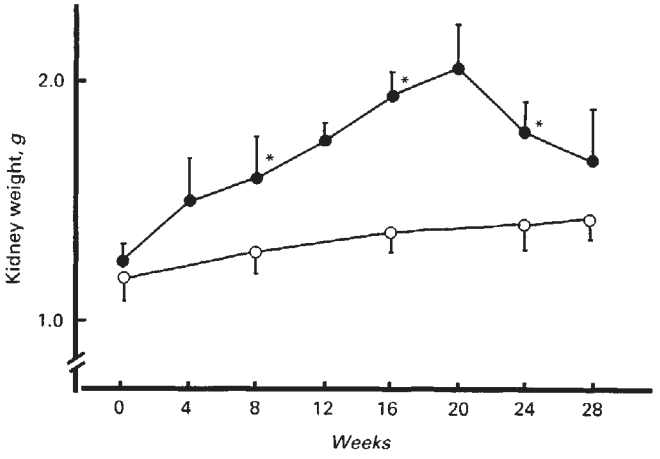

Fig. 3. *Kidney wt (mean of right and left kidneys) in ADR-administered rats* (●—●) *and control rats* (○—○). Values are mean of seven rats ± SD. $*P < 0.001$ compared with the controls.

Kidney wt gradually increased, reached a peak at week 20, and then slightly decreased (Fig. 3). Enlarged kidneys were granular on the surface and pale in color. Light microscopic examinations revealed that glomeruli and tubulointerstitium

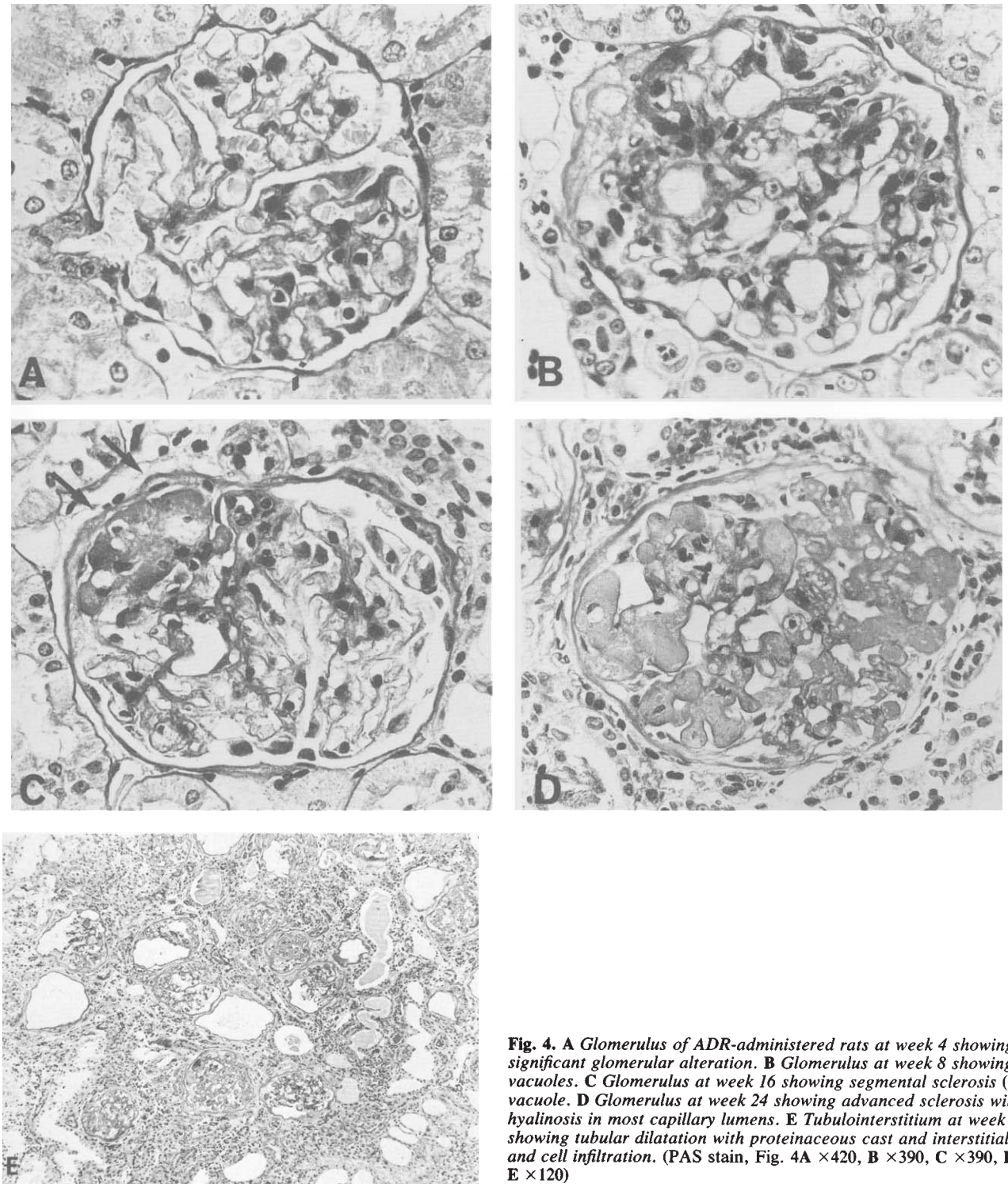

Fig. 4. **A** *Glomerulus of ADR-administered rats at week 4 showing no significant glomerular alteration.* **B** *Glomerulus at week 8 showing several vacuoles.* **C** *Glomerulus at week 16 showing segmental sclerosis (←) and vacuole.* **D** *Glomerulus at week 24 showing advanced sclerosis with hyalinosis in most capillary lumens.* **E** *Tubulointerstitium at week 28 showing tubular dilatation with proteinaceous cast and interstitial fibrosis and cell infiltration.* (PAS stain, Fig. 4**A** ×420, **B** ×390, **C** ×390, **D** ×360, **E** ×120)

were fairly intact at week 4 (Fig. 4**A**). At weeks 8 and 12, several vacuoles were seen in some glomeruli (Fig 4**B**), the diameter of which was almost twice as large as that of the capillary lumen. At week 8, less than 10% of all glomeruli contained the vacuoles while more than 30% did so at week 12. Segmental glomerular sclerosis appeared in some glomeruli at week 16 (Fig. 4**C**) and 20. At weeks 24 and 28, extensive glomerular sclerosis, tubular dilatation, dense proteinaceous casts, and interstitial fibrosis with round cell infiltration were observed in most of the ADR-treated rats (Figs. 4**D** and **E**). Small arteries and arterioles showed no remarkable changes. In the capillary lumen of the sclerosing glomeruli, fibrin deposition was occasionally present. Figure 5 shows the degree of glomerular sclerosis in cortical and juxtamedullary portions of the

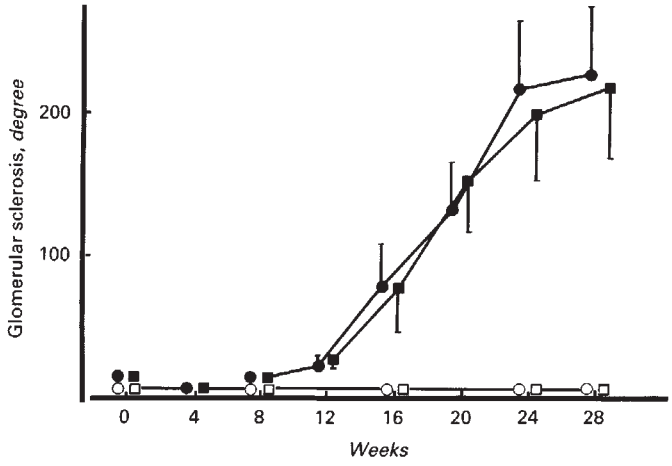

Fig. 5. *Degree of glomerular sclerosis in cortical portion (●—●) and juxtamedullary portion (■—■) of ADR-administered rats and cortical portion (○—○) and juxtamedullary portion (□—□) of control rats.* The values are semiquantitative scores to evaluate the degree of glomerular sclerosis, according to the method of Raij et al [11] and mean of seven rats ± SD.

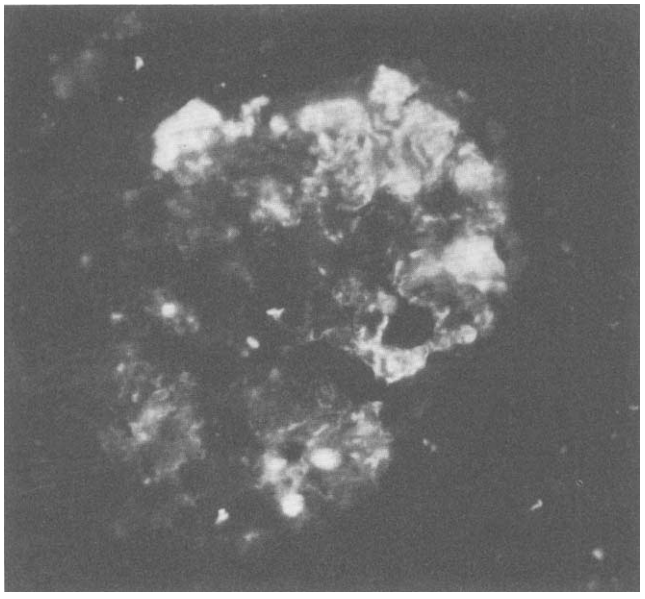

Fig. 6. *Glomerulus stained with FITC-anti-rat IgM in ADR-administered rats at week 20 showing segmental distribution of IgM.* (×380)

kidney. There were no differences in the glomerular lesions between both areas.

Immunofluorescence microscopic studies showed no depositions of IgM, IgG, or C_3 until week 12. From week 16, IgM was present in some glomeruli, in segmental or global distribution (Fig. 6), and corresponded to the sclerosing area of glomeruli determined by light microscopy. IgG and C_3 were also present in a similar pattern, but in a lesser intensity. Table 1 shows the degree of glomerular deposits for IgM, IgG, and C_3 in all the rats studied. the number of rats with intense deposits of IgM increased at the later stage of the observation. A similar tendency was observed in case of deposition of IgG or C_3. Glomerular staining for human IgG after the injection of AHIgG was intense and diffuse in the ADR-treated rats (Fig. 7), but was not observed in the control rats.

Electron microscopic studies revealed degenerative changes in the epithelial cells in the ADR-treated rats. Some visceral and parietal epithelial cells had enriched cellular organelles, and small vacuoles were seen in the cytoplasm at weeks 4 and 8 (Fig. 8). The origin of the vacuoles could not be determined precisely, but it seemed that the vacuole arose in a dilated endoplasmic reticulum. The endothelial and mesangial cells showed no significant alteration at these stages. Large oval or spherical vacuoles were prominent at weeks 12 and 16. Vacuoles in the epithelium increased in number and in size. There was widespread fusion of foot processes as well as increased osmiophilic substance in the cytoplasm adjacent to the glomerular basement membrane (GBM) at week 16 (Fig. 9). Large vacuoles surrounded by thin epithelial cytoplasm were often seen to occupy the urinary space at weeks 20, 24, and 28 (Fig. 10). Sclerotic changes of capillary loops began to appear at week 12. The sclerotic area consisted of mesangial cell proliferation, increased mesangial matrix, deposition of hyaline substances, and lipid-containing cells.

Discussion

In ADR-treated rats, urinary protein appeared shortly after intravenous injection of ADR and increased progressively with time. Furthermore, pathological changes of the kidney developed during long-term observation, and the course resembled that seen in chronic renal diseases in humans. In addition, histological changes such as focal and segmental glomerular sclerosis and IgM-dominant deposition in glomeruli also indicate that this nephropathy is similar to focal and segmental glomerular sclerosis (FGS) seen clinically. FGS-like chronic renal disease has been produced by repeated injection with aminonucleoside (AMNS) in rats [13, 14], in which vacuolated glomerular epithelial cells, capillary collapse, and progressive hyalinization are major histological findings. These are very similar to the findings in ADR-treated rats. However, frequent and continuous administration of AMNS is necessary to maintain urinary protein excretion and for renal lesions to develop in rats. In contrast, administration of ADR led to progressive renal lesions without additional administrations for 28 weeks after two intravenous injections, and terminal renal failure occurred. Bertani et al gave a single injection of ADR (5 mg/kg) and noted after 6 months a focal glomerular sclerosis associated with severe tubular and interstitial changes in 50% of the biopsied specimens [15]. These results indicate that the renal lesions of ADR nephropathy are more self perpetuating than those in the AMNS-treated rats.

Many nephrotoxic drugs have acute effects that may cause acute tubular necrosis, but ADR differs as it has chronic effects on the kidney, as shown in this study. The initial renal lesion induced by ADR may have an essential property to relate with progressive renal destruction. Various immunological or hemodynamic changes caused by ADR may play a role in the chronicity of ADR-induced nephropathy.

The renal lesions in ADR-treated rats varied with the stages: fairly normal appearances at the early stage, vacuolated changes in glomeruli at the middle stage, and either segmental or global sclerosis with tubulointerstitial changes at the late stage. These findings are compatible with previous observa-

Table 1. Immunohistological findings in ADR-administered rats[a]

Time after ADR administration *week*	IgM				IgG				C_3			
	−	+	++	+++	−	+	++	+++	−	+	++	+++
0	7	0	0	0	7	0	0	0	7	0	0	0
4	7	0	0	0	7	0	0	0	7	0	0	0
8	7	0	0	0	7	0	0	0	7	0	0	0
12	7	0	0	0	7	0	0	0	7	0	0	0
16	1	3	3	0	3	4	0	0	4	3	0	0
20	0	2	2	3	0	5	2	0	0	5	2	0
24	0	2	1	4	0	3	4	0	0	6	1	0
28	0	2	1	4	0	3	4	0	0	5	2	0

[a] Seven animals were studied every 4 weeks.
Symbols are: −, negative; +, weak; ++, moderate; +++, intense.

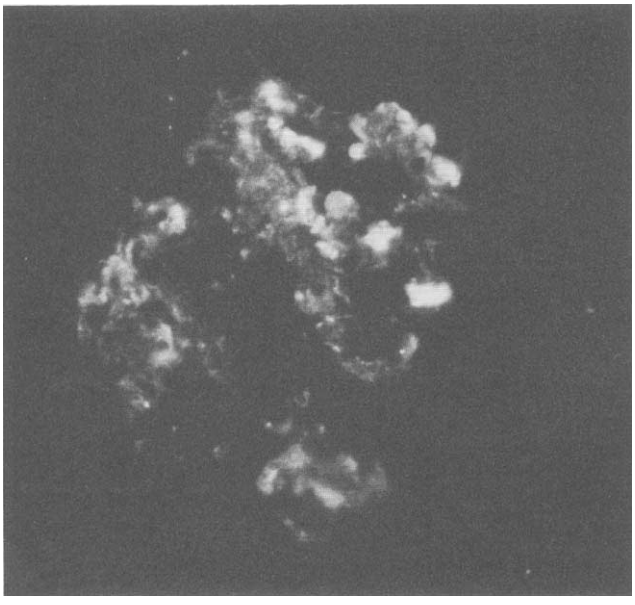

Fig. 7. *Glomerulus stained with FITC-antihuman IgG in ADR-administered rats at week 24,* 12 hr after AHIgG administration, showing increased deposition of AHIgG. (×380)

tions: the minimal alterations at week 4 seen by Bertani et al [4]; vacuolation and swelling of epithelial cells at week 8 reported by Weening and Rennke [5]; and glomerular sclerosis and stromal fibrosis at week 29 as noted by Fajardo et al [6]. In the present study, electron microscopy revealed small vacuoles in the cytoplasm of epithelial cells and focal fusion of the foot processes, even at the early stage. These vacuoles became larger and increased in number at the later stage. These findings indicate that the epithelial vacuolation is an initial lesion in the kidney and progresses into an irreversible state. The epithelial degeneration may play an important role in the progress of glomerular deterioration. Ryan et al suggested that segmental changes of FGS in human and animal models are well explained by differing degrees of epithelial cell injury leading to specific segmental area of denuded glomerular basement membrane [16, 17]. Epithelial cells retain glomerular function by producing GBM or by acting as a filtration barrier in association with the endothelium and GBM. Severe epithelial injury increases the transcapillary flux of macromolecules. The elevated transglomerular traffic may cause an intraglomerular accumulation of the macromolecules, which ultimately increases the matrices in the mesangial area. An overloading of the mesangial region leads to an increase in matrix deposition and to a glomerular sclerosis. Indeed, the elevated uptake of AHIgG found in the glomeruli indicates an increased glomerular accumulation of the macromolecules in ADR-treated rats.

The antineoplastic effect of ADR at the cellular level is an inhibition of RNA synthesis by drug binding to DNA [18]. Giroux et al reported that DNA-bound ADR was less nephrotoxic than free ADR [19], thereby indicating that the renal lesions in ADR-treated rats are directly related to pharmacological effects. Another mechanism of tissue damage from ADR is the formation of toxic oxygen [20]. This mechanism may become important in light of the observation that glomerular injury and glomerular permeability in nephrotoxic serum nephritis is related to the formation of oxygen free radicals [21]. In humans, however, consistent ADR nephrotoxicity is not evident, in contrast to animals. Species-related variations in toxicity of anthracycline have been noted in the kidney and heart [22]. Unique long-term toxic effects on kidneys have been observed only in rats and rabbits, while effects on the heart were noted in humans, rabbits, monkeys, and hamsters [2].

IgM, IgG, and C_3 were observed in glomeruli of ADR-treated rats at the late stage. On the other hand, none of the previous reports has demonstrated a positive immunofluorescence in ADR-treated rats [4, 5, 7]. This discrepancy is probably due to length of the observation period from ADR administration to death. Those studies were completed within 10 weeks at the longest, a time when no positive immunohistology was seen in our study.

The deposition of immunoglobulin and complement raises the question of whether immunological mechanisms may contribute to renal deterioration in ADR-treated rats. Verosa, Miller, and Michael noted deposition of immunoglobulin and complement in glomeruli undergoing sclerosis and suggested that immunological mechanisms may participate in the progression of glomerular injury, regardless of the primary disease [23]. However, the immune deposition in ADR-treated rats was IgM dominant and was located on the sclerosing area of glomeruli late in the course. An elevated uptake of AHIgG was found in the same area. Further, electron microscopic examination revealed no electron dense deposits that would suggest im-

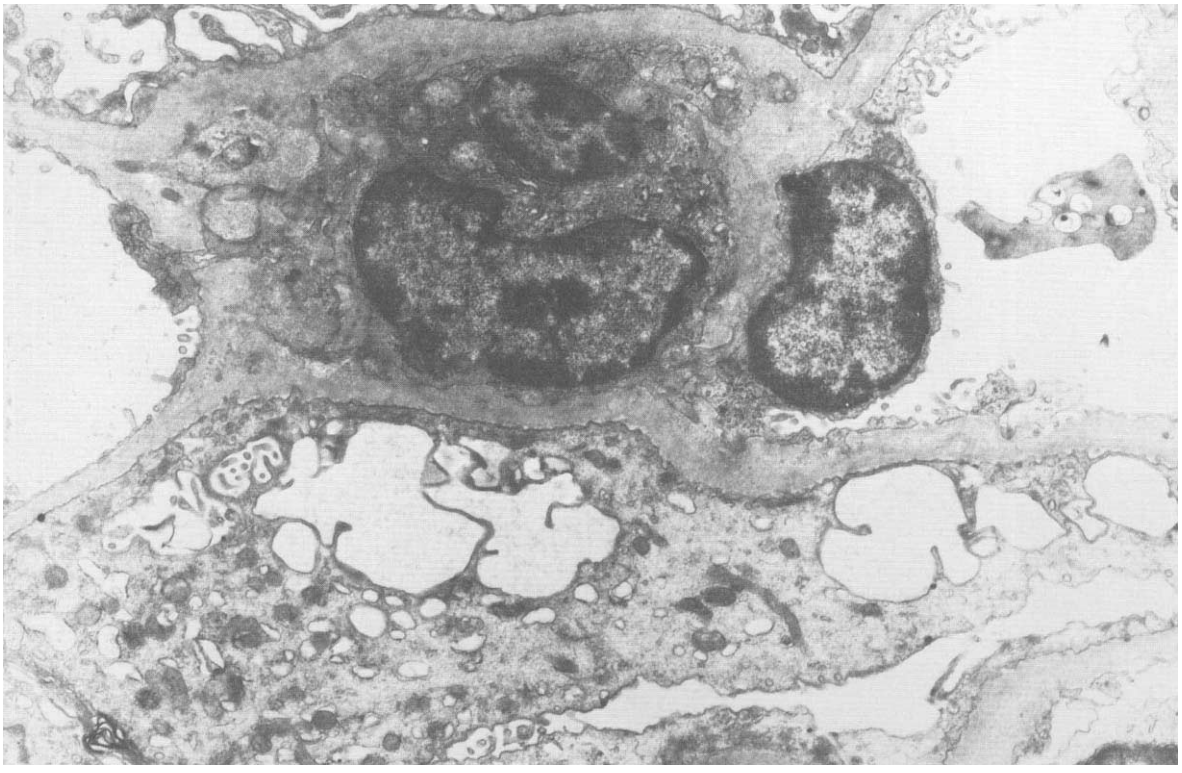

Fig. 8. *Glomerulus in an ADR-administered rat at week 8.* The podocyte has small vacuoles, probably originating from the endoplasmic reticulum. The mesangial and endothelial cells show no remarkable changes. (×9200)

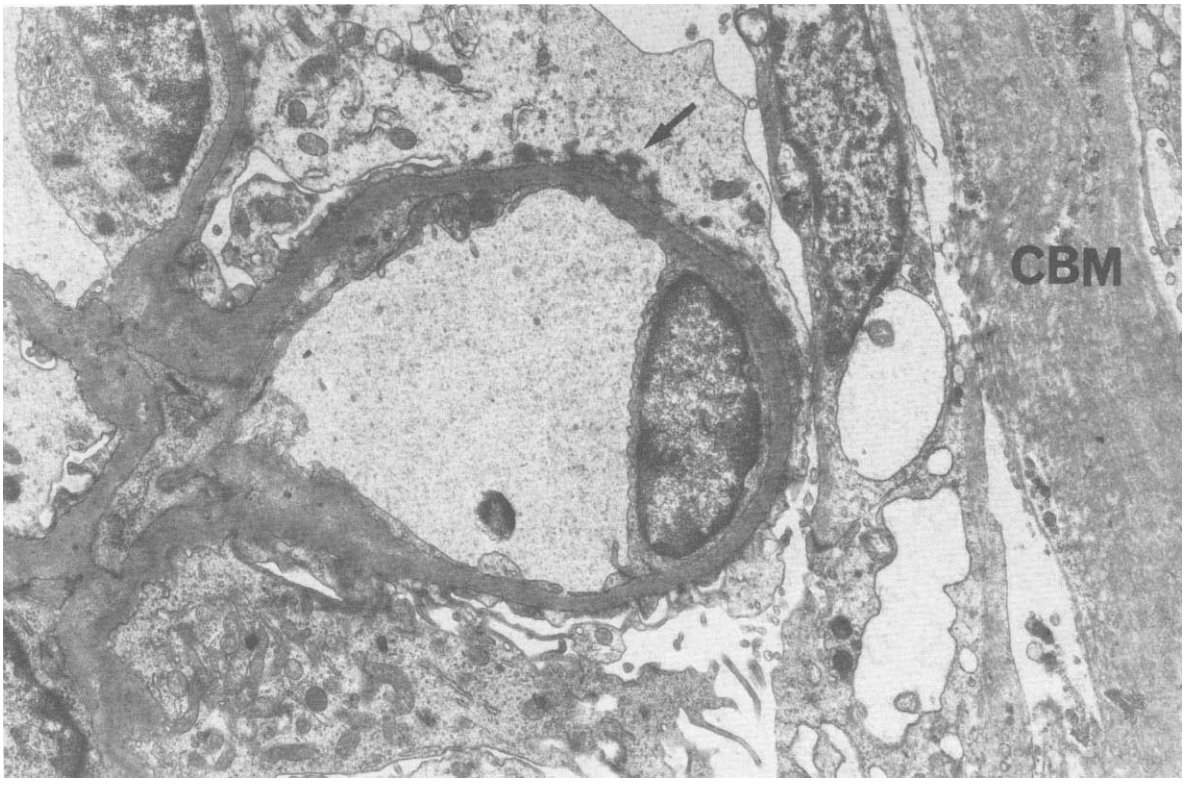

Fig. 9. *Glomerulus in an ADR-administered rat at week 16.* There are widespread fusion and increased osmiophilic substances (←) in the cytoplasm adjacent to the GBM. The parietal epithelial cell also shows vacuolar degeneration. Abbreviation: CBM, capsular basement membrane. (×7650)

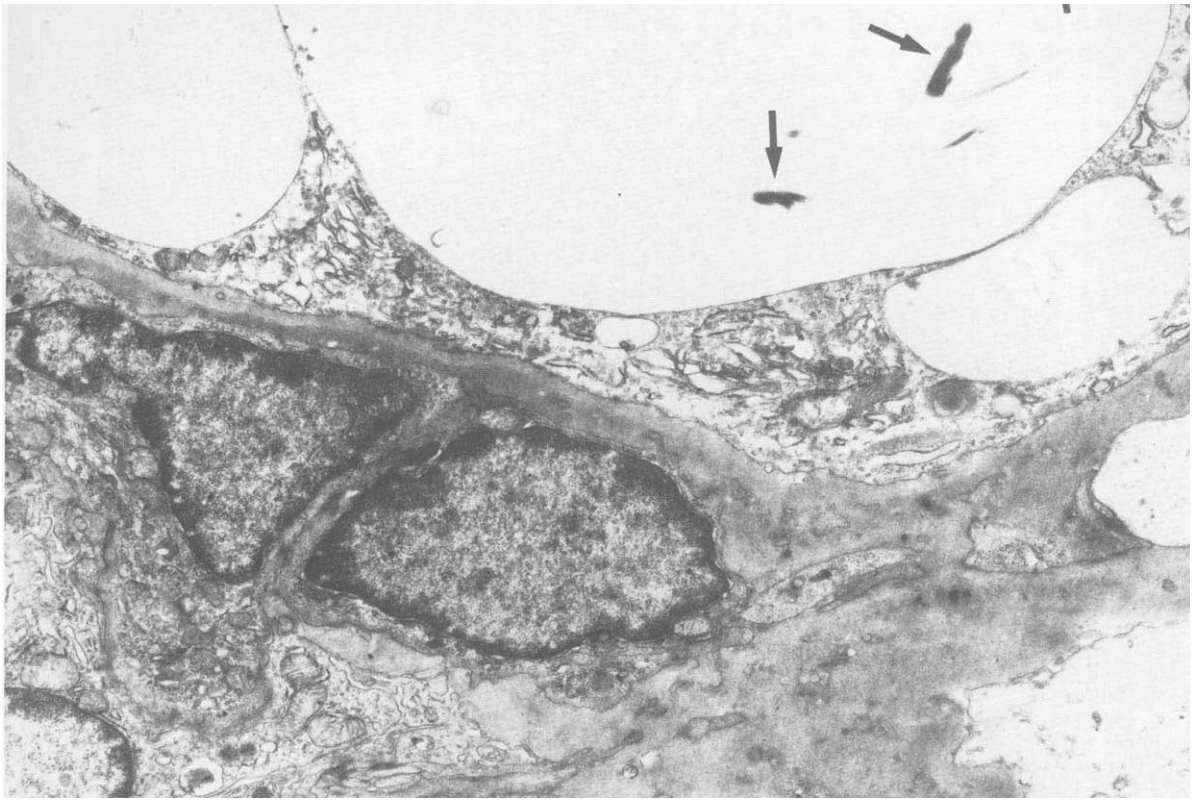

Fig. 10. *Glomerulus in ADR-administered rat at week 28.* The podocyte is ballooned up by large vacuoles which are separated by a veil of thin cytoplasm. In the empty spaces, fibrin strands (←) are evident. (×9100)

mune-complex deposition. These findings indicate that the glomerular capillary wall undergoing sclerosis is open to glomerular perfusion and large-size protein accumulates in these areas. Thus, the deposition of immunoglobulin may be an effect of prior damage rather than a mechanism related to glomerular damage.

Hypertension may aggravate renal deterioration in ADR-treated rats, although it was too mild to cause hypertensive vascular lesions. Superimposed hypertension has been reported to be an accelerating factor in glomerular sclerosis [24, 25]. Diseased kidneys are susceptible to hemodynamic changes caused by hypertension [26]. Bristow et al reported that intravenous ADR caused cardiac and peripheral vascular hemodynamic abnormalities associated with an increased release of histamine and catecholamine into the circulation, and that histamine antagonists and adrenergic blockers prevented the renal lesions [27]. This report is pertinent to recent observations in which hemodynamic changes in the glomerulus were found to be linked to focal and segmental glomerular sclerosis [28, 29]. Further investigations are necessary to clarify the mechanisms related to hypertension and the contribution to the renal deterioration.

In conclusion, in the ADR-treated rats, there was a chronic progressive renal deterioration during a long observation. Although the cause of this nephropathy differed from that in cases of chronic renal disease seen clinically, both show similarities with regard to the progress of renal deterioration. Thus, this model should be useful for the study of chronic glomerular disease.

Acknowledgments

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