ABSTRACT

Previous reports have suggested that, in proteinuria induced by adriamycin (ADR), the functional size barrier of the glomerular basement membrane (GBM) is altered as the result of a sieving defect, whereas the functional charge barrier of the glomeruli remains intact. The aim of this study was to reevaluate the effect of ADR on anionic constituents in the glomerular capillary wall (GCW). Kidneys of nephrotic rats, induced by the injection of 7.5 mg/kg ADR, and controls were resected, and cortices were isolated 24 h and 10 days postinjection, fixed with formaldehyde, and embedded in paraffin. For the histochemical evaluation of sialyl residues, deparaffinized sections were treated with biotin-labeled peanut agglutinin (PNA), before or after neuraminidase treatment. PNA binding was visualized by the avidin-biotin-peroxidase complex and interacted with hydrogen peroxide and diaminobenzidine. For electron microscopy, kidney cortices were fixed with glutaraldehyde and embedded in araldite or LR-white. The postembedding localization of anionic sites was carried out by cationic colloidal gold (CCG), directly applied on thin LR-white sections. Although in the 24-h ADR group, kidney functions and glomerular morphology were generally unaltered, the 10-day ADR group exhibited severe proteinuria, hypoalbuminemia, and massive fusion of intercalated foot processes of the podocytes. Intense PNA binding was observed after neuraminidase treatment in the GCW of the controls. This was gradually decreased in the 24-h ADR kidneys and further decreased in the 10-day ADR, indicating a gradual decrease in glomerular sialic acid content. CCG binding in the controls was mainly restricted to the GBM, exhibiting a threefold higher concentration at the lamina rara externa as compared with the lamina rara interna. In the 24-h ADR, the CCG binding was practically similar to that of the controls, whereas in the 10-day ADR, there was a drastic reduction in CCG binding. The results indicate that ADR-induced nephrosis is associated with a reduction in the content and/or expression of the polyanionic constituents of the GCW, including sialoglycoconjugates of the podocytes and sulfated proteoglycans of the GBM.

Key Words: Adriamycin nephrosis, glomerular polyanions

Adriamycin (ADR) was previously reported to cause experimental nephrotoxicity in rats as expressed by a nephrotic syndrome. As such, it constitutes a useful experimental animal model for studying the pathogenesis of proteinuria (1).

On the basis of clearance analyses, it was suggested that, in ADR-induced proteinuria, the functional size barrier of the glomerular basement membrane (GBM) is altered as the result of a sieving defect, whereas the functional charge barrier of the glomeruli remains intact (2). These findings were supported by histochemical-ultrastructural analyses with polycationic markers, which indicated that the distribution of polyanions in the GBM of the ADR-treated animals is not altered by the drug (2).

We have previously introduced a new polycationic histochemical probe, cationic colloidal gold (CCG), which enables an efficient localization of anionic sites on tissue sections on the light and electron microscope levels (3,4). This polycationic marker was recently shown to provide a useful postembedding histochemical probe for the localization and evaluation of GBM polyanions at the ultrastructural level of resolution (5-7).

Using this approach, we have previously shown that, in rat kidneys, the CCG interacts mainly with anionic constituents of the GBM, but not with membrane polyanions of the podocytes (7). In this study, we used CCG as a polycationic histochemical probe to analyze the normal distribution of polyanions in the glomerular capillary wall of rat kidneys and the alterations induced in these constituents by ADR.

Because the functional integrity of the glomerular filtration unit is also influenced by the electronegativity of the visceral epithelial foot processes (8), which are mainly contributed by membrane sialoglycoproteins (9), we also used the lectin peanut agglutinin (PNA), in combination with neuraminidase, for the
localization of membrane sialoglycoconjugates. This lectin was selected on the basis of its ability to localize the sugar sequence βGal(1-3)GalNAc (10), which in most mammalian tissues, is exposed after the removal of terminal sialyl residues by neuraminidase (11).

MATERIALS AND METHODS

Tissue Preparation

Male Wistar rats (Bellinson, Petah-Tikva, Israel) were divided randomly into three groups. The rats in each group were housed in individual metabolic cages and had free access to water and rat chow. After an acclimatization period of 5 days, two groups received a single dose of ADR (Farmam, Milan, Italy), 7.5 mg/kg body wt., through the tail vein. The third group, which served as the control, received an equivalent volume of 0.9% NaCl. Urinary protein excretion was determined for the first 24 h of the study. One group of rats was euthanized 24 h after ADR injection. The control and the other ADR recipient group were euthanized 10 days after the injection. On the day of euthanasia, the rats underwent light ether anesthesia, and blood was obtained by cardiac puncture. After the rats were euthanized, the kidneys were weighed and the cortices were separated from the medulla. Slices of the left kidney were fixed in 1% glutaraldehyde and 1% paraformaldehyde (GA)-fixed kidney slices were treated with 1% bovine serum albumin for 10 min at 37°C, to block endogenous peroxidase reactivity. The examination of all sections was carried out with a JEM-100B (JEOL, Tokyo, Japan) electron microscope at 80 kV.

Fixation and Embedding

Tissue blocks of 0.5 cm³ of the GA-fixed kidney slices were embedded in paraffin for light microscope analyses. For electron microscopy (EM), 1-mm³ blocks of GA-fixed kidney cortices were washed with PBS, dehydrated in ethanol, and embedded in LR-white (Polysciences, Washington, PA). For EM morphology, similar tissue blocks were postfixed with 1% OsO₄ in barbital-acetate buffer, pH 7.4, for 1 h at 4°C, dehydrated in ethanol and propylene oxide, and embedded in araldite.

Light Microscope Histochemistry

Five-micrometer-thick sections of the paraffin-embedded tissues were mounted on microscope slides. Deparaffinization was carried out by xylenol and ethanol. After rehydration with PBS, the sections were treated with 2% H₂O₂ for 10 min at 37°C, to block endogenous peroxidase reactivity.

For the localization of sialic acid residues, we used the lectin PNA, which binds mainly to the disaccharide β-galacto(1-3)N-acetylgalactosamine (10), which is exposed after the removal of sialic acid residues by neuraminidase (11). Neuraminidase treatment was carried out by the incubation of the deparaffinized tissue sections for 1 h at 37°C with 1 U/mL neuraminidase (Clostridium perfringens origin; Sigma, St. Louis, MO) in acetate buffer, pH 6.5. The histochemical localization of PNA binding was carried out by the ABC method (13). To block nonspecific binding, the sections were covered for 10 min with 1% bovine serum albumin solution in PBS, the excess solution was shaken off, and the slides around the tissue section were blotted. The sections were then incubated for 1 h at room temperature with 50 μg/mL biotin-labeled PNA (Vector, Burlingame, CA) in PBS. Control sections were similarly incubated with PNA without neuraminidase treatment.

At the end of incubation, the lectin-labeled sections were washed three times with PBS and incubated with avidin-biotin-peroxidase complex (Vector) for 30 min at 25°C, followed by three washes with PBS (13). The visualant, horse-radish peroxidase, was activated by incubation for 8 min in PBS solution containing diaminobenzidine and H₂O₂ and counterstained with 0.2% methyl green. The sections were then washed in tap water, dehydrated with ethanol and xylene, and cover slipped with Merkoglass (Merk, Darmstadt, Germany). The incubation of the PNA with 0.2 M solutions of β-galactose, before and during the application of the lectin solution to the tissue section, served as a specific control.

Statistical Analysis

Data are presented as mean ± SE. Comparisons between groups were made by the use of analysis of variance with the t-test for unpaired and paired data as needed (two tailed); P < 0.05 was considered significant.

RESULTS

Effect of Adriamycin Injection on Kidney Functions

The average body weights of the three groups were practically similar: 320 ± 12 g in the controls; 324 ± 13 g in the 24-h ADR; and 317 ± 12 g in the 10-day ADR. Twenty-four hours after the injection of ADR, the average body weight of the rats did not change (322 ± 12 g). At 10 days, however, although the control rats' body weight increased to 354 ± 10 g (P < 0.01), the 10-day ADR rats' weight decreased significantly to 251 ± 18 g (P < 0.01). Similarly, the average weights of the left and right kidneys of the control group (1,124 ±
32 and 1,092 ± 32 mg, respectively) were not different from those of the 24-h ADR rats (1,061 ± 21 and 1,016 ± 21 mg), whereas 10 days after ADR injection, the kidney weights decreased significantly to 980 ± 36 and 949 ± 38 mg (P < 0.05) respectively.

The biochemical parameters examined for kidney functions, including urinary proteins and plasma proteins, albumin, cholesterol, creatinine and urea, are summarized in Table 1. Twenty-four hours after the ADR injection, there were no considerable changes in all biochemical parameters studied, as compared with the control group. However, 10 days after the injections, protein excretion by the control group remained similar to the basal excretion, whereas the 10-day ADR rats developed severe proteinuria, as expressed by the excretion of 270 mg/dL protein. Completing the nephrotic syndrome expression in the 10-day ADR rats may be attributed to the massive weight loss of these animals.

### Light Microscope Morphology and Lectin Histochemistry

Hematoxylin-eosin staining of paraffin sections did not reveal changes in the kidney morphology in the 24-h ADR rats as compared with the control. In the 10-day ADR group, however, there were considerable morphologic changes, mainly expressed in the massive tubular accumulation of proteins and focal glomerular sclerosis.

In order to analyze possible ADR-induced changes in the content or distribution of sialoglycoconjugates in the glomeruli, we used the lectin PNA in deparaffinized sections to localize the disaccharide β-galactoside(1-3)N-acetylgalactosamine, exposed after the removal of sialyl residues by neuraminidase. The analysis of PNA binding to kidney sections without neuraminidase treatment revealed that in both the control and ADR-treated kidneys, the PNA intensely bound to some of the tubuli, but failed to bind to the glomeruli (Figure 1B). However, after neuraminidase treatment, the PNA bound similarly to the tubuli, but also exhibited intense binding to the glomeruli and blood vessel endothelium (Figure 1A). The binding intensities of PNA to neuraminidase-treated kidneys were high in the control group (Figure 1A) but were slightly reduced in the 24-h ADR group (Figure 1C) and further reduced in the glomeruli of the 10-day ADR group (Figure 1D). There was no PNA binding in the control sections that were labeled in the presence of β-galactose, serving as a competing sugar.

### EM Morphology

EM analysis of thin araldite sections of the 24-h ADR kidneys did not reveal considerable morphologic changes in the glomerular structure and organization of the podocyte foot processes (Figure 2B) as compared with the control (Figure 2A). On the contrary, the 10-day ADR kidneys exhibited drastic alterations

### TABLE 1. Urinary and plasma biochemical parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Urinary Proteins</th>
<th>Plasma day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>First Day (mg/dL)</td>
<td>Day 10 (mg/dL)</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>20.6 ± 3.5</td>
<td>23.7 ± 3.5</td>
</tr>
<tr>
<td>10-day ADR</td>
<td>7</td>
<td>18.7 ± 1.6</td>
<td>270.0 ± 59.9</td>
</tr>
<tr>
<td>P&lt;</td>
<td>NS</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* a P < 0.001 versus Day 1.

* b NS, not significant.
Figure 2. Electron micrographs of araldite sections of kidneys of a control rat (A) and of rats euthanized 24 h (B) and 10 days (C) after ADR injection. Massive fusion of epithelial foot processes is seen in the 10-day ADR kidney (C), whereas the morphology of the 24-h ADR kidney (B) is not affected, as compared with the control. Original magnification, ×25,000.

Figure 3. Electron micrographs of LR-white sections of kidneys of a control rat (A) and of rats euthanized 24 h (B) and 10 days (C) after ADR injection, labeled with CCG. In the control (A), the CCG particles are mainly seen on the GBM, whereas the membranes of the epithelial and endothelial cells are practically unlabeled. The density of CCG particles in the LRE is considerably more intense as compared with that in the LRI. The distribution of CCG particles on the 24-h ADR kidney (B) is generally similar to that of the control, whereas that of the GBM in the 10-day ADR kidney is considerably low (C). In contrast, a high density of attached CCG is seen on the basement membrane of the Bowmann's capsule (arrow). Original magnification, ×18,000.

in the glomerular morphology, mainly expressed in the extensive loss of podocyte architecture and massive fusion of their intercalated foot processes (Figure 2C). No detachment or shedding of epithelial cells from the GBM could be detected.

Binding of CCG to the GBM

Analyses of the binding of CCG to kidney glomeruli were carried out on thin LR-white sections at pH 7.4. The binding of CCG to the glomeruli of the control kidneys was mainly restricted to the GBM, as well as to basement membranes of other blood vessels, tubuli, and Bowmann's capsule. There was also some binding of CCG to the heterochromatin of nuclei and to cell cytoplasm, mainly in association with rough endoplasmic reticulum and polyribosomes. There was also binding of CCG to the extracellular matrix of the mesangium. In contrast, there was no CCG binding to the plasma membrane of any of the glomerular cells, including the podocyte foot processes (Figure 3).

The distribution of the binding of CCG to the GBM was highly asymmetrical, exhibiting considerably higher labeling intensity in the LRE as compared with the LRI (Figure 3A). There was relatively poor CCG binding in the lamina densa. Morphometrical analysis of the density distribution of CCG particles in the different GBM regions (Figure 4) indicated that the average gold particle density in the LRE (19.5 ± 0.8/μm length of GBM) was more than 2.5 fold that of the LRI (7.7 ± 0.4/μm; P < 0.001). The distribution of the attached CCG particles was generally random, but in most instances, there was less binding at the GBM regions facing the slit diaphragms.

The binding of CCG to the GBM of the 24-h ADR kidneys (Figure 3B) exhibited generally a distribution pattern similar to that of the control group (Figure 3A).
The average density of attached CCG particles on the LRE (28.3 ± 1.3/μm) was about 2.5-fold higher than that of the LRI (11.2 ± 0.75/μm; P < 0.001). However, in this group, the average density of attached CCG, on both the LRE and the LRI, was 44% higher than that of the control group (P < 0.001) (Figure 4).

The binding intensity of CCG to the GBM of the 10-day ADR kidneys (Figure 3C) was drastically reduced, as compared with the control (P < 0.001) and 24-h ADR (P < 0.001) kidneys (Figure 4). Similar to the control, the 2.5:1 ratio between the labeling density of the LRE (5.5 ± 0.8/μm) and LRI (2.0 ± 0.3/μm) was preserved. Unlike the GBM, the binding intensity of CCG to basement membranes of other kidney elements, including Bowman’s capsule and tubuli, was not affected by the ADR treatment (Figure 3C).

DISCUSSION

The use of CCG as a postembedding marker is suggested to provide a better insight as to the distribution of GBM polyanions at the ultrastructural level of resolution. Unlike most previously used polycationic markers, which are introduced by perfusion through unfixed kidneys, the postembedding approach is not subjected to perfusion artifacts and provides free and equal excess to all glomerular capillary wall components on the surface of the thin section.

As indicated by the results, the CCG, which was used at a physiologic pH (7.4), did not label polyanionic constituents known to exist in the podocyte membrane, such as the major sialoglycoprotein, podocalyxin (9). Similar observations were reported by Goode et al. (5), who demonstrated that positive staining of the podocyte glyocalyx with CCG can be observed at pH below 2.5. We, therefore, used the lectin PNA as a probe for the evaluation of membrane sialyl residues. This lectin interacts with the sugar sequence β-galactose(1-3)N-acetylgalactosamine (10), which in many mammalian cells exists as a penultimate disaccharide that is exposed only after the removal of terminal sialyl residues by neuraminidase (11). Our results indeed show that, in the rat kidney, the PNA binds to the glomerulus only after neuraminidase treatment. This indicates that glomerular cells in the rat kidney contain PNA-binding sugars only in their “masked” configuration. Therefore, in the glomerulus, the binding of PNA after neuraminidase treatment may be suggested as a specific histochemical probe for the localization of sialyl residues. With this approach, we have shown that the content of sialglycoconjugates of the glomerulus is already reduced 24 h after ADR injection and is further reduced 10 days after ADR treatment. The reductions in sialic acids in ADR nephrosis are in agreement with results from previous reports (2, 14) that demonstrated reduction of colloidal iron binding at pH 1.8, a procedure known to be specific for sialyl residues. The reduction in PNA binding in the 10-day ADR observed in this study may be attributed mainly to a reduction of visceral epithelial cell surface membranes, consequent to the obliteration of their foot processes. However, the fact that some decrease in PNA labeling was already observed in glomeruli of the 24-h ADR, where no ultrastructural changes of the epithelial foot processes could be detected, may reflect abnormal sialylation of the visceral epithelial cell membranes, rather than a reduction of membrane surface area. Decreased sialic acid content in glomerular epithelial cell membranes was previously demonstrated in ADR nephrosis (15), as well as in puromycin nephrosis (16).

In the GBM, the CCG binding to polyanions was highly asymmetrical, exhibiting a 2.5-fold higher density of attached CCG particles at the LRE as compared with the LRI. We have previously shown that, at neutral pH, the CCG may be used as an efficient postembedding histochemical marker of membrane-bound heparan sulfate, but not of membrane sialylglycoconjugates (4). Glycocalyx polyanions of glomerular epithelium and endothelium were shown to bind CCG only at pH below 2.5 (5). Most of the polyanionic sites of the GBM are attributed to the high content of heparan sulfate (17). However, recent studies using CCG in combination with glycosaminoglycan-degrading enzymes, have demonstrated that other glycosaminoglycan molecules, such as hyaluronic acid and chondroitin sulfate, also contribute to the GBM electronegativity (5).

The possibility that anionic sites in the LRE are also contributed by glycocalyx polyanions of the podocyte foot processes may be ruled out by the observation that the major epithelial membrane polyanion, podocalyxin, is practically absent from the basal surface of the epithelial foot processes facing the GBM (18). Furthermore, sialic acid residues in rat glomeruli were shown to be equally distributed throughout the entire
thickness of the GBM (19). We may therefore suggest that the high concentration CCG-binding polyanions reflects a greater concentration of sulfated proteoglycans at the LRE region of the GBM. The presence of polyanionic constituents in the glomerular capillary wall was previously intensively investigated with various polycationic markers at the ultrastructural level (20). These included colloidal iron (21), colloidal thionin (22), ruthenium red (23), cationized ferritin (17,24), lysozyme (25), and polyethyleneamine (PEI) (26,27). In most studies, in which the polycatonic reagents were introduced by perfusion, the densities of anionic sites in the LRI and LRE regions of the GBM were reported to be similar. This distribution differs from that observed in this study, as well as in other recent analyses, using CCG as a postembedding histochemical marker of ultrathin sections (5,7). The latter reports clearly show a considerably higher polyanion density at the LRE, as compared with the LRI, that reached a 2.5:1 ratio in our study. We, therefore, suggest that the postembedding approach used in our study provides a more reliable view of GBM polyanion distribution, because it avoids the possibility of perfusion artifacts that may be caused either by filtration pressure or by the cross-linking of unfixed polyanionic constituents of the GBM by the polycationic probes (28).

Our results are in controversy with previous reports regarding the effect of ADR on the distribution of GBM anionic sites. In our study, 10 days after the ADR administration, there was a drastic reduction in the density of GBM polyanions, as determined by CCG binding. Previous analyses of ADR-treated kidneys, using PEI as polycationic probe (2,14), have demonstrated that the distribution of anionic sites in the GBM is practically unaffected by ADR. It is possible that, unlike CCG, the PEI also interacts with polyanionic constituents other than heparan sulfate that do not interact with CCG. Therefore, our results may reflect a reduction in the sulfated proteoglycans of the GBM, rather than a general loss of polyanions. The reduction in CCG binding in the GBM is in direct correlation with the development of proteinuria and the obliteration of visceral epithelial foot processes. The increase in CCG binding in the 24-h ADR probably reflects early structural changes that make the GBM polyanions more available to the polycationic probe.

Interestingly, in all cases, the asymmetrical binding of CCG in the GBM was preserved, despite a loss of anionic constituents in the ADR-treated kidneys. The earlier decrease in the density of stialic acid residues in the 24-h ADR kidneys, as compared with the polyanions in the GBM (noticed only in the 10-day ADR group), suggests a closer association of the latter with the proteinuria.

REFERENCES


