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Glomerulonephritis Induced by High Doses of Ovalbumin

Studies by Electron Microscopy, Immunofluorescence and Immuno-Electron Microscopy

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Summary. Experimental glomerulonephritis was produced in 16 rabbits by intravenous injections of ovalbumin in high doses (0.1 g/day during the first week, 0.2 g × 6/day during the second). The animals were killed on day 14. At that time all animals had 2–4+ proteinuria and a serum C3 level reduced to about 50% of the control level; 11 animals had a significantly raised blood urea level. In all rabbits the antigen had induced severe proliferative glomerulonephritis. Electron microscopy showed that many of the cells accounting for the hypercellularity were monocytes. Surprisingly, electron dense deposits were few and small, mainly on the subendothelial and subepithelial aspects of the glomerular basement membrane. In all the animals ultrastructural immunoperoxidase technique revealed deposits containing ovalbumin, rabbit IgG and C3. With immunofluorescence sparse deposits were occasionally seen. It is concluded that a severe experimental glomerulonephritis can be produced in a state of antigen excess, with the deposition of immune complexes being minimal. Immuno-electron microscopy is essential, however, in detecting even the smallest amounts of deposited immune reactants.

Key words: Ovalbumin – Glomerulonephritis – Immune complex disease – Immune deposits – Immuno-electron microscopy.

Introduction

Recently Germuth et al. (1975) induced experimental immune complex glomerulonephritis in rabbits by immunizing them with frequent injections of ovalbumin in high doses. Interestingly, a fatal disease resulted despite the virtual absence of glomerular immune deposits. We undertook a detailed morphological study to further characterize the features of this experimental disease. Our aim was

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to search for immune reactants deposited or otherwise localized in glomeruli and to detect any other morphological or immuno-histochemical changes. For this study we used light microscopy, immunofluorescence, electron microscopy and ultrastructural immunoperoxidase technique.

Materials and Methods

Animals and Immunization Procedure

Glomerulonephritis was produced in 16 male albino New Zealand rabbits, weighing 2.0–2.5 kg. Fine powdered ovalbumin (E. Merck, Darmstadt, West Germany) was dissolved in phosphate-buffered saline (PBS) to make a solution with 100 mg ovalbumin in 1 ml. The animals were injected intravenously with a single daily dose of 0.1 g of ovalbumin for 1 week and with 0.2 g ovalbumin every 4 h for the next week. One animal died spontaneously on the 10th day; the others were killed on the 14th day.

Serum and Urine Assays

Serum was collected on days 0, 7, 10 and 14 for determination of C3, free ovalbumin and urea. Serum C3 levels were measured by single radial immunodiffusion (Mancini et al., 1965) in serum taken before an ovalbumin injection. The sera were tested for the presence of circulating free ovalbumin by the precipitation test of Ogden et al. (1967). The test was performed on serum taken immediately before an ovalbumin injection. Serum urea levels were determined by the method of Chaney and Marbach (1962). Urine samples were obtained at the same intervals for measurement of proteinuria. Urinary protein was determined with Albustix® (Ames Company, Slough, England). In the urine of six animals rabbit endogenous albumin, ovalbumin and rabbit IgG were assayed by single radial immunodiffusion.

Light and Electron Microscope Techniques

Kidney tissue was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, embedded in paraffin, sectioned at 3–4 μm, and stained with haematoxylin and eosin, periodic acid-Schiff, Masson's trichrome and Jones' periodic acid-silver methenamine methods. For ultrastructural studies small fragments of cortical tissue were fixed for 2 h in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, postfixed in phosphate-buffered 2% osmium tetroxide, dehydrated through a series of acetones, and embedded in Epon 812. Ultrathin sections were cut on an LKB Ultrotome III, stained with lead citrate and uranyl acetate, and examined in the JEM 100 C electron microscope.

Antisera

Both fluorescein-conjugated and unlabelled goat anti-rabbit IgG and C3 sera were purchased from Cappel Laboratories, Cochranville, Pennsylvania, U.S.A., and goat anti-rabbit gammaglobulin from Behringwerke AG, Marburg-Lahn, West Germany. Goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was provided by Miles Laboratories, Inc., Elkhart, Indiana, U.S.A. Anti-ovalbumin was produced in rabbits. The IgG fraction, obtained by DEAE column chromatography and giving a single line in immunoelectrophoresis, was conjugated with FITC as previously described (Clark et al., 1963) and with horseradish peroxidase (Reinheitsgrad I, Boehringer, Mannheim, West Germany) according to the two step procedure of Avrameas and Ternynck (1971). Antiserum to rabbit C3 was produced in guinea-pigs with the method of Mardiney and Müller-Eberhard (1965). Double diffusion gel precipitation and immunoelectrophoresis showed the serum to be monospecific. Its IgG fraction was conjugated with HRP as described above (Avrameas and Ternynck, 1971).

Immunofluorescence Microscopy

Kidney tissue was snap-frozen in liquid nitrogen, sectioned at 4 μm in a cryostat, dried in air, washed in PBS, fixed in acetone at −20° C, and stained with monospecific, fluorescein-conjugated antisera to ovalbumin, and to rabbit IgG, gammaglobulin and C3. Controls included a section from an untreated rabbit and one with blocking with monospecific, unconjugated antiserum.

Immuno-Electron Microscopy

Blocks of renal cortical tissue were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at +4° C for 1 h. The tissue was washed in phosphate buffer at +4° C for 5 days, snap-frozen in liquid nitrogen and cut at 30 μm in a cryostat. Sections were then incubated for 12 h at room temperature in HRP-conjugated monospecific anti-rabbit IgG, C3 or anti-ovalbumin sera. The sera, containing 5 mg of antibody in 1 ml, were diluted 1:10. The sections were washed in phosphate buffer at +4° C for 10–12 h, and subjected to the diaminobenzidine reaction of Graham and Karnovsky (1966). They were then washed in phosphate buffer at +4° C for 45 min, treated with phosphate-buffered 2% OsO_4 for 5 min, dehydrated, embedded in Epon 812, and sectioned for electron microscopy. Controls consisted of sections previously treated with unlabelled antisera, or stained with unspecific HRP-labelled sera, as well as sections in which only the endogenous peroxidase activity was demonstrated.

Electrophoretic Studies of Tissue Homogenates

Renal tissue was studied by electrophoresis for the presence of immune complexes. Pieces of cortical tissue were minced, washed five times in PBS, and packed into a circular well of 5 mm ∅ punched in an agarose electrophoresis plate. Electrophoresis was performed at pH 8.4 with 250 V for 90 min. This was followed either by counterelectrophoresis against anti-ovalbumin serum at 250 V for 30 min or by immunodiffusion with anti-rabbit IgG serum.

Results

Findings in the Sera and Urines

In Fig. 1 are compiled the data on serum urea, serum complement (C3) and urinary protein. On day 10 a significant increase in mean serum urea was noted. By day 14 a further increase had taken place. On day 14 the mean serum C3 value, expressed as a percentage of the C3 value of pooled control rabbit serum, was significantly decreased. Most animals had 2–3+ proteinuria on day 10 and 2–4+ proteinuria on day 14. In urines of six animals the proteins were identified and assayed (Table 1). In 5 out of 6 animals small amounts of rabbit IgG were present in the urine on days 7, 10 and 14.

In all animals free ovalbumin was present in the serum from day 10 on, although not detectable on day 7.

Morphological Changes

In all animals light microscopy showed a diffuse proliferative glomerulonephritis. This consisted of considerable proliferation of endocapillary cells leading to

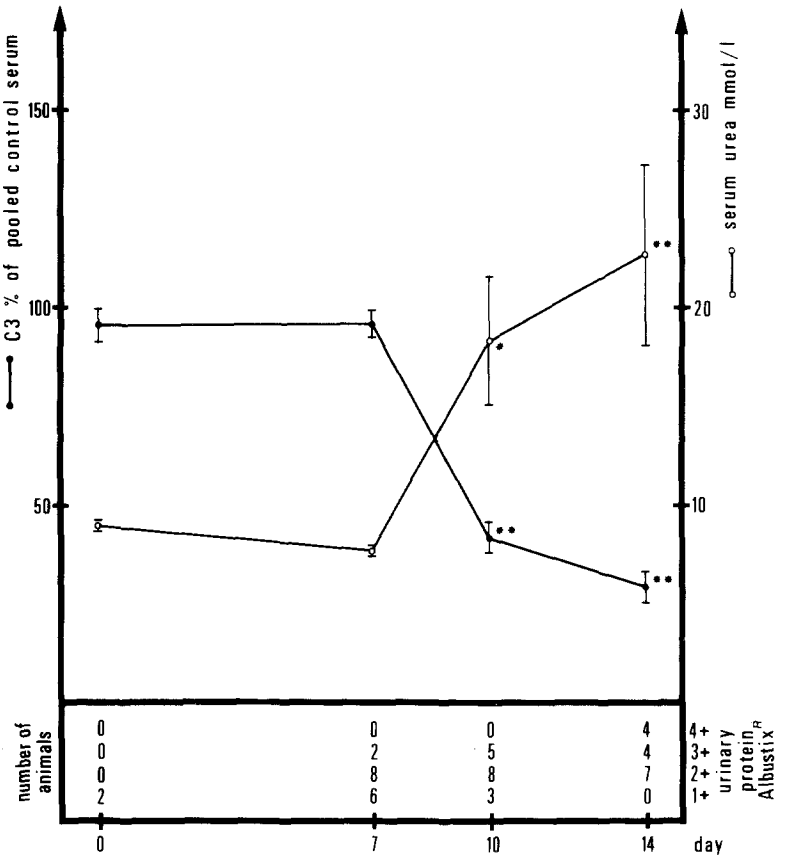

Fig. 1. Serum urea, serum complement (C3) and urinary protein (Albustix®) during the experiment. Mean ± SE. Statistically significant compared with day 0 at *P*-level: * <0.05; ** <0.01

Table 1. Concentrations of rabbit albumin and ovalbumin in the urines of six rabbits during the experiment. Mean ± SE

	0 Day	7 Day	10 Day	14 Day
Rabbit albumin (mg/ml)	0	0.85 ± 0.18	1.37 ± 0.49	2.78 ± 0.48[a]
Ovalbumin (mg/ml)	0	0.55 ± 0.15	1.12 ± 0.23	0.98 ± 0.14

[a] Statistically significant compared with day 10 at *P*-level 0.001

obliteration of the capillary lumina (Fig. 2). With light microscopy alone, no distinction could be made between endothelial, mesangial and other possible types of cells. Not infrequently, a mitotic figure was present among the proliferating cells. In some glomeruli these changes were accompanied by foci of tuft necrosis and by occasional polymorphonuclear leucocytes. No crescent formation was seen. The basement membranes appeared normal in silver-stained

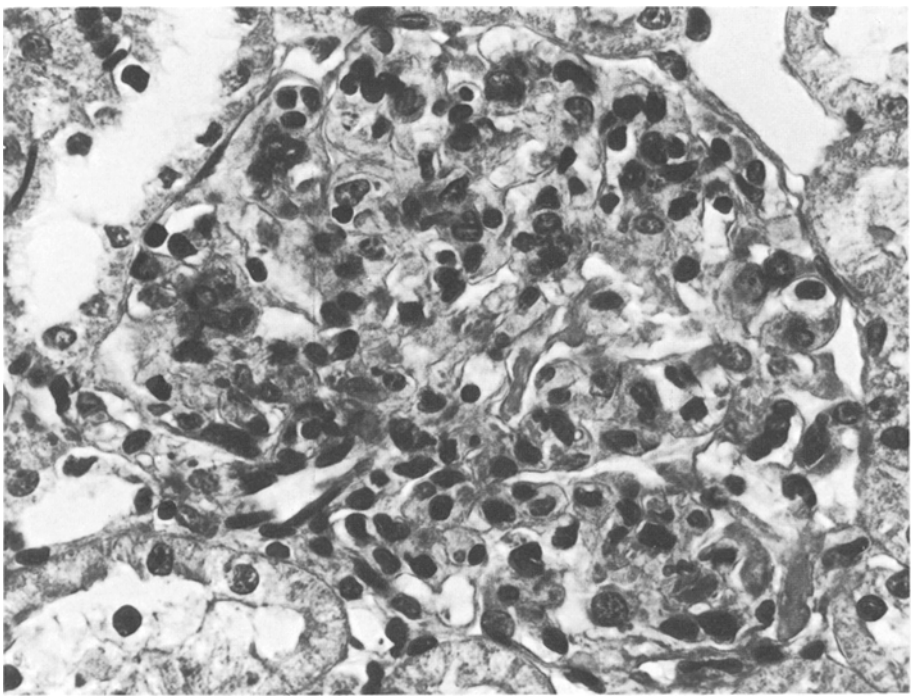

Fig. 2. Representative glomerulus from an immunized rabbit showing proliferative glomerulonephritis. Haematoxylin and eosin. × 500

sections. The lesions described varied somewhat in severity within one kidney, but were essentially the same in all the experimental animals.

In electron microscopy the most conspicuous finding was increased cellularity. Both mesangial and endothelial cells participated in the proliferation, causing widening of the mesangial areas and occlusion of the capillaries (Fig. 3). The mesangial cell appeared to be the main proliferating cell type, although not all cells could be identified with sufficient accuracy. A third cell type, present both in mesangial areas and in obliterated capillaries, accounted for part of the hypercellularity. These cells had a slightly indented nucleus and cytoplasma containing several lysosomes as well as other organelles in abundance, i.e., the morphological features typical of blood monocytes (Fig. 4).

The basement membranes were normal in structure except for occasional areas of decreased electron density and slightly irregular linings. Sparse electron-dense deposits could be detected. These were always small and located on either the subepithelial (Fig. 6A) or subendothelial (Fig. 6C) aspect of the basement membrane or, less frequently, in the mesangium. Deposits of subepithelial type were often wedge-shaped, lying under the slit membrane between two adjacent foot processes. Very rarely 'humps' were seen. Other findings included local fusion of foot processes, sparse polymorphonuclear leucocytes, occasional mitoses and localized areas of necrosis.

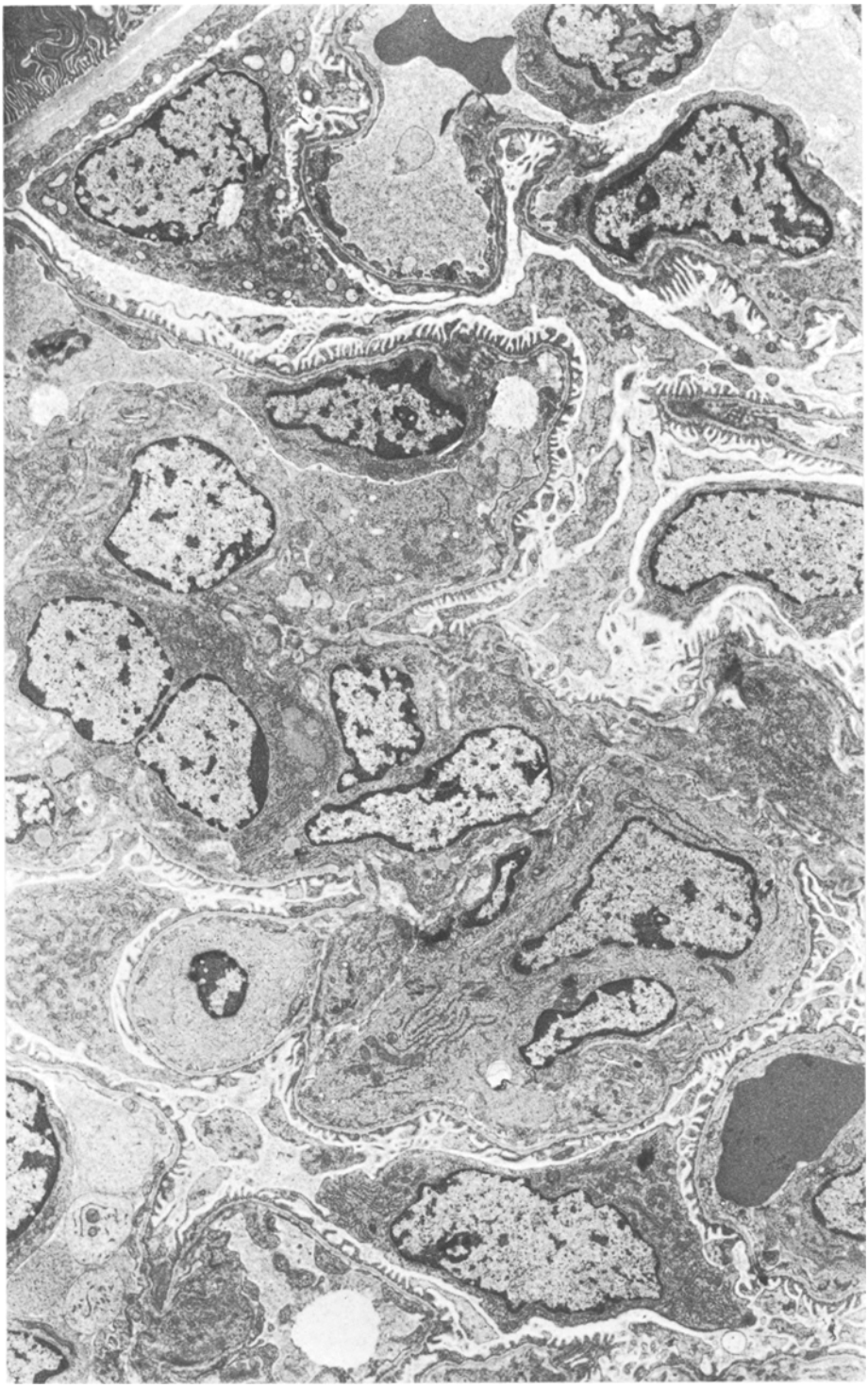

Fig. 3. Survey electron micrograph of a glomerulus in which marked hypercellularity due to proliferation of endocapillary cells has led to mesangial widening and capillary occlusion. ×4,700

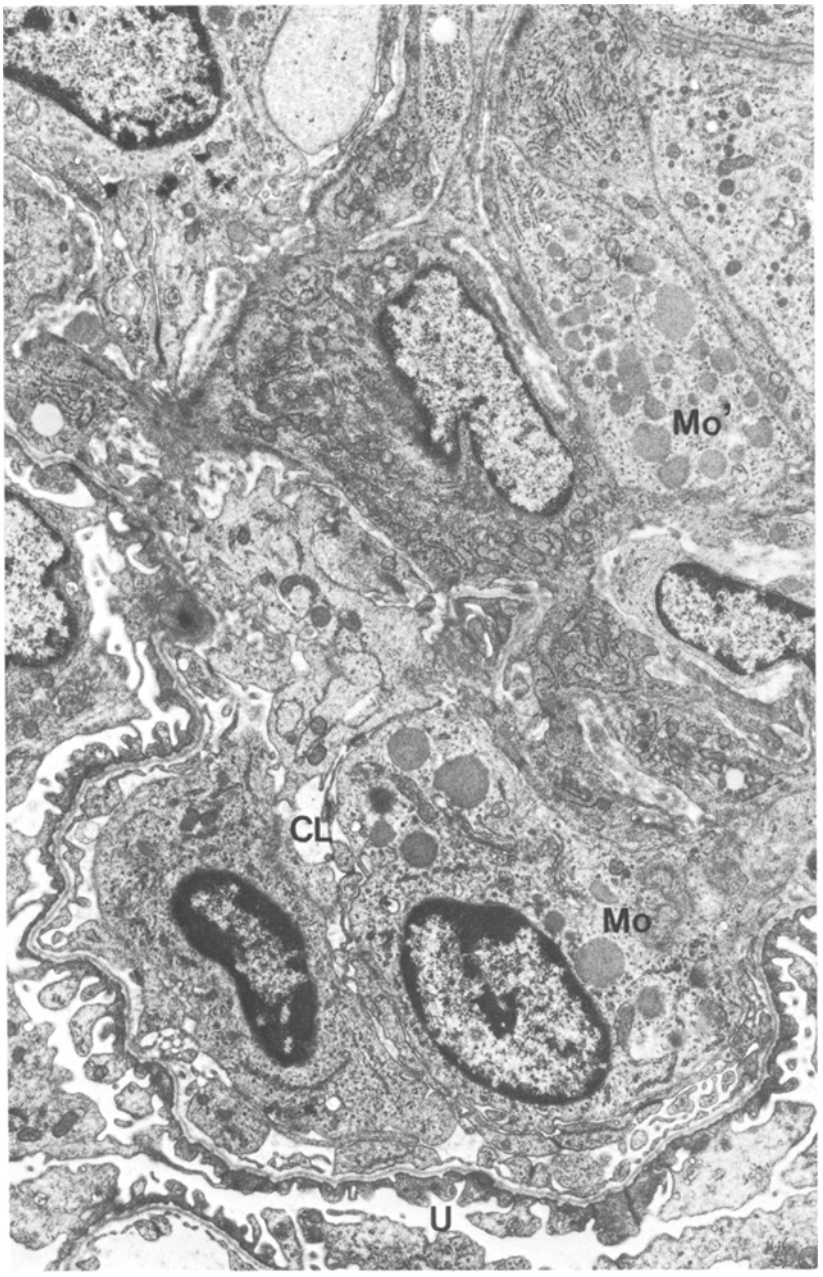

Fig. 4. Glomerular infiltration with monocytes. One of the two (*Mo*) in a capillary lumen (*CL*), the other (*Mo'*) in the mesangium (*M*). (*U*) Urinary space. ×6,400

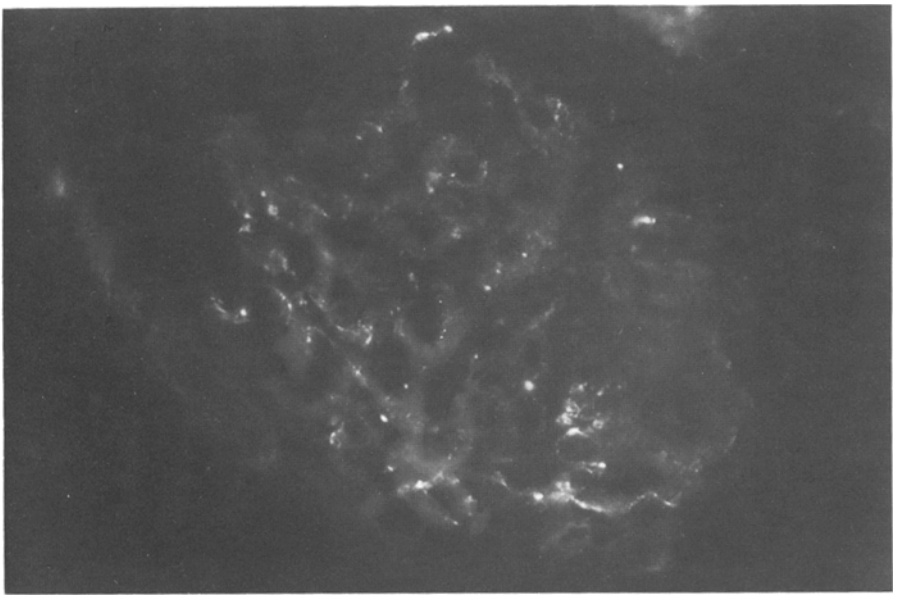

Fig. 5. Immunofluorescent staining with antiserum to rabbit IgG. Sparse granular fluorescence, localized in capillary walls. ×380

Immunohistochemical Findings

Staining with FITC-labelled antiserum specific for rabbit IgG and γ-globulin showed very sparse granular fluorescence in the peripheral capillary walls (Fig. 5). Its location and intensity were the same with the two antisera. In some of the animals the finding was negative. Antisera to C3 and ovalbumin also produced fluorescence in the same location, but this was less intense and less extensive than with the anti-IgG serum. In a few animals no C3 or ovalbumin was detected.

The ultrastrutural immunoperoxidase technique revealed immune deposits in all animals. These closely resembled the deposits found in conventional electron microscopy and stained positively with HRP-labelled antisera to rabbit IgG, C3 and ovalbumin (Figs. 6 and 7). The same reactants were also detectable in the plasma of the peripheral capillary portions and in the urinary space. The glomerular basement membrane gave a positive reaction with all the antisera tested. This reaction was most conspicuous on the epithelial side of the lamina densa. In control specimens the only notable feature was an occasional weak staining of the plasma membrane of epithelial cells (Fig. 7C).

Findings in Tissue Electrophoresis

Electrophoresis of renal cortical homogenates revealed rabbit IgG. In counter-electrophoresis a fraction with the same motility could be precipitated with anti-ovalbumin serum.

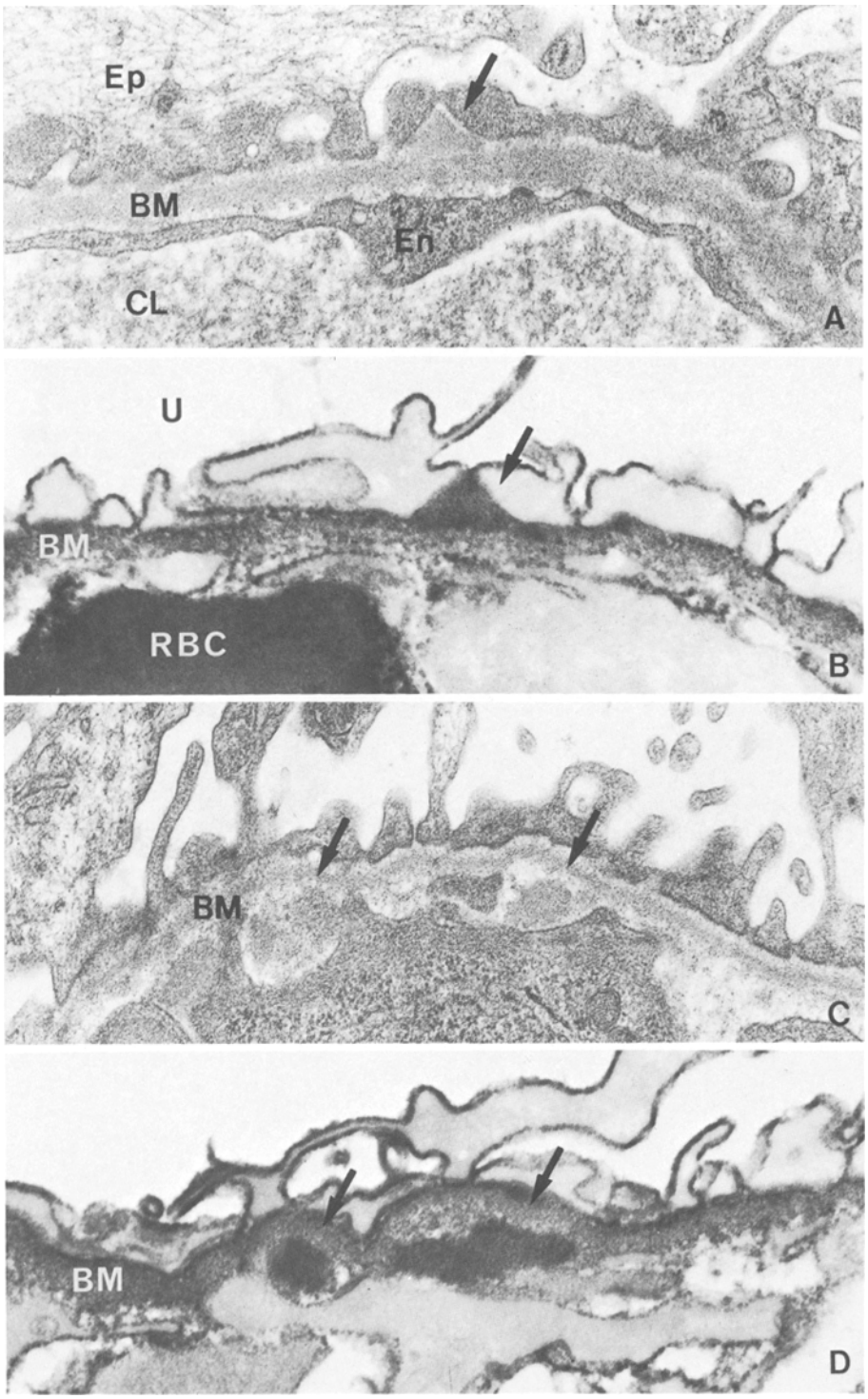

Fig. 6 A–D. Immune deposits (arrow) in subepithelial (**A** and **B**) and subendothelial (**C** and **D**) location. Staining with HRP-labelled antiserum to rabbit IgG (**B** and **D**) shows a positive reaction in the deposits as well as in a red blood cell (*RBC*), basement membrane (*BM*) and plasma membrane of epithelial cells (*Ep*). (*CL*) capillary lumen; (*En*) endothelial cell; (*U*) urinary space. All ×33,000

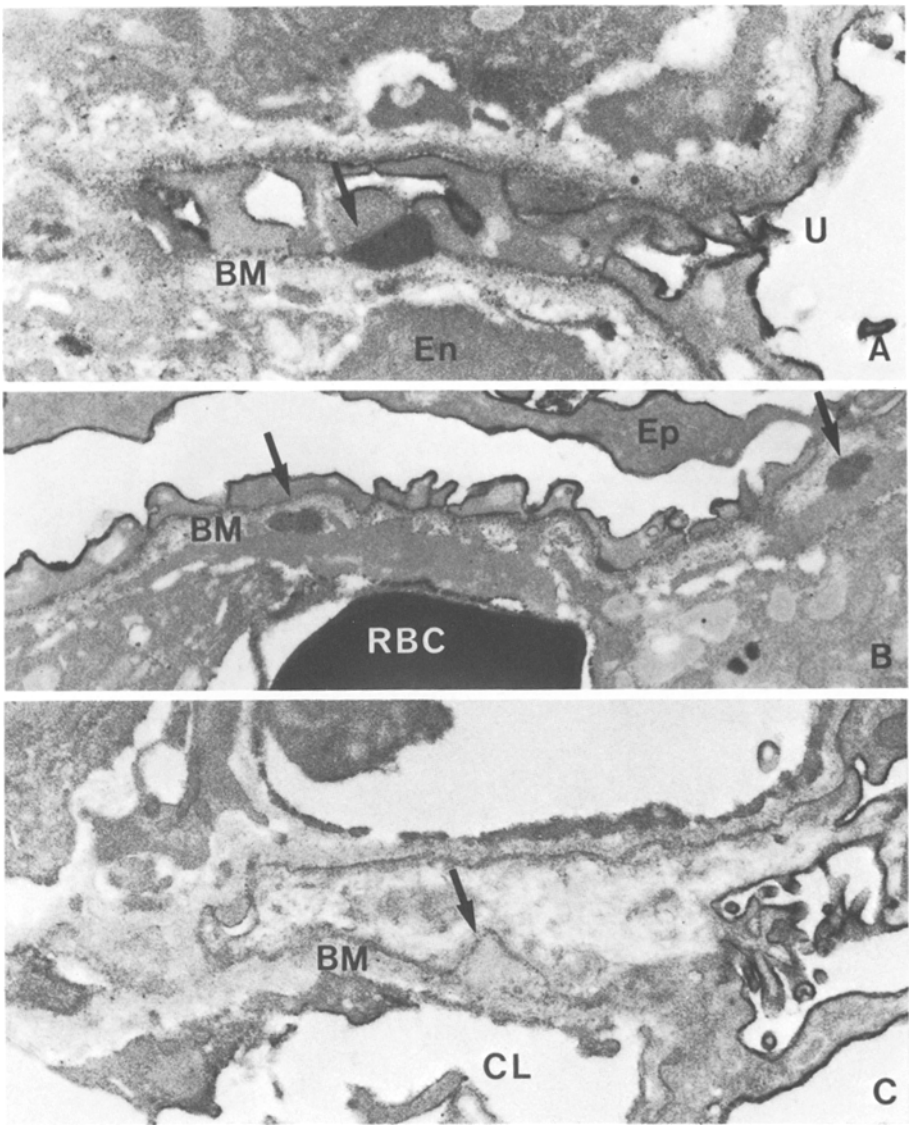

Fig. 7 A–C. A positive reaction with HRP-labelled anti-ovalbumin serum in subepithelial (**A**) and subendothelial (**B**) deposits (arrow). Other structures stained are a red blood cell (*RBC*), the plasma membrane of epithelial cells (*Ep*) and, weakly, the basement membrane (*BM*). In **C** an unstained deposit in a control section treated with unlabelled antiserum before incubation with HRP-labelled antiserum to rabbit IgG (see Fig. 6). (*CL*) Capillary lumen. (*En*) Endothelial cell. (*U*) Urinary space. **A** and **C** ×17,900. **B** ×14,700

Discussion

The experimental study reported here was designed to investigate the morphological and immuno-histochemical characteristics of glomerulonephritis produced in antigen excess, the antigen given being a low molecular weight heterologous protein rapidly eliminated by glomerular filtration. The presence of immune complex glomerulonephritis was suggested by the coincidence of the rise in serum urea levels with the decrease in serum C3 levels, urinary excretion of protein and histological changes. The predominance and increasing amounts of rabbit albumin in the urine rules out the possibility that the only protein excreted is the injected ovalbumin; rather, it suggests that the permeability of the glomerular capillary wall is altered. This view is supported by the finding of rabbit IgG (with a molecular weight of 150,000) in the urines of 5 out of 6 animals. Detection of the circulating free antigen (ovalbumin) in the serum from day 7 on, and immuno-histochemical recognition of the antigen in addition to the antibody in most of the immune deposits indicated a state of antigen excess. The finding in tissue electrophoresis of rabbit IgG precipitable with anti-ovalbumin indicates the presence of tissue-bound immune complexes.

Morphologically, the glomerulonephritis described here was characterized by extensive proliferation of endocapillary cells and occasional polymorphonuclear cells, and by local areas of necrosis. The nature of the proliferating cells could not always be determined, but most of them were without doubt of mesangial origin. In addition, the glomerular hypercellularity was partly due to monocytes, encountered in mesangium and obliterated capillaries. The presence of monocytes has been described previously in experimental glomerulonephritis and they are suggested to aid in eliminating the products of tissue damage (Shigematsu, 1970; Shigematsu and Kobayashi, 1976) and antigen-antibody complexes (Okumura et al., 1971). According to another view, the monocytic infiltration could be connected with the mechanism of cellular immunity (Schreiner et al., 1978).

Electron-dense glomerular deposits containing antigen, antibody and complement components are traditionally regarded as strong evidence of the presence of an immune complex glomerulonephritis. In this study, electron microscopy showed sparse, and usually small, subepithelial, subendothelial and mesangial deposits. These scanty deposits were much more easily detectable with the ultrastructural immunoperoxidase technique than with immunofluorescence or ordinary electron microscopy, and in every case were shown to contain components of immune complexes (rabbit IgG, ovalbumin, C3). Nevertheless, the sparsity of these immune deposits was typical of the finding, and in this respect our results are in agreement with those of Germuth et al. (1975). Possibly because of the immunoperoxidase technique that we used, however, we observed immune deposits in all animals and thus more often than Germuth and his colleagues. Regarding the occurrence of immune deposits, the continuous antigen overload in this experimental model may have dissolved the complexes formed, preventing an otherwise more extensive deposition (Valdes et al., 1969).

The results of the present study cannot be used to evaluate the pathogenetic role of immune complexes. The view that immune complex deposits are not

per se at least of central importance is favoured by their sparse occurrence in the presence of severe histological changes of other types. Small complexes formed when antigen is present in excess and filtering profusely through the glomerular capillary wall are possibly the toxic factors acting on glomerular tissue with subsequent inflammation. In this study we were struck by the presence of monocytes in the glomeruli. This suggests that in addition to the well-recognized mediator mechanism, the complement system, monocytes may play a role in the course of events leading to severe glomerular alterations.

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