# ELECTRON MICROSCOPIC LOCALIZATION OF THE NEPHROTOXIC ANTIBODY IN THE GLOMERULI OF THE RAT AFTER INTRAVENOUS APPLICATION OF PURIFIED NEPHRITOGENIC ANTIBODY-FERRITIN CONJUGATES*

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It has been shown in a previous paper (1) that intravenously injected, ferritin-conjugated, nephrotoxic antibodies localize preferentially at the endothelial side of the glomerular basement membrane. The present report gives further data concerning the localization of nephrotoxic antibody using isolated conjugates free from uncoupled antibody in amounts large enough to produce nephritis.

## *Materials and Methods*

*Rats.*—Male Wistar rats (H. Bosse, Greven, Germany) weighing 140–160 g were used. Kidneys were usually examined separately. From 24 hr to 3 days after the injection of the conjugate or at the time of the onset of proteinuria, the left kidney was removed under slight ether anesthesia, so that proteinuria of later onset could also be demonstrated. The other kidney was examined when the rat was sacrificed.

*Determination of Proteinuria.*—Collection of urine and quantitative estimation of proteinuria (biuret method) were carried out as described in a previous report (2).

*Application of Nephrotoxic Antibody.*—Globulin fractions containing nephrotoxic antibody were injected into the tail vein. Because of loss of antibody activity due to coupling to ferritin (3), the conjugates had to be injected in large amounts to induce nephritis. Usually not more than 80–100 mg of ferritin in the form of antibody-ferritin conjugate was injected at one time. In rats receiving larger quantities of ferritin, the injections were distributed over a 24–36 hr period. None of the rats received more than three injections. To keep the injected amount of conjugate as small as possible, only potent globulin fractions were selected for conjugation. The gamma globulin obtained from the rabbit nephrotoxic serum (NTS) was usually able to initiate immediate and lasting proteinuria in a dose of 1–4 mg. The globulin fractions from duck NTS were less potent. We have not yet succeeded in producing immediate nephritis by the injection of purified ferritin-labeled duck nephrotoxic antibody.

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In order to get an approximation of the amount of conjugate needed to cause nephritis, the kidney-fixing ability of the conjugates was compared with that of the globulin fraction used for the conjugation by a titration method using fluorescent antibody (4). 1 ml of serial dilutions of purified conjugates and uncoupled globulin fractions were injected intravenously into the rats. 3 hr later the kidneys were removed and stained for fixed nephrotoxic globulin with fluorescein-labeled anti-globulin. Comparison of the end dilution at which a distinct fluorescence was observed allowed a rough estimation of the loss of antibody activity. Calculation of the amount of antibody globulin conjugated to ferritin was done as previously described (1) assuming a ratio of globulin to ferritin of 1 to 1 on a molecular basis (5).

As controls, rats were injected with similar doses of ferritin, ferritin mixed with uncoupled nephrotoxic antibody, or ferritin conjugated to normal bovine globulin. In some experiments ferritin-labeled nephrotoxic antibody globulin was injected into rats which had previously received free nephrotoxic globulin.

*Antisera to Rat Glomeruli (NTS).*—Antisera were prepared as described in a previous report (2) with the following modification: The first four injections were given in complete Freund's adjuvant at 3 wk intervals.

Gamma globulins of NTS were prepared by a fractionation with Rivanol followed by a repeated precipitation with ammonium sulfate (6). The final precipitate was dissolved in a small volume of phosphate-buffered saline (PBS), dialyzed exhaustively against PBS, filtered through a Millipore filter, and stored at −20°C until use.

*Preparation of Ferritin.*—Ferritin was prepared from horse spleen after the method of Granick (7) but the final crystallization with cadmium sulfate was omitted. The crude ferritin preparation obtained by precipitation with ammonium sulfate was dialyzed for 24–48 hr against running water. The precipitate was removed by repeated centrifugation at 15,000 rpm for 30 min. The ferritin was spun down at 40,000 rpm for 2–3 hr in a Spinco ultracentrifuge, model L, rotor 40. The pellet was dissolved in distilled water, adjusted to a concentration of 10–30 mg/ml and centrifugation was repeated four times to remove all apoferritin. The final product was checked for purity in an analytical ultracentrifuge and with polyacrylamid gel electrophoresis. The ferritin was passed through a Millipore filter and stored at 4°C. The ferritin obtained in this simple manner is pure and free of apoferritin. The average yield is much higher than by crystallization with cadmium sulfate.

*Preparation of Antibody-Ferritin Conjugates.*—Three different coupling agents were used: toluene-2,4-diisocyanate (TC), *p,p'* difluoro-*m,m'* dinitrodiphenyl sulfone (FNPS) and *o*-dianisidine (DB). Conjugation was performed as described in previous publications (8, 5, 9). According to our experience, disproportionately high losses of antibody activity occurred if more than 30% of the ferritin present in the reaction mixture was conjugated (1, 3). Therefore, independently of the method used, we tried not to exceed this limit.

*Purification of Conjugates.*—To remove uncoupled antibody globulin, the crude conjugates were centrifuged twice at 40,000 rpm for 1–2 hr. The pellet was dissolved with buffer and electrophoresis performed in Geon X-427 to separate the antibody-ferritin from the uncoupled ferritin. The conjugate was concentrated either by dialyzing against Aquacide (Calbiochem, Lucerne, Switzerland) or by ultracentrifugation, or by both methods.

The purity of isolated conjugates was tested by immunoelectrophoresis and by intravenous injection into the rat of 2 ml of the last supernatant after ultracentrifugation. The kidneys were removed 2 hr later and stained with fluorescein-labeled anti-globulin to detect unconjugated nephrotoxic antibody. The conjugates purified and isolated in the above manner were usually free of uncoupled nephrotoxic antibody in the case of rabbit NTS. Nephrotoxic globulin from the duck conjugated to ferritin was not free of uncoupled antibody.

All isolated conjugates still contained small amounts of uncoupled ferritin. The percentage of free ferritin was determined densitometrically after separation in agar gel electrophoresis

and staining with Prussian blue. The amount of antibody globulin in the purified conjugate was calculated as previously described in detail (1).

To avoid gross bacterial contamination, 60 $\mu$g of penicillin and 100 $\mu$g of streptomycin/ml was added to the preparations. At the end of purification and isolation, the conjugate was sterilized by passage through cellulose filters (Millipore). Only with these precautions was it possible to inject amounts of conjugate large enough to induce nephritis.

*Fluorescent Antibody.*—Preparation of antisera against rabbit globulin, duck globulin, ferritin, and rat $\beta_{1C}$, conjugation to fluoresceinisothiocyanate, purification of conjugates, and immunohistochemical procedure were described previously (1, 2).

*Electron Microscopic Studies.*—Kidneys were either fixed in phosphate-buffered 1% $OsO_4$ containing 0.25 M sucrose and then embedded in methacrylate, or they were prefixed in buffered 5% glutaraldehyde, postfixed in 1% $OsO_4$, and embedded in Epon according to Luft (10). Ultrathin sections were cut with a LKB Ultotome and stained with uranyl acetate. The sections were examined and photographed in a Hitachi HU-1 and a JEM-7 electron microscope.

For the determination of the fixed antibodies in the glomerular basement membrane (BMFAb), ferritin molecules per 1000 A were counted in different parts of each investigated animal. The thickness of the sections was determined by the interference color as usual (11, 12). The methacrylate-embedded sections revealed a thickness of about 300 A and the Epon-embedded sections a thickness of about 500 A. The concentration of BMFAb was expressed in ferritin molecules per 3000 $m\mu^2$ of filtration surface.

Disappearance rates were calculated from semilogarithmic plots with the number of the BMFAb in the ordinate and the days postinjection in the abscissa.

## RESULTS

### *In Vivo Effect of Purified Antibody-Ferritin Conjugates*

A total of 56 rats were injected with purified conjugates. 42 animals received conjugates prepared with the globulin fraction of rabbit NTS, and 14 conjugates with duck NT globulin. The latter conjugates were prepared without exception with FNPS as coupling agent. All three conjugation methods were suitable in obtaining purified conjugates potent enough to induce nephritis.

Of the injected rats, 18 died within 24 hr after the injection of purified conjugate. As death also occurred among controls which received only ferritin or ferritin-conjugated to normal gamma globulin, we assume that the high death rate, especially in the initial experiments, can be largely attributed to contamination with bacterial endotoxin. This assumption was confirmed by isolating Gram-negative bacteria from all toxic preparations. We tried, therefore, to reduce bacterial contamination by handling the preparations more carefully and by adding antibiotics. Before application, the conjugates were passed through Millipore filters.

Of the injected rats, 14 developed proteinuria. In five rats the onset was immediate, in the remaining nine animals proteinuria started after a latent period of 2–8 days. Five rats of this group died within 4 wk. Of the surviving nine animals, eight rats showed a normal amount of protein in the urine at the end of the 4th wk. Only one rat developed a lasting proteinuria. Nephritis in

this animal had started immediately, and the amount of protein excreted at the end of the 4th wk was 42 mg/24 hr.

Injection of about 5–40 mg of nephrotoxic globulin conjugated to ferritin was necessary to cause nephritis (Table I). No immediate onset of proteinuria was observed if less than 20 mg of conjugated nephrotoxic globulin was applied. Compared with the same globulin fraction, which caused an immediate proteinuria already in an amount of 1 mg prior to conjugation, 20–40 times more globulin had to be injected after coupling to ferritin.

Similar results were obtained with the semiquantitative immunofluorescent technique. To demonstrate heterologous globulin in the glomeruli by fluorescent

TABLE I

*Onset of Proteinuria and Amount of Injected Purified Conjugate*

| Day of onset of proteinuria | No. of animals | Amount of purified conjugate injected | | Maximal amount of proteinuria per 24/hr |
|---|---|---|---|---|
| | | Ferritin* | NT globulin | |
| | | *mg* | *mg* | *mg* |
| 1 | 5 | 130.0 to 340.0 | 23.4 to 39.3 | 18 to 186 |
| 2 | 2 | 85.5 and 111.0 | 12.4 and 14.4 | 25 and 89 |
| 3 | 2 | 30.0 and 329.0 | 3.1‡ and 43.9 | 25 and 39 |
| 4 | 1 | 74.0 | 11.2 | 22 |
| 6 or later | 4 | 25.5 to 283.0 | 4.7 to 41.1 | 47 to 88 |

* Total ferritin, i.e., ferritin of conjugate plus free ferritin.

‡ In this rat, receiving an amount of 3.1 mg of NT globulin, the left arteria renalis was clamped during the injection of conjugate.

antibody, usually more than 20 times the calculated amount had to be injected.

The only rat which developed proteinuria after injection of conjugated duck antibody was injected with 43.9 mg nephrotoxic globulin coupled to ferritin. The onset of nephritis occurred after a latent period. To induce immediate nephritis, 4 mg of the uncoupled globulin of this fraction was needed.

As controls nine rats were injected with 50–270 mg ferritin alone or ferritin conjugated to bovine gamma globulin. After the injection of high doses, protein concentration in the urine increased during the first days but never exceeded 13 mg/24 hr.

*Immunofluorescent Studies*

In rats injected with purified conjugates, accumulation of nephrotoxic globulin and ferritin could be shown in the same capillary pattern as after application of uncoupled nephrotoxic globulin. The only difference seen was a somewhat more pronounced staining of the mesangial region after injection of the conjugate (Figs. 1 and 2). There was good correlation between the intensity of staining and the development of proteinuria (Table II).

*Electron Microscopic Studies*

*I. Localization of Ferritin Molecules.—*

*The glomerular basement membrane:* The injection of ferritin or ferritin conjugated to bovine gamma globulin never caused proteinuria nor did it cause lasting localization of ferritin molecules in the basement membrane. Depending on the amount of injected ferritin or inert conjugate, ferritin molecules could be detected several hours after the application accumulated within the capillary lumen of the glomeruli (Fig. 4). Individual molecules were scattered irregularly throughout the basement membrane and the mesangial matrix. Usually more ferritin molecules were found in the mesangial matrix than in the adjoining basement membrane (Fig. 5). At the end of the first day, almost

TABLE II

*Comparison between Proteinuria and Fluorescent-Staining Intensity for Ferritin, Nephrotoxic Globulin, and Host $\beta_{1C}$ in Glomerular Pattern after Injection of Purified Conjugates*

| Proteinuria | Nephrotoxic globulin | | Ferritin | | Rat $\beta_{1C}$ | |
|---|---|---|---|---|---|---|
| | Bright | Weak | Bright | Weak | Positive | Negative |
| yes | 13 | 0 | 13 | 0 | 12 | 1 |
| no | 28 | 9 | 27 | 13 | 3 | 16 |

all ferritin molecules had already passed the basement membrane. 2 or 3 days later most ferritin had also disappeared from the mesangial matrix.

After the injection of purified ferritin-conjugated nephrotoxic globulin, the findings in the basement membrane were quite different. Massive accumulation of ferritin molecules occurred within it. The molecules were located preferentially in the endothelial side (Fig. 3). Fixed ferritin-labeled nephrotoxic antibody molecules remained there for at least several weeks, though decreasing in number with time. The distribution of molecules along the capillary loop was not uniform. Most times more antibody molecules had reacted with the peripheral part of the basement membrane than with the axial region (Fig. 6). No pronounced difference in localization could be demonstrated between rabbit and duck nephrotoxic antibody.

In the nephrotoxic rats so far investigated, the ferritin-labeled antibody once fixed to the basement membrane remained at the reaction site (Figs. 7 and 8). No migration of ferritin towards the epithelial side of the basement membrane could be observed, though the number of the fixed molecules progressively decreased.

*Glomerular cells:* Besides this specific reaction of labeled nephrotoxic antibody with the basement membrane, nonspecific localization of ferritin molecules occurred within cells adjoining the basement membrane. After injection

of nephrotoxic conjugates as well as after application of ferritin or inert conjugates, ferritin molecules were picked up by cells and remained there, visibly, for at least several weeks. The most striking findings were the segregation of ferritin molecules within small vesicles, vacuoles, and dense bodies (Figs. 5 and 6). Vesicles filled with ferritin occurred preferentially within mesangial

TABLE III

*Persistence of Ferritin-Conjugated Rabbit Nephrotoxic Antibody in the Glomerular Basement Membrane*

| Rat No. | Day of examination* | No. of BMFAb molecules per 3000 mμ² | Half-disappearance time |
|---|---|---|---|
| | | | *days* |
| 170 | 7 | 24.2 | 5.8 |
| | 21 | 4.4 | |
| 161 | 3 | 26.2 | 6.9 |
| | 17 | 6.3 | |
| 176 | 1 | 28.1 | 7.0 |
| | 18 | 5.0 | |
| 186 | 1 | 21.3 | 10.2 |
| | 29 | 3.0 | |
| 212 | 1 | 9.9 | 11.9 |
| | 30 | 1.8 | |
| 70 | 3 | 4.9 | 13.9 |
| | 47 | 0.5 | |
| 71 | 3 | 8.3 | 17.8 |
| | 48 | 1.5 | |

* For the first determination, the left kidney was removed under slight ether anesthesia; the right kidney was examined when the rat was sacrificed.

cells, sometimes within endothelial, and seldom within epithelial cells. Most epithelial cells did not contain any ferritin. In a few cells ferritin molecules could also be shown scattered irregularly throughout the cytoplasm without connection to distinct cytoplasmic fine structures. These molecules were found even several weeks after the administration of ferritin.

*Half-disappearance time of basement membrane-fixed ferritin-conjugated antibody (BMFAb):* The half-disappearance time of the fixed ferritin-labeled nephrotoxic antibody was estimated by counting ferritin molecules in the same animal at different times. The left and right kidney of seven individual rats were examined at intervals of 14–45 days (Table III). The three rats sacrificed on day 17, 18, and 21 revealed a half-disappearance time of about 6–7 days, while the half-disappearance time of the BMFAb in the four animals killed on day 29, 30, 47, and 48 was calculated as 10–18 days. The thus calculated half-disappearance time is only of limited significance because of the insufficient

data available. But it seems that with increasing time the half-disappearance time of the remaining BMFAb becomes longer.

*Quantitative studies:* Table IV demonstrates the relation between the number of BMFAb and the development of proteinuria. An average of more than 20 rabbit antibody molecules was found per 3000 m$\mu^2$ basement membrane in animals which developed immediate proteinuria. In a few cases, a significant proteinuria could be induced with an average of 5 molecules. In some animals

TABLE IV

*No. of Ferritin Molecules Localized in the Glomerular Basement Membrane of Animals with and without Proteinuria*

| Rat No. | NT globulin | Coupling agent | Day of onset of proteinuria | Day of examination (left kidney) | No. of ferritin molecules per 3000 m$\mu^2$ of basement membrane | |
|---|---|---|---|---|---|---|
| | | | | | Average | Maximal |
| 175 | Rabbit | FNPS | 1 | 1 | 31.5 | 39.0 |
| 176 | " | FNPS | 1 | 2 | 28.1 | 54.7 |
| 186 | " | FNPS | 1 | 2 | 21.3 | 77.2 |
| 211 | " | TC | 1 | 3 | 26.7 | 47.5 |
| 70 | " | FNPS | 2 | 3 | 4.9 | 7.4 |
| 193 | " | FNPS | 2 | 2 | 4.7 | 6.8 |
| 152 | Duck | FNPS | 3 | 3 | 55.8 | 114.1 |
| 302* | Rabbit | FNPS | 3 | 3 | 19.9 | 28.1 |
| 212 | " | TC | 4 | 2 | 9.9 | 15.7 |
| 170 | " | FNPS | 6 | 7 | 24.2 | 37.6 |
| 71 | " | FNPS | 7 | 3 | 8.3 | 12.1 |
| 366 | " | DB | No | 1 | 14.2 | 24.1 |
| 161 | " | TC | No | 2 | 26.2 | 31.2 |
| 363 | " | DB | No | 3 | 7.1 | 12.6 |

* The left kidney was clamped during the injection of the conjugate and the right kidney was examined.

however, also listed in Table IV, a concentration of 8–26 BMFAb failed to induce any detectable kidney damage. It may be of some significance that among the listed rats without proteinuria no deposits of host complement could be demonstrated, not even in glomeruli showing as many as 26.2 BMFAb/ 3000 m$\mu^2$ of basement membrane.

Only in one case were we able to induce nephritis by injection of labeled duck antibody. Proteinuria in this animal appeared on the third day. The basement membrane examined at this time for fixed ferritin molecules showed a heavy accumulation of BMFAb. Both the average and the maximal counts per 3000 m$\mu^2$ found in this rat were higher than in any other animal injected with ferritin-labeled nephrotoxic antibody from the rabbit. This is the more surprising

as, in contrast to the rabbit antibody, the preparations of conjugates with duck antibody were not absolutely free of uncoupled nephrotoxic globulin as outlined above.

*II. Morphological Changes.*—

In diseased animals a marked swelling of endothelial cells and an increase of mesangial cells associated with an increase of ribosomes and endoplasmatic reticulum could be shown. Detachment of the endothelial cells from the basement membrane was also detectable. Besides these observations, already reported by others, an interesting finding was that, in the early stage between the basement membrane and the detached endothelium, an amorphous material appeared with an electron density similar to the basement membrane. In some cases peculiar, microtubuli-like, structures could be found in this layer, indicating a new formation of the basement membrane. Most times the endothelial side of the basement membrane proper was clearly marked by the fixed ferritin molecules (Fig. 10). In rats developing longer lasting proteinuria, a fusion of the foot processes could be shown (Fig. 9). But in general all the mentioned morphological changes were not severe.

## DISCUSSION

Intravenous injection of purified ferritin-conjugated nephrotoxic antibody can cause nephritis in rats. The fluorescent antibody technique shows the same capillary pattern in rats receiving nephrotoxic globulin or ferritin-conjugated antibody.

By electron microscopy the exact reaction site of the ferritin-labeled nephrotoxic antibody could be demonstrated. Even if nephritogenic amounts of conjugate were applied, the fixed molecules were restricted to the inner part of the basement membrane. Only occasionally were a few single ferritin molecules present within the lamina densa of the glomerular basement membrane.

These findings are partially in agreement with earlier results reported by others (17, 18) obtained with an in vitro technique using ferritin-conjugated antibodies against the injected nephrotoxic globulin. Andres and coworkers (17) have found ferritin molecules spread uniformly throughout the entire basement membrane. Arhelger and coworkers (18) have seen ferritin molecules concentrated in the less dense zones on either side of the lamina densa of the basement membrane. The explanation for this difference is not entirely clear. We have not tried the in vitro method. As the passage of molecules through the glomerular basement membrane is directed from the capillary lumen towards the urinary space, it is conceivable that ferritin-conjugated nephrotoxic antibody accumulates on the endothelial side of the membrane. We are aware that, in our experiments, the injected amounts of purified ferritin-conjugated

nephrotoxic globulin were relatively small, causing only slight kidney damage. It is possible that after application of higher doses of conjugate an accumulation of ferritin will be seen in a broader zone of the glomerular basement membrane.

It is further of interest that the concentration of fixed ferritin was usually higher in the peripheral part of the capillary loop than in the axial region. This observation suggests that a protein is preferentially excreted through the peripheral part of the basement membrane, and that a partial damage of the basement membrane may be sufficient for the appearance of proteinuria.

The once-fixed antibody remained unchanged at the original site of reaction for several weeks at least, but the number of molecules decreased rapidly in the course of time. The calculated half–disappearance time of 6–18 days was much shorter than the half–disappearance time of about 20 days (15, 16) and of 52.5 days (13) reported by others, using $^{131}$I-labeled nephrotoxic globulin. A change in quality, possibly a lower avidity, of the antibody conjugated to ferritin may be the reason for this behavior. A migration of ferritin molecules once fixed at the endothelial side of the basement membrane towards the epithelial cells could not be demonstrated.

There is also a discrepancy between our findings and those of the abovementioned authors (17, 18) concerning glomerular cells. According to the observations of both working groups, ferritin molecules were also present to a lesser degree in a uniform distribution in the cytoplasm of the adjacent epithelial and endothelial cells. Andres and coworkers discussed the possibility that this distribution of ferritin in the cytoplasm of glomerular cells may reflect the presence of nephrotoxic globulin. Arhelger and coworkers assumed a specific reaction. Our results do not support this interpretation. After intravenous injection of the ferritin-conjugated nephrotoxic antibody, a random distribution of ferritin could be demonstrated only as an exception. Most of the endothelial and epithelial cells were free of ferritin. If ferritin occurred in the cytoplasm it was nearly always accumulated within membrane-limited vacuoles or vesicles. Those ferritin-containing vesicles were observed mainly, and sometimes in great numbers, in mesangial cells. The uptake of ferritin by cells was as high in control rats receiving ferritin-conjugated bovine gamma globulin as in nephritic rats. Segregation of ferritin within vacuoles and vesicles was described by Farquhar and coworkers (14) in a study of the permeability of the glomerular basement membrane with ferritin as a tracer. The unspecific accumulation of ferritin in glomerular cells, therefore, seems to play no active part in the onset of nephrotoxic nephritis.

Segregation of ferritin within vacuoles were not described by authors using the in vitro technique. It is possible that uncoupled nephrotoxic globulin is taken up by cells in a lesser degree. Another reason could be that, due to the heavy loss of nephrotoxic antibody activity by conjugation to ferritin (3), we

had to inject at least 20 times more of the calculated amount of conjugate to cause proteinuria. It is conceivable that the injection of such large doses resulted in a higher uptake of ferritin.

As expected, the highest amount of BMFAb was found in rats developing immediate proteinuria. At least 40–80 rabbit BMFAb per 3000 $m\mu^2$ filtration surface was needed to cause immediate nephritis if one assumes that the highest concentration of reacted antibody was more important than the average amount. The average amount of BMFAb in these rats was about 20–30 molecules/3000 $m\mu^2$. If the calculation is based on the same values used by Unanue and Dixon (13), i.e. a total filtration surface of 14,000 $mm^2$ for both rat kidneys and a molecular weight of 140,000 for a rabbit antibody, a total amount of about 30 $\mu g$ BMFAb was found in the kidneys of those rats developing immediate proteinuria. This value is considerably lower than the amount of 150 $\mu g$ kidney-fixing antibody obtained by Unanue and Dixon (13) using $^{131}I$-labeled nephrotoxic globulin from the rabbit. When comparing the results one has to take into consideration that, in our experiments, only a relatively mild nephritis was observed.

Only in one of our rats receiving ferritin-conjugated nephrotoxic antibody from the duck have we been able to induce nephritis. Though proteinuria occurred in this rat after a latent period of 3 days, we observed to our surprise, in the glomerular basement membrane of this animal, the highest concentration of all of fixed ferritin molecules. The maximal concentration was 114 BMFAb/3000 $m\mu^2$, the average amount 56 molecules. As we have not yet succeeded in removing the entire uncoupled antibody from the purified conjugated duck nephrotoxic globulin, the actual concentration of BMFAb in this rat was even higher.

We realize the limited significance of this single observation. Nevertheless, taking into account previous findings (19), it seems that more duck antibody is necessary to cause kidney damage than rabbit antibody, if $gamma_2$ antibody is involved. Unanue and Dixon (13), on the contrary, obtained no evidence of higher nephrotoxicity for the rabbit antibody.

Animals developing proteinuria after a latent period showed a great variation in the concentration of BMFAb. Though in one rat, 31 BMFAb/3000 $m\mu^2$ filtration surface did not induce proteinuria, in another animal developing nephritis after a latent period of 2 days a maximal concentration of only 6.8 BMFAb was found. Differences in the response of the individual host to the injected heterologous proteins may account for this unexpected observation.

## SUMMARY

Nephritis in rats was induced by intravenous injection of purified ferritin-conjugated rabbit and duck nephrotoxic globulin.

Using the fluorescent antibody technique, the same capillary pattern was

found as that in glomeruli of rats receiving uncoupled nephrotoxic globulin.

Electron microscopy revealed a heavy accumulation of the basement membrane–fixed antibody almost exclusively at the endothelial side. A higher concentration of ferritin was demonstrable in the peripheral basement membrane.

The once-fixed antibody remained at the site of reaction though decreasing with time. The half–disappearance time seemed to be shorter than that of the uncoupled nephrotoxic globulin.

No difference in localization was observed between rabbit and duck antibody.

At least 40 basement membrane–fixed antibody molecules from the rabbit per 3000 $m\mu^2$ of filtration surface were needed to cause immediate nephritis. To induce nephritis using duck antibody, a greater number of basement membrane–fixed antibody seemed to be necessary.

No evidence of specific reaction with constituents of glomerular cells was obtained.

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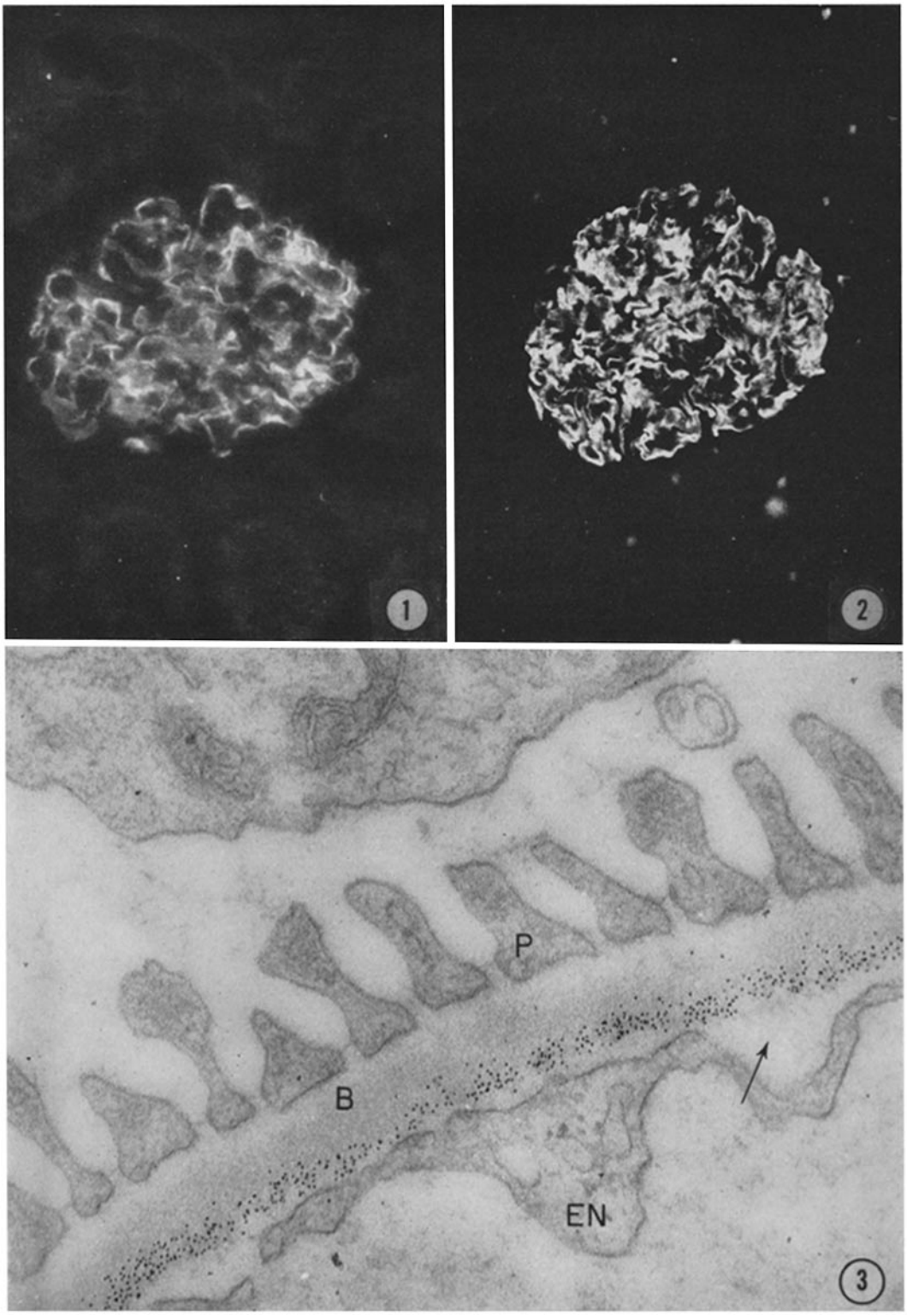
## EXPLANATION OF PLATES

### PLATE 93

FIGS. 1 and 2. Kidney section from animals receiving purified rabbit nephrotoxic globulin conjugated to ferritin. Fig. 1 stained with fluorescent anti-rabbit globulin, 1 day after the injection. Fig. 2 stained with fluorescent anti-ferritin, 3 days after the intravenous application of the conjugate. The glomeruli show the same capillary pattern as known from rats receiving uncoupled nephrotoxic globulin. × 200.

FIG. 3. Part of a glomerulus from an animal receiving 12 mg of rabbit nephrotoxic globulin conjugated to ferritin, examined 3 days after the injection. No proteinuria. Heavy accumulation of ferritin molecules is seen in the endothelial side of the basement membrane (B). No particles are visible within the adjoining cells. The endothelial cell (EN) appears to be swollen and is partially separated (arrow) from the basement membrane. Epithelial foot processes (P) are not altered. × 80,000.

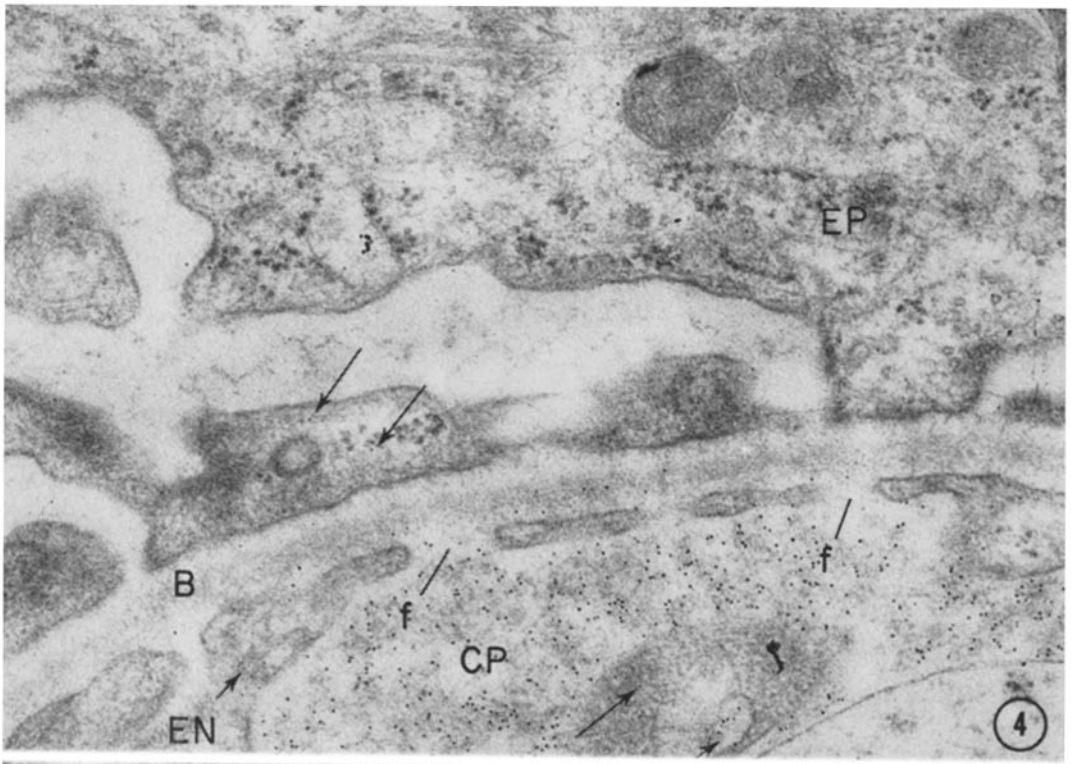


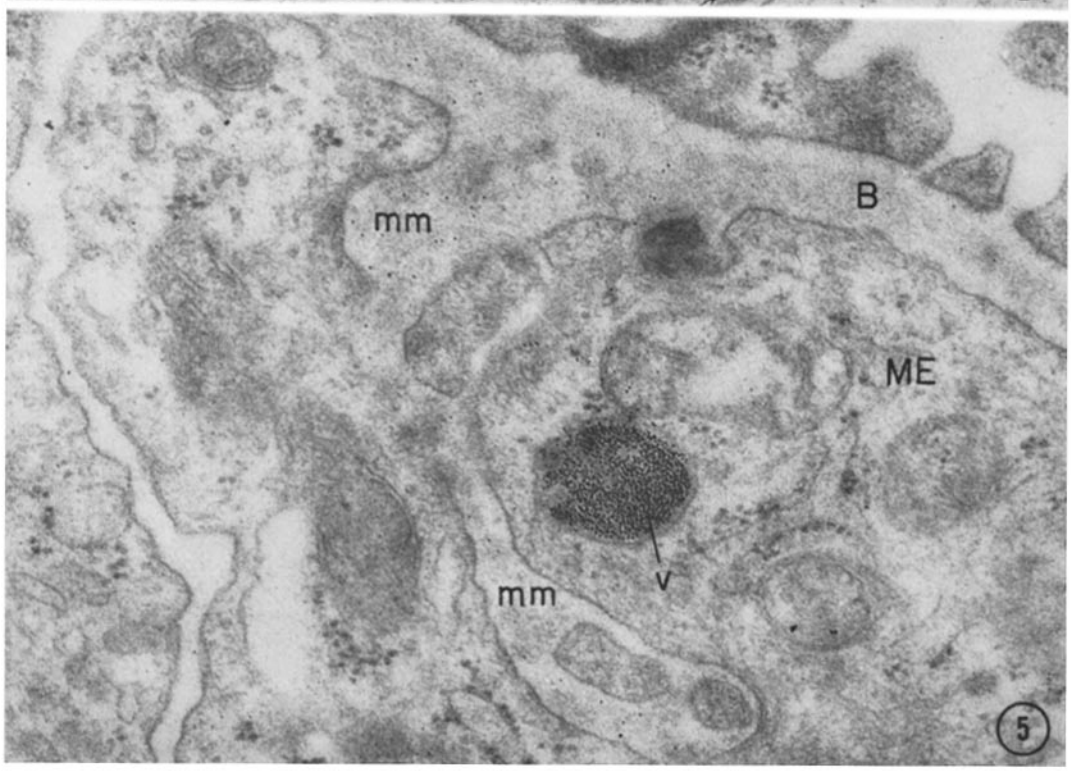

(Vogt et al.: Localization of nephrotoxic antibody)

PLATE 94

FIG. 4. Control animal 2 hr after intravenous injection of 100 mg of ferritin. The electron micrograph shows a part of the peripheral region of a capillary loop. Numerous ferritin molecules are seen in the capillary lumen (CP). A considerably lower number of particles are also present in the basement membrane (B) which they appear to penetrate through the fenestrae (f). Single molecules (arrows) can also be seen at this early stage within the cytoplasm of endothelial (EN) and epithelial (EP) cells. × 50,000.

FIG. 5. The same glomerulus as that in Fig. 4, showing the distribution of ferritin in the axial part of the glomerular loop. Few molecules are present in the basement membrane (B) at this early stage. A somewhat higher concentration of randomly distributed ferritin molecules is seen within the mesangial matrix (mm). A membrane-limited vesicle (v) packed with ferritin is located within the cytoplasm of a mesangial cell (ME). × 50,000.

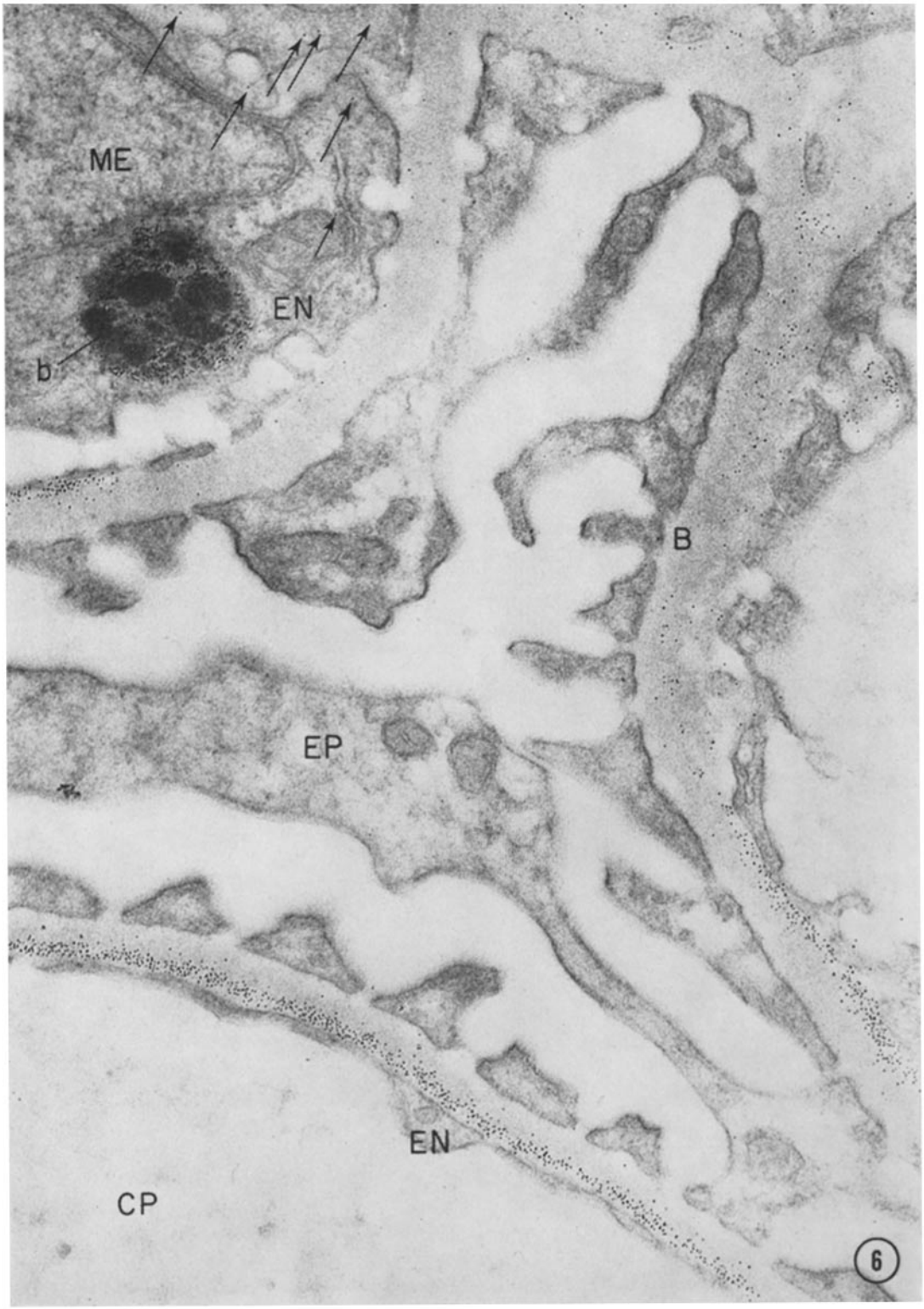



(Vogt et al.: Localization of nephrotoxic antibody)

PLATE 95

FIG. 6. Cross-section through a glomerular capillary from the same kidney as that in Fig. 3. The concentration of ferritin molecules throughout the basement membrane (B) is not uniform. Much more ferritin has accumulated in the peripheral than in the axial part of the basement membrane. A dense body (b) containing numerous ferritin molecules is seen within an endothelial cell (EN). A few single molecules (arrows) are also randomly scattered in the cytoplasm of the glomerular cells. Practically no ferritin molecules are present in the epithelial cell (EP). × 50,000.



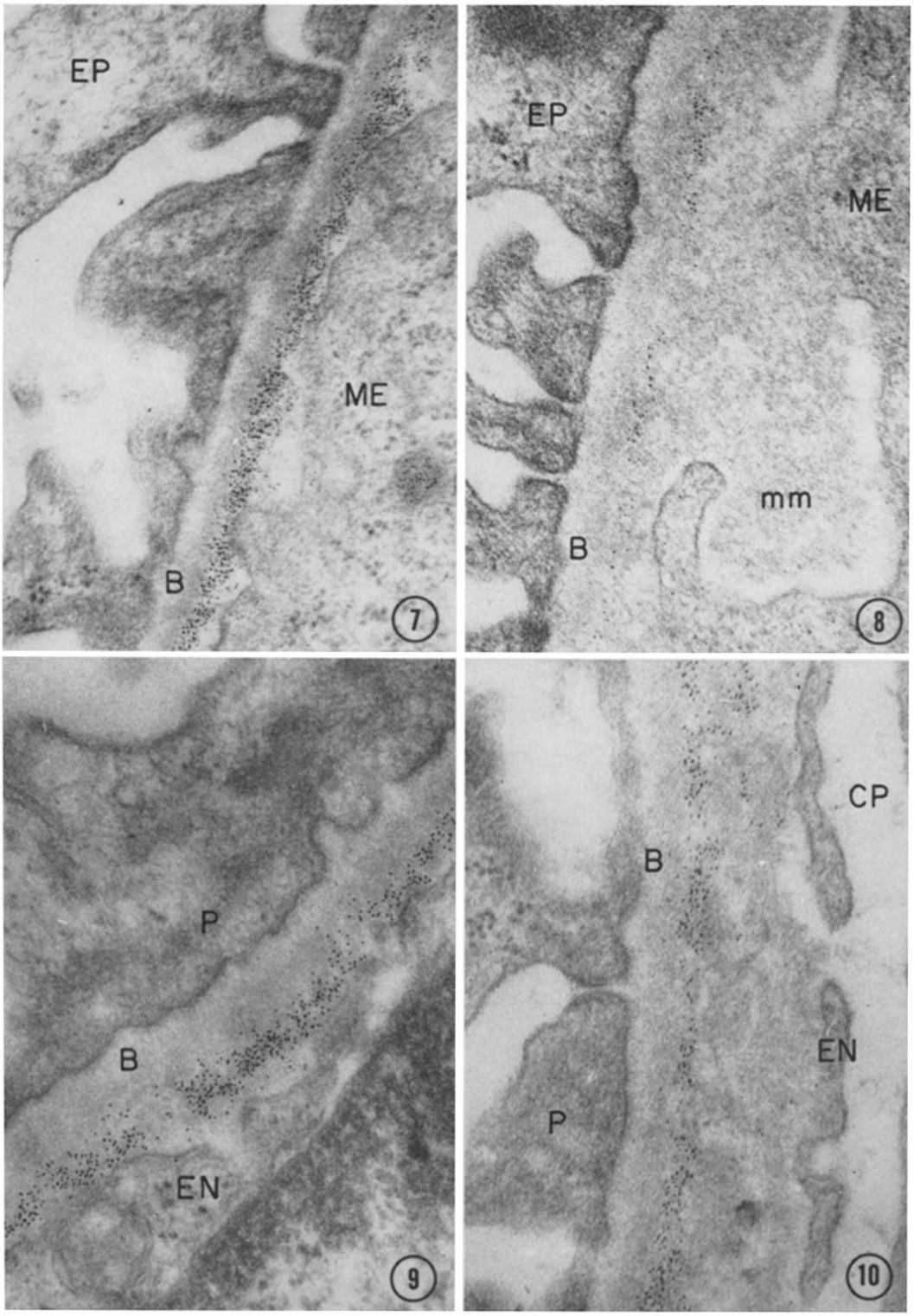

(Vogt et al.: Localization of nephrotoxic antibody)

PLATE 96

FIGS. 7 and 8 show sections of the glomerular basement membrane (B) from the same animal examined on day 1 and day 29, respectively, after the injection of 29 mg of rabbit nephrotoxic globulin conjugated to ferritin. The rat developed proteinuria of immediate onset. The same localization of ferritin is seen in both sections, but the number of molecules 28 days later has markedly decreased. × 40,000.

FIG. 9. Section through the peripheral part of a glomerular loop from a rat developing immediate proteinuria after the injection of 23.4 mg of rabbit nephrotoxic globulin conjugated to ferritin. The kidney was removed 26 hr after the injection. There is typical accumulation of ferritin molecules on the endothelial side of the basement membrane (B). The epithelial foot processes (P) have fused and a continuous epithelial cytoplasm covers the basement membrane. The cytoplasm of the endothelium (EN) is slightly swollen. × 60,000.

FIG. 10. This electron micrograph shows a section of a part of the glomerular basement membrane (B) from a rat receiving 27.5 mg of rabbit nephrotoxic globulin conjugated to ferritin. Onset of proteinuria came 6 days after the injection of the conjugate. The left kidney was removed 1 day later. Considerable broadening of the basement membrane (B) caused by a new formation of basement membrane–like material between the endothelium and the original basement membrane. The endothelial side of the basement membrane proper is clearly marked by the fixed ferritin molecules. × 80,000.




(Vogt et al.: Localization of nephrotoxic antibody)