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Subcellular Distribution of Gentamicin in Proximal Tubular Cells, Determined by Immunogold Labeling

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The subcellular distribution of gentamicin in rat renal proximal tubular cells was evaluated by immunogold labeling. The distribution of the drug was monitored from 10 min to 10 days following single (40 mg/kg of body weight) and multiple (5 and 20 mg/kg/12 h) injections of gentamicin. Animals were killed on day 11, and cubes of renal cortex tissue were fixed overnight in cold phosphate-buffered glutaraldehyde (0.5%), dehydrated in ethanol, and embedded in Araldite 502 epoxy resin. Ultrathin sections were made and incubated with sheep antigentamicin and then with protein A-gold (15 nm) complex. At 10 min after a single injection, the labeling was found over the brush border membrane and over the membranes of endocytic apical vesicles of proximal tubular cells. After 1 h, a similar distribution was observed and the labeling was also seen over small lysosomes located close to the brush border membrane. At 24 h, gold particles were found over large lysosomes of proximal tubular cells. Following 10 days of treatment, lysosomes of proximal tubular cells were densely labeled with gold particles. The labeling was distributed uniformly over the lysosomes, although a lower density of labeling was observed over the myeloid bodies inside the lysosomes. Necrotic proximal tubular cells showed labeling over intact lysosomes and also in the cytoplasms of the cells, in the mitochondria, and in the nucleoli. The various control experiments demonstrated the high specificity of these results. The present immunocytochemical study better documents the subcellular disposition of gentamicin in proximal tubular cells, as previously evaluated by subcellular fractionation and autoradiography. This technique will be useful for better understanding the relationship between drug disposition and drug-induced toxicity.

The nephrotoxicity of aminoglycosides has been extensively investigated, but the mechanism by which these drugs induce cellular disturbances and necrosis remains to be elucidated. These drugs, which are essentially eliminated by glomerular filtration, are partially reabsorbed by proximal tubular cells (15). They concentrate into lysosomes (7), where they induce a lysosomal phospholipidosis (9) characterized by the appearance of myeloid bodies (8).

The incorporation of aminoglycosides within the lysosomes of renal cortical cells has been demonstrated by subcellular fractionation (16). The binding of aminoglycosides to brush border membranes and their subsequent accumulation into lysosomes of proximal tubular cells have been previously shown by autoradiography (3, 15). Moreover, Giurgea-Marion et al. (7), by using subcellular fractionation, have observed that gentamicin remains associated with the lysosomes of proximal tubular cells throughout a 9-day treatment.

The objective of the present study was to evaluate the subcellular disposition and accumulation of gentamicin in renal cortical cells by immunogold labeling on ultrathin sections. Gentamicin distribution in the renal proximal tubular cells was monitored from 10 min to 10 days following either a single injection or several injections.

MATERIALS AND METHODS

Animals and treatment. Female Sprague-Dawley rats (Charles River Breeding Laboratories Inc., Montréal, Québec, Canada) weighing between 175 and 200 g were used. They had free access to food and water throughout the experiment. The first group of rats received a single intravenous injection of gentamicin (kindly donated by Schering Canada Inc., Pointe-Claire, Québec, Canada) at a dose of 40 mg/kg of body weight and were killed exactly 10 min, 1 h, and 24 h following the injection. Briefly, four animals per group were anesthetized with pentobarbital sodium (50 mg/kg), and a catheter (PE-50, Intramedic; Clays-Adams, Parsippany, N.J.) was inserted into the right jugular vein for infusion of the antibiotic. The catheter was then removed, and the animals were returned to their cages until sacrifice. Previous experiments showed that pentobarbital sodium had no influence on the tissular accumulation of gentamicin. Another group of rats were treated intraperitoneally with gentamicin at dosages of 5 and 20 mg/kg/12 h during 10 days. Four rats per group were killed 15 to 18 h after the last injection. Normal untreated rats were also used as controls.

Tissue sampling and fixation. At the time of sacrifice, animals were killed by decapitation and bled. Midline abdominal incisions were made, the left kidneys were removed, and the cortices were dissected. Small pieces of cortex were cut into blocks of approximately 1 mm^3 in 0.5% phosphate-buffered glutaraldehyde and left overnight in the same fixative. Blocks were then washed in 0.1 M phosphate buffer, pH 7.4, dehydrated in ascending grades of ethanol, and embedded in Araldite 502 epoxy resin (J.B. EM Services Inc., Pointe-Claire, Québec, Canada). Ultrathin sections (silver to light gold) were obtained with an Ultracut E ultramicrotome (Leica Canada Inc., Québec, Québec, Canada) and mounted on Formvar-coated nickel grids.

Immunogold labeling. For the subcellular localization of gentamicin, the protein A-gold technique was used (1, 13). The grids were first floated for 20 min on a drop of phosphate-buffered saline (PBS) containing 0.25% bovine serum

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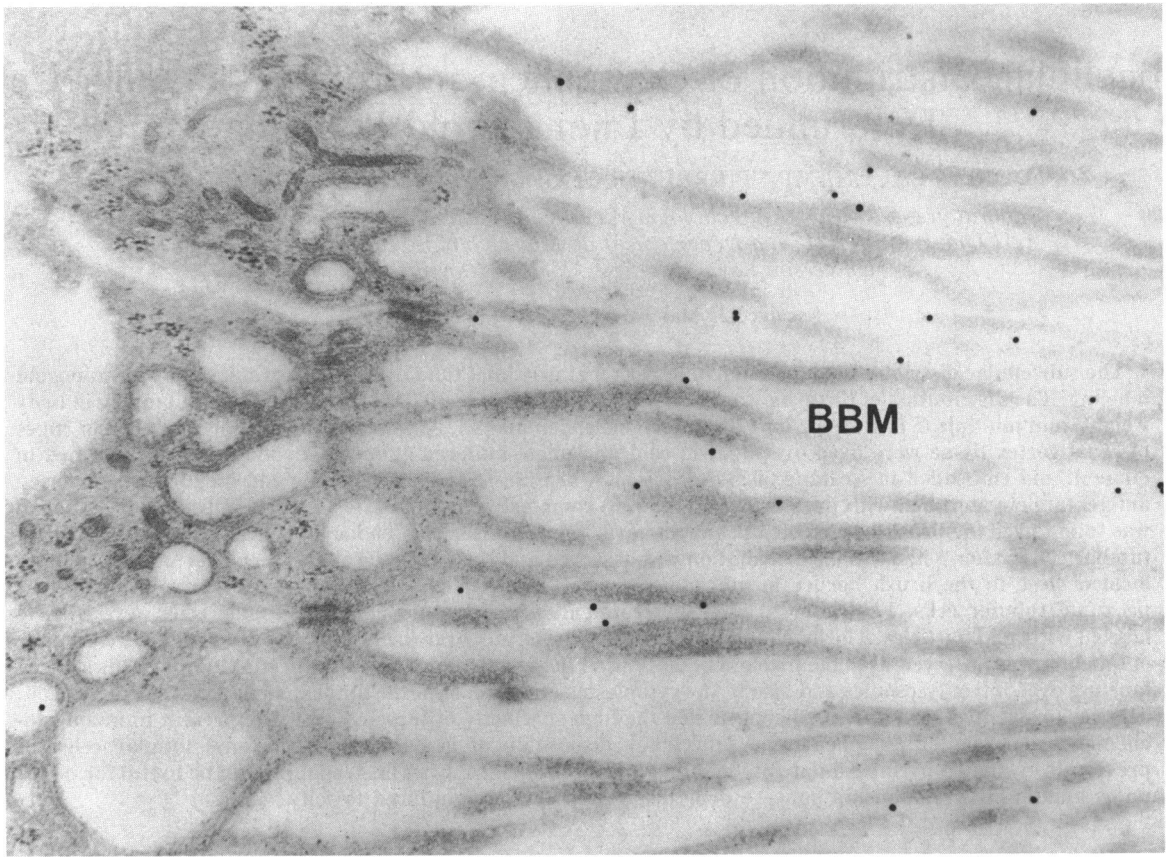

FIG. 1. Subcellular localization of gentamicin in proximal tubular cells 10 min following a single intravenous injection of 40 mg/kg. Gold particles were seen associated with the brush border membrane (BBM) at the apical part of the cell. Magnification, ×44,317.

albumin. The grids were then placed on a drop of sheep antigentamicin (CortexBiochem, San Leandro, Calif.) diluted 1/30 to 1/500. These dilutions were found to yield optimal results, with high labeling and low background levels. Incubation with gentamicin antisera was carried out for 60 min at room temperature in a moist chamber. The sections were then rinsed with PBS to remove unbound antibody molecules and transferred onto a drop of protein A-gold (15-nm-diameter particles) complex diluted 1/10 with PBS–0.02% polyethylene glycol 20000 for 30 min. At the end of this incubation, the sections were washed twice with PBS, rinsed with distilled water, and dried. Staining with uranyl acetate and lead citrate was performed before examination with a Philips EM 300 electron microscope at 60 kV.

Immunogold controls. Several concurrent control experiments for assessing the specificity of the immunolabeling were performed as follows: (i) incubation with protein A-gold alone to identify its nonspecific absorption to the section; (ii) absorption of antibody with its specific antigen before performing the labeling protocol to verify the specificity of the antigen-antibody interaction; (iii) incubation with unlabeled protein A before applying the protein A-gold complex to verify the specificity of the immunoglobulin G-protein A interaction; (iv) replacement of the specific antibody by normal serum; and (v) immunogold labeling of the renal cortical tissue of untreated animals.

RESULTS

The first group of animals were given a single intravenous injection of gentamicin at a dose of 40 mg/kg and killed 10 min, 1 h, and 24 h after the injection. At 10 min after the injection, the labeling was found essentially over the brush border membrane (Fig. 1). No labeling was observed in the basolateral membrane or in any other subcellular components of proximal tubular cells. At 1 h following the injection, the labeling was seen over the brush border membrane, over the membrane of endocytic vesicles, and also over small lysosomes located close to the brush border membrane (Fig. 2). Large lysosomes located at the basal part of the cells were not labeled at this time. At 24 h, gold particles were found over the matrix of all lysosomes of proximal tubular cells. No specific labeling was seen over the brush border membrane or over the membrane of endocytic vesicles (Fig. 3). There was no specific gold labeling found in other tubular cells of the renal cortex, such as the distal tubular cells.

Another group of animals were treated intraperitoneally with gentamicin at dosages of 5 and 20 mg/kg/12 h during 10 days and were killed 15 to 18 h after the last injection. Typical signs of aminoglycoside nephrotoxicity were observed in proximal tubular cells. These cells were filled with large lysosomes containing numerous myeloid bodies. The changes were more severe in the renal cortices of animals treated with gentamicin at a dosage of 20 mg/kg/12 h than in

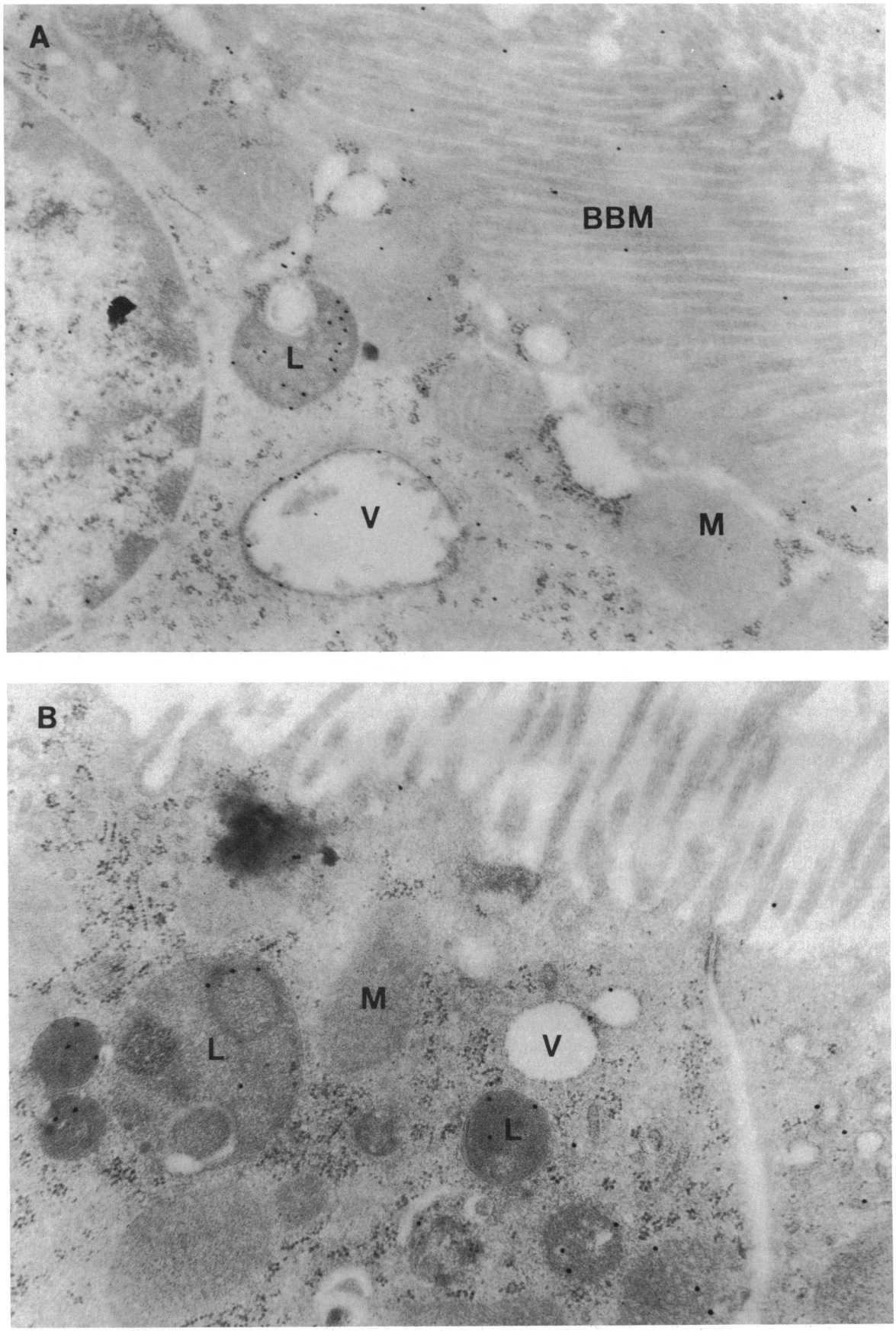

FIG. 2. Subcellular localizations of gentamicin in proximal tubular cells 1 h following a single intravenous injection of 40 mg/kg. Gold particles were seen on the brush border membrane (BBM) and on the membrane of endocytic vacuoles (V) (A) and over small lysosomes (L) located at the apical part of the cell (A and B). M, mitochondria. Magnifications, ×36,258 (A) and ×44,317 (B).

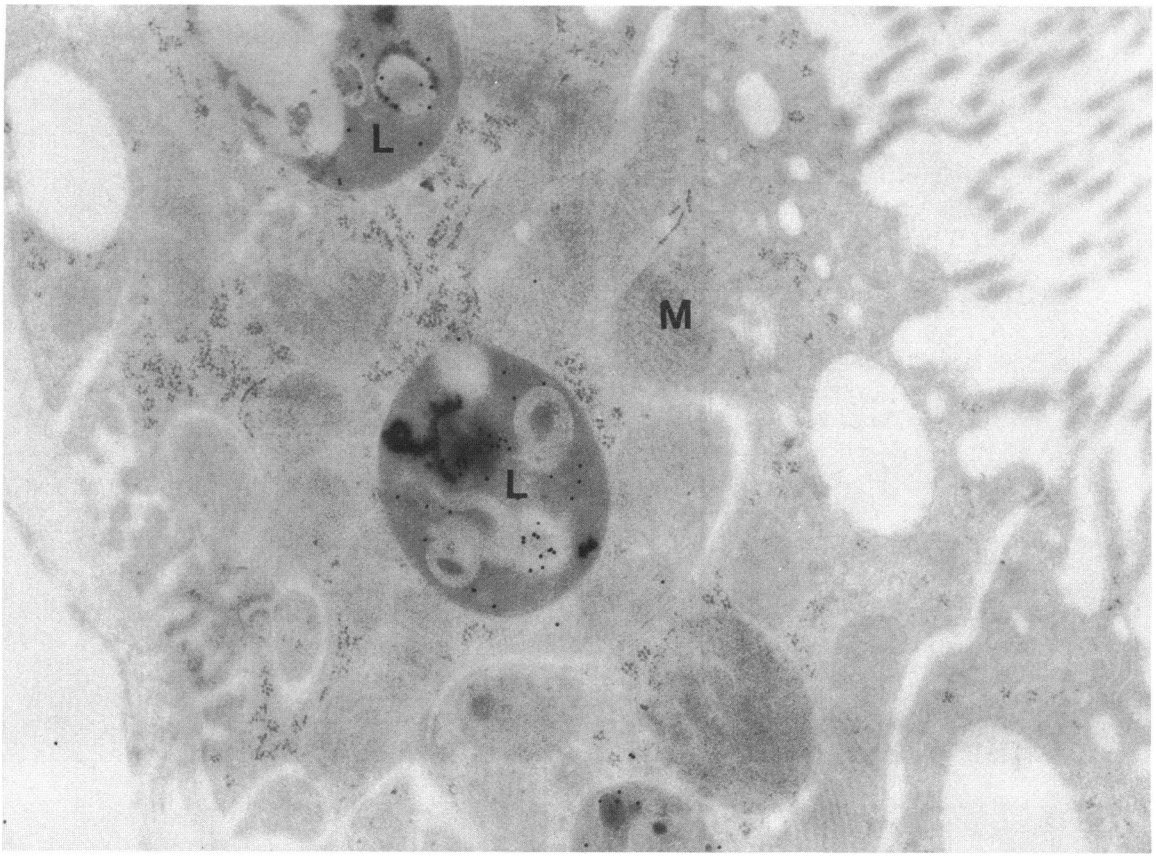

FIG. 3. Subcellular localization of gentamicin in proximal tubular cells 24 h following a single intravenous injection of 40 mg/kg. Gold particles were distributed throughout the matrix of lysosomes (L). M, mitochondria. Magnification, ×36,258.

those of animals treated at a dosage of 5 mg/kg/12 h. Myeloid bodies did not appear as electron-dense material with the typical tightly and loosely apposed lamellae usually seen in proximal tubular cells, since the renal tissue was not post-fixed with osmium tetroxide. In fact, fixation with osmium tetroxide interferes with the subsequent immunogold labeling. Myeloid bodies appeared as clear material in the lysosomes of proximal tubular cells (Fig. 4). Gold particles were seen essentially over the lysosomes of proximal tubular cells of animals treated with gentamicin at dosages of 5 and 20 mg/kg/12 h. Figure 4 shows lysosomes in a proximal tubular cell of an animal treated with gentamicin at a dosage of 20 mg/kg/12 h during 10 days. Lysosomes were densely labeled with gold particles. However, the density of gold particles on myeloid bodies was lower than that observed over the matrix of the lysosomes (Fig. 4). Similar observations were made for the renal cortices of animals treated with gentamicin at a dosage of 5 mg/kg/12 h. Moreover, necrotic cells were also seen in the proximal tubules of animals treated with gentamicin at a dosage of 20 mg/kg/12 h. Although intact lysosomes labeled with gold particles were found in these necrotic cells, gold particles were also observed in the cytoplasms, in mitochondria, and in the nucleoli of necrotic cells (Fig. 5). All these observations were similar for the renal cortices of all animals studied.

The control experiments proved the high specificity of the labeling. The absorption of the antibody with its antigen resulted in the abolition of the labeling. The labeling was also absent under the other control conditions tested. Moreover, no labeling was observed over any subcellular sites of untreated control kidneys.

DISCUSSION

The accumulation of aminoglycosides into the renal cortex has been reported for human kidneys (5) as well as for the kidneys of experimental animals (10). The uptake of aminoglycosides by proximal tubular cells has been demonstrated by different approaches such as micropuncture (11, 14) and autoradiographic experiments (3, 15, 17). The association of aminoglycosides with lysosomes of cortical renal cells has been shown by subcellular fractionation (16). Moreover, Giurgea-Marion et al. (7), using subcellular fractionation, have observed that aminoglycosides are still associated with lysosomes throughout a 9-day treatment.

The subcellular distribution of aminoglycosides has never been evaluated by immunogold labeling on ultrathin tissue sections. This technique has several advantages over autoradiography and subcellular fractionation. In fact, with autoradiography the radiolabeled drug must be injected into animals, and a long exposure time is needed when autoradiography is done on ultrathin sections. For the subcellular fractionation, the tissue must be homogenized, centrifuged, and separated into different fractions, and markers of several cell compartments must be localized among the different fractions. Immunogold labeling is a highly specific, rapid, and sensitive method. Moreover, more than one antigen can be localized on the same tissue section by using gold

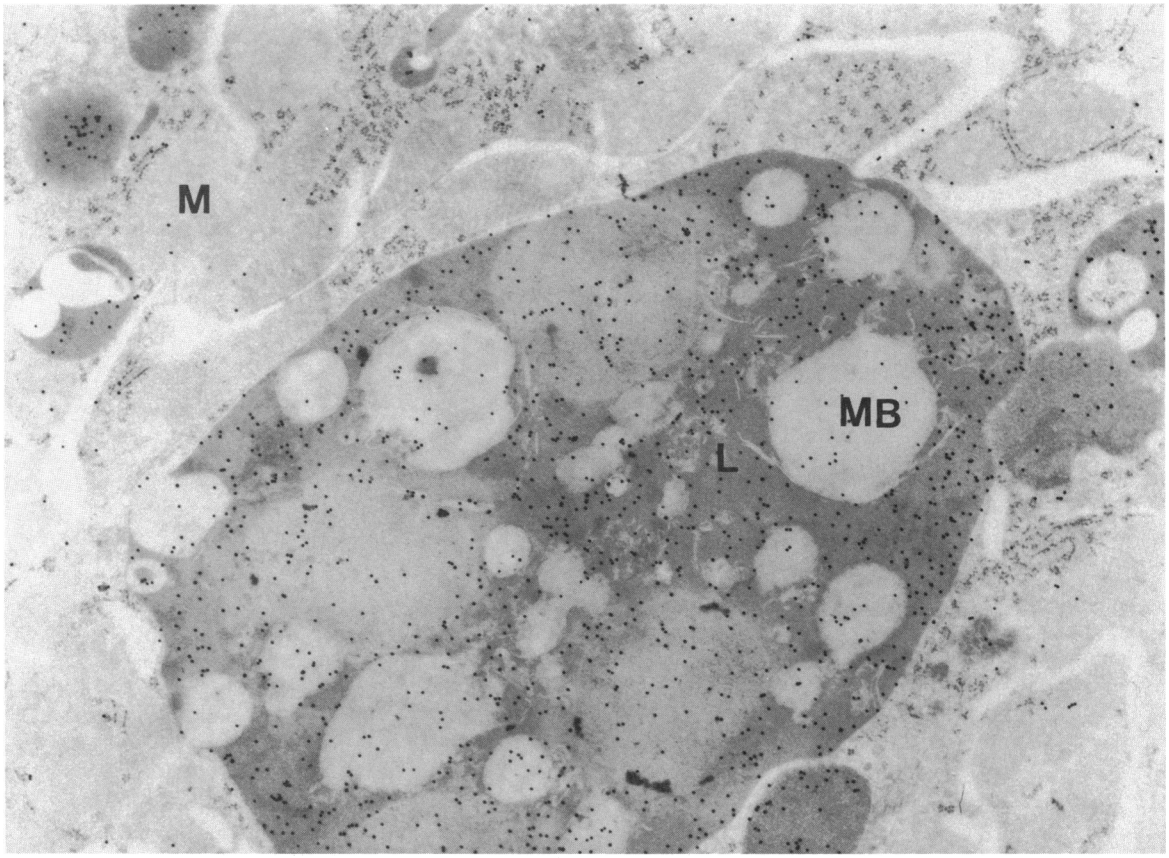

FIG. 4. Subcellular localization of gentamicin in proximal tubular cells following a 10-day treatment of gentamicin at a dosage of 20 mg/kg/12 h. Gold particles were distributed throughout the matrix of the lysosomes (L). A lower density of gold particles was seen on myeloid bodies (MB). M, mitochondria. Magnification, ×22,158.

particles of different sizes. Since the radiolabeled drug is not needed, this technique can also be used with human tissue.

Our results support the evidence that gentamicin is incorporated into proximal tubular cells via the brush border membrane, as previously shown by autoradiographic studies (3, 15, 17). In fact, 10 min after gentamicin injection, gold particles were found on the brush border membrane and on the membranes of endocytic apical vesicles. No labeling was seen on the basolateral membranes of proximal tubular cells. Several studies reported that aminoglycoside uptake via the basolateral membrane of proximal tubular cells may contribute to the overall cellular accumulation of these drugs (2, 12, 14). Williams and Hottendorf (18), using isolated brush border and basolateral membrane vesicles, showed that the basolateral as well as the brush border surfaces of proximal tubular cells may contribute significantly to the renal accumulation of aminoglycosides.

Our results show that gentamicin is incorporated into proximal tubular cells by endocytosis into small vesicles that fuse to primary lysosomes. In fact, 1 h after the injection, gentamicin was found on the membranes of endocytic apical vesicles, and once transferred into lysosomes, the drug was seen over the matrix of these lysosomes (Fig. 2A and B). These observations support the hypothesis that aminoglycosides bind to membrane receptors before they accumulate into lysosomes. It is not yet clear whether gentamicin is free in the matrix of the lysosomes or is still bound to these cell membrane receptors inside the lysosomes. Tissue culture models of proximal tubular cells may elucidate this question.

After 10 days of treatment, the gold particles were still distributed over the lysosomes of proximal tubular cells. No other subcellular sites were labeled. Giurgea-Marion et al. (7) showed that the lysosomal compartment remains the major site of intracellular gentamicin storage in spite of the marked changes in the biophysical, biochemical, and morphological properties of the lysosomes. Our results also show that the subcellular distribution of the drug did not change throughout the treatment duration.

Gold particles were less numerous over the myeloid bodies than over the matrix of the lysosomes of proximal tubular cells of animals treated with gentamicin at dosages of 5 and 20 mg/kg/12 h during 10 days. This might suggest that once transferred into the lysosomes, the aminoglycoside is not bound into the matrix of the lysosomes and that only a fraction of the drug is fixed to the accumulated phospholipids (myeloid bodies) inside the lysosomes. Another possibility is that the immune reaction may have failed, particularly over myeloid bodies which are composed of tightly apposed phospholipid membrane. The mode of insertion of several aminoglycosides with phosphatidylinositol monolayers has already been shown by conformational analysis (4). Other experiments either using Lowicryl as embedding material or using frozen sections must be done to better document these observations.

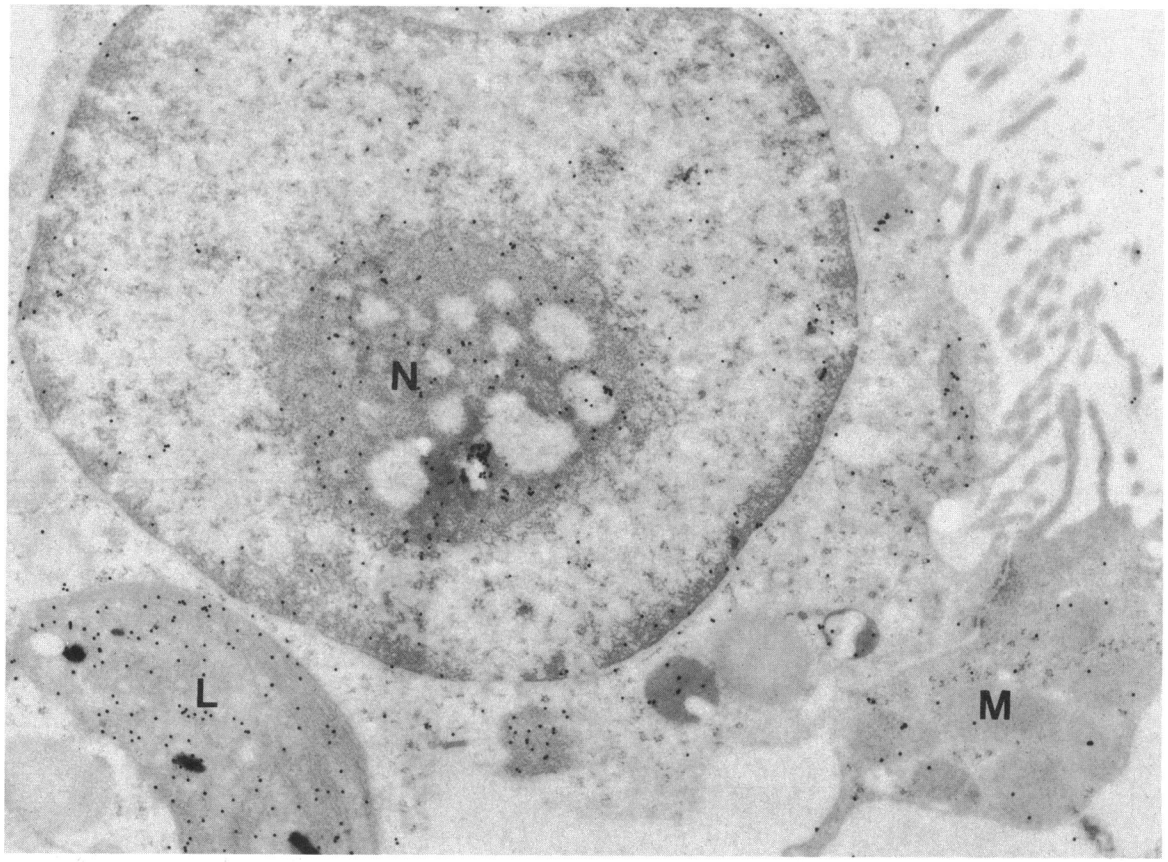

FIG. 5. Subcellular localization of gentamicin in a necrotic proximal tubular cell following a 10-day treatment at a dosage of 20 mg/kg/12 h. Gold particles were distributed throughout the cytoplasm of the cell, on mitochondria (M), and on the nucleolus (N). L, lysosomes. Magnification, ×22,158.

One interesting observation made in the present study is that of the subcellular distribution of gentamicin within necrotic cells. These cells were seen only in the renal cortices of animals treated at a dosage of 20 mg/kg/12 h during 10 days. Gold particles were found over intact lysosomes, in mitochondria, and in nucleoli. This has never been observed previously. The relationship among aminoglycoside-induced renal phospholipidosis, cellular dysfunction, and necrosis is not well understood. It has been suggested that labilization or rupture of the lysosomes with release of hydrolases into the cytoplasm of the cell may be responsible for cellular damage and death (6). On the basis of the results of the present study, which show that gentamicin accumulates into lysosomes throughout a 10-day treatment and is present within other subcellular structures such as mitochondria and the nucleoli of dead cells, we believe that lysosomal membrane rupture might be an important factor responsible for cell death following aminoglycoside treatment.

The present study shows that immunogold labeling can be used to study the subcellular disposition of drugs within tissue. In fact, the subcellular distribution of gentamicin previously evaluated by autoradiography and subcellular fractionation was confirmed in the present study. Moreover, immunogold labeling gave us more insight into the subcellular localization of the drug and into the mechanism of cellular necrosis.

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