ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1992, p. 2204–2210 Vol. 36, No. 10
0066-4804/92/102204-07$02.00/0


# Subcellular Localization of Tobramycin and Vancomycin Given Alone and in Combination in Proximal Tubular Cells, Determined by Immunogold Labeling

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Received 21 January 1992/Accepted 17 July 1992

**The subcellular localization of tobramycin and vancomycin in the renal cortices of rats was determined with ultrathin sections by immunogold labeling. Four groups of four rats each were treated for 10 days with saline (NaCl, 0.9%), tobramycin at dosages of 20 mg/kg of body weight per 12 h intraperitoneally, vancomycin at dosages of 25 mg/kg/12 h subcutaneously, or the combination tobramycin-vancomycin. On day 11, the animals were killed, and cubes of renal cortex were fixed overnight in phosphate-buffered glutaraldehyde (0.5%), dehydrated in ethanol, and embedded in Araldite 502 resin. Ultrathin sections were made and incubated with sheep antitobramycin antibody followed by protein A-gold (15-nm diameter) complex or rabbit antivancomycin antibody followed by gold (30-nm diameter)-labeled goat anti-rabbit antibody. For the double labeling, incubations were made on opposite sides of the grid. Tobramycin was detected over the lysosomes of proximal tubular cells, but the labeling was concentrated into small areas in the matrix of the lysosomes. Vancomycin was seen over the lysosomes of proximal tubular cells and was distributed uniformly throughout the matrix of the lysosomes. In rats treated with tobramycin-vancomycin, both drugs were still detected in lysosomes of proximal tubular cells. It is concluded that tobramycin and vancomycin accumulate in lysosomes of proximal tubular cells throughout 10 days of treatment and that vancomycin has no effect on the subcellular distribution of tobramycin.**

The behavior of aminoglycosides in the kidney has been studied extensively. These drugs are eliminated by glomerular filtration, and a percentage of filtered drug is reabsorbed by proximal tubular cells and ultimately accumulates in lysosomes (1, 9, 22). The effect on lysosomes has been termed a lysosomal phospholipidosis characterized by inhibition of phospholipase enzymes and phospholipid accumulation (12). The propensity of the modern vancomycin preparation to cause nephrotoxicity appears modest (7, 8, 15, 21, 24); however, in studies with animals and in clinical studies with adults, vancomycin appears to amplify the risk of aminoglycoside nephrotoxicity (2, 8, 13, 14, 17, 24, 27). In animal studies, vancomycin is shown to accumulate in renal tissue (2, 13, 14, 17, 27). However, there are few data as to the behavior of vancomycin in the kidney and the influence of the concomitant aminoglycoside on the behavior of vancomycin and other glycopeptides.

The intralysosomal accumulation of aminoglycosides in proximal tubular cells has been previously shown by subcellular fractionation (9) and autoradiography (3, 22). It has also been observed that gentamicin remains associated with lysosomes throughout 9 days of treatment (9). The subcellular distribution of gentamicin, measured from 10 min to 24 h following a single injection and after 10 days of treatment, has recently been determined by immunogold labeling in our laboratory (1). The latter study clearly showed that gentamicin bound to the brush border membrane and to the membrane of endocytic vacuoles before accumulating in the lysosomes of proximal tubular cells. Up to now, the subcellular site of vancomycin accumulation in renal cells has never been investigated.

In order to better understand the nephrotoxic interactions between aminoglycosides and vancomycin, we used immunogold labeling to study the subcellular distribution of tobramycin and vancomycin, given alone and in combination, in the renal cortex.

## MATERIALS AND METHODS

**Animals and treatments.** 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 rats were separated into four groups of four each and were treated for 10 days with saline (NaCl, 0.9%), tobramycin (kindly donated by Eli Lilly Canada Inc., Scarborough, Ontario, Canada) at a dosage of 20 mg/kg of body weight per 12 h intraperitoneally, vancomycin (kindly donated by Eli Lilly Canada Inc.) at a dosage of 25 mg/kg/12 h subcutaneously, or with the combination tobramycin-vancomycin. All animals were killed on day 11.

**Tissue sampling and fixation.** Animals were killed by decapitation and bled. A midline abdominal incision was made, the left kidney of each animal was removed and bisected, and the cortex was dissected. A small piece of cortex was cut into small blocks of 1 $mm^3$, the blocks were placed into drops of 0.5% phosphate-buffered glutaraldehyde, and they were left overnight in the same fixative at 4°C. The 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 sec-

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tions (silver to light gold) were cut with an ultramicrotome, Ultracut E (Reichert-Jung; Leica Instruments GmbH, Montréal, Québec, Canada), mounted on nickel grids, and processed for immunocytochemistry.

**Immunogold labeling.** To determine the subcellular localization of tobramycin and vancomycin, the protein A-gold complex (19, 20) and antibody-gold complex (25) techniques were used. Each grid was first floated on a drop of phosphate-buffered saline (PBS) containing 0.25% bovine serum albumin (Sigma Chemical Company, St. Louis, Mo.) for 20 min. Each grid was then placed on a drop of sheep antitobramycin (Cortex Biochem, San Leandro, Calif.) diluted 1/500 to 1/900 or rabbit antivancomycin (East-Acres Biologicals, Southbridge, Mass.) diluted 1/25 to 1/500. These dilutions gave optimal results and a low level of background activity. Incubations with the antisera were carried out for 60 min at room temperature in a moist chamber. The sections were then rinsed with PBS to remove unbound antibody and incubated for 30 min onto a drop of protein A-gold (15-nm diameter) complex diluted 1/10 with PBS-polyethylene glycol 20000 (0.02%) (Fisher Scientific Inc., Québec, Québec, Canada) for sheep antitobramycin or onto a drop of goat anti-rabbit–gold (30-nm diameter) (Cederlane Laboratories Ltée, Hornby, Ontario, Canada) diluted 1/10 with PBS-polyethylene glycol (0.02%) for rabbit antivancomycin. At the end of the incubation, the sections were washed twice with PBS, rinsed with distilled water, and dried. The double labeling was performed on opposite sides of the grid to eliminate the nonspecific interactions. Staining of the grids with uranyl acetate and lead citrate was performed before they were examined 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 or goat anti-rabbit–gold alone to identify the nonspecific absorption of each into the sections, (ii) absorption of each antibody with its specific antigen before 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, (v) absorption of each antibody with an excess of the opposite antigen before immunogold labeling to verify the cross-reactivity between antibodies, and (vi) immunogold labeling of each antibody on renal cortical tissue of untreated animals.

## RESULTS

A first group of rats was treated with tobramycin at doses of 20 mg/kg/12 h for 10 days. On day 11, specific signs of aminoglycoside toxicity in proximal tubular cells were seen. In fact, lysosomes containing numerous myeloid bodies were observed. Figure 1 shows the subcellular localization of tobramycin in proximal tubular cells. Gold particles were essentially distributed over the lysosomes of these cells. Gold particles were not distributed uniformly over the lysosomes, as previously observed with gentamicin (1), but were distributed over specific small areas inside the lysosomes. It was impossible to identify the nature of the labeled structures inside the lysosomes, in part because of the conditions used in the present study for the fixation of the tissue. No other subcellular sites in proximal tubular cells or in other cortical cells were labeled with gold particles in this group.

Another group of rats was treated with vancomycin at doses of 25 mg/kg/12 h for 10 days. On day 11, larger lysosomes were seen in proximal tubular cells than in tubular cells of control animals. No myeloid bodies were seen inside the lysosomes. Gold particles were found essentially over the lysosomes of proximal tubular cells, and the particles were uniformly distributed throughout the matrix of the lysosomes (Fig. 2). No other subcellular sites in proximal tubular cells or in other cortical cells were labeled with gold particles in this group.

A third group of rats was treated with tobramycin-vancomycin for 10 days. Antitobramycin antibodies were labeled with 15-nm-diameter gold particles, and antivancomycin antibodies were labeled with 30-nm-diameter gold particles. Gold particles (15- and 30-nm diameters) were seen over the lysosomes of proximal tubular cells in the renal cortices of animals given tobramycin-vancomycin (Fig. 3). The subcellular localization of both drugs in animals given the combination was similar to that in animals treated with tobramycin or vancomycin alone. Although few gold particles were seen in the cytoplasm of the cell, control experiments showed that this binding was not specific, and it was considered the background activity. The subcellular distribution of these drugs given alone was not modified in animals given the combination.

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. The absorption of antibodies with an excess of the opposite antigen did not modify the labeling. Moreover, no labeling was observed when sections prepared from the renal cortices of animals treated with tobramycin were incubated with rabbit antivancomycin antibodies. Similarly, no labeling was observed when sections prepared from the renal cortices of animals treated with vancomycin were incubated with sheep antitobramycin antibodies. Finally, no labeling over any subcellular site of untreated control kidneys was observed. All these observations were similar for all animals of each experimental group.

## DISCUSSION

The present study shows that tobramycin and vancomycin accumulate in the lysosomes of proximal tubular cells after 10 days of treatment and that the subcellular localization observed in animals treated with each drug alone was not modified in animals treated with the combination.

Subcellular fractionation was used by Tulkens and Trouet (26) to study the uptake and the intracellular accumulation of aminoglycosides in lysosomes of cultured rat fibroblasts. The association of aminoglycosides with lysosomes of proximal tubular cells following several days of treatment has also been demonstrated by subcellular fractionation (9, 10). Giurgea-Marion et al. (9) showed that gentamicin was associated with the lysosomal fraction following 9 days of treatment. The subcellular localization of aminoglycosides on the brush border membrane a few minutes following the injection and in the lysosomes of proximal tubular cells several hours following the injection was shown by autoradiography (3, 22). The subcellular distribution of gentamicin has been previously determined by immunogold labeling (1). This technique has several advantages over autoradiography and subcellular fractionation, as previously discussed (1). In fact, no radiolabeled drug is needed, and the results can be seen immediately after the incubation with the antibodies. Immunogold labeling is a very specific, rapid, and sensitive

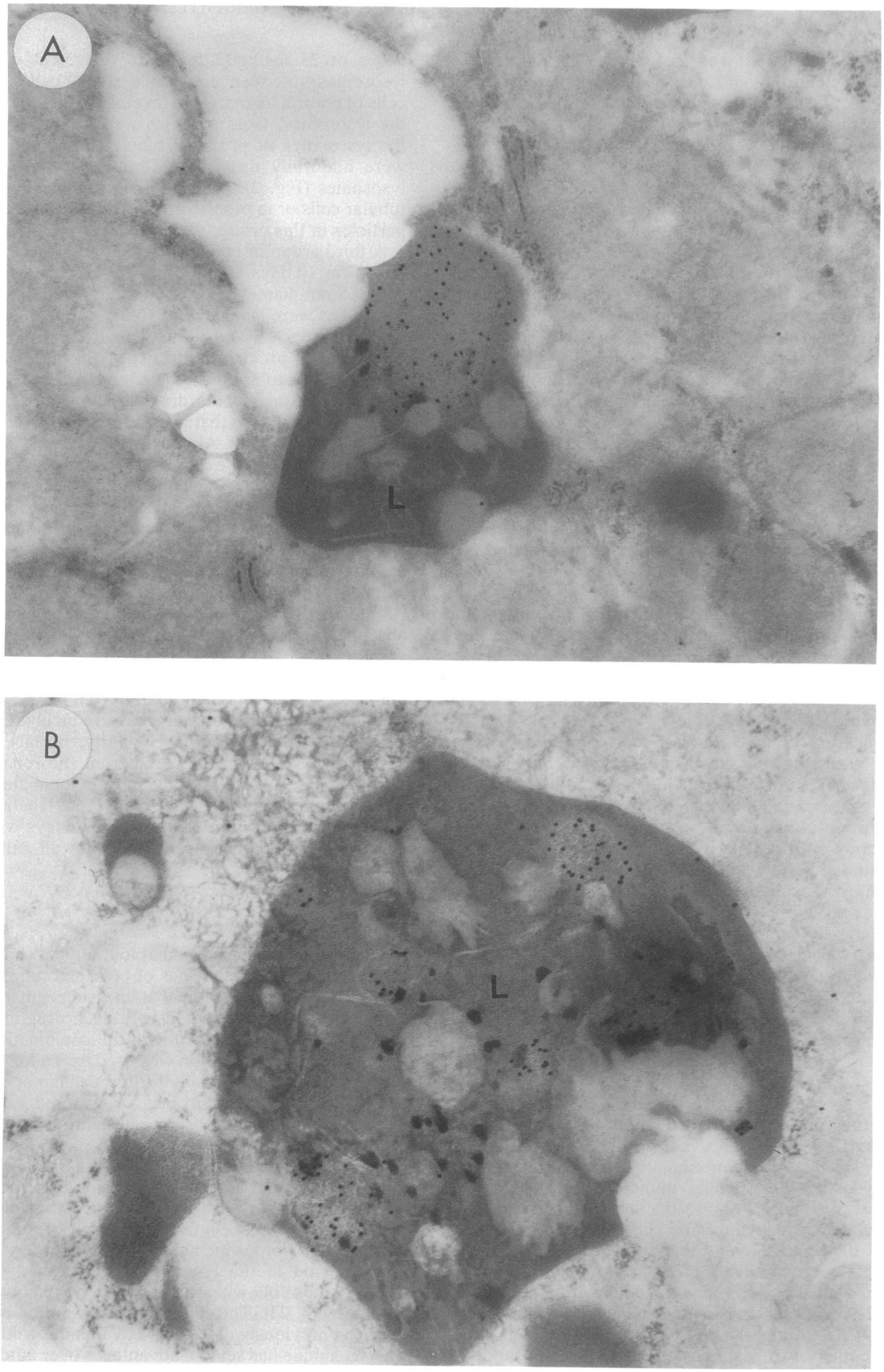


FIG. 1. Subcellular distribution of tobramycin in proximal tubular cells following treatment at doses of 20 mg/kg/12 h for 10 days. Gold particles (15-nm diameter) were located essentially in the lysosomes (L) of proximal tubular cells. The drug was not distributed uniformly throughout the matrix of the lysosomes, but was found over small areas in the lysosomes. Magnification, ×40,128.

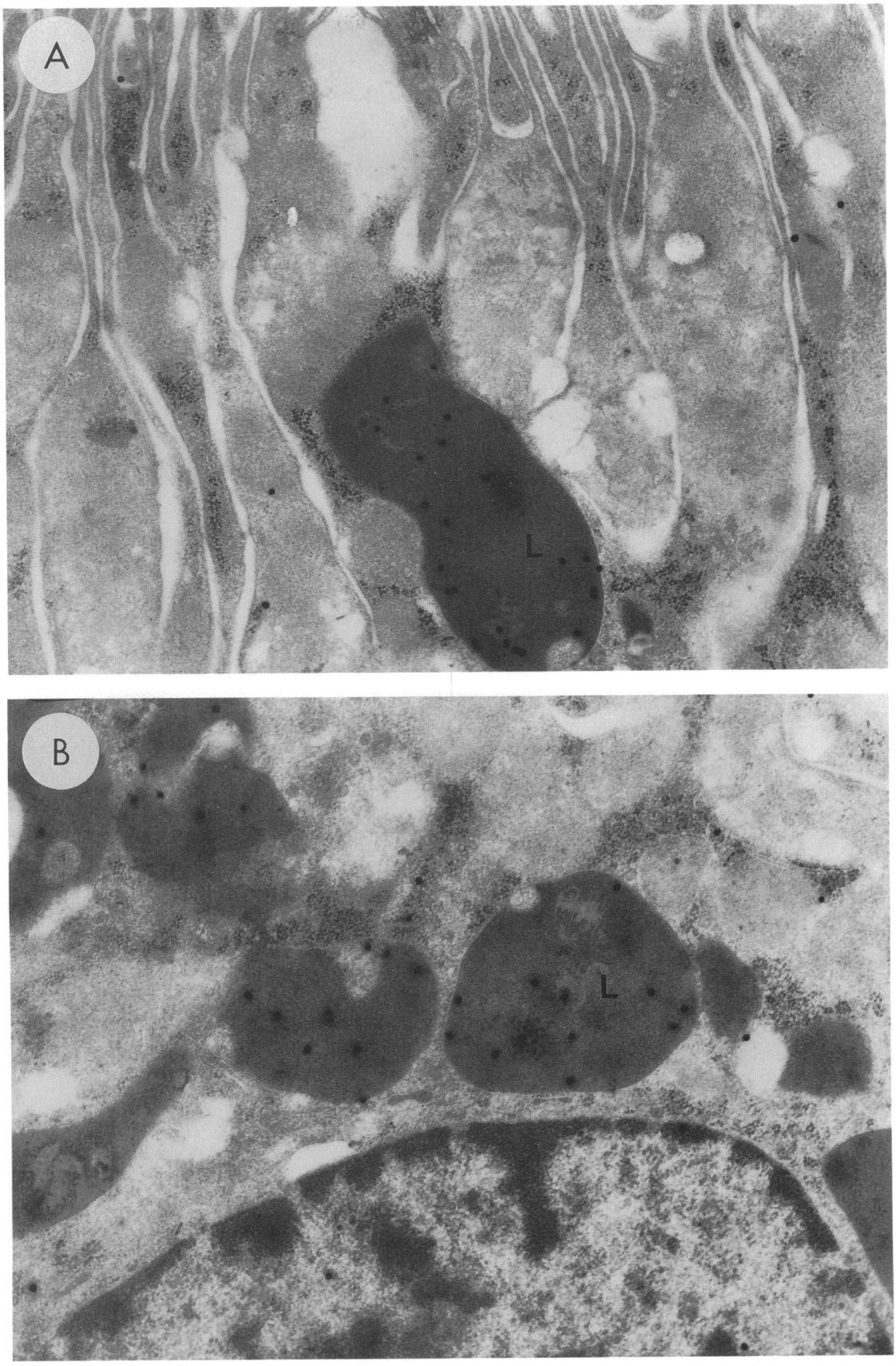


FIG. 2. Subcellular distribution of vancomycin in proximal tubular cells following treatment at doses of 25 mg/kg/12 h for 10 days. Gold particles (30-nm diameter) were located over the matrix of the lysosomes (L). The drug was distributed uniformly in the lysosomes. Magnification, ×33,026.

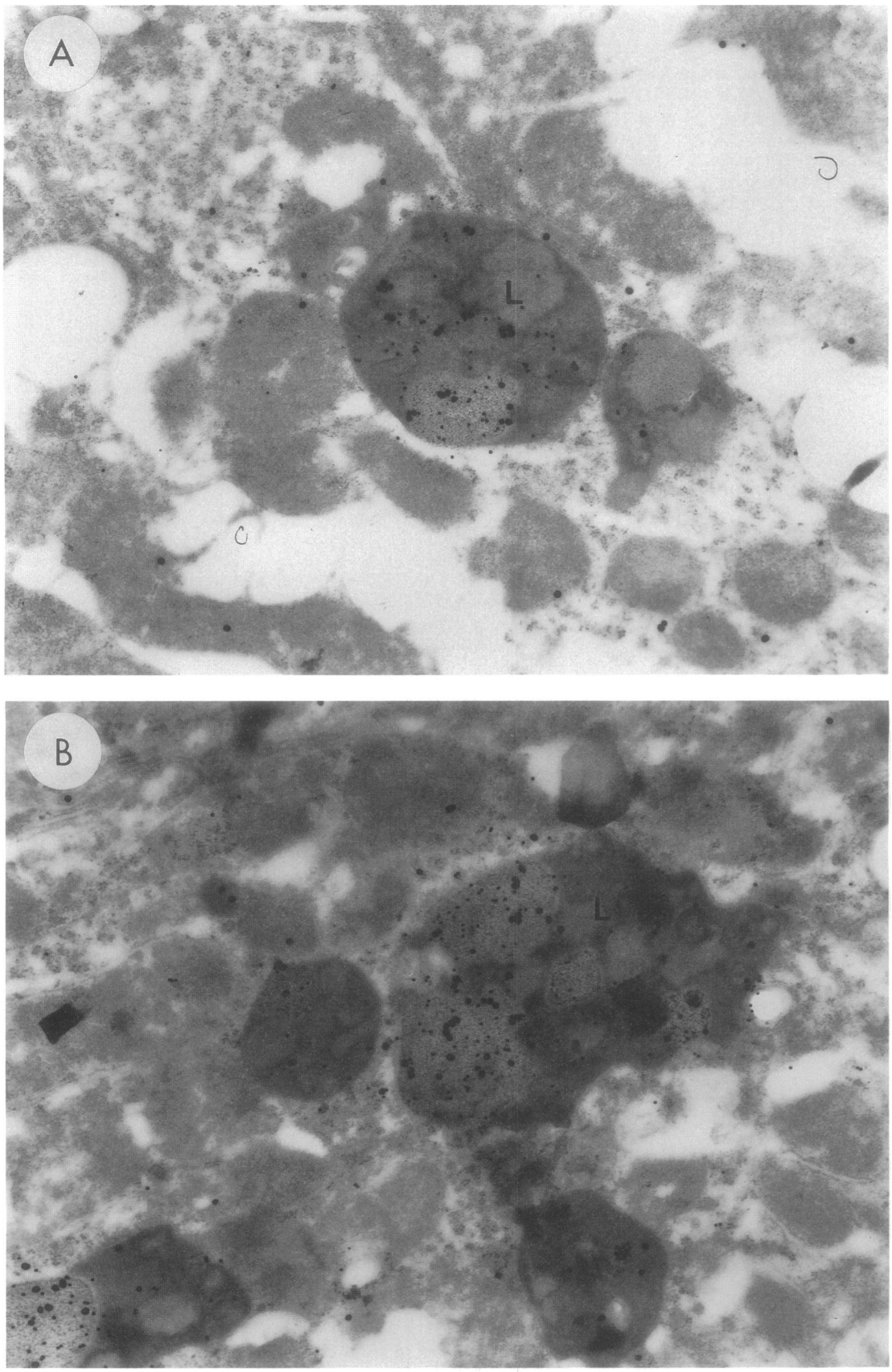


FIG. 3. Subcellular distribution of tobramycin (20 mg/kg/12 h) and vancomycin (25 mg/kg/12 h) given in combination for 10 days. Tobramycin antibodies were labeled with 15-nm-diameter gold particles, while vancomycin antibodies were labeled with 30-nm-diameter gold particles. Both drugs were located in the lysosomes (L) of proximal tubular cells. The subcellular distribution of tobramycin and vancomycin given in combination was similar to that observed in the renal cortices of animals give each drug alone. Magnification, ×32,832.

method. Moreover, more than one antigen can be localized on the same tissue section by using gold particles of different sizes. Since radiolabeled drug is not needed, this technique can also be used on human tissue.

The subcellular distribution of gentamicin in proximal tubular cells from 10 min to 10 days following a single injection or multiple injections was recently investigated in our laboratory (1). In fact, 10 min after a single injection of gentamicin, gold particles were seen over the brush border membrane and over the membrane 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 at the apical part of proximal tubular cells. At 24 h, gold particles were found over larger lysosomes of proximal tubular cells and were uniformly distributed over the matrix of the lysosomes. Following 10 days of treatment, lysosomes of proximal tubular cells were densely labeled with gold particles.

The present study shows that tobramycin accumulates in the lysosomes of proximal tubular cells, as shown for gentamicin with the same immunogold technique. However, differences between the intralysosomal distributions of gentamicin and tobramycin were observed. In fact, gentamicin was distributed uniformly over the lysosomes, although a lower density of labeling over the myeloid bodies inside the lysosomes was observed. In contrast, tobramycin was not distributed uniformly throughout the matrix of the lysosomes but was specifically distributed over small areas inside the lysosomes. This might be due to differences in the handling of slightly different molecules by the cell. It must be pointed out that various factors influence the interactions of aminoglycosides with anionic phospholipids, and the degree of interaction is not just a direct function of the number of cationic amino groups in the molecule but is also influenced by their positions and spatial arrangements (4, 5). All these factors might be responsible for possible differences in handling of these drugs by the cell and for the differences in the intralysosomal distribution of these drugs. The sensitivity of the immunogold labeling done on ultrathin plastic sections is another factor that may limit the detection of these antibiotics within the cell. Other experiments on tissue embedded in Lowicryl or on frozen sections must be done to better document these observations.

The subcellular distribution of vancomycin has never been investigated. It has been shown that vancomycin accumulates in the renal cortices of experimental animals (2, 13, 14, 17, 27). A recent study on the mechanism of vancomycin transport in the rabbit kidney reported that vancomycin entered the cells by mediated transport across the basolateral membrane but not across the brush border membrane (23). Although the mechanism of vancomycin nephrotoxicity is not well understood, the accumulation of vancomycin in the lysosomes of proximal tubular cells did not induce histopathological changes similar to those observed following aminoglycoside therapy. The present study demonstrates that vancomycin given at doses of 50 mg/kg/day accumulates in the lysosomes of proximal tubular cells after 10 days of treatment, but we have no information on the handling of vancomycin by proximal tubular cells before its accumulation in lysosomes. Thus, vancomycin might induce toxicity to other subcellular sites during its cellular transport.

Several studies showed that the combination of an aminoglycoside with vancomycin is more nephrotoxic than each drug given alone. In fact, Sorrell and Collignon (24) reported from a prospective study of the efficacy and safety of vancomycin for patients that nephrotoxicity and ototoxicity were confined to patients receiving an aminoglycoside plus vancomycin. Rybak et al. (21) observed recently that the factors associated with increased risk of nephrotoxicity in patients receiving vancomycin were concurrent therapy with an aminoglycoside, length of treatment with vancomycin, and trough concentrations of vancomycin in serum. On the other hand, Downs et al. (6) failed to identify underlying illness or concurrent risks that may have contributed to the development of nephrotoxicity associated with vancomycin, including combined therapy with an aminoglycoside. Mellor et al. (15) also noted no evidence of synergistic toxicity between vancomycin and aminoglycosides in a prospective study performed to assess the toxicity of vancomycin. Pauly et al. (18) concluded from a retrospective review of patients receiving aminoglycosides and vancomycin that the length of therapy, concentrations of aminoglycosides (trough) and vancomycin (peak and trough) in serum, neutropenia, peritonitis, increased age, liver disease, concurrent amphotericin B therapy, and male sex are associated with nephrotoxicity. Nephrotoxicity has also been reported to be uncommon in pediatric patients receiving combined therapy with vancomycin and gentamicin, particularly when concentrations of gentamicin in serum are within therapeutic ranges (16).

In animal experiments, the combination of vancomycin and tobramycin was toxic earlier and was more severely toxic than tobramycin or vancomycin given alone (2, 8, 13, 14, 17, 27). Results from our laboratory demonstrated that vancomycin enhanced tobramycin-induced inhibition of sphingomyelinase activity in the renal cortex and that more-severe cellular and tubular alterations were observed histologically (2). By contrast, a lack of potentiation of vancomycin and gentamicin nephrotoxicity was observed in newborns, probably because of the decreased accumulation of either antibiotic in the kidney (11).

The results of the present study, which show that vancomycin and tobramycin accumulate at the same subcellular site in proximal tubular cells after 10 days of treatment but are handled differently by the cells (1, 3, 9, 22, 23), support evidence that vancomycin might interfere with the aminoglycoside-phospholipid interaction in the lysosomes. In fact, changes have been previously observed in the ultrastructure of myeloid bodies in rats treated with the combinations of vancomycin plus tobramycin (2) and vancomycin plus gentamicin (17) compared with structures of myeloid bodies in animals given tobramycin or gentamicin alone. These biochemical and histological observations suggested that both drugs disturb lysosomal function. Although the results of the present study show that the target sites of aminoglycoside and vancomycin in the renal cortical cells are the same, the mechanisms of their toxicity might be different.

Further investigations of the mechanism of the lysosomal sequestration of vancomycin and tobramycin, given alone or in combination, determined by immunogold labeling at different doses and times following a single intravenous injection, as was recently done for gentamicin (1), must be done to better understand the interactions between these molecules at the subcellular level.

## ACKNOWLEDGMENTS

This study was supported by The Kidney Foundation of Canada and by the Medical Research Council of Canada (grant MA-10157). Denis Beauchamp was a scholar of the P.M.A.C. Health Research Foundation.

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