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The Distribution of Cytochrome P-450 Monooxygenase in Cells of the Rabbit Lung: An Ultrastructural Immunocytochemical Characterization

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SUMMARY

The cytochrome P-450 monooxygenase system of the mammalian lung is known to be associated with the microsomal subcellular fraction and has been demonstrated in two pulmonary cell types rich in endoplasmic reticulum: Clara cells and type II pneumocytes. However, analysis of ultracellular fractions, isolated cell preparations, or light microscopic immunohistochemical studies of tissue sections has permitted only limited resolution of the distribution of this enzyme system within the 40 or more cell types of the lung. Therefore, we have used the greater resolving power of transmission electron microscopy and immunogold labeling to characterize the cellular and subcellular distribution of the cytochrome P-450 system in the lung. In Lowicryl-embedded sections of lung from adult rabbits, antisera (1:10,000) against the constitutive pulmonary microsomal cytochrome P-450 monooxygenase isozymes 2 and 5 and NADPH-cytochrome P-450 reductase (anti-2, anti-5 and anti-R) bound specifically to regions known to be rich in agranular endoplasmic reticulum (AER) in the cytoplasm of Clara cells. The plasma membranes of bronchiolar Clara cells, the tips of microvillae of ciliated cells, secretory granules of goblet cells, and the cell membrane and pinocytotic vesicles of endothelial cells were all intensely labeled with anti-2 and anti-5 but not with anti-R, even at a 10-fold higher concentration. The intensity of labeling of AER in Clara cells with anti-R and anti-2, but not anti-5, appeared to correlate positively with the cellular content of secretory granules. The Golgi membranes of ciliated cells were labeled intensely with anti-5 only. The plasma membrane of type II pneumocytes was not labeled by any of the antisera, but with anti-2 or anti-5 there was labeling of AER-associated vacuoles, the membranous residue of lamellar bodies, and, to some extent, mitochondria; at 1:5,000 but not 1:10,000 dilution, staining with anti-R was qualitatively similar. Type I pneumocytes, ciliated cell cytoplasm, and nuclei were essentially unlabeled. Immunoblots (Western) of tracheal homogenates yielded no evidence for epitopes other than those in microsomal fractions from whole lung. Contact blots of fresh whole trachea, before but not after lavage, bound anti-2 and anti-R. Thus, we have demonstrated for the first time that components of the pulmonary cytochrome P-450 monooxygenase, although localized in the AER-rich regions of the Clara cells and type II pneumocytes, are not restricted to these cell types or to the endoplasmic reticulum. Labeling of the endothelium, plasma membranes, and the tracheal extracellular matrix indicates the possibility of cytochrome P-450-mediated metabolism at the luminal interface in the airways and the vasculature.

Throughout the airways of the rabbit lung, a relatively high proportion of nonciliated epithelial (Clara) cells have a notably high content of AER (1). Furthermore, the pulmonary microsomal fraction of the rabbit has a high content of cytochrome P-450 monooxygenase components as compared to other species (2). Light microscopic immunocytochemistry with antibodies to cytochrome P-450 components show this cell type to be immunoreactive (3-9), and cell fractions enriched in Clara cells exhibit monooxygenase activity (10). However, much of the cellular and subcellular pulmonary distribution remains to be demonstrated, in part, because of the limitations of the techniques that were available: resolution of the numerous pulmonary cell types (~40) in paraffin or frozen sections is limited, the isolation of individual types of intact pneumocytes is quite difficult (11), and subcellular fractionation of the lung yields very impure membrane or organelle preparations.

The major constitutive pulmonary cytochrome P-450 monooxygenase components are cytochrome P-450 isozymes 2 and 5, which have distinct substrate specificities, and NADPH-cytochrome P-450 reductase, which for most substrates is required for the transfer of electrons from NADPH to cytochrome P-450 (12). Despite the extensive characterization of the metabolic activity of this enzyme system with various endogenous (steroids, fatty acids) and exogenous lipophilic compounds

ABBREVIATIONS: AER, agranular endoplasmic reticulum; ER, endoplasmic reticulum; TEM, transmission electron microscopy; PBS, phosphate-buffered saline; anti-2, anti-5, and anti-R, antisera to cytochrome P-450 isozyme 2, isozyme 5, and NADPH-cytochrome P-450 reductase, respectively; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(polycyclic hydrocarbons, aromatic amines), its role in pulmonary function and susceptibility is not clear. The delineation of the relative cellular and subcellular distribution would further our understanding of both the monooxygenase system and potential nonrespiratory functions of various pneumocytes, which is the purpose of the present study. By modifying fixation, embedding and immunogold staining techniques for TEM analysis of the lung, we have demonstrated qualitative differences in cellular, subcellular, and extracellular distribution of the three monooxygenase components.

Materials and Methods

Electron microscopic immunocytochemistry. Lungs and tracheas of seven adult (25 weeks old) male New Zealand White rabbits were used in this study. Five were from a specific pathogen-free colony (Dutchland Laboratories, Inc., Denver, PA) and were killed by carbon dioxide inhalation and exsanguination. Two were not from a specific pathogen-free colony and were killed within 1 hr of arrival at the laboratory by exsanguination following anesthesia with sodium pentobarbital (30 mg, intravenously). The trachea and lungs were removed by thoracotomy and fixed for 1 hr by infusion via a tracheal cannula with 1% paraformaldehyde in 0.1 M phosphate buffer (395 mOsm at pH 7.4) at a water pressure of 30 cm. The fixed trachea and right apical lobes were dissected as previously described (13). Cross-sections or longitudinal sections of airway tissue which contained terminal bronchiole-alveolar duct sections were used. These tissue specimens were stored in phosphate buffer prior to embedding. The blocks were washed in buffer, dehydrated with *N,N*-dimethylformamide (50–90%), and infiltrated with Lowicryl K4M (Polysciences, Warrington, PA) (14). Tissue was then transferred to polyethylene capsules filled with fresh Lowicryl. Sealed capsules were polymerized with UV irradiation at 4° in a foil-lined box with a lamp-to-tissue distance of 10 cm for 1 hr. Blocks were sectioned on a Sorvall MT 5000 microtome using a diamond knife. The sections were mounted on nickel grids. Immunocytochemical characterization was carried out using a modification of the procedure described by Roth *et al.* (15) for protein A-gold with the exception that colloidal gold-labeled rabbit anti-goat immunoglobulin was used in place of protein A-gold. Grids were floated in droplets of 1% ovalbumin in PBS and then incubated in drops of primary antisera for 18–24 hr at 4° in a moist chamber. Antibodies against purified pulmonary cytochrome P-450 isozymes 2 and 5 (previously called P-450I and P-450II, respectively) and cytochrome P-450 reductase were produced in goats, and the IgG fractions have been previously characterized (16); the whole antisera were used in this study. Dilutions of primary antiserum ranged from 1:500 to 1:50,000, but unless otherwise indicated, the dilution used was 1:10,000. All primary antisera were diluted in 1% ovalbumin in 0.1 M phosphate buffer, pH 7.4 (PBS). Following incubation, the grids were washed and incubated in drops of gold-labeled rabbit anti-goat immunoglobulin (Jansson Life Sciences Products, Beerse, Belgium). Monodispersion of the immunogold was verified using Formvar (Electron Microscopy Sciences, Fort Washington, PA)-coated grids treated with poly-L-lysine (1 mg/ml) prior to incubation in the diluted immunogold for 30 sec to 2 min. Minimal clumping of the beads was observed. Two sizes of colloidal gold were used: 10 nm and 40 nm. The colloidal gold-labeled antiserum was diluted with 0.1 M phosphate buffer, either 1:10 or 1:20. After a wash in PBS, the grids were stained with aqueous uranyl acetate. Controls included substitution of primary antisera with either 0.1 M PBS or preimmune goat serum diluted to the same concentration as primary antisera. The range of dilutions was large enough to include both positive and negative reactions. The effect of dilution from 1:1,000 to 1:50,000 was qualitatively the same for the three antisera. At dilutions less than 1:10,000, the amount of label on the surrounding plastic increased without a compensatory increase in cytoplasmic labeling, whereas at greater dilutions the cytoplasmic labeling markedly decreased. The Clara cell and the ciliated cell of the terminal bronchiole, because of their high and low content of AER (8), respectively, provided positive and near negative end-points for evaluating the specificity of staining under various conditions. More intense labeling was observed with the 10-nm than the 40-nm colloidal gold-labeled second antibody. All specimens were examined using Zeiss EM 10 or Hitachi H-600 transmission electron microscopes at 60 or 80 kV.

Immunoblotting. Normal buffered saline was used to lavage tracheas at 4°. Differential centrifugation of the lavage fluid at 400, 10,000, and 100,000 × *g* yielded the cytosol and three particulate fractions: intact cells (primarily erythrocytes) which were discarded, a submicrosomal fraction, and a microsomal fraction. A 25% homogenate in 1.15% KCl/HEPES, pH 7.4, was prepared from the tracheal epithelium that was removed by scraping with the edge of a glass slide; immunoblots (17) stained by the immunoperoxidase bridge/3,3′-diaminobenzidine method were as previously described (18). Contact blots of unfixed tracheal lumen were obtained before or after lavage; the trachea was cut vertically along the dorsal side and wrapped snuggly with parafilm around a cylinder of nitrocellulose supported by a glass tube and left at 4° for 30 min before being immunostained. The effect of this procedure on the integrity of the epithelium was confirmed by scanning electron microscopy, i.e., the cells appeared undamaged. The dot blots (19) of pulmonary microsomes (20) before and after fixation for 10 min with 1% paraformaldehyde in 100 mM sodium phosphate, pH 7.4, were immunostained for isozyme 2, 5 and reductase. The range of 3.9 ng to 12.8 μg of microsomal protein yielded some values above and below the linear range of detection. The limit of detection and relative staining for each enzyme before and after fixation were examined.

Results

Bronchiolar Epithelial Cells

Optimization of immunostaining in the Clara cell. Three antisera against cytochrome P-450 isozymes 2 and 5 and NADPH-cytochrome P-450 reductase (anti-2, anti-5, and anti-R) bound specifically to regions rich in endoplasmic reticulum in the Clara cell of the distal airway. Consistent with the known distribution of AER (1), the binding of anti-2 (Fig. 1a), anti-5 (Fig. 1b), or anti-R (Fig. 1, c and d) was somewhat greater in the apical than in the basal cytoplasm. The amount of immunolabeling over mitochondria, nucleus, and secretory granules appeared comparable to that observed in control sections treated with preimmune goat serum (Fig. 1e); no labeling was observed when PBS was substituted for primary sera (Fig. 1f). The plasmalemmal surface was labeled by anti-2 and anti-5 (Fig. 1, a and b) but not by anti-R (Fig. 1d), even at 1:1000 dilution (not shown). Labeling of what appeared to be the Golgi apparatus was often apparent with anti-5 (see Fig. 3b) but less so with anti-2 or anti-R.

In a few instances there were marked differences in the intensity of labeling of adjacent Clara cells (Fig. 2, a–c). This is shown with anti-R (Fig. 2, a and b), but was not observed with anti-5 (Fig. 2c). Compared to the more heavily labeled cells, the poorly labeled ones contained fewer cytoplasmic components such as mitochondria and secretory granules; the abundance of AER was difficult to assess because of the mild tissue processing used so as to preserve antigenicity. Non adjacent Clara cells that differed in their content of mitochondria and secretory granules and that were labeled with anti-2 are shown in Fig. 2, d and e. The cytoplasmic staining as well as the labeling of the plasma membrane was more dense (Fig. 2d) in the cell with the higher content of cytoplasmic components.

Ciliated cells. The cytoplasmic labeling of the ciliated cell with all three antisera was considerably less than in the Clara cell. However, intense labeling of the plasmalemma, especially

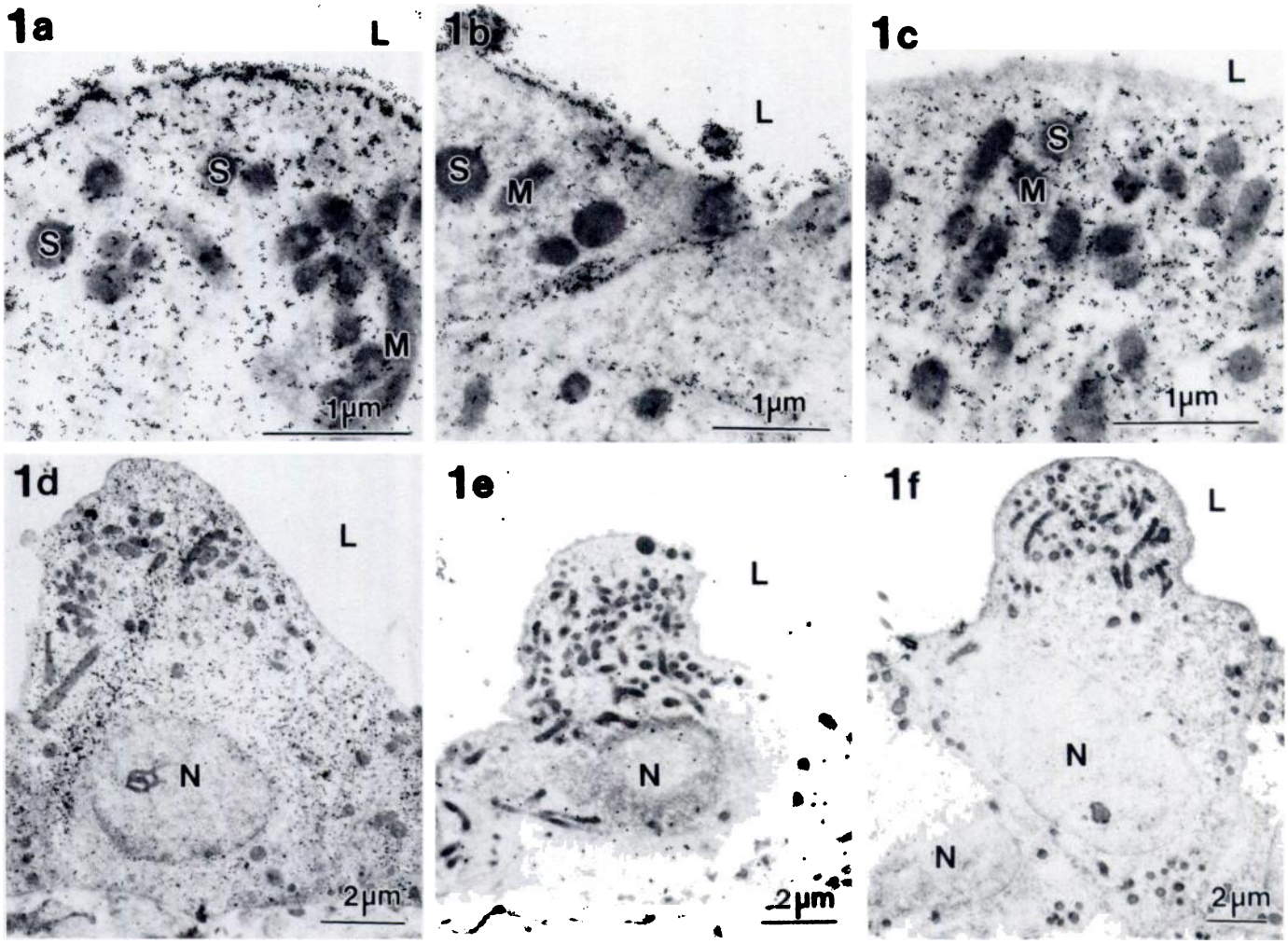

Fig. 1. Clara cells from terminal bronchioles. All three antisera labeled cytoplasmic regions rich in AER. *N*, nuclei of Clara cells; *L*, airway lumen; *M*, mitochondria; *S*, secretory granules. The primary antisera were: (a) anti-2, and (b) anti-5, (c) anti-R, (d) anti-R, (e) preimmune goat serum, and (f) PBS substitute. the size of gold particle on the secondary antibody was 10 nm for all sections except e, 40 nm.

the microvillar surface, was observed with anti-2 (Fig. 3a) and anti-5 (Fig. 3b) but not with anti-R (Fig. 3d). The membrane segments between cilia and microvillae were not as heavily labeled. Another similarity to Clara cells (Fig. 3b) was the intense cytoplasmic labeling with anti-5 over what appeared to be the Golgi apparatus (Fig. 3, b and c).

Interalveolar Septum

Epithelial cells. The plasma membrane of type II alveolar epithelial cells did not label with anti-2 (not shown), anti-5 (Fig. 4, a and b) or anti-R (Fig. 4, c and d); the labeling there was comparable to that seen with PBS or preimmune goat serum (Fig. 4e). Binding of anti-5 (Fig. 4, a and b) and anti-2 (not shown) appeared focal (see Fig. 4, *arrowheads*), particularly with 10-nm gold particles. Labeling was observed around vacuolated structures apparently associated with ER and mitochondria, as well as with membranous residues of lamellar bodies (Fig. 4, a, b, and d). The mild fixation without osmication resulted in poor preservation of the lamellar body and precluded a complete evaluation of these structures. Labeling with anti-R at 1:10,000 dilution (Fig. 4c) and at 1:5000 (Fig. 4d) was not strong. The labeling ratios of the cytoplasm versus the nucleus were 3.6 and 3.5 in Fig. 4, c and d, respectively, and 1.3 for the control (Fig. 4c). We estimate a 2.7-fold greater cytoplasmic labeling (versus the nucleus) in these anti-R-labeled sections as compared to the control. The type I pneumocyte was essentially negative for all three antisera (Fig. 5 a–c, see *arrows*).

Endothelial cells. In capillary endothelial cells, anti-2 and anti-5 produced intense labeling of both the cytoplasm and the cell surface (Fig. 5, a and b). Larger vessels associated with conducting airways exhibited similar labeling (Fig. 5, d and e). Endocytotic vesicles (Fig. 5, d and e, *open arrows*) along the luminal surface and in the cytoplasm were also labeled. No binding of anti-R (Fig. 5, c and f) in any endothelial cell was observed, even at high concentrations of antisera (1:1000; not shown).

Trachea and proximal bronchi. Clara cells and ciliated cells of the trachea and proximal airways exhibited labeling similar to that observed in these cell types in the distal airways, except that the labeling in the apical cytoplasm of the Clara cell was less pronounced, i.e., more evenly distributed throughout the cytoplasm (not shown), and the plasma membrane, except for the microvillae, was essentially unlabeled, unlike the Clara cells of the lower airways (not shown). Goblet cells, particularly over the mucous droplets, were densely labeled with anti-2 (Fig. 6a) or anti-5 (not shown) but not with

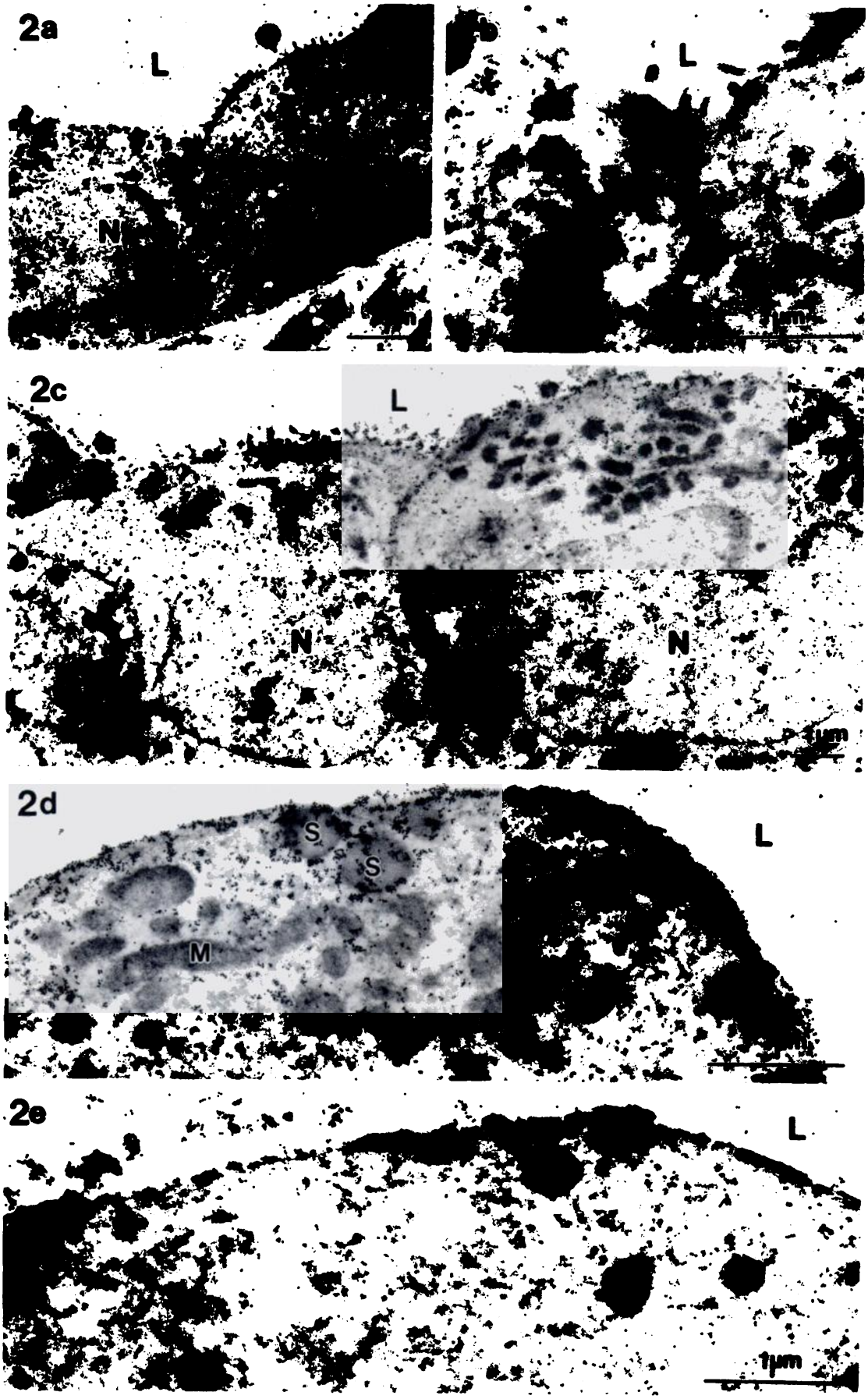

Fig. 2. Clara cells from terminal bronchioles. Labeling varies in density in adjacent cells or between cells having different contents of mitochondria and secretory granules. *N*, nuclei of Clara cells; *S*, secretory granules; *M*, mitochondria, *L*, airway lumen. Primary antisera were: (a) anti-R, 1:5000; (b) anti-R, 1:5000; (c) anti-5; (d) anti-2; and (e) anti-2. All gold particles are 10 nm.

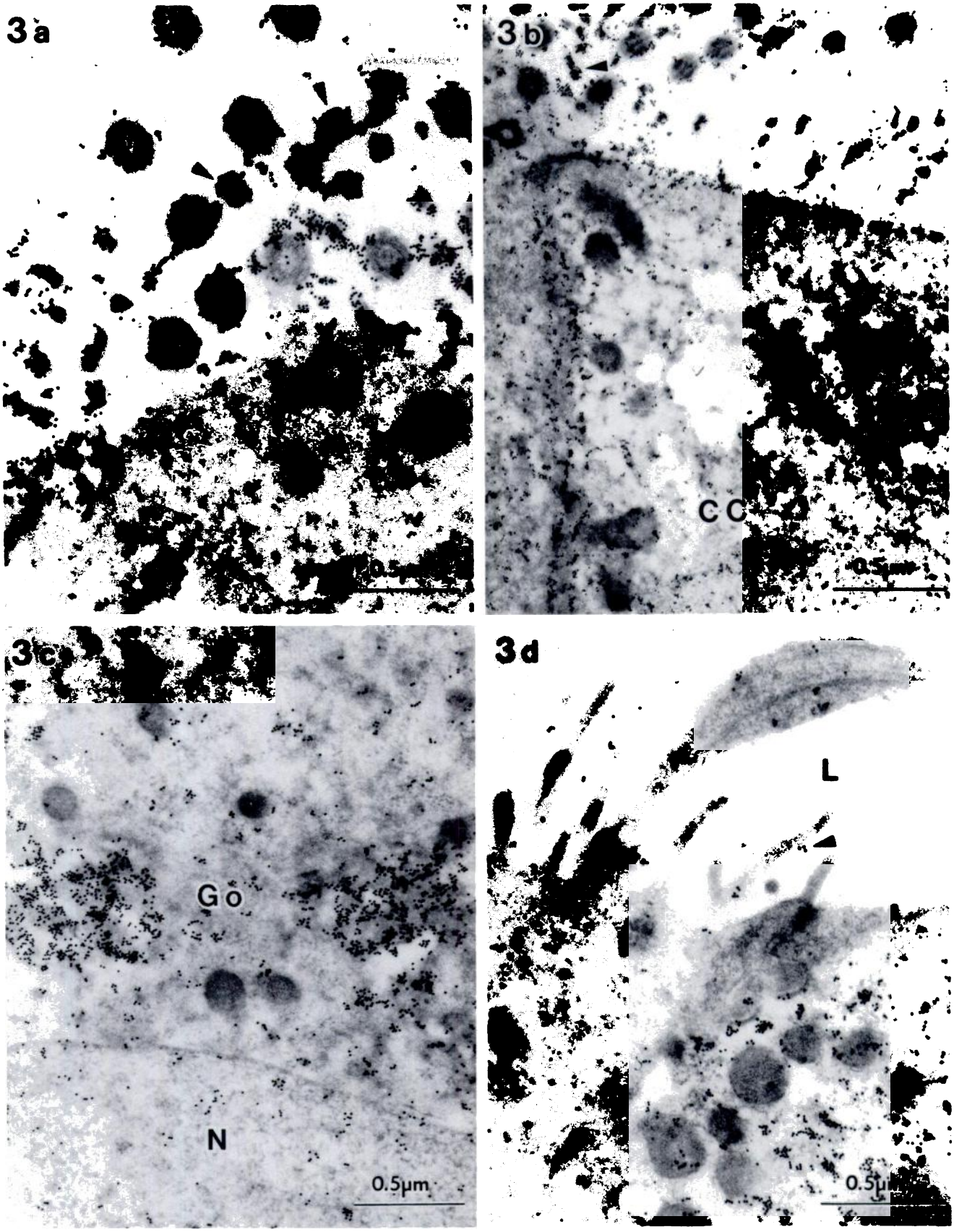

Fig. 3. Ciliated cells from terminal bronchioles. The majority of the label is on microvilli (►) and Golgi apparatus (*Go*). *N*, nucleus of ciliated cell; *CC*, Clara cell; *L*, airway lumen. Primary antisera were : (a) anti-2; (b) anti-5; (c) anti-5; and (d) anti-R. All gold particles are 10 nm.

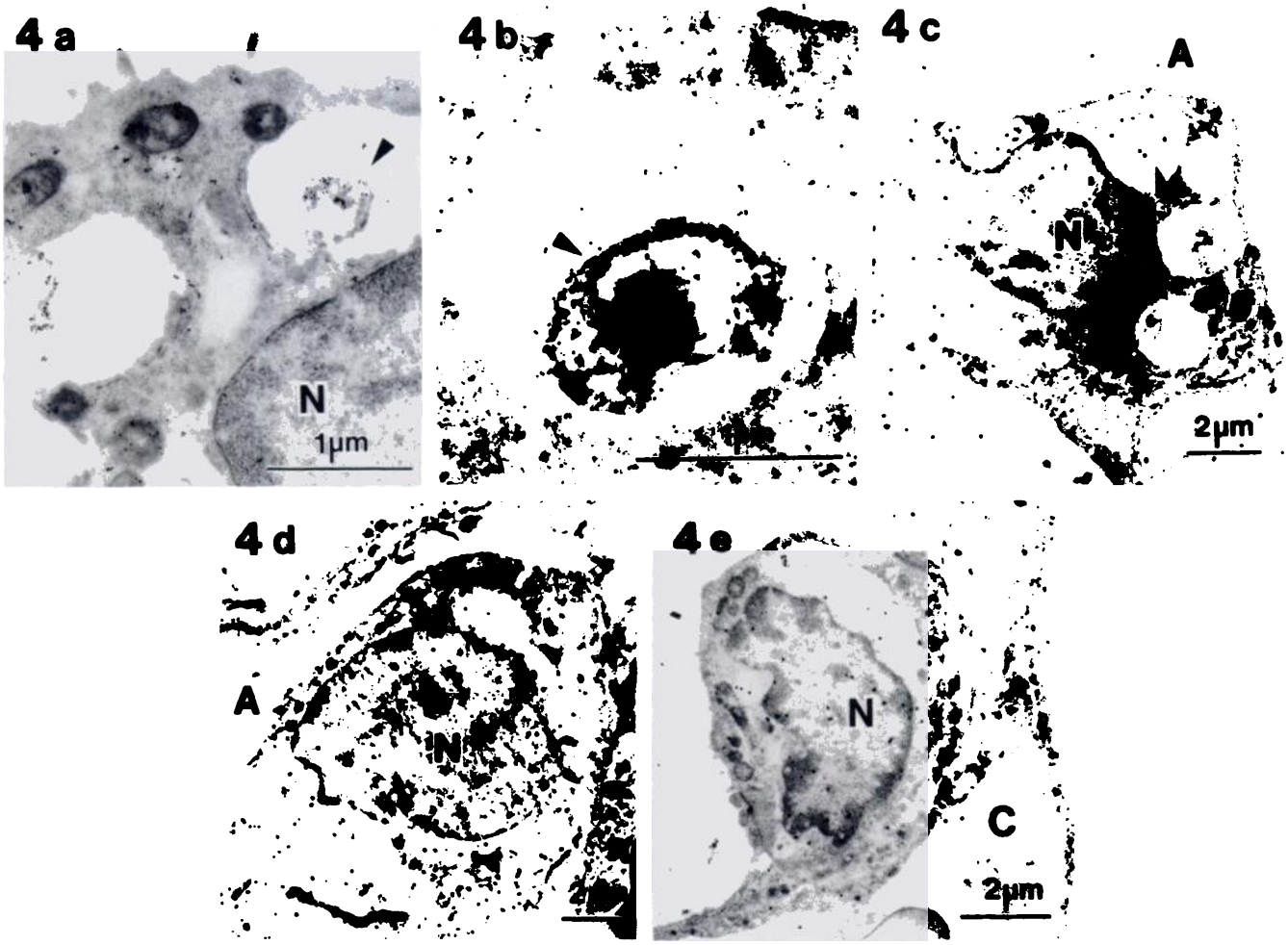

Fig. 4. Type II pneumocytes from interalveolar septa. Labeling is primarily in the residue of lamellar bodies (▶). *N*, nuclei of type II cells; *A*, alveolar airspace; *C*, pulmonary capillary lumen. Primary antisera were (a) anti-5, (b) anti-5, (c) anti-R, 1:10,000; (d) anti-R, 1:5000; and (e) preimmune goat serum. Gold particle sizes are: a and b, 10 nm; c–e, 40 nm.

anti-R, even at a 1:1000 dilution (Fig. 6b); the portion of an adjacent Clara cell shown is densely labeled (Fig. 6a).

Immunoblotting

Dot blots. Because of the lack of labeling with anti-R at 1:10,000 dilution except in the Clara cell, the effect of fixation of pulmonary microsomes with 1% paraformaldehyde for 1 hr on the relative reactivity of anti-2, anti-5, and anti-R was determined in an immunostained dot blot. Serial dilutions of fixed and unfixed microsomes over a 32-fold range were examined and no loss of reactivity due to fixation was apparent (not shown).

Contact blots. To determine whether or not the immunoreactivity observed on the epithelial surface was inherently extracellular, fresh (unfixed) tracheas before (Fig. 7, A and B) or after (Fig. 7, C and D) lavage with saline were placed around a cylinder of nitrocellulose that was then immunostained for isozyme 2 (Fig. 7, A and C) and reductase (Fig. 7, B and D). These epitopes were readily transferred to the membrane (Fig. 7, A and B) as indicated by the dark immunoperoxidase stain. Lavage prior to blotting significantly diminished the reactivity (Fig. 7, C and D). The epithelial surface appeared intact upon examination by scanning electron microscopy (not shown).

Immunoblot (Western). The electrophoretic mobilities (apparent monomeric molecular weights) of the immunoreactive bands resolved from cell-free tracheal lavage (*L*) or tracheal epithelial homogenate (*H*) were the same as those of isozyme 2 and reductase in pulmonary microsomes (*M*) (Fig. 8); no additional immunoreactive species were identified. Two pellets (10,000 and 100,000 × *g*) were recovered by differential centrifugation of the lavage. The majority of the immunoreactivity sedimented with the 100,000 × *g* pellet (Fig. 8, *LM*) which contained small membrane fragments as determined by TEM. Some reactivity was observed in the 10,000 × *g* fraction which contained large cellular debris, but no cross-reactivity was observed in the cytosol (Fig. 8, *LC*).

Discussion

The components of the rabbit pulmonary cytochrome P-450 monooxygenase system are considered to be associated primarily with the ER and to be located in Clara and type II cells (5, 8–10). In this study we have confirmed the cytoplasmic localization of these components in the Clara and type II cell. In addition, with the higher resolution of electron microscopic immunocytochemistry we have demonstrated for the first time the presence of epitopes of cytochrome P-450 isozyme 2 or isozyme 5 in the plasma membranes and Golgi apparatus of Clara and ciliated cells; in the plasma membranes, caveoli, and

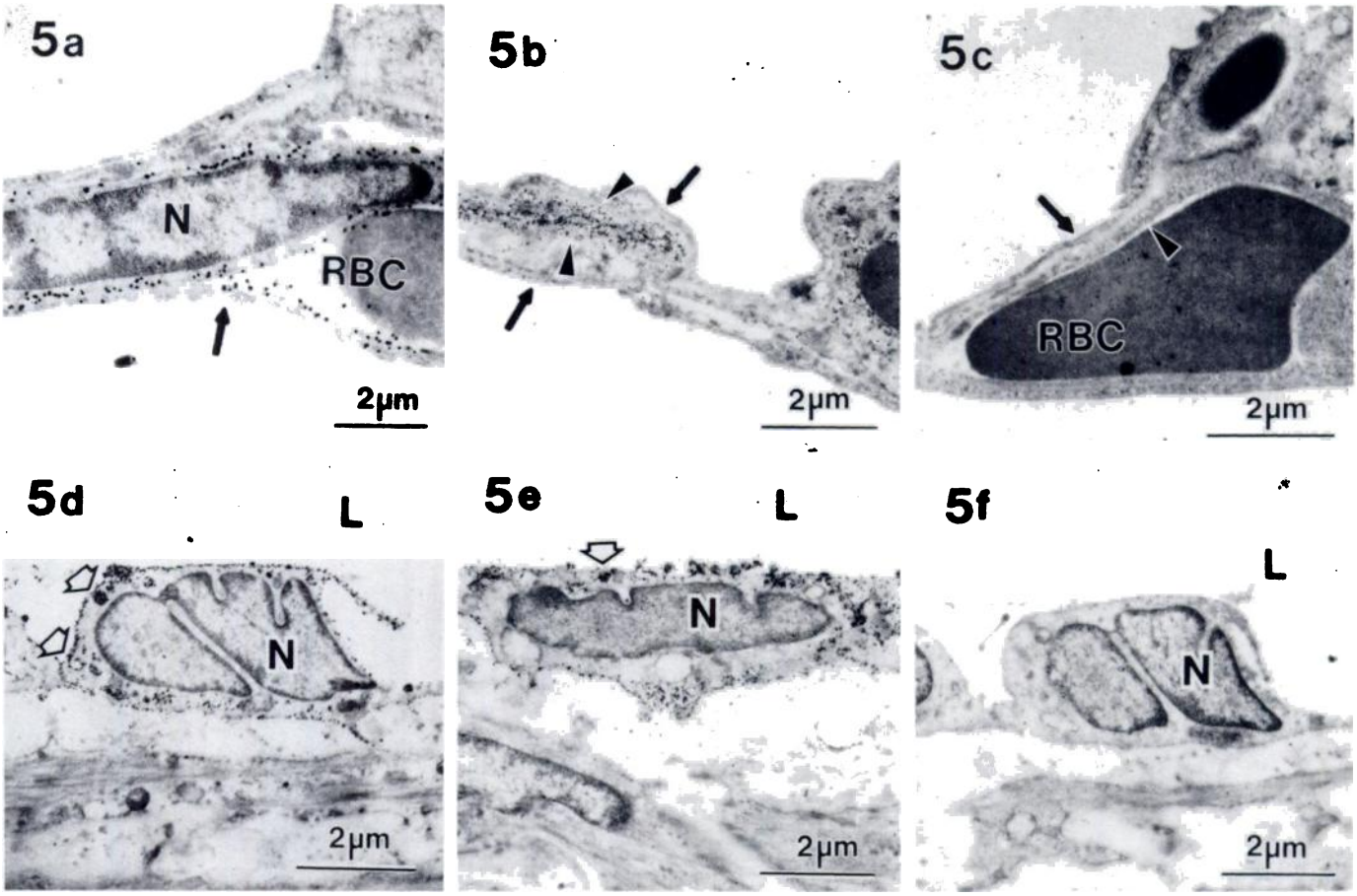

Fig. 5. Type I epithelial cells and endothelial cells of interalveolar septa and pulmonary artery. Cytoplasm of type 1 pneumocytes (*arrows*) is unlabeled compared to capillary endothelium (*arrowheads*). *N*, nuclei of endothelial cells; *RBC*, red blood cells in pulmonary capillary lumen; *L*, lumen of pulmonary artery; *Open arrow*, pinocytotic vesicles. Primary antisera were: (a) anti-2, (b) anti-5, (c) anti-R, (d) anti-2, (e) anti-5, and (f) anti-R. All gold particles are 10 nm.

cytoplasm of endothelial cells; and in secretory granules of goblet cells. We have also detected extracellular intact reductase and isozyme 2. These results bring into question our understanding of the relationship between cellular susceptibility and the sites of activation/inactivation of pulmonary toxicants and carcinogens by this enzyme system.

The cytoplasm of the Clara cell was labeled with anti-R, but little (in type II cell) or no reactivity (in goblet cells, endothelial cells, or plasma membrane) of this antisera was observed elsewhere. Although NADPH-cytochrome P-450 reductase is obligatory for most monooxygenase activities toward xenobiotics, electron transport can proceed via the NADH-cytochrome b_5 reductase/cytochrome b_5 pathway (21) which may prove more relevant for endogenous substrates yet to be identified. The detection of cytochrome b_5 and NADH-cytochrome *c* reductase in plasma membranes of intestinal microvilli, erythrocytes (22), and rat hepatocytes (23) led Bruder *et al.* (22) to propose that these redox enzymes are generally present in cell membranes. Clearly, the pulmonary distribution of these reductive enzymes should be determined.

The limited labeling with anti-R, however, may be due to a lack of sensitivity or to adequate preservation of epitopes as suggested by the following observations: 1) cell fractions enriched in type II cells (>80%) have NADPH-cytochrome *c* reductase activity and immunoreactive protein comparable to that in Clara cell fractions (11); 2) undigested reductase protein (monomeric molecular weight of 72,000) was recovered by lavage of the tracheal lumen (Fig. 8); 3) the lumenal surface of trachea embedded in paraffin reacted with anti-R (8), although not as strongly as with anti-2 or anti-5. Thus, the embedding conditions for Lowicryl, more than for paraffin, may selectively solubilize the reductase which is not as well protected by the membrane as is the cytochrome (24). Just as epitopes may differ in lability, cells may differ in their retention of ultrastructure; for example, the type II cell, in addition to having less AER than the Clara cell, appears to be less well preserved by this method of processing the tissue.

In a series of preliminary experiments (not shown), we evaluated other fixation, embedding, and staining protocols. The preservation/availability of epitopes depended on mild fixation and embedding (Lowicryl) conditions. Osmication of the tissue allowed clear visualization of cellular membranes, but this treatment was detrimental to immunoreactivity. Thus, simultaneous resolution of fine membrane structure and immunolabeling in a single section was not feasible.

In spite of the limitations of immunocytochemistry, the detection of epitopes in organelles or various cell types is not compromised by contamination as is the case with subcellular fractionation or cell isolation. This has been recognized in studies of subcellular fractions where the content of monooxygenase components in plasma membrane or Golgi apparatus (25), for example, is nearly comparable to the level of contam-

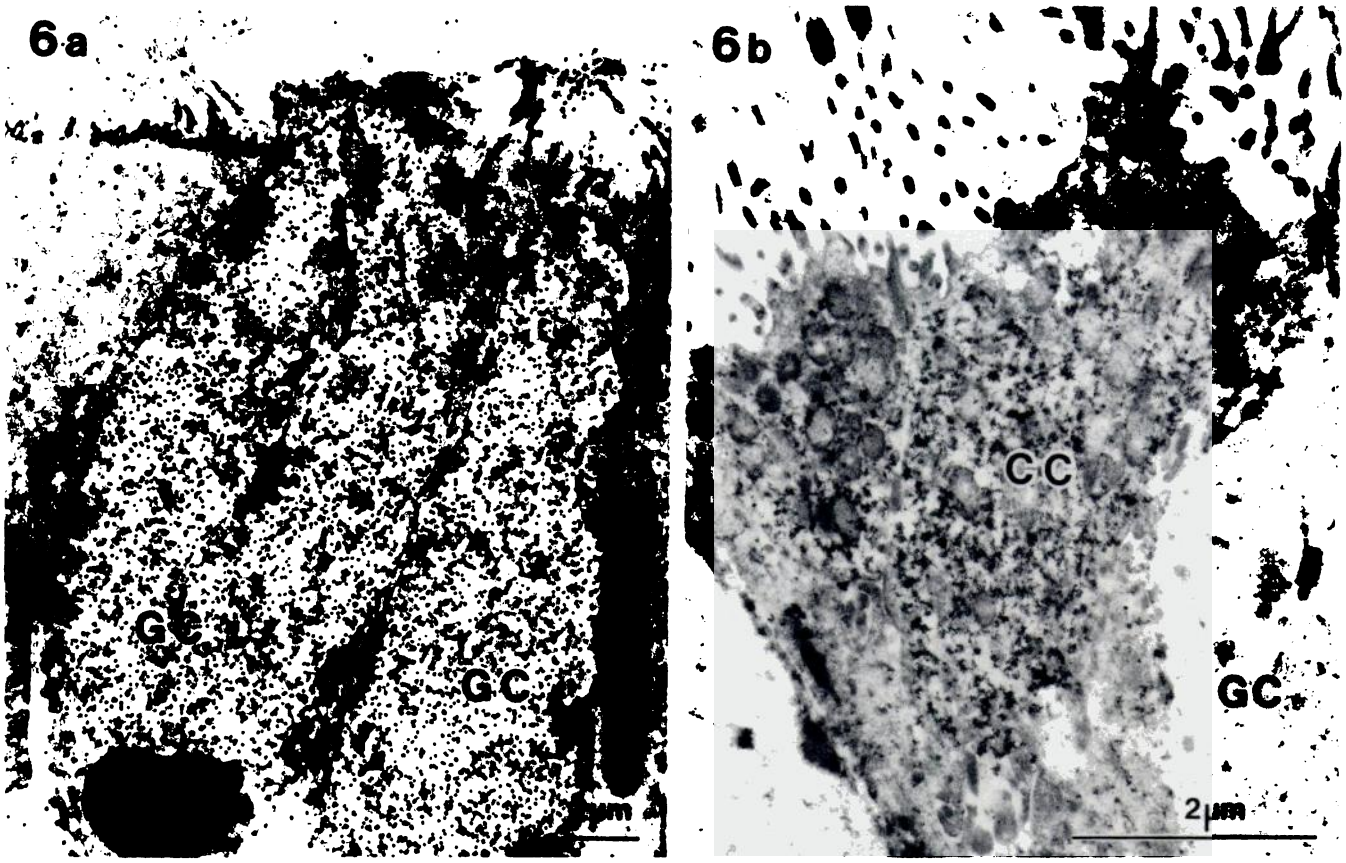

Fig. 6. Tracheal epithelial cells. Both mucous goblet cells (*GC*) and Clara cells (*CC*) were labeled by anti-2. Only Clara cells were labeled by anti-R. Primary antisera were: (a) anti-2, and (b) anti-R, 1:1000. Gold particle sizes are: (a) 40 nm and (b) 10 nm.

ination by ER (26); and in membranes other than ER, these enzymes appeared to be more labile (22, 23, 27). Those investigations, which are consistent with this study, did not definitively demonstrate NADPH-cytochrome *c* reductase in normal mammalian plasma membranes. However, KB cells, a human oral carcinoma line, do have a surprisingly high level of this enzyme in plasma membrane (28).

Despite the questionable distribution of the reductase, the presence of epitopes of cytochrome P-450 in plasma membranes, and in Golgi and goblet cell secretory granules does indicate a potential for monooxygenase activity. Unfortunately, monooxygenation of commonly used substrates by non-ER fractions is so low as to be compromised by the impurity of the fraction; e.g., benzo(*a*)pyrene hydroxylase in rat hepatic plasma membrane fraction (29). Nevertheless, the potential for complexes of cytochrome P-450 and substrate/metabolites in the plasma membrane (whether they are formed or transported there) to elicit extracellular responses has been considered in the case of a hepatic plasma membrane-bound complex of a cytochrome P-450-halothane metabolite that was implicated in the immune reaction to halothane (30). The plasma membrane of isolated rat hepatocytes has been found to be immunoreactive with the antibody to rat cytochrome P-450b (31), which is homologous to rabbit isozyme 2. We have examined immunogold-labeled rabbit hepatic sections and, with anti-2 and anti-5 (but not anti-R), have observed labeling of the plasma membranes of hepatocytes and cells lining the bile canniculi.[1]

The recovery from tracheal lavage of immunoreactive proteins of apparent monomeric molecular weight identical to those of isozyme 2 or reductase with little evidence of proteolysis is remarkable, especially in the case of reductase, thus implying that the extracellular as well as intracellular epitopes detected *in situ* represent the intact proteins. The extracellular tracheal membrane fragments may be from normal cell turnover and mucociliary clearance or, more directly, from secretion by mucous goblet cells. Enzyme activities used as markers for mitochondria, Golgi apparatus, or ER have been detected in the olfactory mucus of the rhesus monkey and putatively were secreted by Bowman's gland (32). Of course, *in vivo*, the extracellular activity of these enzymes would depend on the availability of cofactors [NAD(P)H] in the mucus.

The selective anti-5 labeling of what appears to be the Golgi apparatus in ciliated cells and, to some degree, in Clara cells, suggests the metabolism of a secretory product by isozyme 5 but not by isozyme 2. It would appear that the two isozymes are sorted differently in the processing of the ER membranes. The apparent lack of isozyme 2 in the Golgi apparatus of Clara and ciliated cells is consistent with the apparent lack of transport of a homologue of isozyme 2 from the ER to the Golgi apparatus of rat hepatocytes (33). If the Clara cell is the progenitor of the ciliated cell (34), then staining with anti-2 and anti-5 in the plasma membrane of the Clara cell and in portions of the ciliated cell membrane may correspond to the degree of preservation of that membrane in the process. It is interesting that the plasma membrane of tracheal (versus bronchiolar) Clara cells is noticeably less immunoreactive, perhaps representing a transitional phase of this cell type.

[1] C. J. Serabjit-Singh, S. J. Nishio, R. M. Philpot, and C. G. Plopper, unpublished observations.

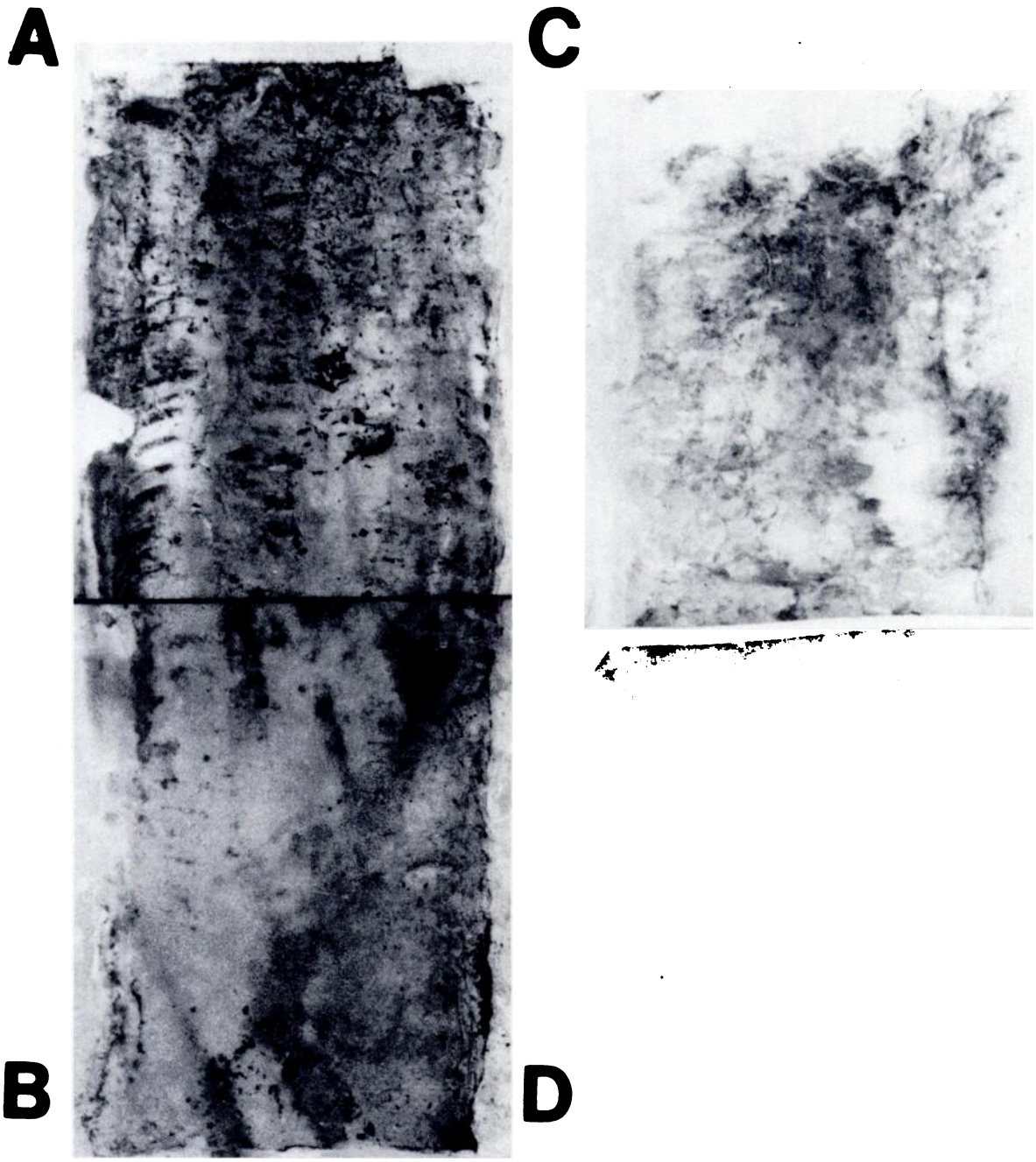

Fig. 7. Tracheal lumen blotted directly onto nitrocellulose. A and B, unwashed trachea; C and D lavaged trachea. A and C were immunostained with anti-2 (1:8000) and B and D with anti-R (1:1000). The immunoperoxidase-bridged antibody technique with 3,3′-diaminobenzidine as substrate was used (18).

The apparent lack of reductase, but not cytochrome P-450, in Clara cells that have few secretory granules (perhaps as a result of dedifferentiation) may be an artifact of the technique as discussed above. Alternately, it suggests a lack of coordination in the regulation of the cytochrome P-450 and the putative electron-transport protein. This would seem to be the case in microsomal fractions, where the activity of an isozyme is limited by the microsomal reductase content (35), suggesting an overproduction of cytochrome P-450. However, at given concentrations of cytochrome P-450 and reductase in microsomes, the extent of expression of activity differs with different substrates (35); ostensibly, if the appropriate substrate were found, the activity might not be limited by the constitutive level of reductase. The ratio of enzymes, and thus the specific activity, is unlikely to be constant among the cells of a tissue as heterogeneous as the lung. This is qualitatively reflected by the differences in the apparent ratio of epitopes.

Previously, we demonstrated immunoperoxidase labeling of the pulmonary vasculature in paraffin-embedded sections by light microscopy; anti-2 and anti-5 produced strong labeling, whereas labeling with anti-R was poor (8). In contrast, Dees *et al.* (4) observed no vascular labeling for isozyme 2 in frozen sections of lung; the pulmonary vasculature of untreated mouse (6), untreated rat (3, 4, 7) or minipig (4) has not been shown to immunostain for monooxygenase components. Monooxygenase activity of aorta from rabbit, monkey, and man (36) toward

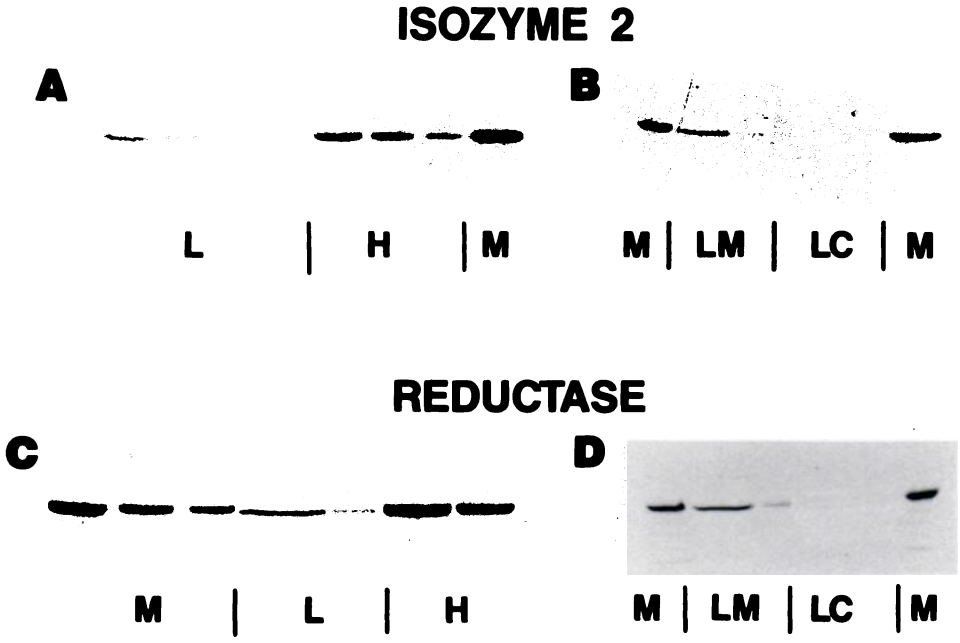

Fig. 8. Western blots of tracheal lavage fractions and homogenate. Preparation of the microsomal fraction (*M*) from lung, the homogenate (*H*) from unwashed trachea, and the microsomal (*LM*) and cytosolic (*LC*) fractions from tracheal lavage (*L*) is described in the text. The amounts of protein (μg) applied per track are: A 100, 75, 50, 20, 63, 30, 10, and 10; B and D, 5, 50, 20, 50, 20, and 5; C, 5, 2, 1, 100, 50, 63, 30, respectively, from the left. A and B were immunostained for form 2 and C and D for reductase.

exogenous substrates has been reported. However, a role for cytochrome P-450 monooxygenase in the vascular metabolism of the endogenous substrate, arachidonic acid, to vasoactive compounds has been suggested (37, 38) and is possibly the function of isozyme 2 and isozyme 5 in the endothelium throughout the pulmonary vasculature. However, in the aorta, removal of the lumenal endothelium did not appreciably alter the amount of monooxygenase enzymes (including the reductase) detected by Western blots or enzymatic activity (39). Thus, the relative content of monooxygenase components in smooth muscle and endothelium appears to vary among vascular beds, perhaps corresponding to site-specific functions of this enzyme system.

The rabbit does have a uniquely high level of microsomal cytochrome P-450 monooxygenase as well as a high proportion of AER-rich Clara cells and thus differs from other species, especially primates. It should be noted, however, that there are immunoreactive homologues of isozymes 2 and 5 in the lungs of all other species examined (guinea pig, hamster, rat, mouse, monkey) (18, 40). It will be interesting to see if any correspondence between morphology and enzyme distribution is conserved among species.

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