Environmental Health Perspectives
Vol. 93, 225–232, 1991

Ultrastructural Immunohistochemical Localization of Clara Cell Secretory Protein in Pulmonary Epithelium of Rabbits

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Highly purified Clara cells (93 ± 3%) isolated from the lungs of rabbits were used to produce an antiserum against Clara cell secretory proteins. This antiserum was used to identify and study the biosynthesis and secretion of [^{35}S]methionine-labeled proteins from isolated Clara cells. The antiserum recognized one major secretory protein with apparent molecular weight of 6 kDa and reacted weakly with a higher molecular weight protein of about 180 kDa. Biosynthesis and secretion of these proteins was not detected in preparations of isolated alveolar type II cells or alveolar macrophages. Immunocytochemical localization of the antigen with colloidal gold indicated a dual localization in bronchiolar Clara cells. Gold labeling was found over the osmiophilic secretory granules of Clara cells and smooth endoplasmic reticulum. In tracheal Clara cells, labeling was found mostly in association with secretory granules and relatively little in association with the smooth endoplasmic reticulum. Labeling was also found over the lamellar bodies of type II cells, although the reaction was weak. Labeling of ciliated cells, alveolar type I cells, capillary endothelial cells, and alveolar macrophages was not distinguishable from background. These data indicate that Clara cells of both the bronchioles and trachea of rabbits synthesize and secrete the low molecular weight protein previously called Clara cell secretory protein (CCSP). This antigen does not belong to that group of surfactant proteins whose molecular weights range from 26 to 40 kDa.

Introduction

Most of the studies involving the toxicologic pathology of Clara cells have been concerned with cells found in the bronchioles of the lungs (*1*), with little thought to the fact that Clara cells also exist in the upper airways and trachea of numerous mammalian species (*2–4*). A contributing difficulty has been the identification of Clara cells in the upper airways. Clara cell identification has depended almost exclusively on their location as nonciliated cells in the distal airways and their morphological appearance under the electron microscope; that is, the presence of abundant smooth endoplasmic reticula and characteristic cytoplasmic osmiophilic granules (*2*). Unfortunately, the classification of nonciliated cells in the upper airway as Clara cells is often impossible because of fundamental differences in the overall appearance of the cell in the upper and lower airways. In the bronchioles the epithelium is low and cuboidal, whereas the upper airway epithelium is tall, columnar, and pseudostratified (*2,4*). In addition, the abundance of smooth endoplasmic reticulum and osmiophilic granules in Clara cells of the upper versus distal airways may vary enormously, thus making identification of Clara cells very difficult. This problem is even more pronounced when morphological appearance is obscured by the action of toxic agents. There is a need for a Clara cell-specific reagent that can be used for the identification of Clara cells throughout the airways.

Recently, we incubated isolated Clara cells in the presence of [^{35}S]methionine and examined proteins synthesized and secreted by those cells. From these experiments we identified the major secretory product of rabbit lung Clara cells to be a low molecular weight protein (apparent Mr = 6 kDa under reducing conditions on SDS gels) (*5*) that appears to have thiol protease inhibitory activity (*6*). We have shown that this low molecular weight protein is present in pulmonary lavage effluents, and it seems reasonable to assume that at least a proportion of this extracellular protein should arise from Clara cells of the pulmonary airways. We do not know which other pulmonary cells, if any, contribute

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this protein to the extracellular lining. A similar, but not identical, protein has been identified in lavage effluents from the lungs of rats and localized immunocytochemically to Clara cells (*7,8*).

A number of papers have reported the immunohistochemical localization of several different antigens in Clara cells. Surfactant-associated proteins have been detected in Clara cells by using antiserum developed against the 26 to 40 kDa (reduced) group of proteins purified from pulmonary surfactant (*9*). As expected from their close association with surfactant lipids, these antigens were localized in alveolar type II cells, and their presence in bronchiolar Clara cells must be considered somewhat of a surprise in view of current opinions regarding the absence of surfactant in bronchiolar Clara cells. The report by Walker et al. (*9*) is by no means unique regarding the presence of surfactant proteins in Clara cells. An earlier report by Balis et al (*10,11*), in which antisera had been developed against both high and low molecular weight forms of surfactant proteins, also showed reactivity with bronchiolar Clara cells.

Recently, an immunocytochemical method was used to demonstrate the presence in Clara cells of the steroid-binding protein uteroglobin (*12*), and similar methodology has also revealed the presence of the low molecular weight protease inhibitor antileukoproteinase (*13,14*). Curiously, each of these antigens has been localized to the cytoplasmic osmiophilic granules of the Clara cell. These osmiophilic granules are thought to be storage sites for materials secreted by the cell. If the cytoplasmic osmiophilic granules of Clara cells are indeed storage sites for secretory materials, then at least three antigens including surfactant-associated proteins, uteroglobin, and antileukoprotease may be localized to these granules.

We have prepared an antiserum in goats against isolated Clara cells and found it to be highly specific for Clara cell secretory proteins. In this investigation we demonstrate its specificity and, using the immunogold procedure, show that it could be used for the identification of Clara cells not only in the lower airways but also in the trachea of the rabbit. This antiserum may prove useful for the study of toxic agent interactions with Clara cells of the upper and lower airways.

Materials and Methods

Animals

Male New Zealand white rabbits (2 kg) (Dutchland Laboratory Animals, Denver, PA) were killed with an overdose of sodium pentobarbital (150 mg) dissolved in isotonic saline (4 mL) containing heparin (50 mg), injected into a marginal ear vein. The lung were perfused *in situ* through the pulmonary artery with 500 mL Junod's medium (*15*) at 37°C. Throughout the perfusion the lungs were gently inflated and deflated by using a syringe and cannula inserted into the trachea. The lungs were then removed from the thorax, rinsed externally with HEPES balanced salt solution (HBSS) (*5*) and lavaged six times via the trachea with HBSS (at 37°C) to remove free cells and the pulmonary extracellular lining.

Isolation of Lung Cells

Alveolar macrophages were sedimented from the lavage fluid at 580*g* for 10 min at 4°C and washed once with HBSS. Mixed lung cells were isolated by protease digestion, tissue mincing, and filtration as previously described (*5*).

Clara cells were isolated from mixed lung cells by using the method of Patton et al. (*5*), involving continuous Percoll gradient centrifugation followed by centrifugal elutriation. A final 1-hr incubation in uncoated plastic dishes permitted substantial reduction of contaminating alveolar macrophages. This step was omitted from cells used in preparation of antiserum. Clara cells do not attach to uncoated plastic, and alveolar macrophages do attach during this time period. Approximately 6×10^6 Clara cells were routinely obtained from two rabbits.

Clara cells for the preparation of antiserum were isolated as described above except that immediately following elutriation, the cells were cultured *in vitro* for a period of 18 hr. This period allowed the cells to recover from the isolation procedure and also provided a substantial increase in purification of the cells. Most non-Clara cells, including type II cells, did not attach to the collagen/fibronectin matrix. In general, Clara cells removed from the culture dishes were above 90% in purity.

Aliquots of 3×10^6 cells, suspended in 2 mL Ham's F12 medium containing 10 μg/mL bovine pancreatic insulin, 10 μg/mL human transferrin, 25 ng/mL epidermal growth factor, 0.5 μL/mL 0.4% bovine hypothalamus extract, 60 μg/mL pencillin, 60 μg/mL streptomycin, 50 μg/mL gentamicin, 100 μg/mL kanamycin, and 0.25 μg/mL FungizoneR (Amphotericin-B) (*16*), were placed in 35-mm plasatic culture dishes that had been treated for 1 to 2 hr at room temperature with 2 mL of FAV solution. The cells were then incubated in a CO_2 incubator at 37°C in a humid atmosphere containing 95% O_2 and 5% CO_2. After 18 hr of incubation, the unattached cells were removed by three 2-mL washes of Ham's F12 medium with gentle pipetting, swirling, and aspiration. The attached cells (93 ± 3% Clara cells, n = 4 separate preparations) were recovered from the dishes by incubating with 0.02% bovine pancreatic trypsin type III, 0.2 mM ethylenediamine tetraacetic acid (EDTA) in HBSS for 15 to 20 min to 4°C. The cells were further loosened by repetitive pipetting and transferred to a centrifuge tube. To this suspension was added 0.1 mL/mL of a solution containing 1 mg/mL soybean trypsin inhibitor type I-S and 50 μg/mL deoxyribonuclease I. The cells were centrifuged at 580*g* for 10 min. The supernatant was removed and stored at −70°C until sufficient cells had been collected for mixing with adjuvant and injection into goats.

Type II cells were isolated from the suspension of type II cells obtained from the last and least dense 18 mL of the Percoll density gradient. These cells were loaded into the elutriation chamber at a flow rate of 8 mL/min, and the first 200 mL was discarded. The flow rate was increased to 14 mL/min, and the next 200 mL, which was enriched in type II cells, was collected. The cells were washed once with HBSS. Final purification of each type was achieved by differential adherence, as described above for the Clara cells. Approximately 50 $\times 10^6$ type II cells were routinely obtained from two rabbits.

Characterization of Cells

Cells were counted by using a hemocytometer. Viability of cells was determined by using the trypan blue exclusion method (*18*). Slides of cells for histochemical examination were made in a cytocentrifuge (Shandon Elliot Cytospin, Shandon Southern Instruments Inc., Sewickley, PA). Granulocytes and macrophages were identified by using Diff-Quik aqueous Wright-Giemsa stain. Clara cells were routinely identified by using the NBT stain of Devereux and Fouts (*19*). Slides were fixed in 10% formalin in phosphate buffer (pH 7.0) for 45 sec, rinsed in HBSS, and then incubated with 0.1% NBT and 0.1% NADPH in HBSS for 10 min at 37°C. The cells were counterstained with 1% aqeous methyl green for 4 min. The proportion of purple-staining Clara cells in each preparation was determined by counting at least 500 cells. Type II cells, basal cells, ciliated cells, and goblet cells were identified by using the modified Papanicolaou (PAP) stain with 50% aqueous Harris hematoxylin and without acid alcohol (*20*).

Preparation of Anti-Clara Cell Antiserum

A two-year-old female alpine mixed-breed goat, from which preimmune serum had been collected, was innoculated with Clara cells (8.97×10^6 Clara cells, 90.8% pure, containing 1.08 mg protein) emulsified in Freund's complete adjuvant. Goat serum obtained before and after immunization was absorbed with rabbit liver acetone powder (rehydrated by mixing 2.5 g powder with 2 mL PBS) (absorption performed by using 2.5 g powder per 10 mL of goat serum). Two successive absorptions were necessary and sufficient to remove serum reactivity with all rabbit liver, kidney, and thymus antigens as determined by immunoblotting of organ proteins separated on SDS polyacrylamide gels.

Radiolabeling of Cells and Electrophoresis

Aliquots of cells (3×10^6) suspended in 2.0 mL F12 complete medium were placed in 35-mm plastic culture dishes. 200 μCi of [^{35}S]methionine was added to each culture dish. The cells were then incubated at 37°C in a humid atmosphere containing 5% CO_2. After 4 hr of incubation, no significant attachment had occurred. The cells were sedimented by centrifugation at 2000g for 5 min and washed once with 0.9% NaCl buffered with 0.01 M sodium phosphate (PBS). The cells were again sedimented and the supernatant removed. The labeled proteins associated with the cells and released into the incubation media were precipitated with trichloracetic acid and boiled for 3 min in electrophoresis sample buffer containing SDS and β-mercaptoethanol. Total incorporated radioactivity was determined, and gradient gel electrophoresis over 7 to 20% acrylamide, staining, and fluorography were all performed as previously described (*5*).

Protein Immunoblotting

Proteins from the SDS-polyacrylamide gels were transferred to nitrocellulose membranes according to the method of Towbin et al. (*21*). Nonspecific sites were blocked with 3% (w/v) bovine albumin fraction V (BSA) and 10% fetal bovine serum (FBS) (*6*).

Immunocytochemistry

For immunocytochemical localization of antigens recognized by the Clara cell antiserum, five adult New Zealand white rabbits from a specific pathogen-free colony (Dutchland Laboratories, Inc., Denver, Pennsylvania) were killed by CO_2 inhalation and exsanguination. The trachea and lungs were removed by thoracotomy, fixed by infusion via tracheal cannula with 1% formaldehyde in 0.1 M phosphate buffer (395 mOsm at pH 7.4) at a fluid pressure of 30 cm. Blocks were cut from the fixed lungs and areas containing terminal bronchiole-alveolar duct junctions were selected for processing. Tissue blocks were washed in buffer, then dehydrated in *N,N*-dimethylformamide (50–90%), and infiltrated with Lowicryl K4M (Polysciences, Warrington, Pennsylvania) (*22*). Tissue was then transferred to polyethylene capsules filled with fresh Lowicryl. Sealed capsules were polymerized with UV radiation for 45 min at 4°C in a foil-lined box with a lamp-to-tissue distance of 10 cm.

The blocks were sectioned with a Sorvall MT5000 ultramicrotome using a diamond knife. The sections were mounted on nickel grids. Immunocytochemical labeling was performed by using a modification of the procedure described by Roth et al. (*23*) for protein A gold except that gold-labeled immunoglobulin was substituted. Grids were floated in droplets of 1% ovalbumin in 0.1 M phosphate buffered saline (PBS) (pH 7.4) and incubated in drops of primary antisera for 18 to 24 hr at 4°C in a moist chamber. Dilutions of primary antiserum used in this study ranged from 1:1000 to 1:50,000. All primary antisera were diluted in 1% ovalbumin in PBS. Following incubation the grids were washed and incubated in drops of rabbit anti-goat immunogold (Janssen Life Sciences Products, Beerse, Belgium). Anti-goat immunoglobulin labeled with two sizes (10 nm and 40 nm) of colloidal gold were used. Colloidal gold-labeled antiserum was diluted in PBS at either 1:10 or 1:20. Controls included substitution of primary antisera with 0.1 M PBS, normal goat serum diluted to the same

concentration as primary antisera, or a series of dilutions of primary antisera in a single staining series. All specimens were examined by using a Zeiss Em 10 or Hitachi H-600 transmission electron microscopes at 60 or 80 kv.

Results and Discussion

Isolation of Pulmonary Cells

Rabbit lungs were digested with protease as described in "Materials and Methods." The resulting digest was fractionated by using a combination of density gradient centrifugation and elutriation to yield cell fractions highly enriched in either Clara cells or alveolar type II cells. Alveolar macrophages were isolated from pulmonary lavage effluents by using differential centrifugation. The cellular composition of the unfractionated lung digest as well as of the three cell fractions are listed in Table 1.

The major identifiable cell types in the lung digest were type II cells (33%), Clara cells (4%), and granulocytes (18%). The granulocytes were presumably interstitial, as the circulating cells were removed by perfusion prior to protease digestion. A large proportion of cells (38%) could not be identified at the light microscopic level.

The Clara cell preparations contained 84% Clara cells, 9% granulocytes, and 4% alveolar type II cells. Other cell types were also present but amounted to less than 3% of the total. Type II cell preparations contained 80% type II cells, 2% Clara cells, and 13% unidentified cells. Alveolar macrophages obtained from pulmonary lavage consisted of 100% macrophages and a fraction of a percentage of unidentified cells.

Synthesis and Release of Proteins by Specific Pulmonary Cell Fractions

The purpose of these studies was to compare proteins synthesized by isolated, nonciliated bronchiolar epithelial cells of the distal airways with two other cell types of the distal lung. The various cell fractions were incubated with [^{35}S]methionine for 4 hr.

[^{35}S]methionine was incorporated into numerous proteins with molecular weights ranging from less than 10 kDa to over 200 kDa (Fig. 1). All cells synthesized and released a major protein with molecular weight of approximately 48 kDa. Besides this 48 kDa protein, the major proteins released by Clara cells had molecular weights of 6, 38, 54, 60, 98, 180, and 190 kDa; the major proteins released by type II cells had molecular weights of 27, 41, 51, 57, 71, 78, 97, 155, and 170 kDa; and the major proteins released by alveolar macrophages had molecular weights of 63 and 91 kDa. The pattern of major secretory proteins was quite different with each cell type.

We have shown previously (5) that a 6-kDa protein is the principle secretory protein of the Clara cells. Because of previous reports of common proteins produced by both Clara cells and type II cells, we were particularly interested in determining whether this protein was also synthesized and secreted by type II cells. Over 40% of the total protein-associated [^{35}S]methionine released by Clara cells was found in the 6-kDa protein (Fig. 2). A very faint band was detectable from the type II cell preparations, but this could have arisen from the small degree of contamination with Clara cells.

Identification of Secreted Clara Cell Antigens

The antiserum prepared against isolated Clara cells reacted with only one major intracellular Clara cell antigen, and this protein had an apparent molecular weights of about 6 kDa. Weak reaction was observed with a protein with molecular weights of about 180 kDa, but this was seen only upon prolonged development of the blots (Fig. 2) and virtually disappeared when the antiserum was diluted in the range of 1:1000 and beyond, dilutions found to be effective in the immunocytochemical studies. Similar reaction was seen with proteins released by Clara cells. The 6-kDa protein ap-

Table 1. Composition of epithelial cell preparations from rabbit lungs.

Cell type	Lung cells	Clara cells	Type II cells	Alveolar macrophages
Clara cells[a]	4 ± 2[b]	84 ± 5	2.0 ± 1	0
Type II cells[c]	33 ± 5	4 ± 2	79 ± 2	0
Basal cells[c]	2 ± 1	0.6 ± 0.7	1 ± 0.4	0
Ciliated cells[c]	1 ± 0.6	0.4 ± 0.3	0.2 ± 0.2	0
Goblet cells[c]	0.8 ± 0.0	1 ± 1	0.2 ± 0.1	0
Macrophages[d]	3 ± 1	0.6 ± 0.7	1 ± 0.8	99.7 ± 0.1
Granulocytes[d]	18 ± 1	9 ± 3	3 ± 1	0.1 ± 0.0
Miscellaneous[e]	38 ± 8	− 0.3 ± 0.8	13 ± 2	0.2 ± 0.0
Viability	87 ± 2	92 ± 3	78 ± 8	98

[a]Clara cells identified by using NBT stain.
[b]Proportion of each cell preparation that can be identified as a known cell type or as viable by exclusion of trypan blue (mean ± SD, n = 4).
[c]Epithelial cells identified by using the modified Papanicolaou stain.
[d]Blood-derived cell types identified by using Diff-Quik aqueous Wright-Giemsa stain.
[e]Cells not identified.

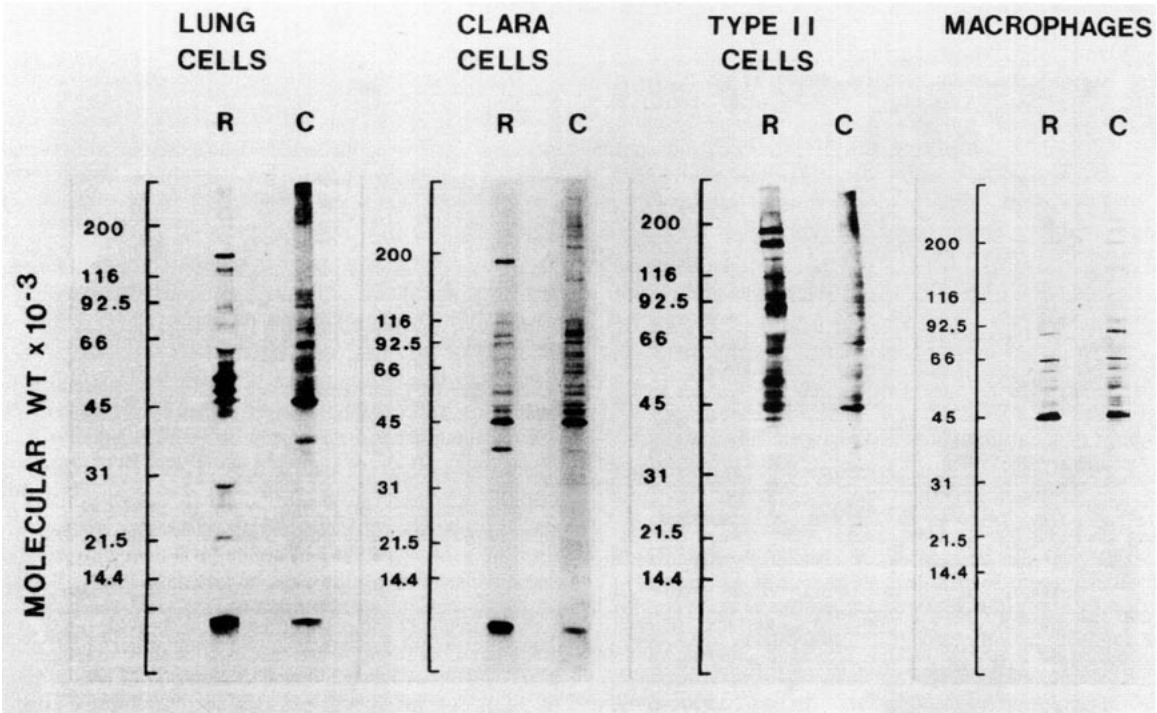

FIGURE 1. Fluorographs of ^{35}S-labeled proteins synthesized and released by isolated pulmonary cells. Mixed lung cells, Clara cells, type II cells, and alveolar macrophages were incubated with [^{35}S]methionine for 4 hr. Fluorographs were prepared of TCA-precipitable released (R) and cell-associated (C) proteins following SDS-PAGE under reducing conditions as described in "Materials and Methods."

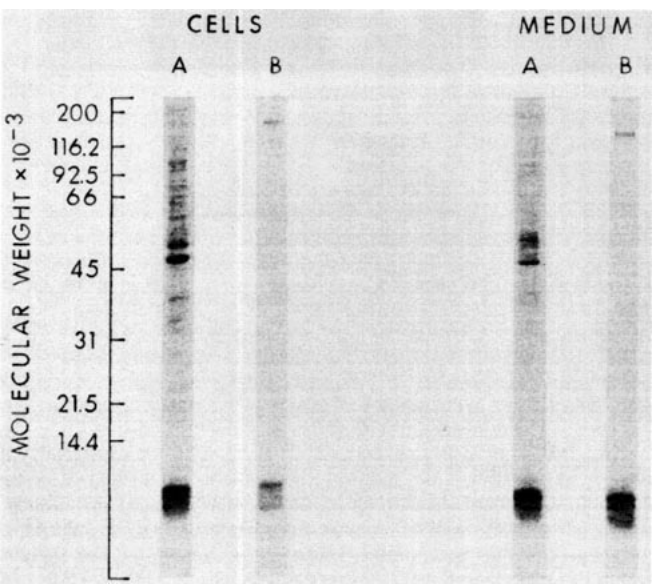

FIGURE 2. Fluorographs (*A*) and immunoblots (*B*) of ^{35}S-labeled proteins synthesized and released by isolated Clara cells incubated in the presence of [^{35}S]methionine. Proteins were electrophoresed on linear gradient polyacrylamide gels in the presence of SDS under reducing conditions. Immunoblots were prepared with Clara cell antiserum developed in a goat and twice absorbed with rabbit liver acetone powder.

peared to be a major component of the Clara cells and was easily detectable by autoradiography, Coomassie blue straining, and immunoblotting. Only Clara cell preparations and mixed lung cells gave significant reaction with the Clara cell antiserum when used in a western blot (data not shown).

Immunocytochemical Localization of Clara Cell Antigens

The antiserum developed against Clara cells appeared to be highly specific for Clara cells, according to immunohistochemical staining with colloidal gold (Fig. 3). Compared with substitution controls (Fig. 3*A*), gold labeling was most dense over Clara cells (Fig. 3*B* and 3*C*). At lower dilutions the label was found over both the cytoplasm and the granules of Clara cells (Fig. 3*B*). There was little gold labeling over the nucleus of the cells, but a heavy density at the cytoplasmic surface (Fig. 3*D* and 3*E*). At higher dilutions (such as 1:50,000), the antiserum almost exclusively labeled Clara cell cytoplasm and granules (Fig. 3*C*). Increasing the dilution of the antiserum decreased the gold labeling of the cytoplasm and the plasma membrane, but did not substantially reduce the gold labeling of the granules (compare Fig. 3*D* and 3*E*). Ten-fold dilution reduced the cytoplasmic gold labeling substantially, except over the osmiophilic granules (compare Fig. 3*D* and 3*E*).

Gold labeling of alveolar macrophages was not distinguishable from background, an observation consistent with our inability to detect the Clara cell secretory protein in macrophages incubated with [^{35}S]methionine (Fig. 3*F*). Gold labeling of ciliated cells was limited primarily to cilia and to microvilli at the luminal surface (Fig. 3*G*). This reaction product was generally in clumps and appeared to be associated with the microvilli. Small

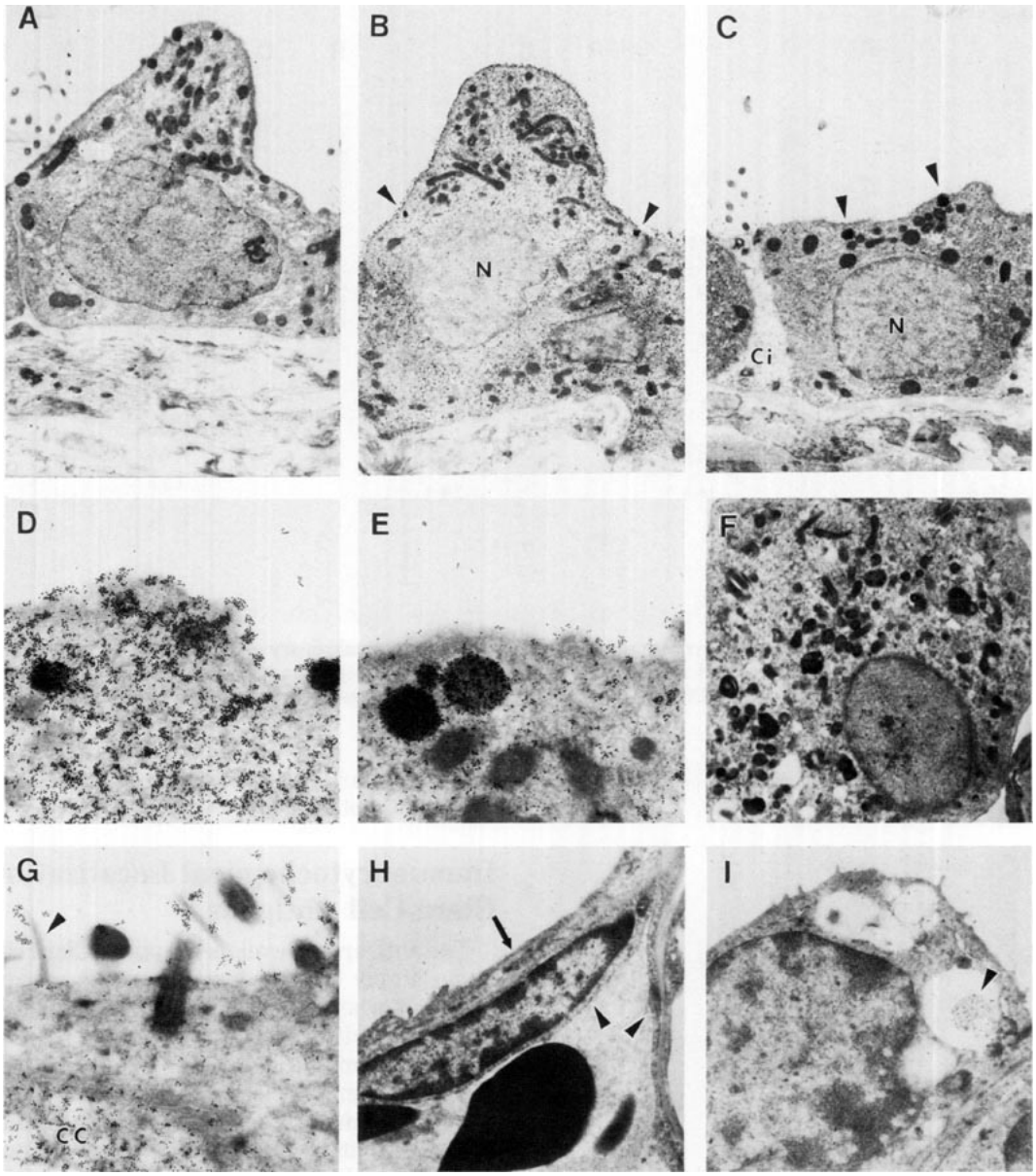

FIGURE 3. Electron micrographs of immunocytochemical labeling of rabbit lung sections with Clara cell antiserum. The antiserum was developed in goats against highly purified Clara cells isolated from the lungs of rabbits. The antiserum was twice absorbed with rabbit liver acetone powders. (*A*) Labeling of bronchiolar Clara cells when preimmune serum (diluted 1:5000) was substituted for primary antiserum. ×5000. (*B*) Bronchiolar epithelium labeled with antiserum diluted 1:10,000. The heaviest labeling was found over secretory granules (arrowheads). ×5000. (*C*) Bronchiolar epithelium labeled with antiserum diluted 1:50,000. Labeling over granules (arrowhead) was heavy, but the amount of cytoplasmic labeling in Clara cells was reduced. The Clara cell's nucleus (N) and ciliated cell (Ci) were unlabeled. ×5000. (*D*) Apex of Clara cell from section labeled with antiserum diluted 1:50,000. ×22,500. (*E*) Apex of Clara cell from section labeled with antiserum diluted 1:50,000. ×25,000. (*F*) Alveolar macrophage reacted with antiserum diluted 1:10,000. ×6,250. (*G*) Apex of ciliated cell labeled with antiserum diluted 1:10,000. Microvilli (arrowhead) were labeled at the base and the surface. Compared to Clara cells (CC), cytoplasm of ciliated cells had minimal labeling. ×24,000. (*H*) Interalveolar septum from section reacted with antiserum diluted 1:50,000. Capillary endothelium (arrowheads) and type I cell cytoplasm (arrow) were not heavily labeled. ×7,500. (*I*) Type II cell in section labeled with antiserum diluted 1:50,000. The primary labeling was observed in the material within the lamellar bodies (arrowhead). ×10,000.

focal sites of reactivity were seen at the base of microvilli and distributed througout the cytoplasm, however, the density was considerably less when compared to neighboring Clara cells (Fig. 3*G*). Gold labeling of type I cells and endothelial cells was only slightly elevated above background (Fig. 3*H*). The distribution was random in these cells and did not appear to be associated with any specific cellular compartment. Gold labeling of

type II cells was restricted to the material within lamellar bodies (Fig. 3*I*), and even here labeling was only marginally above background. There was little gold-labeling over any organelles identifiable within the cytoplasm or over the components of the plasma membrane.

In the tracheal epithelium, gold labeling was primarily in nonciliated cells. In nonciliated cells, immunolocalization occurred in apical secretory granules (Fig. 4). Minimal gold labeling occurred over microvilli of nonciliated cells, mucous cells, and ciliated cells. Unlike the cilia of ciliated cells in the bronchiolar regions of the lungs, cilia of tracheal ciliated cells did not label. Granules of mucous cells were poorly labeled.

A low molecular weight protease inhibitor has been localized immunohistochemically to the osmiophilic granules of Clara cells (*13*). We have measured the protease inhibitory activity of Clara cell secretory protein (CCSP) and found antithiol protease activity but against trypsin and elastase the activity was only very weak (*6*). Whether or not CCSP is the same as that protein suggested to be present in the osmiophilic granules of Clara cells has yet to be determined, in view of this weak antitrypsin activity. Uteroglobin has also been localized immunohistochemically to the osmiophilic granules of rabbit Clara cells (*12*). Several similarities exist between uteroglobin and the major Clara cell secretory protein including molecular weight and N-terminal amino acid sequences (*6*). Lopez de Haro et al. (*24*) report that CCSP and uteroglobin must be identical based upon similarities in antibody and progesterone binding. A low molecular weight protein has been reported (*25–28*) to be associated with extracellular surfactant in pulmonary lavage effluents, but CCSP appears to be immunochemically different. We have isolated a 6-kDa surfactant protein using the method of Claypool et al. (*25*), and although the molecular weight of this protein is close to that of the Clara cell protein, antisera reactive with the Clara cell protein did not react with the low molecular weight surfactant protein (*6*). In addition, our anti-Clara cell serum did not react with any proteins in the 26 to 40 kDa surfactant protein range in type II cells. It therefore seems unlikely that the same surfactant proteins known to be present in type II cells are also present in Clara cells. However, it is possible that development of antiserum against Clara cells might not have led to the appearance of appropriate antibodies, particularly if the putative immunogens in question were not present in sufficient quantity. Analysis of the autoradiographs of proteins synthesized and secreted by Clara cells does indicate that proteins of unknown function with molecular weights between 26 and 40 kDa were minimally detectable in Clara cells, and a 38-kDa protein was released by them.

In summary, we have compared proteins synthesized and secreted by bronchiolar Clara cells with those synthesized and secreted by alveolar type II cells and alveolar macrophages. We found that, in general, the major proteins synthesized and secreted by these three pulmonary cell types are quite distinct from each other. Using immunocytochemistry, we have shown that the

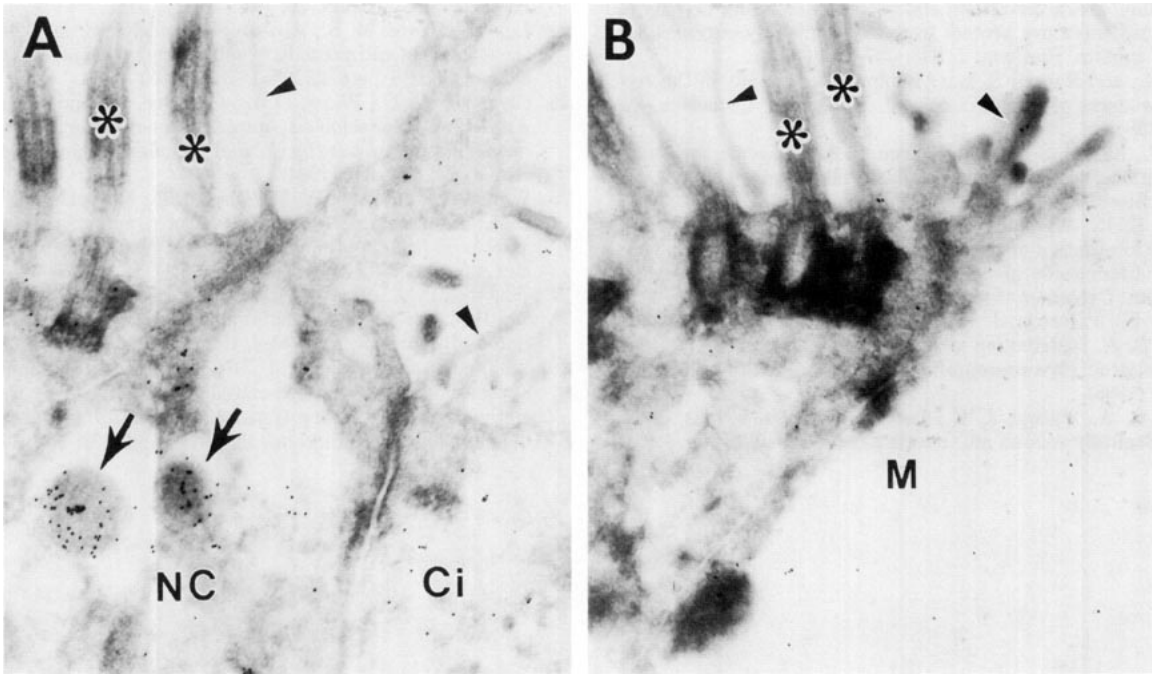

FIGURE 4. Tracheal epithelium. Antigen was localized within apical secretory granules (arrows, *A*) of the nonciliated cells (NC). There was minimal labeling of apical microvilli (arrowheads, *A* and *B*) on either mucous cells (M), ciliated cells (Ci), or nonciliated cells (NC). Cilia (*) were unlabeled. Granules of mucous cells (M) were poorly labeled. (*A* and *B*) Antiserum diluted 1:20,000. Protein G gold (10 m). ×30,000.

major secretory protein of bronchiolar Clara cells is located primarily in nonciliated cells but could be present to a very small degree in other secretory cells of the pulmonary epithelium, cells such as type II cells and mucous cells. However, we would point out that localization of antigens in Clara cells must be done with care. Our titer of antibodies was so high that we were easily able to dilute out the effect of nonspecific binding and reduce the background gold labeling to very low values. This antiserum may prove useful in studies correlating agent-induced toxicity with target cells in the pulmonary epithelium.

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