

Endogenous Mammalian Lectin Localized Extracellularly in Lung Elastic Fibers

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ABSTRACT An affinity-purified antibody preparation raised against a β -galactoside-binding lectin from bovine lung was used to localize a similar lectin in rat lung by immunofluorescence and by electron microscopy after on-grid staining visualized with colloidal gold conjugated second antibody. The endogenous mammalian lectin was found in smooth muscle cells and squamous alveolar epithelial (type I) cells and was concentrated extracellularly in elastic fibers of pulmonary parenchyma and blood vessels. The extracellular localization of this lectin suggests that it, like others, functions by interaction with extracellular glycoconjugates.

Lectins are a family of carbohydrate-binding proteins originally identified in plants. They have also been found in a variety of vertebrate tissues, which in turn has stimulated studies of their endogenous functions (1, 2). Some of the vertebrate lectins are integral membrane proteins that are believed to play a role in the hepatocyte uptake of partially degraded circulating glycoproteins (3). Others (1, 2) are soluble proteins that may be extracted from tissues with simple buffers.

The most extensively investigated soluble vertebrate lectins are β -galactoside-binding proteins that are widely distributed in both chicken (4–6) and mammalian (7–9) tissues. One approach to determining their function has been to localize them immunohistochemically. Most of these studies have been done with chicken tissues, which contain two β -galactoside-binding lectins called chicken-lactose-lectin-I (CLL-I)¹ and chicken-lactose-lectin-II (CLL-II) (6).

CLL-I has been studied most extensively in muscle where it was discovered. It is most prominent between days 12 and 16 of embryonic development (10, 11), at which time it is largely localized intracellularly. Later, it is externalized into the extracellular matrix around developing myotubes (12). In pancreas, another tissue in which CLL-I has been localized, it is also extracellular, around exocrine acini (13). CLL-I's localization suggests a role in binding glycoconjugates in the extracellular matrix.

¹ *Abbreviations used in this paper:* CLL-I, chicken-lactose-lectin-I; CLL-II, chicken-lactose-lectin-II; PBS, 75 mM Na₂HPO₄/KH₂PO₄, pH 7.2; PBS + GS, PBS containing 2.5% goat serum; TBS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.6; TBS-WASH, TBS containing 2.5% goat serum, 0.2% bovine serum albumin, 0.05% Tween 20.

CLL-II, also present in many tissues (11), has been examined most extensively in adult chicken intestine in which it is localized in the secretory granules of the goblet cells (14). When it is externalized, it is into the mucin coating the intestine, rather than into the extracellular matrix (14).

A β -galactoside-binding lectin has been identified in several mammalian tissues including bovine (7, 8) and rat (9, 15) lung. Like CLL-I, it is a dimer under nondissociating conditions. To immunohistochemically localize the mammalian lectin, we first purified it from bovine lung and raised an antiserum against it. Rat tissues were chosen for the immunohistochemical studies since they are more readily available than bovine tissues, and antisera show extensive cross-reactions among such mammalian lectins (16). Here, we show that this endogenous lectin is found in both alveolar and smooth muscle cells, and is concentrated extracellularly in elastic fibers. This extracellular localization suggests that it, like CLL-I, may function in the organization of extracellular glycoconjugates.

MATERIALS AND METHODS

Lectin Purification

Lectin was extracted from adult bovine lung by homogenization in 5 vol of 75 mM NaCl, 75 mM Na₂HPO₄/KH₂PO₄, pH 7.2 (PBS) containing 4 mM β -mercaptoethanol, 2 mM EDTA, and 300 mM lactose, followed by centrifugation at 100,000 g for 1 h. The lectin was initially purified by affinity chromatography on asialofetuin-derivatized Sepharose and was specifically eluted from the affinity column with 300 mM lactose, as previously described (6). Further purification was achieved by preparative isoelectric focusing performed for 96 h at 4°C in a LKB isoelectric focusing apparatus (LKB Instruments, Rockville, MD), as previously described (11). Protein was determined by the method of Bradford (17).

For comparative purposes, small amounts of lectin were prepared from lungs

of 90-d-old Sprague-Dawley male rats (Charles River Laboratories, Wilmington, PA) by affinity chromatography on asialofetuin-derivatized Sepharose.

Electrophoresis and Peptide Mapping

Purity of the lectin preparations was evaluated by PAGE in SDS under reducing conditions (18) using a 12.5% polyacrylamide sample gel. To dissociate the lectin into subunits, we boiled it for 10 min immediately before electrophoresis. Staining of the gels was generally done with silver nitrate (19) to increase the probability of detecting trace contaminants in the material used for immunization or for preparation of columns for affinity purification of antibody. To quantitate the intensity of bands by densitometry, we stained gels with Coomassie Blue. Elution of protein from gel slices was performed with an ECV-80 electroelution apparatus (CBS Scientific Co., Del Mar, CA).

Chymotryptic peptide maps were prepared from protein bands resolved on polyacrylamide gels based on the method of Elder et al. (20), as described previously (6). The bands, identified by staining with Coomassie Blue, were cut from the gel, iodinated with ^{125}I , and digested with 50 μg of chymotrypsin (Sigma Chemical Co., St. Louis, MO) in 1 ml of 0.05 M NH_4HCO_3 , pH 8.0, for 24 h at 37°C. The peptides were eluted from the gel slice by diffusion, separated by electrophoresis in one dimension and chromatography in another dimension, and visualized by radioautography.

Antibody Preparation and Characterization

Rabbit antiserum against the bovine lectin purified through the isoelectric focusing step was raised by procedures like those described previously (6), except that seven boosts were given. Rabbit antiserum raised against rat laminin was the generous gift of E. Ruoslahti (La Jolla Cancer Research Foundation).

AFFINITY PURIFICATION OF ANTIBODY: Bovine lectin purified through the isoelectric focusing stage was immobilized by conjugation of 1.2 mg of the purified protein to 4 ml of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's instructions. Rabbit antiserum was adsorbed by repeated passage of 5 ml of serum over the column that was then washed with PBS and eluted with 0.2 M HCl adjusted to pH 2.2 with 2 M glycine. Fractions of 0.6 ml were collected directly into tubes containing 0.15 ml of 1 M K_2HPO_4 and dialysed against PBS overnight at 4°C.

IMMUNOBLOTTING: The molecules in rat lung and in purified rat lectin preparations that bound the affinity-purified antibody were determined after solubilization by boiling for 10 min in 2% SDS, 0.75 M β -mercaptoethanol, 10% glycerol in 0.02 M Tris hydrochloride, pH 6.8, and electrophoresis in SDS in a 12.5% polyacrylamide slab gel. Proteins were then transferred to nitrocellulose sheets (21) in an electrophoretic blotting chamber (CBS Scientific, Del Mar, CA) at 2 V/cm, 50 mA for 30 min at room temperature followed by 24 V/cm, 400 mA for 2.5 h. The sheets were incubated in 150 mM NaCl, 50 mM Tris-HCl, pH 7.6 (TBS), containing 2.5% normal goat serum (Colorado Serum Co., Denver, CO), 0.2% bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO) and 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) (TBS-WASH) overnight at 4°C to block nonspecific binding of antibody. All subsequent incubations and washes were performed at room temperature. After a 1-h incubation in 20 $\mu\text{g}/\text{ml}$ affinity-purified antibody or 100 $\mu\text{g}/\text{ml}$ preimmune rabbit IgG diluted in TBS-WASH, the sheets were washed for 30 min with two changes in TBS-WASH. The bound antibody was visualized by incubation in a 1:40 dilution (in TBS-WASH) of goat anti-rabbit IgG (Calbiochem-Behring, La Jolla, CA), washed as above, and incubated in a 1:40 dilution (in TBS-WASH) of rabbit peroxidase-anti-peroxidase (Cappel Laboratories, Cochranville, PA) for 1 h. The sheets were finally washed in TBS and developed in a fresh solution of TBS containing 0.2% H_2O_2 and 0.16 mg/ml 4-chloro-1-naphthol (Polysciences Inc., Warrington, PA).

Light Microscope Immunohistochemistry

TISSUE PREPARATION: Sprague-Dawley male rats were sacrificed by decapitation and the lungs were inflated *in situ* by injection of 3% paraformaldehyde, 0.1% glutaraldehyde in PBS, pH 7.2, through the trachea. After 15 min, the lungs were excised and 2-mm³ pieces were immersed in the same fixative for an additional 45 min. The pieces of lung were then transferred to 0.3% paraformaldehyde, 30% sucrose in PBS and stored at 4°C overnight. The tissue pieces were quick-frozen and mounted on a microtome chuck using O.C.T. compound. 1–2 μm frozen sections were cut at –35°C in a H/I Bright Cryostat (Hacker Instruments, Inc., Fairfield, NJ) fitted for sectioning with glass knives with an LKB "Ralph" knife adapter (LKB Instruments, Bromma, Sweden). Sections were picked up on slides that had been cleaned then dipped while warm in a mixture of 0.5% gelatin and 0.05% chromic potassium sulfate heated to 50°C. Sections on slides were stored at –20°C before staining.

FLUORESCENCE HISTOCHEMISTRY: Slides were washed before stain-

ing for 1 h in two changes of PBS containing 2.5% normal goat serum (PBS + GS). PBS + GS was used throughout the staining procedure for washes and as a diluent for antisera.

In experiments with antibodies against the β -galactoside-binding lectin, slides were then incubated with 10 $\mu\text{g}/\text{ml}$ affinity-purified antibody or an equivalent amount of preimmune rabbit IgG for 20 min. In experiments using antiserum against rat laminin, slides were incubated with immune or normal rabbit serum diluted 1:100 for 20 min. After antibody incubation, the slides were washed for 30 min in two changes of PBS + GS and incubated in rhodamine-conjugated goat anti-rabbit IgG (Cappel Laboratories, Inc., Cochranville, PA) diluted 1:50. After two 15-min washes in PBS without GS, slides were drained and mounted with coverslips using 90% glycerol.

Slides were examined with a Leitz Dialux epifluorescence microscope using a Leitz 40 \times (NA 1.3) oil immersion lens. Photomicrographs were taken with a Wild MPS 45 camera and Kodak Tri-X film. Exposure of experimental and control slides and printing were done under identical conditions.

Electron Microscope Immunohistochemistry

TISSUE PREPARATION: Tissue was obtained as described for light microscopy. To determine the optimal fixation procedure, we fixed small pieces of tissue for 1 h at room temperature in freshly prepared 3% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, to which either 0.1%, 0.5%, or 1% glutaraldehyde was added. Tissue blocks were then rinsed in 0.1 M sodium cacodylate buffer, pH 7.4, and a portion of each sample was postfixated in 1% OsO_4 in cacodylate buffer for 1 h at room temperature. All samples were dehydrated in ethanol and embedded in epon 812 by standard procedures. Thin sections were cut and picked up on uncoated nickel grids for immunostaining.

PREPARATION OF COLLOIDAL GOLD-IGG COMPLEX: Colloidal gold with a mean diameter of 4 nm, prepared by the reduction of chloroauric acid with sodium borohydride (22), was generously provided by C.-M. Chang (Scripps Clinic and Research Foundation, La Jolla, CA). Goat anti-rabbit IgG was coupled to colloidal gold using a modification of published procedures (23, 24). Before coupling, the goat anti-rabbit IgG (heavy and light chains specific, Cappel Laboratories, Cochranville, PA), 10 mg/ml in 0.02 M PBS, pH 7.3, was dialyzed overnight against 200 vol of glass distilled H_2O . The pH of the colloidal gold solution was adjusted to approximately 8.5 with 2% K_2CO_3 . For coupling, 1/10 vol of IgG was added to the colloidal gold slowly with stirring. After 5 min, 1/10 vol of 10% NaCl was added dropwise followed by 1/10 vol 0.1% polyethylene glycol-20,000 (Sigma Chemical Co., St. Louis, MO) in 0.9% NaCl. The solution was then diluted 10–20-fold with TBS-containing 1% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MO) and 0.01% polyethylene glycol-20,000. The solution was centrifuged at 60,000 g in a 50 Ti fixed angle rotor (Beckman Instruments, Fullerton, CA) for 30 min at 4°C.

Centrifugation under these conditions generally resulted in a homogenous, loosely packed, red-orange pellet of fully conjugated colloidal gold. Occasionally, a portion of the gold was not stabilized and formed a tightly packed, dark purple dot at the bottom of the pellet. To separate the gold-IgG complex from the unstabilized material, we kept the tube vertical for ~10 min at room temperature so that the fully stabilized portion could slide away from the purple dot. Most of the supernatant was then carefully removed and the colloidal gold-IgG complex was collected with a Pasteur pipet in as small a volume as possible, then diluted approximately 20–30-fold in TBS containing 1% bovine serum albumin and 0.01% polyethylene glycol-20,000 before use.

STAINING AND MICROSCOPY: All buffers used were dispensed from syringes fitted with a 0.22 μm Millex filter (Millipore Filter Corp., Bedford, MA) immediately before use. Grids containing thin sections of lung tissue were floated on drops of affinity-purified antibody using dilutions ranging from 1–10 $\mu\text{g}/\text{ml}$ in TBS-0.3% bovine serum albumin for 2 h at room temperature. Control grids were incubated with twice the concentration of an affinity purified antibody raised against a slime mold lectin, which does not react with rat tissues, or with twice the concentration of preimmune IgG. Samples were washed three times for 20 min each in TBS-bovine serum albumin, then transferred to drops of colloidal gold conjugated goat anti-rabbit IgG for 2 hours at room temperature. Grids were then rinsed twice for 10 min in TBS-bovine serum albumin, twice for 10 min in TBS, and twice for 5 min in glass distilled H_2O . The sections were then counterstained with saturated aqueous uranyl acetate and lead citrate before examination in a Zeiss EM 10 electron microscope.

RESULTS

Lectin Purification

Lectin was purified from extracts of bovine lung by affinity chromatography on an asialofetuin-Sepharose column fol-

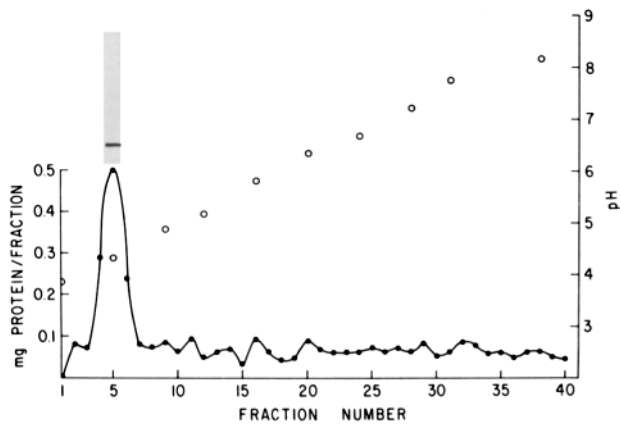


FIGURE 1 Final purification of bovine β -galactoside-binding lectin by isoelectric focusing. Bovine β -galactoside-binding lectin, purified by affinity chromatography on asialofetuin-Sepharose, was purified further in an isoelectric focusing column containing ampholytes over a pH range of 4-9. Protein content (●) and pH (○) of all fractions were determined. Fractions 4-6 were pooled and an aliquot containing $\sim 1 \mu\text{g}$ protein was electrophoresed and visualized with a silver stain (inset), as described in Materials and Methods.

lowed by preparative isoelectric focusing (Fig. 1). Upon PAGE, the lectin migrated as a single band with $M_r \sim 14,000$ (Fig. 1). No impurities were detected with a highly sensitive silver stain even using more protein or longer development times than in the example shown in Fig. 1. This highly purified antigen was used for immunization, and an aliquot was coupled to a Sepharose column for affinity purification of antibody.

For comparative purposes, lectin was also purified from rat lung by affinity chromatography. Recovery was similar to that using bovine lung but less tissue was used, so that we harvested only small amounts of lectin. The rat lectin showed one major protein band with $M_r \sim 14,500$ when examined by PAGE (Fig. 2), but there was also a clear minor band with $M_r \sim 29,000$ and a small amount of protein trailing behind the major band. On the basis of microdensitometry (Fig. 2), the ratio of protein in the band with $M_r \sim 14,500$ to that with $M_r \sim 29,000$ was determined to be 85:15. When the minor band was electrophoretically eluted from the gel, boiled in solubilizing buffer under reducing conditions and electrophoresed on another polyacrylamide gel, it again had an apparent $M_r \sim 29,000$. Therefore, it is not simply a dimer that had not been dissociated by the original solubilization, although the possibility that it is a stable dimer cannot be excluded.

Because the rat lectin preparation that had been purified by affinity chromatography contained a minor band with $M_r \sim 29,000$, we examined preparations of the bovine lectin that had been purified only by affinity chromatography but had not been subjected to isoelectric focusing. It, too, had a minor band with $M_r \sim 28,000$. This band is apparently separated from the major one by isoelectric focusing (Fig. 1) and was not included in the protein used for immunization or affinity purification of antibody. Since it binds to an asialofetuin-Sepharose column and is specifically eluted with lactose, it, too, behaves like a carbohydrate-binding protein. However, it remained possible that it was associating with the column only indirectly, by binding the lectin.

To evaluate the relationship between the protein bands with $M_r \sim 29,000$ and $M_r \sim 14,500$ in the rat lung lectin

preparation, we excised the bands and analyzed their peptide maps. Many similar peptides were generated from each band (Fig. 3, *a* and *b*), but each had two prominent peptides not found in the other (Fig. 3, *a* and *b*). To further evaluate the apparent similarities, we mixed aliquots of the two preparations containing equal amounts of radioactivity and examined the map of the mixture (Fig. 3*c*). Most peptides in the mixture migrated together (Fig. 3*c*), but the differences were, again, observed. The marked similarities in the maps strongly support the inference that the larger molecule is a closely related carbohydrate-binding protein, but the nature of the relationship is not presently clear.

Characterization of the Antilectin Antibodies

The affinity-purified antibody was reacted with nitrocellulose blots of SDS extracts of adult rat lung that had been electrophoresed in SDS under reducing conditions, as well as with the purified rat lectin that had the properties shown in Fig. 2. The antibody bound two bands with apparent $M_r \sim 14,500$ and $29,000$ in both the crude rat lung extract and the purified rat lectin (Fig. 4). In some lung preparations, a very faint narrow band was also seen near the top of the gel. No significant staining was observed with IgG prepared from preimmune serum (Fig. 4). Based on this analysis, we conclude that the antibodies raised against the highly purified bovine lectin are extremely specific for the rat lectin. Although the minor band from bovine lectin was not included in the protein used for immunization (Fig. 1), antibody raised against the major band reacts well with the minor band in affinity-purified lectin preparations from bovine (not shown) and rat lung (Fig. 4). This is consistent with the other evidence that it is a closely related carbohydrate-binding protein. In the immunohistochemical studies, we will refer to the major and minor band together as the β -galactoside-binding lectin.

Localization of β -Galactoside-binding Lectin and Laminin by Immunofluorescence

The antibody raised against the β -galactoside-binding lectin bound to localized areas in sections of rat lung (Fig. 5*a*). Specificity of antilectin staining is indicated by the absence of detectable staining with IgG prepared from preimmune serum (Fig. 5*c*) or immune IgG that had been adsorbed with highly purified lectin (Fig. 5*d*). Because the lectin localization suggested that it might be concentrated extracellularly, we com-

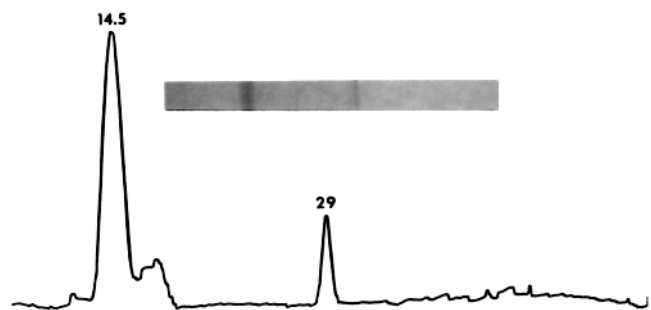


FIGURE 2 Electrophoresis of rat β -galactoside-binding lectin after purification by affinity chromatography. Lectin was purified by affinity chromatography on asialofetuin Sepharose and elution with lactose. A $10\text{-}\mu\text{g}$ aliquot was dissociated, electrophoresed on a slab gel in SDS and the gel was stained with Coomassie Blue (inset). The gel was scanned with a microdensitometer to determine the intensity of the bands with $M_r \sim 14,500$ (14.5) and $M_r \sim 29,000$ (29).

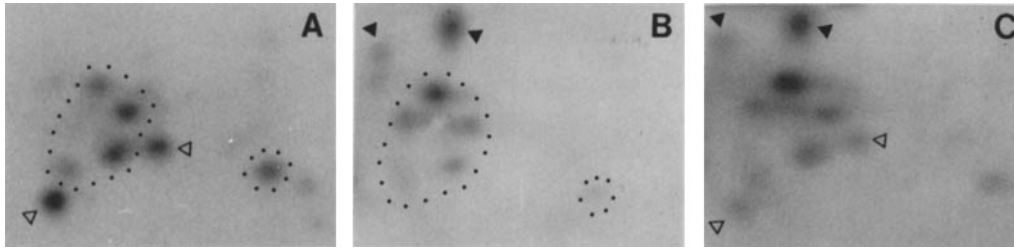


FIGURE 3 ^{125}I -labeled chymotryptic peptide maps of the two protein bands of the rat β -galactoside-binding lectin. The bands with M_r \sim 14,500 (A) and 29,000 (B) of rat β -galactoside-binding lectin (Fig. 2) were digested with chymotrypsin and mapped as described in Materials and Methods. A mixture of equal amounts of both bands is shown in C. Areas of similarity are circled and differences are noted with arrows. The origin was located at the lower left hand corner of the maps. Electrophoresis was in the vertical plane and chromatography in the horizontal plane.

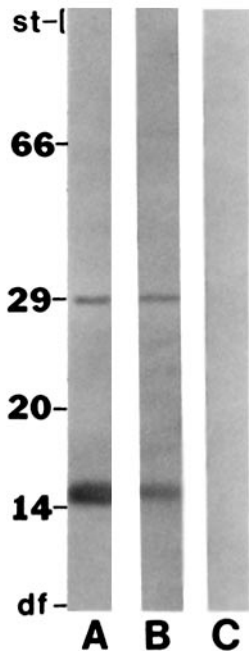


FIGURE 4 Identification of antigens in rat lung by immunoblotting. Affinity purified rat lung lectin (A) or crude extracts of rat lung (B and C) were dissociated and electrophoresed on polyacrylamide gels in SDS. Proteins were then transferred from the gels to nitrocellulose blots and reacted with affinity-purified immune IgG (A and B) or preimmune IgG (C). Bound antibody was visualized as described in Materials and Methods. The numbers on the left indicate migration of standards (Sigma Chemical Co.) with M_r \sim 14,000, 20,000, 29,000, and, 66,000. *st*, the stacking gel; *df*, the dye front.

pared the pattern of antilectin staining with that in parallel sections reacted with antiserum raised against rat laminin, a basement membrane protein (24). Staining with antilectin was not as sharp and linearly defined as staining with antilaminin (Fig. 5e), indicating that, if the lectin were indeed extracellular, it was not primarily associated with basement membrane. Because the lung has so many fine cellular processes and a complex microscopic anatomy, precise localization required observations with the electron microscope.

Electron Microscope Immunohistochemistry

In preliminary studies, we compared the effects of various fixation procedures on morphology and preservation of antigenicity. We found that fixation with 0.5% glutaraldehyde, 3% paraformaldehyde preserved the tissues well and, yet, allowed for ready detection of antibody staining even with postfixation in 1% OsO_4 . This procedure was routinely used in all further studies. Antibody staining in the same distribution was also observed with all the other fixation procedures that we tried, but increasing the glutaraldehyde concentration to 1% markedly diminished it. Osmication also diminished staining somewhat but did not influence its overall distribution.

The highest concentrations of antibody staining were routinely found in elastic fibers. Both elastic fibers of lung parenchyma (Fig. 6a), and the walls of blood vessels (Fig. 7a) were heavily stained when the sections were reacted with affinity-purified antibody that had been raised against the mammalian lectin. In contrast, control sections showed no significant staining (Figs. 6b and 7b).

Because of its prominent location at one extracellular site, we carefully evaluated the possibility that lectin was also present at other extracellular sites, such as bands of collagen or basement membranes. Whereas faint staining was occasionally observed at these sites, it was never reliably different from that observed in controls. Therefore, we conclude that, at least under the fixation conditions used, the only prominent extracellular site of accumulation of this lectin in lung tissue is in elastic fibers. These findings are consistent with the pattern of staining observed by immunofluorescence (Fig. 5a).

Lectin was also present intracellularly. Staining was found in the cytoplasm of squamous alveolar epithelial (type I) cells (Fig. 8a) and smooth muscle cells (Fig. 8b). Since smooth muscle cytoplasm is more abundant than alveolar epithelial cytoplasm, and also appears to be more heavily stained, it is the major intracellular site of lectin accumulation. Alveolar cells stained with a control antibody preparation were negative (not shown) as were smooth muscle cells (Fig. 8c).

Because lectin accumulated at an extracellular site in elastic fibers, we assume that it is secreted into that site by the cells that make it. Since smooth muscle cells are known to synthesize and secrete elastin (25), it seemed possible that they might also secrete the lectin into the elastic fibers. We, therefore, sought to determine if the intracellular lectin in smooth muscle cells was present in structures resembling secretory vesicles. Occasionally, some intracellular lectin in smooth muscle cells was localized in regions that were at least partially membrane-bound (Fig. 9), but the vast majority appeared to be free in the cytoplasm as in Fig. 8. It remains possible, however, that some of the lectin that appears to be free in the cytoplasm is actually contained within vesicular structures that are not preserved by the fixation procedures that we used.

DISCUSSION

A major finding of this study is that an endogenous mammalian β -galactoside-binding lectin becomes localized extracellularly in elastic fibers of lung. All the elastic fibers of this tissue contain the lectin, including those of the lung parenchyma and blood vessels. Lectin is also found intracellularly

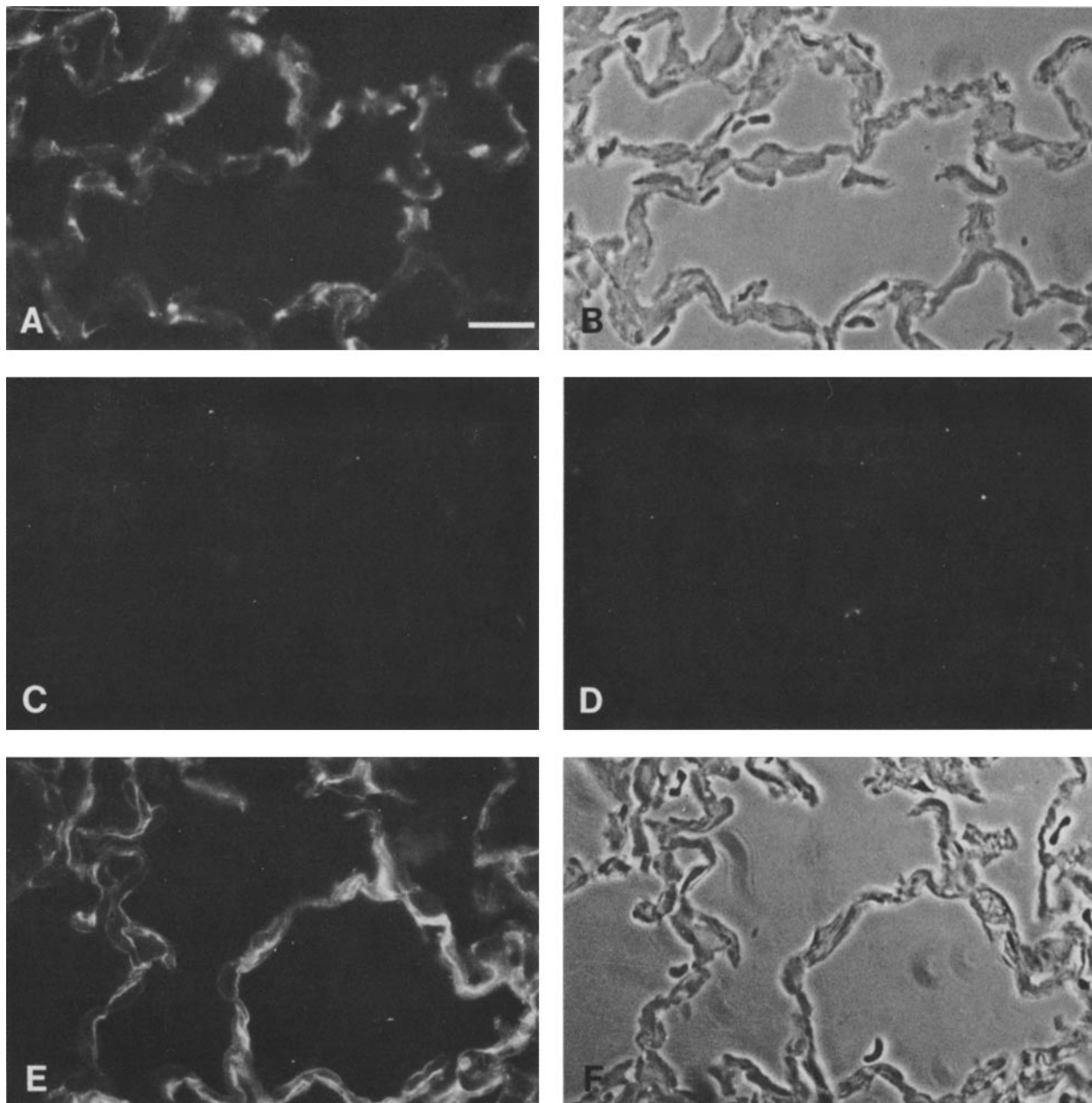


FIGURE 5 Immunohistochemical localization by fluorescence microscopy of the endogenous β -galactoside-binding lectin and of laminin in rat lung. Sections of rat lung were stained with affinity-purified antilectin IgG (A), preimmune IgG (C), antilectin IgG adsorbed twice with $10 \mu\text{g}$ heat denatured pure lectin (D), or rabbit antilaminin (E) and visualized with rhodamine conjugated goat anti-rabbit IgG. B and F are phase-contrast photographs of sections shown in A and E, respectively. Bar, $5 \mu\text{m}$. $\times 2,000$.

in smooth muscle cells and squamous alveolar epithelial (type I) cells, but it is not known whether one or both of these cell types give rise to the lectin in the elastic fibers. Smooth muscle cells are known to be a source of elastin (25), the major structural protein of elastic fibers, but the relationship of the lectin in those fibers to the elastin, if any, has not yet been determined. Powell and Whitney (15) found that the lectin in rat lung reached maximal levels of activity per milligram of lung protein between days 10 and 13 after birth, a time when elastin levels were also rising but had not yet peaked. This raises the possibility that the synthesis of elastin and the lectin are coordinately regulated, but the fact that lung contains several cell types, at least two of which synthesize the lectin,

makes interpretation of such overall tissue composition very difficult.

The finding that this endogenous vertebrate lectin is concentrated in a specific extracellular site is consistent with previous work showing externalization of other vertebrate lectins. For example, CLL-I—which is homologous to the lectin studied here since it is a dimeric protein of similar size and specificity—is externalized into the extracellular matrix of developing chicken muscle (12) and has been identified in the extracellular matrix around pancreatic acini (13). At the latter site, an extracellular localization was confirmed by electron microscopy using thin frozen sections and ferritin labeling. However, the nature of the extracellular material

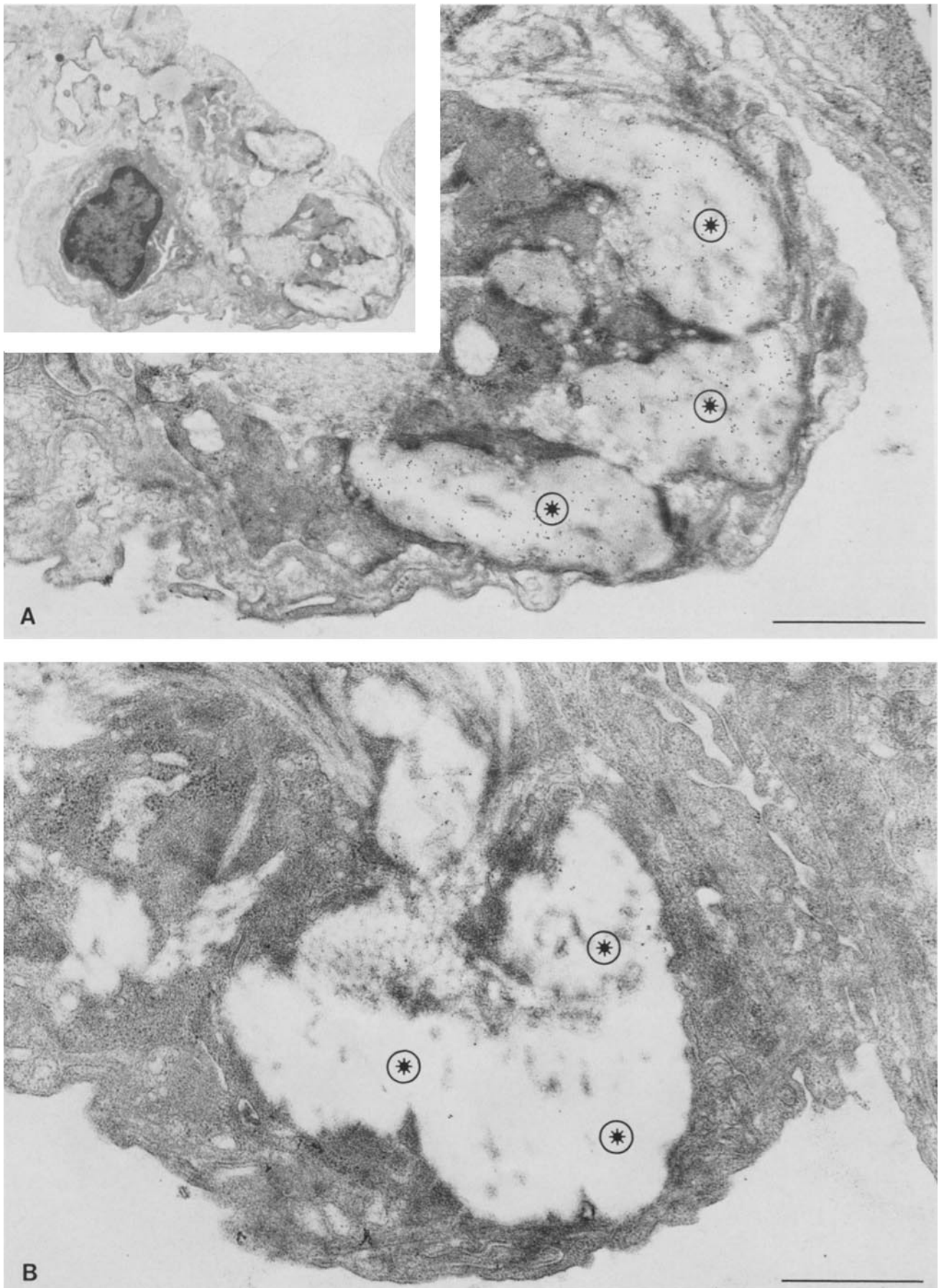


FIGURE 6 Immunohistochemical localization by electron microscopy of endogenous β -galactoside-binding lectin in elastic fibers (*) of a pulmonary alveolus. Sections were reacted either with affinity purified rabbit anti-lectin (A) or control rabbit IgG (B) followed by colloidal gold conjugated goat anti-rabbit IgG. (Inset) A larger field ($\times 7,200$) to assist in orientation. Bar, 1 μ m. (A) $\times 29,000$. (B) $\times 26,000$.

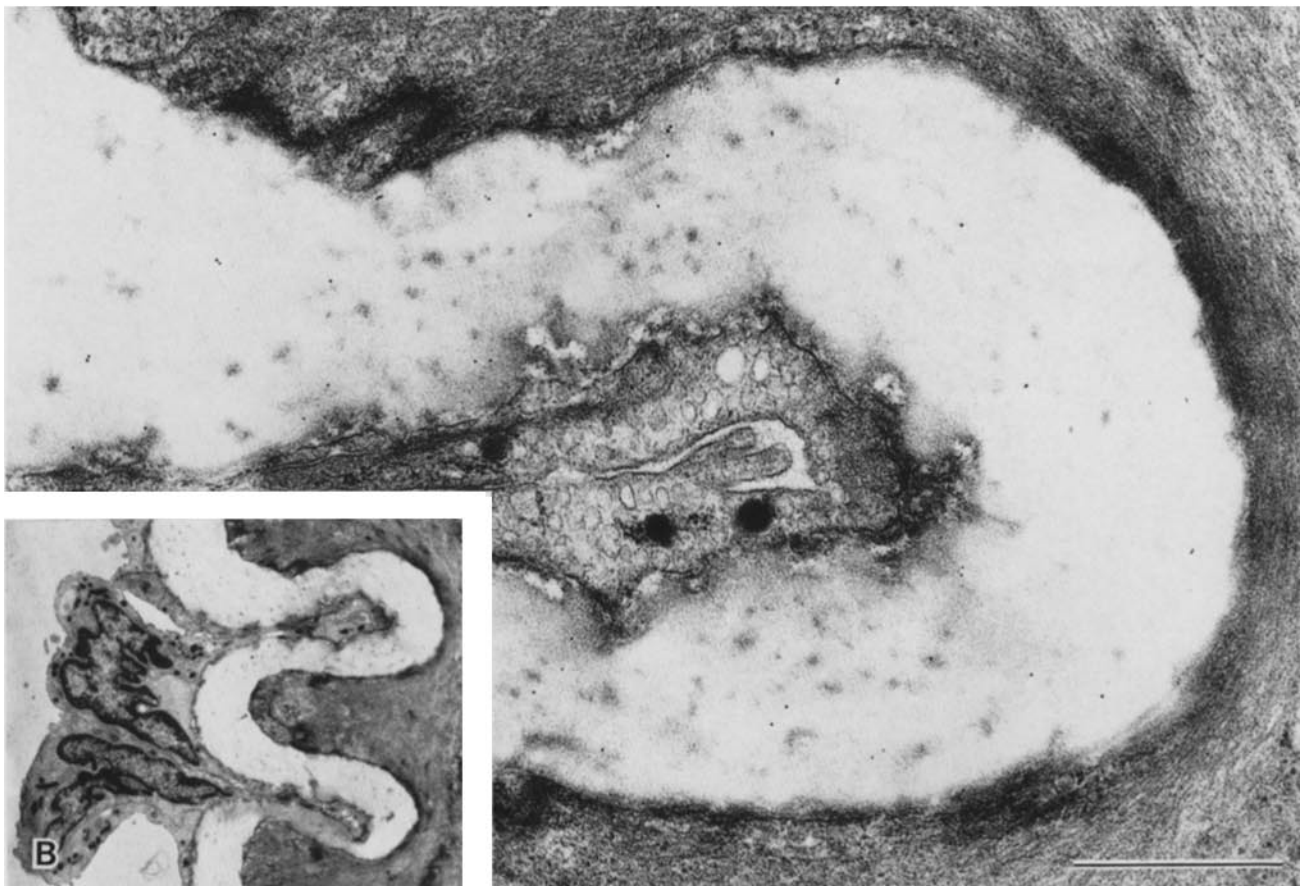
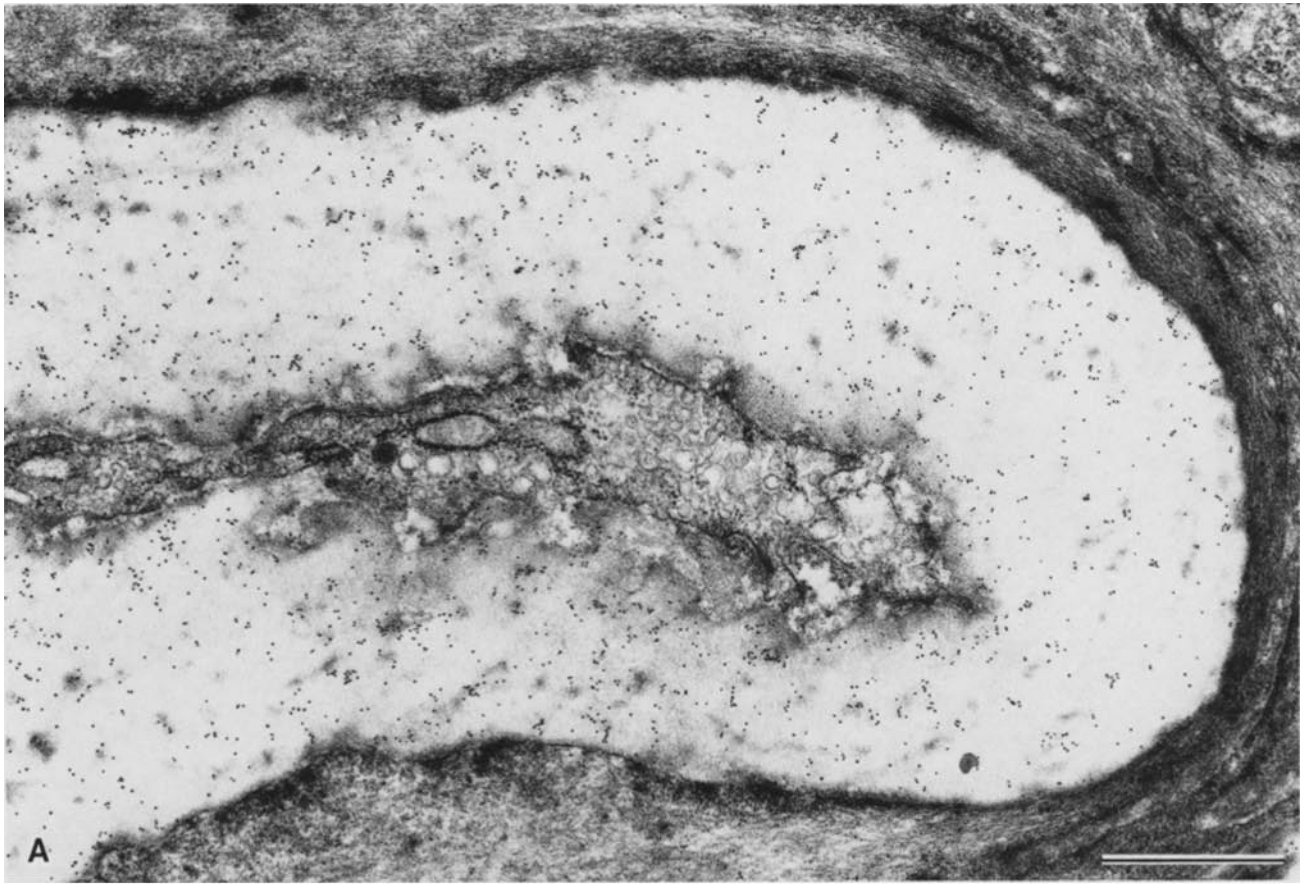


FIGURE 7 Immunohistochemical localization by electron microscopy of β -galactoside-binding lectin in elastic fibers in the wall of a blood vessel in rat lung. Sections were reacted either with affinity-purified rabbit antilectin (A) or control rabbit IgG (B) followed by colloidal gold conjugated goat anti-rabbit IgG. (Inset) A larger field ($\times 4,600$) to assist in orientation. Bars, $1 \mu\text{m}$. (A) $\times 24,000$. (B) $28,000$.

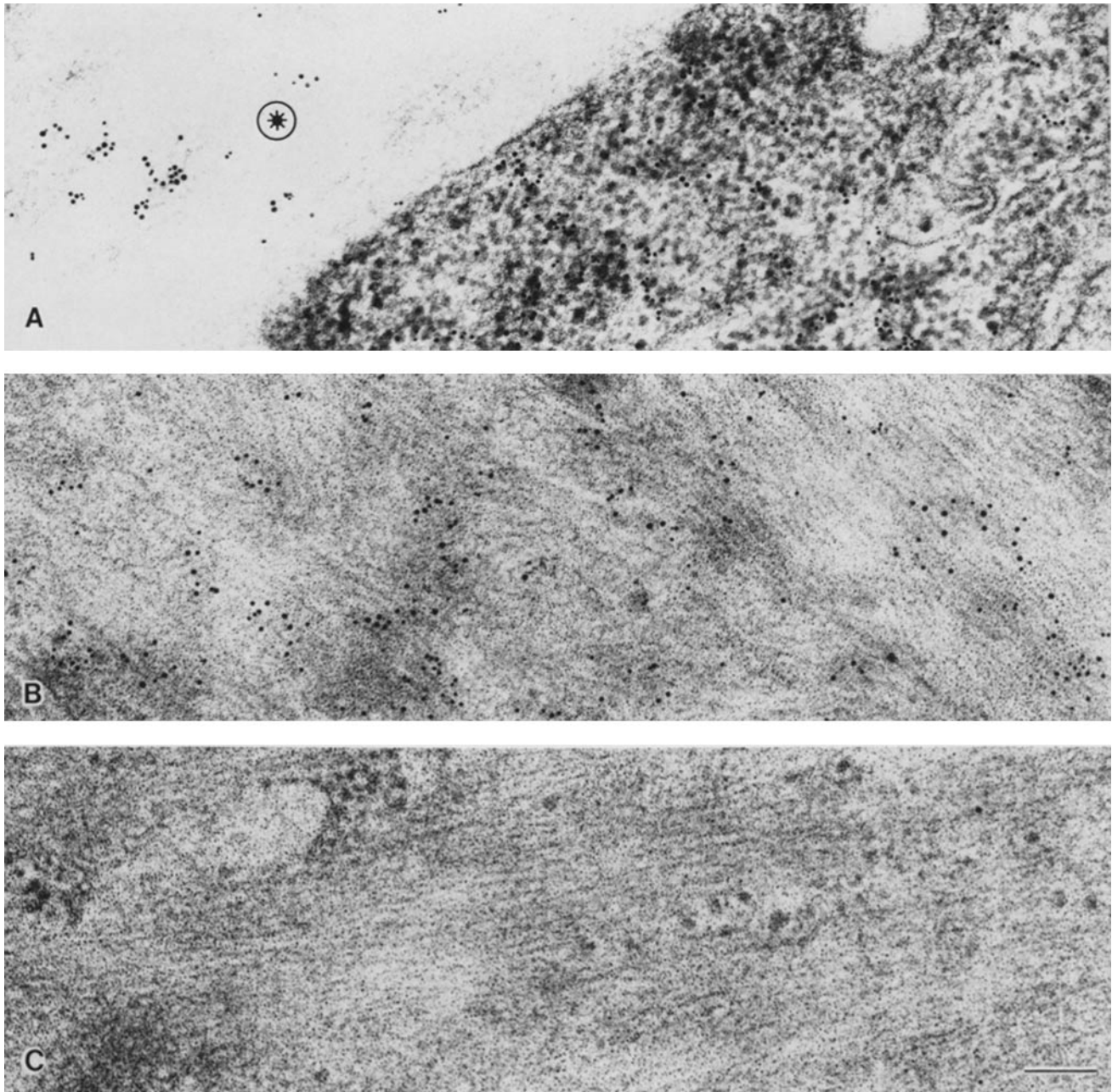


FIGURE 8 Immunohistochemical localization by electron microscopy of endogenous β -galactoside-binding lectin in cytoplasm of a squamous alveolar epithelial (type I) cell (A) and a smooth muscle (B) cell. Sections including alveolar (A) or smooth muscle cells (B and C) were reacted with affinity purified rabbit antilectin (A and B) or control rabbit IgG (C) followed by colloidal gold conjugated goat anti-rabbit IgG. A portion of an elastic fiber is denoted "*." Bar, 0.1 μ m. \times 113,000.

with which the lectin was associated was difficult to determine because morphological preservation with that technique was of much lower quality than that achieved here. Neither skeletal muscle matrix nor pancreatic matrix are especially rich in elastic fibers so that the extracellular localization of CLL-I could well be different from that observed in rat lung.

The extracellular localization of the mammalian lectin also supports the hypothesis that soluble lectins in general tend to be externalized, presumably to interact with extracellular and cell surface glycoconjugates (2). For example, another endogenous lactose-binding lectin from chicken, CLL-II, has been shown to associate with the secretory granules of the goblet cells of the intestine (14) and to be externalized, perhaps in association with mucins. Chicken-heparin-lectin (27) is exter-

nalized by skeletal muscle cultures (28). A lectin from *Xenopus laevis* becomes associated with extracellular material of cleavage stage embryos (29). Furthermore, in *Dictyostelium discoideum*, two developmentally regulated lectins, discoidin I and discoidin II, are found extracellularly (30), the latter apparently in association with a spore coat polysaccharide.

Of the glycoproteins of elastic fibers with which the endogenous lectin might associate, the two most likely candidates are presumed to be components of elastic fiber microfibrils. In bovine ligaments, these materials have M_r \sim 150,000 and M_r \sim 300,000 (31). The microfibrils (32, 33) are believed to play a role in orienting the elastin precursors that then become enzymatically cross-linked into a stable polymer. This raises the possibility that the lectin also participates, perhaps by

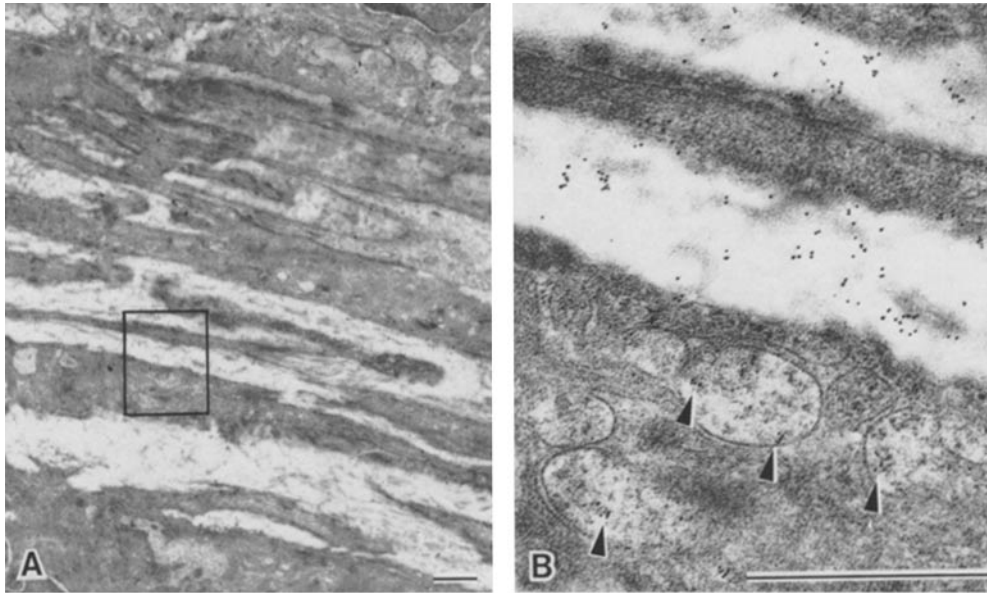


FIGURE 9 Immunohistochemical localization by electron microscopy of endogenous β -galactoside lectin in the wall of a respiratory bronchiole containing multiple bands of elastic fibers and smooth muscle cells. The section was stained with affinity purified rabbit anti-lectin followed by colloidal gold conjugated goat anti-rabbit IgG and shown at magnifications of $\times 5,700$ (A) and $\times 33,000$ (B). Arrowheads indicate rarely seen accumulations of colloidal gold in regions that are at least partially membrane-bound. Bars, 1.0 μm .

binding to the microfibrillar glycoproteins. Direct interaction of the lectin's carbohydrate-binding site with elastin seems to be precluded because no sugar residues have been detected on this protein.

A vertebrate β -galactoside lectin with $M_r \sim 29,000$ under dissociating and reducing conditions has not been observed in rat (15), bovine (8, 16), or human (15, 16) tissues prepared by methods like those used here, although it is detectable in the original preparation of bovine lectin (7). CLL-I (14–16) and electrolectin (34), related carbohydrate-binding proteins, also have subunit molecular weights in the range of 15,000 but no larger subunits were detected by gel electrophoresis under dissociating conditions. Failure to detect the larger band may simply be due to its relative scarcity, but could also reflect other factors, such as differences in its tissue distribution, or the period in development when it is expressed.

The relationship between the two lectin protein bands that we observed is not clear. Because of their marked similarities, they might be identical gene products that are modified after translation. One possibility is that the larger form is a precursor that is proteolytically cleaved to become the smaller. Another possibility, suggested by their size, is that the larger form is a stable dimer resulting from cross-linking of two monomers. Cross-links in elastin are known to be mediated by a lysyl oxidase that oxidizes the ϵ -amino moiety of specific lysyl residues to form reactive aldehydes (35). Similar lysyl cross-links may exist in other proteins (35). This raises the possibility that the lectin may also have been cross-linked. This reaction might have occurred intracellularly, in the extracellular matrix, or artifactually during lectin isolation. The differences in peptide maps between the two bands are not inconsistent with this possibility.

Another unresolved question is the mechanism of lectin secretion. Some lectins like CLL-II (14) or discoidin II (30) appear to be localized in secretory vesicles. Their secretion is presumably by a classical mechanism of vesicle fusion with

the plasma membrane. However, the distribution of the rat lung lectin resembles that of a cytoplasmic protein, and association with vesicular structures is extremely rare. Whether this is a result of inadequate morphological preservation or an indication of a new secretory mechanism remains to be determined.

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