p172: An Alveolar Type II and Clara Cell Specific Protein with Late Developmental Expression and Upregulation by Hyperoxic Lung Injury

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The epithelium of the alveolus and distal airway meets unique requirements, functioning as a gas exchange membrane and barrier to alveolar flooding by vascular contents as well as to bloodstream contamination by airborne toxins and pathogens. Gene products specifically expressed by this epithelium, notably the surfactant apoproteins, have had important clinical application. No cell surface antigen specific for alveolar type II and Clara cells has been described. We report the biochemical characterization, tissue and developmental expression, and upregulation by injury of a 172 kD protein recognized by a monoclonal antibody, 3F9, synthesized in response to immunization with freshly isolated rat alveolar type II cells. p172 is expressed in a polarized fashion by the apical surface of rat alveolar type II and Clara cells. An immunohistochemical survey of various rat tissues and organs reveals lung specificity. p172 is first detectable in rare epithelial cells at 19 days of gestation, a time when the fully differentiated alveolar type II cell is identified by the first detection of lamellar bodies. There is a dramatic increase in p172 expression just prior to birth. Hyperoxic lung injury results in increased expression of p172. The upregulation of p172 by hyperoxia and its cell-specific expression suggests an important adaptive function. **Girod, C. E., D. H. Shin, M. B. Hershenson, J. Solway, R. Dahl, and Y. E. Miller. 1996. p172: an alveolar Type II and Clara cell specific protein with late developmental expression and upregulation by hyperoxic lung injury. Am. J. Respir. Cell Mol. Biol. 14:538–547.**

The alveolar epithelium is a delicate structure which separates atmospheric air and circulating blood. It is exposed to stressful stimuli in the form of oxidants and airborne toxins. This epithelium displays both adaptive and regenerative potential, especially in response to lung injury. The alveolar type I cell, which covers approximately 95% of the alveolar surface area, has the function of maintaining the integrity of the thin epithelial barrier while allowing for gas exchange (1). The alveolar type II cell, the stem cell of the alveolus, synthesizes surfactant and metabolizes oxidants and other toxins.

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Abbreviations: 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium, BCIP/NBT; 3,3′-diaminobenzidine, DAB; *Maclura pomifera lectin*, MPA; surfactant protein-A(-B,-C,-D), SP-A (-B,-C,-D).

Our knowledge of alveolar epithelial adaptation to injury is incomplete. Investigators have studied genes with postulated adaptive functions that are differentially expressed or upregulated during lung injury. For example, type II cells double surfactant protein-A (SP-A) mRNA expression during hyperoxia (2). Increased expression of Na^+-K^+ ATPase by the alveolar type II cell may alleviate the development of pulmonary edema associated with lung injury (3). Oxidative stress leads to 5-fold and 60-fold increases in gene expression of the free radical scavengers ceruloplasmin (4) and metallothionein (5, 6), respectively. With the exception of the surfactant apoproteins, none of these adaptive responses are specific to the lung. Clinical intervention with surfactant demonstrates the utility for additional knowledge regarding alveolar adaptation to injury (7).

We have initiated a program to identify and characterize proteins specifically expressed by respiratory epithelia (8). We have partially characterized the biochemical nature and regulation during development and injury of p172, a type II and Clara cell antigen. We have chosen a rat hyperoxic lung injury model for evaluation of p172 expression during lung injury because it is well characterized and clinically relevant (9–11).

Materials and Methods

Monoclonal Antibody Synthesis and Selection

The 3F9 IgG1 monoclonal antibody was synthesized in response to the immunization of Balb/c mice with freshly isolated rat type II cells, as previously described (8).

Hyperoxic Lung Injury Model

The hyperoxic rat lung injury model has been described in detail elsewhere (12, 13). Briefly, 21-day-old Sprague-Dawley rats were exposed to 8 days of either normoxia or hyperoxia (> 95% F_{IO_2}) at sea level. Four animals from each group were studied as described below.

Western Blot of Normal and Hyperoxic Rat Lung Subcellular Protein Fractions

Four animals from each group were killed, the right lungs isolated, and cytoplasmic and total cell membrane protein fractions prepared as described elsewhere (14). Briefly, normal and hyperoxic lungs were weighed, perfused, and homogenized by a Brinkman Tissue Homogenizer (Syborn Corp., Westbury, NY) in 10 vol of ice-cold homogenizing buffer (consisting of PBS, pH 7.5, with the addition of 0.1 M EDTA, 0.1 mM PMSF, 1 mM *o*-phenanthroline, 1 mg/ml soybean trypsin inhibitor, and 50 KIU/ml aprotinin). Homogenates were centrifuged at 500 × *g* for 10 min to remove nuclei and tissue fragments. The supernatant underwent cell and organelle disruption by Dounce homogenization followed by repeated ultracentrifugation to obtain a supernatant containing the soluble cytoplasmic protein fraction. The pellet which contained the cell membranes was resuspended in the same buffer, re-ultracentrifuged, and resuspended in buffer containing 0.1% Triton X-100. Membrane proteins were solubilized by incubation at 4°C and isolated by ultracentrifugation. A series of western blots were performed using both hyperoxic and normoxic cytoplasmic and cell membrane protein fractions. Increasing dilutions of all four preparations were evaluated for protein concentration by Coomassie blue R-250 staining and SDS-PAGE, using 7.5% gels run in the presence of 3% β-mercaptoethanol, and by a direct protein quantitation with a modified Lowry assay (15) using bovine serum albumin as a standard (Bio-Rad, Hercules, CA). If needed, concentration of the protein preparations was performed using Centricon-3™ centrifugal microconcentrators (Amicon, Beverly, MA) using a 3,000 mol. wt. cut-off. Transfer of gels to Rad-Free™ nitrocellulose membranes (Schleicher-Schuell, Keene, NH) was performed by electrophoresis at 300 mA current for 2 h in an MRA™ (Clearwater, FL) western blot transfer apparatus. Membranes were blocked for 1 h in membrane-blocking buffer (Rad-Free™ colorimetric detection system; Schleicher-Schuell). Exposure to the 3F9 mAb supernatant (1:5 dilution) and the respective positive and negative controls was performed overnight using the Mini-PROTEAN II™ dual-slab cell apparatus (Bio-Rad). The negative controls consisted of lanes containing no primary antibody, 2A3 antibody (an IgG1 mAb negative in normal lung), and 1A6A7 (an IgG1 mAb to human transferrin) (16). Positive controls consisted of polyclonal and mAb to SP-A, kindly provided by James Fisher from the University of Colorado Health Sciences Center and Robert Mason from the National Jewish Center for Immunology and Respiratory Medicine (Denver, CO), respectively. After primary antibody incubation, the nitrocellulose membranes were exposed to: (*1*) three brief washes in distilled water followed by a 5-min rinse in Rad-Free™ wash buffer, Schleicher-Schuell, and repeated three times; (*2*) alkaline phosphatase-conjugated goat anti-mouse IgG for 30 min at room temperature; (*3*) three-wash cycles; and (*4*) 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) color substrate.

Preparation of Adult Rat Tissue for Immunohistochemistry

Normal adult Sprague-Dawley rats were killed with overdosage of sodium pentobarbital (100 mg/kg). After interruption of the aorta below the renal arteries, lungs were perfused with 10% buffered formalin through the right ventricle. Intratracheal instillation with approximately 8 ml of 10% formalin was also performed. Perfusion of other rat organs and tissues was achieved by infusion of 10% formalin via left ventricular injection. The following organs were then removed from four adult rats: lungs, esophagus, stomach, duodenum, jejunum, ileum, colon, heart, brain, adrenal glands, kidney, liver, muscle, spleen, and testis. The tissues were sliced into 10 mm × 4 mm × 4 mm sections and fixed overnight at 4°C in 10% formalin. Tissue blocks were then paraffin-embedded for immunohistochemistry.

The hyperoxic rat lung injury model described above was prepared in similar fashion for immunohistochemistry. Five animals from each group (hyperoxia and normoxia) were studied. Tissue was formalin-fixed by inflation at a constant pressure of 25 cm H_2O, cut into 5-mm sections, and paraffin-embedded.

Preparation of Fetal Rat Lung for Immunohistochemistry

Timed pregnant Sprague-Dawley rats were maintained at the Denver VA Medical Center Animal Facility for greater than 72 h prior to sacrifice. Pregnant animals were killed at days 17, 18, 19, 20, and 21 of gestation. After anesthetizing the dam with intraperitoneal injection of sodium pentobarbital (50 mg/kg), the abdominal cavity was exposed using a midline incision, the gravid uterus removed and incised, and rat fetuses removed. Fetuses and mothers were then killed by intraperitoneal injection of sodium pentobarbital (100 mg/kg). For day-17 and -18 fetuses, the thoracic cavity was removed *en bloc* and fixed overnight in 10% formalin at 4°C. For day-19 to pre-natal fetuses, newborns, and post-natal rats (days 0, 3, 7, and 14), the whole lung was obtained by midline incision of the thoracic cavity and removed by dissection. This tissue was also fixed in 10% buffered formalin and paraffin-embedded.

Immunohistochemistry

Four-μm sections of formalin-fixed and paraffin-embedded tissue were cut and placed on charged, pre-cleaned slides (ProbeOn™ Plus, Fisher Scientific, Pittsburgh, PA). The MicroProbe™ manual staining system (Fisher Scientific) was used for the immunohistochemical studies. Deparaffinization was performed with a 72°C incubation for 15 min followed by treatment with xylene. Rehydration of slides was

then achieved by serial treatments with 100% ETOH, 70% ETOH, and 1× automation buffer, which consists of PBS, Triton X-100, and proprietary preservatives (Biomeda, Foster City, CA). Reagents from the Super-sensitive Biotin-Streptavidin™ system (BioGenex, San Ramon, CA) were used for subsequent immunohistochemistry. The slides were sequentially treated with the following reagents at 37°C: (*1*) protein block (normal goat serum in PBS) for 9 min; (*2*) avidin block for 9 min; (*3*) biotin block for 9 min; (*4*) 3F9 mAb, 1:200 dilution, for 10 min; (*5*) six rinses with automation buffer; (*6*) biotinylated goat anti-mouse IgG for 5 min; (*7*) repeat buffer rinses; (*8*) streptavidin-conjugated alkaline phosphatase for 5 min; (*9*) repeat buffer rinses; (*10*) fast-red substrate at room temperature for 8–12 min; (*11*) three rinses with distilled water; and (*12*) Mayer's hematoxylin counterstain.

For all experiments, intrinsic alkaline phosphatase activity was quenched with addition of 8 μl of 50× levamisole concentrate (BioGenex, San Ramon, CA) per ml of fast-red substrate solution. Tissue slides were then mounted with Crystal/Mount™ (Biomeda, Foster City, CA) and baked at 80°C for 15 min. Slide covers were placed after addition of Permount™ (Fisher Scientific, Pittsburgh, PA). In initial experiments, negative controls included IgG1 mAbs to human aminoacylase-1 (17); subsequently, controls consisted of extra tissue slides skipping incubation with either the primary antibody, secondary antibody, or streptavidin-conjugated alkaline phosphatase.

Double-immunohistochemistry Methods

Double-immunohistochemistry was performed using the 3F9 mAb and a rabbit polyclonal anti-SP-A antibody. Frozen normal rat lung tissues were used because of better SP-A detection under these conditions. First, standard immunohistochemistry was performed, as described in above sections, using the 3F9 mAb/biotinylated goat anti-mouse IgG/streptavidin-conjugated alkaline phosphatase/fast red reagent protocol. Second, standard immunohistochemistry using anti-SP-A polyclonal antibody/biotinylated goat anti-rabbit IgG/streptavidin-conjugated horseradish peroxidase/DAB (3,3′-diaminobenzidine) substrate was performed. Slides were then counterstained with Mayer's hematoxylin and mounted as described above. Controls consisted of extra tissue slides skipping incubation with one of the following: both primary antibodies, both secondary antibodies, streptavidin-conjugated alkaline phosphatase, or horseradish peroxidase. Intrinsic alkaline phosphatase and horseradish peroxidase were quenched with levamisole and 3% hydrogen peroxide, respectively.

Immunoelectronmicroscopy

Cellular localization of p172 was performed by immunoelectronmicroscopy. Adult Sprague-Dawley rats were killed and the lung inflated, perfused with 4% paraformaldehyde, cut into 5 × 5 × 2 mm^3 sections, and fixed in 4% paraformaldehyde for 3–4 h at 4°C. Then sequential tissue washes in increasing sucrose-PBS gradients (10%, 15%, 20% and 25%) for 3 h each were performed. Tissue was embedded in Histo Prep™ frozen tissue embedding media (Fisher Scientific, Fair Lawn, NJ). Six-μm sections were cut using a standard laboratory cryostat and placed on charged, precleaned slides.

To assure adequate adhesion of tissue to slides, the slides were treated with 10% formalin for 2 min, washed six times in automation buffer, and air-dried in a slide warmer at 37°C. Immunohistochemistry was performed as described above with the exception that streptavidin-conjugated horseradish peroxidase with DAB substrate development was used without counterstain. Endogenous horseradish peroxidase was quenched with 3% hydrogen peroxide applied after the secondary antibody. This prevented 3F9 epitope elimination seen if the tissue slides are exposed to hydrogen peroxide before primary antibody incubation. The slides were then treated with 1% osmium tetroxide for 30 min at room temperature and washed three times with distilled water. Increasing ethanol concentrations rapidly dehydrated the tissue, which was then infiltrated with Epon resin in standard fashion and placed in a 60°C oven to allow polymerization. The tissue was then sectioned, counterstained with 3% aqueous uranyl acetate for 10 min, and analyzed with a Phillips CM-12 electron microscope.

Supra-optimal Dilution Study

A supra-optimal dilutional study, as previously described (18), was used to assess expression of p172 during hyperoxic lung injury. Both normoxic and hyperoxic rat lung tissue slides were studied using immunohistochemistry with increasing (supra-optimal) dilutions of the 3F9 mAb. Slides were examined under light microscopy and photomicrographs taken of slides at each dilution. Results were tabulated as positive, trace, or negative expression of p172. A positive result was defined as unequivocal staining in multiple cells in every alveolus. A trace result was faint staining of approximately 1–2 cells per microscopic high-power field (40× objective). A negative result was no discernable staining.

Results

The 3F9 Monoclonal Antibody Detects a 172 kD Cell Membrane Protein in Both Normoxic and Hyperoxic Lung

The normoxic and hyperoxic cell membrane fractions demonstrate a single band at approximately 172 kD, but only under reduced conditions (Figure 1A, only reduced blot shown). The 172 kD protein was not detected in cytoplasmic protein extracts, confirming cell membrane specific expression of the 3F9 antigen (Figure 1B). In this hyperoxic cytoplasmic fraction, a poorly defined band is detected at approximately 50 kD, not only in the 3F9 lane (lane 5) but also in both the 2A3 (lane 6) and 1A6A7 (lane 7). 2A3 and 1A6A7 were used as controls and represent a mAb directed to isolated rat type II cells and a mAb to human transferrin, respectively. This 50 kD band likely represents nonspecific binding of the monoclonal antibodies to an abundant rat antigen in this cytosolic fraction and not the identification of a unique and specific 3F9 antigen.

p172 is Expressed Exclusively in Type II and Clara Cells

Immunohistochemistry was performed on normal adult rat lung tissue using a 1:200 dilution of the 3F9 mAb superna-

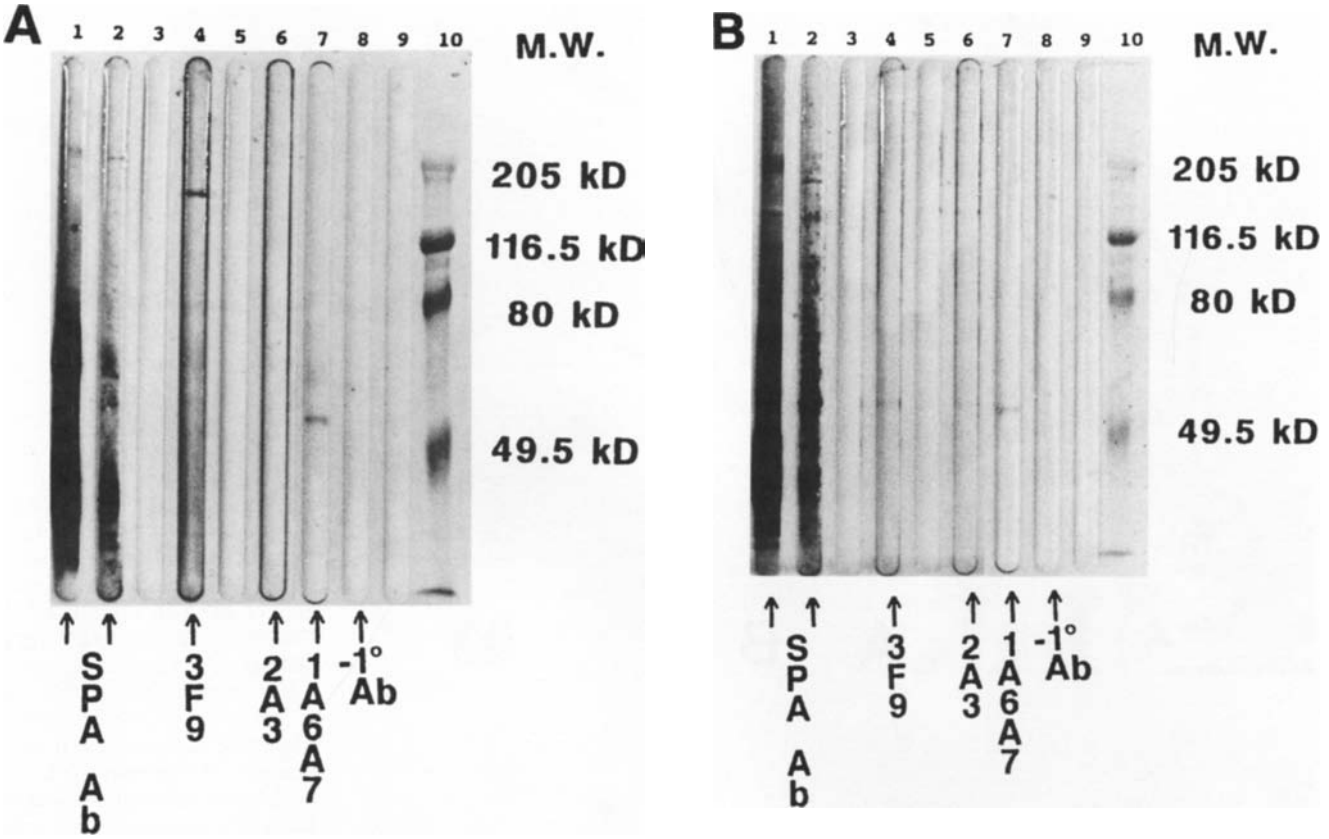

Figure 1. Western blot analysis of adult hyperoxic rat lung (*A*) membrane protein fraction (reduced) and (*B*) cytoplasmic, soluble protein fraction (reduced) incubated with: Lane 1: polyclonal anti-SP-A (1:400 dilution); lane 2: anti-SP-A mAb (1:4 dilution); lane 3: blank; lane 4: 3F9 mAb (1:1 dilution); lane 5: blank; lane 6: 2A3 mAb (1:1 dilution); lane 7: 1A6A7, anti-human transferrin mAb (1:3); lane 8: no primary antibody incubation; lane 9: blank; lane 10: high mol. wt. standards (Bio-Rad). The 3F9 mAb supernatant recognizes a band at an M_r of 172 kD in the reduced membrane fraction only (*A*) and (*B*). Immunostaining of lanes 1 and 2 with anti-SP-A antibodies demonstrates overloading due to relative abundance of SP-A. In (*B*) (hyperoxic cytoplasmic fraction), a poorly defined band is detected at approximately 50 kD, not only in the 3F9 lane (lane 5), but also in both the 2A3 (lane 6) and 1A6A7 (lane 7). 2A3 and 1A6A7 were used as controls and are mAbs directed to isolated rat type II cells and human transferrin, respectively. This 50 kD band therefore likely represents nonspecific binding.

tant. The p172 antigen was exclusively detected in intercalated cells of the alveolar epithelium at the junction of alveolar septae, suggesting immunostaining of type II cells (Figure 2B and C). Staining also occurred in the nonciliated or Clara cells of the bronchiolar epithelium (Figure 2A). The 3F9 antibody was not immunoreactive in the alveolar space, endothelium, connective tissue, or alveolar macrophages.

To corroborate cell specificity, double immunohistochemical studies with a polyclonal antibody to SP-A, a known type II and Clara cell marker, and the 3F9 mAb were performed on the same frozen lung tissue slide. These studies detected co-localization of the p172 and SP-A in specific cells of the alveolar and bronchiolar epithelia that are consistent, respectively, with type II and Clara cells. Most, if not all, SP-A positive cells were also p172 positive, and vice-versa. This cell specificity of p172 was also confirmed using immunoelectron microscopy (Figure 3A, B, and C).

Formalin-fixed tissues showed superior detection of the 3F9 antigen, compared to frozen and paraformaldehyde fixations. Also, exposure of slides to 3% hydrogen peroxide (to quench endogenous peroxidase) prior to primary antibody incubation eliminated p172 antigen detection. Therefore, we used the alkaline phosphatase-fast red reagent system for the p172 immunohistochemical studies.

p172 is Localized Specifically to the Apical Cell Membrane of Type II and Clara Cells

Immunoelectronmicroscopy studies of normal rat lung frozen tissue were performed and demonstrated apical cell membrane linear localization of the p172 antigen exclusively in type II pneumocytes (Figure 3A) and Clara cells (Figure 3B). A low-power photomicrograph (Figure 3C) demonstrates no staining with 3F9 in a monocyte and surrounding type I alveolar cell membrane.

p172 Expression by Immunohistochemical Techniques is Lung-specific

An immunohistochemical survey of multiple rat tissues and organs (lungs, esophagus, stomach, duodenum, jejunum, ileum, colon, heart, brain, adrenal gland, kidney, liver, mus-

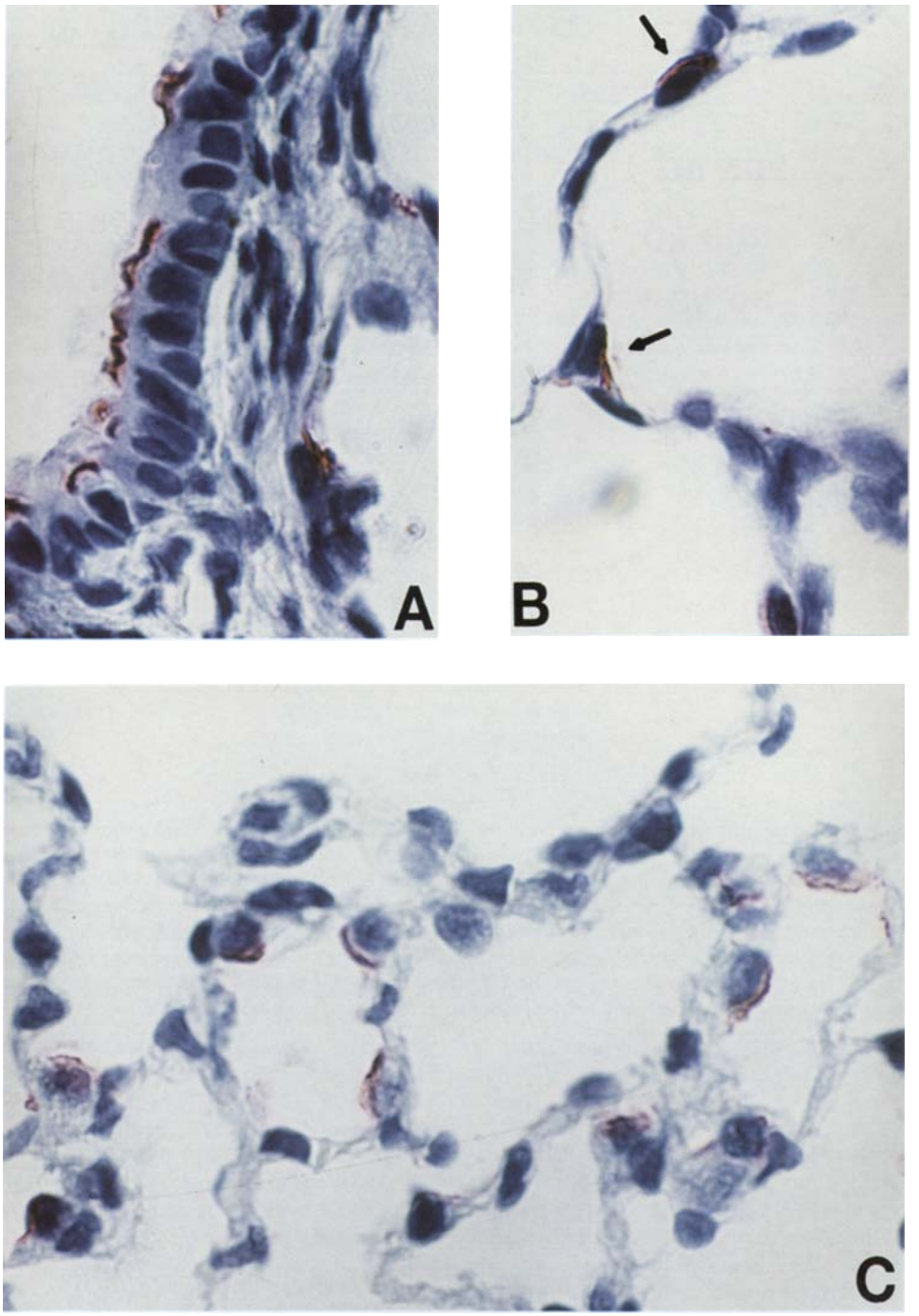

Figure 2. 3F9 mAb/alkaline phosphatase/fast red immunohistochemical staining of normal adult rat lung. (*A*) demonstrates detection of p172 in nonciliated or Clara cells of distal bronchial epithelium. (*B*) demonstrates positive staining in intercalated cells at junctions of alveolar septae suggesting alveolar type II cells (*arrows*). (*C*) represents a larger field of the alveolar epithelium suggesting specific 3F9 antibody binding to apical cell membranes of alveolar type II cells.

cle, spleen, and testis) revealed that the p172 antigen is detectable only in lung. Western blot was also performed on rat kidney cytoplasmic and cell membrane protein fractions, with negative results.

p172 Expression Occurs Late in Fetal Lung Development

Initial expression of p172 during fetal rat lung development was seen by day 19 of the total 21-day gestation period (Figure 4A). p172 is initially localized to rare cells lining tubules of peripheral lung and bronchial epithelium. No evidence of p172 is seen during the glandular stage of fetal rat lung development (days 17 and 18). By day 20 of gestation (Figure 4B), increased expression of the antigen is noted throughout peripheral lung tubules consistent with alveolar structures. Rapid increase of p172 expression throughout the alveolar and bronchial epithelia occurs by day 21 and newborn stages (Figures 4C and D). Evaluation of postnatal rat lung of up to 2 wk shows that peak expression of p172 occurs at the newborn stage.

p172 Expression in Rat Lung Is Upregulated by Hyperoxia

We asked whether increased expression of p172 occurs in hyperoxia-exposed (> 95% F_{IO_2} for 8 days) 21-day-old rats

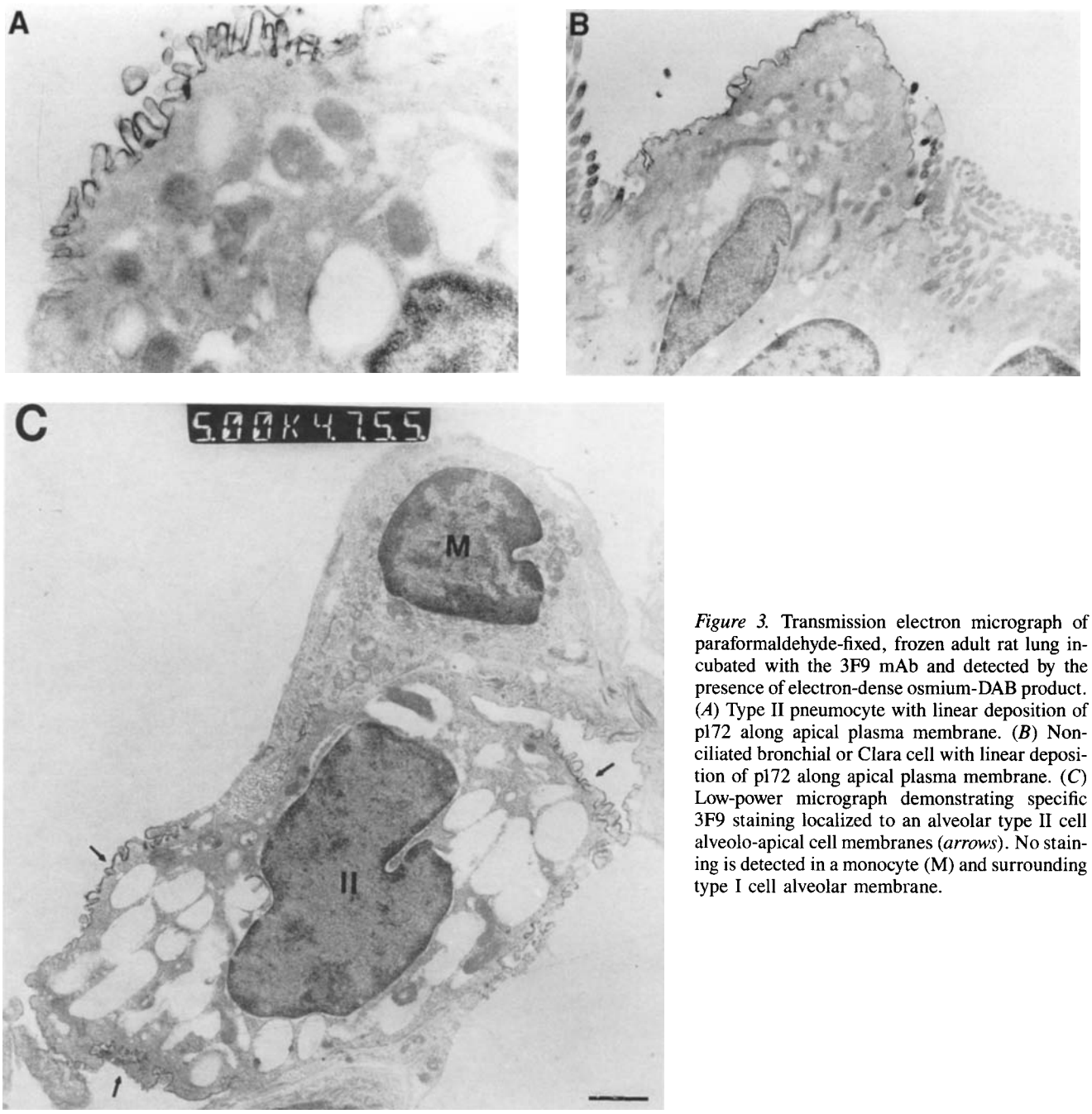

Figure 3. Transmission electron micrograph of paraformaldehyde-fixed, frozen adult rat lung incubated with the 3F9 mAb and detected by the presence of electron-dense osmium-DAB product. (*A*) Type II pneumocyte with linear deposition of p172 along apical plasma membrane. (*B*) Nonciliated bronchial or Clara cell with linear deposition of p172 along apical plasma membrane. (*C*) Low-power micrograph demonstrating specific 3F9 staining localized to an alveolar type II cell alveolo-apical cell membranes (*arrows*). No staining is detected in a monocyte (M) and surrounding type I cell alveolar membrane.

(12, 13). We studied five animals from each group (normoxic and hyperoxic) with immunohistochemistry using the 3F9 mAb. Typical 3F9 alveolar epithelial distribution was found in the normoxic rat lung controls (Figure 5A). In the hyperoxia-exposed animals, apparent increased expression of the p172 antigen was found with more cells expressing the antigen per alveolus (Figure 5B).

To semi-quantitatively evaluate this apparent hyperoxic upregulation, we performed a supra-optimal dilution study (18). This technique utilizes increasing (supra-optimal) dilutions of the 3F9 mAb, allowing for detection of differences in antigen concentration between the normoxic and hyperoxic lung tissues. We detected p172 at higher dilutions of the 3F9 mAb in the hyperoxia-injured animals (Table 1).

p172 upregulation by hyperoxia was demonstrated by comparison of western blots with equal protein concentrations of normal and hyperoxic rat cell membrane protein fractions (Figure 6). Equal protein loading for both normal and hyperoxic lung was demonstrated by Coomassie-stained gel and corroborated by Lowry-based protein assay. In the hyperoxic lung, increased detection of p172 by the 3F9 mAb supernatant was noted in comparison to normal lung.

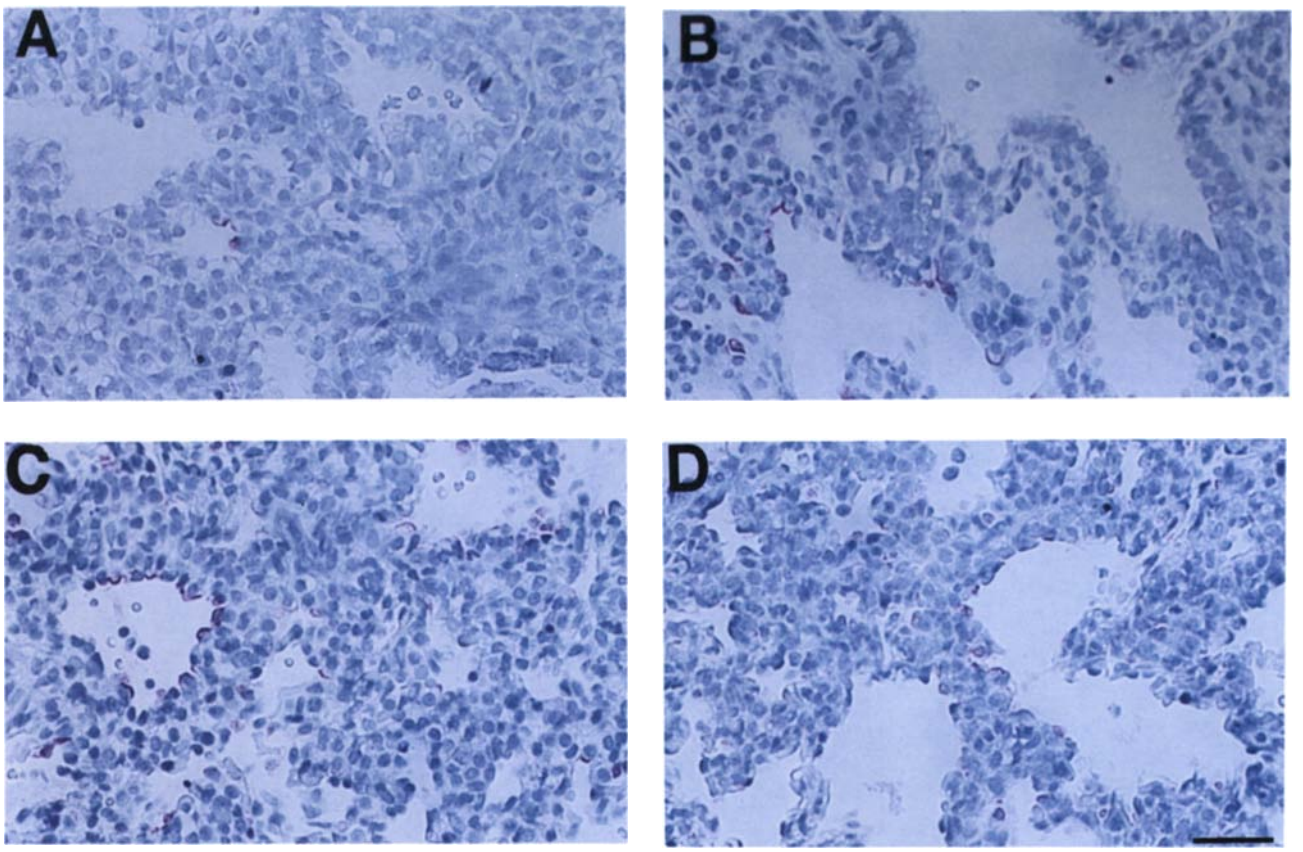

Figure 4. Photomicrographs of formalin-fixed fetal rat lungs immunostained with 3F9 mAb/alkaline phosphatase/fast red reagent. Day 19 fetal lung (*A*) first demonstrates detectable p172 expression in rare cells lining tubules of peripheral lung and bronchial epithelium. From gestational day 20 (*B*), day 21 (*C*), to the newborn period (*D*), increased expression of the antigen is noted throughout peripheral lung tubules consistent with alveolar structures and bronchial epithelium. Magnification bars represent 23 μm.

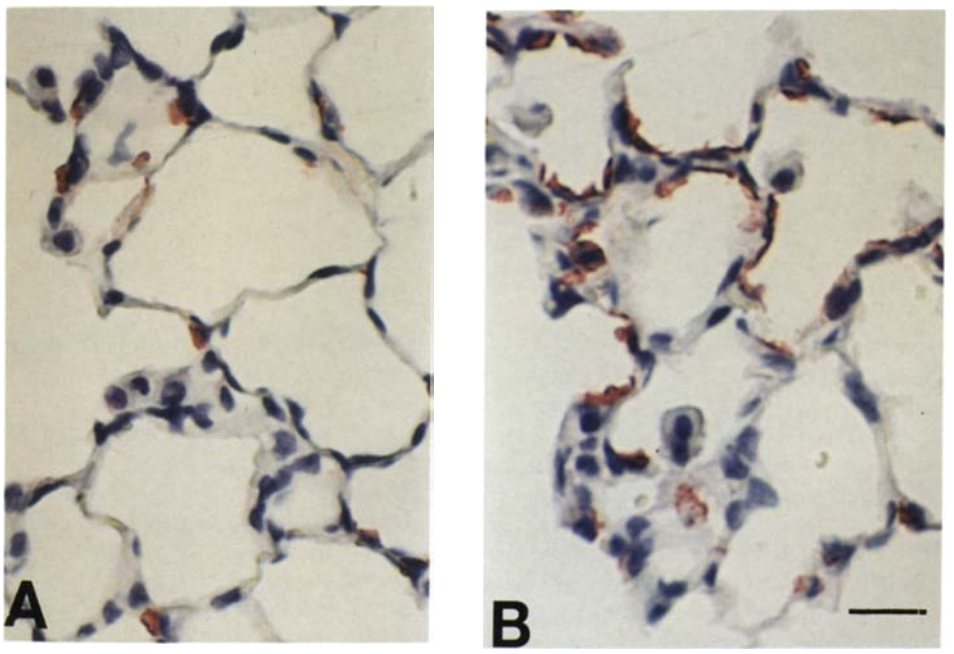

Figure 5. Photomicrographs of formalin-fixed normal and hyperoxic rat lung immunostained with the 3F9 mAb. (*A*) Normal lung demonstrates characteristic pattern of p172 expression in intercalated cells of alveolar epithelium. (*B*) Hyperoxic lung shows greater number of alveolar cells expressing the p172 antigen, as well as increased intensity of immunostaining. Magnification bar represents 25 μm.

TABLE 1

Immunohistochemical detection of p172 with supra-optimal dilutions of the 3F9 mAb

Dilutions	Normoxia*	Hyperoxia
1:200	Positive	Positive
1:400	Positive	Positive
1:800	Positive	Positive
1:1,200	Positive	Positive
1:1,400	Positive	Positive
1:2,000	Trace	Positive
1:2,800	Negative	Positive
1:3,000†	Negative	Positive

* Positive detection = Unequivocal staining of multiple cells in every alveolus. Trace detection = Faint staining of 1–2 cells per high power field (40× lens). Negative detection = No staining seen.

† Experiment performed at a later date demonstrates loss of p172 detection at 1:6,000 and 1:9,000 dilutions in hyperoxic tissue.

Discussion

We describe the characterization of p172, a cell membrane protein expressed by rat type II and Clara cells with unique features which suggest a specialized function. Interesting characteristics of p172 include its biochemical nature, lung-specific expression in type II and Clara cells, apical polarization, late developmental expression, and upregulation during hyperoxic lung injury.

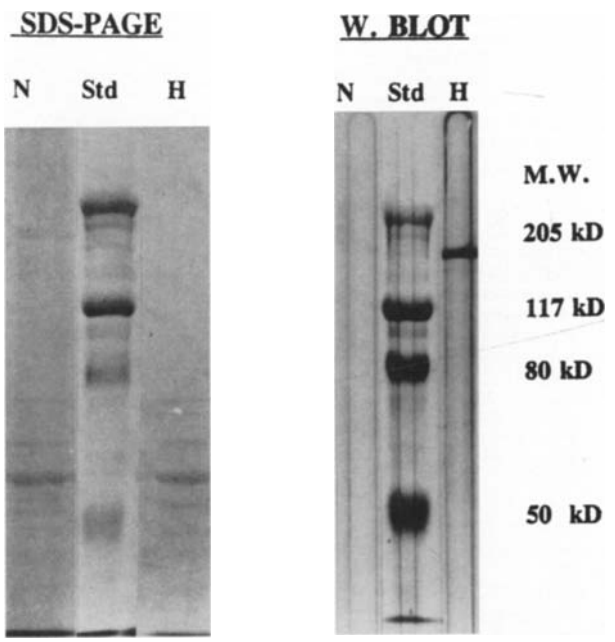

Figure 6. Coomassie-stained 7.5% SDS-PAGE gel and corresponding Western blot of normal and hyperoxic lung cell membrane fractions. SDS-PAGE gel demonstrates equal protein loading for both normal and hyperoxic lung, confirmed by protein quantitation assay using a modified Lowry assay (20) (BioRad). A band consistent with p172 is detected by the 3F9 mAb supernatant and is significantly increased in the hyperoxic fraction when compared to normoxia. Note almost undetectable p172 in normoxic rat lung. This band in normoxic fraction is clearly detected when membranes are overdeveloped (data not shown).

Western blot characterization detected a 172 kD protein localized to the cell membrane fraction of lung and absent in the cytoplasmic protein fraction. We also found that the use of nonreduced conditions during western blots or the addition of hydrogen peroxide during immunohistochemistry led to abolition of p172 detection. These results suggest that the epitope recognized by the 3F9 mAb contains a reversibly oxidizable and reducible moiety such as a sulfhydryl group. Alternatively, p172 may have a critical epitope that is exposed and detected best by the 3F9 mAb after disruption of disulfide bonds by a reduced environment.

The identification of tissue-specific antigens is a means of searching for specialized adaptive functions which has been useful in the hematopoietic and immune systems, but has been largely unexploited within the alveolar epithelium. To date, surfactant-associated proteins are the only other specific type II and Clara cell products used in the study of alveolar epithelial cell differentiation and adaptation during lung injury (19–21). No alveolar type II or Clara cell specific surface antigens have been described. p172 has lung-specific expression by immunohistochemistry. Tissue expression below the limits of detection provided by immunohistochemistry is possible. A western blot of normal rat kidney was negative. Multiple rat tissue western blots were not performed because the immunohistochemical method used provided better sensitivity for p172 identification. p172 expression is clearly highly lung-associated, even if more sensitive techniques eventually demonstrate expression of low quantities in other organs. Indeed, the surfactant-associated protein SP-C has detectable gene expression in brain, liver, and kidney of embryonic rat by reverse-transcriptase polymerase chain reaction (22).

Immunoelectron microscopy and double-immunohistochemistry confirmed cell-specific expression of p172. Most, if not all, SP-A positive cells were p172 positive. p172 is an apical cell membrane protein of type II and Clara cells. No expression was detected in alveolar type I cells, ciliated bronchiolar cells, or alveolar macrophages. This expression pattern is shared with several previously described alveolar type II cell proteins which, in contradistinction to p172, are expressed in organs other than lung: *Maclura pomifera lectin* (MPA)-binding glycoproteins (pneumocin and gp200) and aminopeptidase N. Pneumocin is an apical membrane protein of 165 kD with MPA-binding properties (23, 24). In contrast to p172, pneumocin is detected in liver and kidney. gp200, a 200 kD glycoprotein with MPA-binding properties, is also polarized to the apical cell membrane of the alveolar type II cell (25). MPA-gp200 is detected later than p172 in lung development, day 20 (26). Data regarding tissue specificity for this protein have not been reported. The luminal localization of these type II cell proteins has suggested their possible role in regulation of surfactant metabolism and cell differentiation. Aminopeptidase N is a 146 kD apical cell membrane ectopeptidase of type II and Clara cells which is also detected in the kidney and small bowel (27, 28). Aminopeptidase N is postulated to act as a regulator of bioactive peptide signals that reach the epithelial luminal surface.

Developmental studies of fetal rat lung show that p172 first appears at day 19 of gestation in cuboidal cells of prealveolar or distal epithelial tubules. Previous investigations have reported that day 19 of gestation marks the appearance

of the fully differentiated type II cell with the identification of lamellar bodies (29). This event is preceded by protein expression of SP-A and mRNA expression of SP-A, SP-B, and SP-C (19, 30). By day 20 of gestation, the appearance of morphologically identifiable type I cells occurs (31). Expression of p172 thus occurs at a differentiation junction interposed between these events. The study of p172 expression may lead to further understanding of the process of gene activation and regulation during type II pneumocyte differentiation and type II to type I cell transdifferentiation.

The p172 antigen has increased expression during hyperoxic lung injury. Western blot analysis of whole lung cell membrane fractions of normoxic and hyperoxic rats demonstrates increased p172 in hyperoxic lungs. In addition, inspection of tissue sections by immunohistochemistry reveals that there are apparently more cells expressing p172 in the alveolar space during injury. This could represent the increase in the number of type II cells seen during lung injury, as previously described in chronic oxygen toxicity (32, 33). An alternative explanation is that p172 expression is seen in type II cells transforming into type I cells which have yet to lose type II specific markers.

The question of increased quantitative expression during hyperoxia of p172 on a per-cell basis was addressed by a supra-optimal dilution study (18). This method permits the detection of differences between normal and hyperoxia-exposed rats by using a 3F9 antibody dilution above that used for conventional immunohistochemistry, i.e., a 1:200 dilution. This supra-optimal dilution showed persistent expression of p172 only in hyperoxic animals as higher 3F9 antibody dilutions were utilized, suggesting that more p172 is being expressed per cell during hyperoxia. A more direct method for determination of p172 expression per cell would have been alveolar type II cell isolation with quantitation by western blot. Unfortunately, p172 expression diminishes markedly during the isolation process. Although a quantitative explanation of the relative contribution of increased cell numbers and increased p172 expression on a per-cell basis is not currently available, it appears that both are operative.

Alveolar type II cells manifest a relative resistance to, and increased survival during, lung injury when compared to type I cells. This relative tolerance to injury is vital for the preservation and repair of the alveolar epithelium (34). The exact adaptive factors and functions that account for this resistance remain unclear. Other genes or factors that are increased during hyperoxia and are hypothesized to contribute to the injury-resistant phenotype include: surfactant and surfactant-associated proteins (35), ceruloplasmin (4), metallothionein (6), sodium conductive pathways (3, 36), and the sodium-ascorbic acid co-transport system (37). p172 upregulation could represent an additional adaptive response of the type II cell to injury.

We have described an alveolar type II and Clara cell membrane protein with specific developmental and injury regulation. Elucidation of the regulation of expression and function of this alveolar epithelial protein will increase our knowledge of alveolar and distal airway epithelial differentiation and adaptation to lung injury.

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