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Postnatal Changes in the Expression and Distribution of Pulmonary Cytochrome P450 Monooxygenases during Clara Cell Differentiation in Rabbits

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SUMMARY

Previous studies have indicated that both cytodifferentiation of Clara cells and the onset of pulmonary cytochrome P450 activity are postnatal events. However, the relationship between these two events during lung development remains poorly understood. To determine how these events interrelate, we examined rabbit Clara cells during postnatal differentiation, with the following goals in mind: 1) to identify the patterns of intracellular expression of cytochrome P450 monooxygenase isozymes 2B and 4B and cytochrome P450 reductase, 2) to describe the biogenesis of the organelles with which these isozymes are associated, namely smooth and rough endoplasmic reticulum, and 3) to compare the patterns of expression with cytochrome P450 activity in the whole lung over the same period. Lungs of rabbits ranging in age from 24 days gestational age (DGA) to 25 weeks postnatally were studied. Ultrastructural morphometry showed that smooth endoplasmic reticulum averaged <5% of the Clara cell volume in late gestational (24–30 DGA) and neonatal rabbits [0–7 days postnatally (DPN)], grew to 20–30% of the cell volume in 14–21-DPN animals, and approximated adult levels (>40%) in 28-DPN rabbits. In contrast, rough endoplasmic reticulum decreased from >10% of the cell volume at 27 DGA to <5% in adults. All postnatal animals showed considerable heterogeneity in the abundance of smooth endoplasmic reticulum among individual cells. Immunohistochemistry revealed that cytochrome P450 reductase appeared in Clara cells earlier (28 DGA) than did either isozyme 2B or 4B (1 DPN). Each antigen was detected first in the apical borders of the cells, then throughout the cytoplasm in a few cells by 7 DPN, and finally in adult abundance by 28 DPN. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting showed that cytochrome P450 protein concentrations increased postnatally. Cytochrome P450 heme protein was not detected spectrophotometrically in the lungs of animals younger than 3 DPN but increased to approximately 70% of adult levels by 28 DPN. Likewise, cytochrome P450 activity (measured as ethoxy- and pentoxyresorufin *O*-dealkylation) was not detected in animals younger than 2 DPN but increased to approximately 75% of adult levels by 28 DPN. We conclude that, in rabbits, 1) pulmonary cytochrome P450 monooxygenase activity begins in the perinatal period and attains adult levels after 28 DPN, 2) cytochrome P450 protein expression appears and differentiates postnatally in Clara cells, 3) the appearance of smooth endoplasmic reticulum and the expression of cytochrome P450 protein do not occur uniformly in differentiating Clara cells even in the same bronchiole, and 4) the biogenesis of endoplasmic reticulum precedes the expression of cytochrome P450 protein in differentiating Clara cells.

The adult mammalian lung is a major target for bioactivated environmental toxicants and carcinogens. Of the >40 cell types within the lung, the nonciliated bronchiolar (Clara) cell is susceptible to injury from the widest range of compounds, including furans (1, 2), polycyclic aromatic hydrocarbons (3), and chlorinated hydrocarbons (4). This susceptibility is thought to result from the abundance of P450 monooxygenases in Clara cells (5–7) and the activity of these monooxygenases for a wide range of substrates (8, 9). Indeed, recent studies have demonstrated a close correlation between the susceptibility of Clara cells to injury and the pulmonary activity of substrate-specific P450 isozymes (3, 10). What is not known, however, is how these findings relate to the susceptibility of immature animals during pre- and postnatal development. Existing information about the P450 monooxygenase system indicates that the presence of P450 protein (or mRNAs for P450 proteins) does not necessarily ensure that the system is active. For example, significant P450 monooxygenase activity has not been detected in the lungs of fetuses or newborns (11–16) even though the lungs have been found to contain P450 mRNAs (17–19) and

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ABBREVIATIONS: P450, cytochrome P450; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; DPN, day(s) postnatal(ly); DGA, day(s) gestational age.

P450 proteins (14, 20). Furthermore, P450 proteins reach adult levels within Clara cells almost immediately after birth (20), whereas both P450 activity and Clara cell differentiation occur only after a significant period of time postnatally (21). Despite these important findings, little information exists about the development of the P450 system in relation to morphological changes associated with Clara cell maturation. In addition, much of the previous work has been performed in different species. For these reasons, we have undertaken a systematic study to define the temporal relationship between Clara cell maturation, the expression of P450 protein, and the onset of pulmonary monooxygenase activity in a single species, the rabbit.

Materials and Methods

Animals and lung preparation. Male New Zealand White rabbits, free of chronic respiratory disease as judged by gross examination and histopathology, were used in this study. Postnatal animals were obtained from respiratory disease-free colonies maintained by the University of California, Davis, or by Dutchland Laboratories (Denver, PA). Animals were anesthetized with pentobarbital sodium (1 mg/kg), tracheotomized, and killed by exsanguination. For ultrastructural morphometry, the thorax was opened and the lungs were infused with glutaraldehyde/paraformaldehyde in cacodylate buffer (22) (adjusted to pH 7.4 and 330 mOsm), at 30 cm of water pressure, via a tracheal cannula. After 2–3 hr, the fixed lungs and trachea were removed from the chest and stored in the same fixative until processing. For immunohistochemistry, lungs were fixed for 1 hr with 1% paraformaldehyde in 0.1 M phosphate buffer, via a tracheal cannula. The lungs were removed, sliced, and stored in phosphate buffer before embedding in paraffin. For gel electrophoresis, immunoblotting, and P450 activity assays, lungs were collapsed after thoracotomy, the pulmonary arterial trunk was catheterized, the lungs were expanded with air, and blood was flushed from the lungs through the pulmonary arteries with phosphate-buffered saline. Lungs were considered free of blood when they appeared white on the pleural surfaces and blood was no longer present in the fluid leaving the heart through an incision in the left atrium.

Prenatal animals were obtained by laparotomy from time-mated pregnant rabbits anesthetized with acepromazine (1 mg/kg), Rompun (5 mg/kg), and ketamine (50 mg/kg). As each fetus was removed, it was injected with pentobarbital sodium (1 mg/kg), the trachea was cannulated, and the thorax was opened. The fetal lungs were prepared for each assessment as described above. Gonads from all animals 14 DPN or younger were examined histologically, to determine sex. Only males were included in this study.

Ultrastructural morphometry was performed on lungs from at least three animals at each of the following ages: 24, 27, and 30 DGA; 1–2, 7, 14, 21, and 28 DPN; and adult (12–25 weeks postnatally) (Table 1). Immunohistochemistry and Western blot analysis were performed with three to nine animals at each of the following ages: 28 DGA; 1–2, 7, 14, and 28 DPN; and adult (12–25 weeks postnatally). P450 monooxygenase activities were assayed in three to five pools of lungs from two to five animals or four to six individual animals at the following ages: 28 DGA and 1, 2, 3, and 7 DPN. P450 monooxygenase activities were assayed in lungs of six to 10 animals at 14 and 28 DPN and adults (12–25 weeks postnatally).

TABLE 1

Rabbit ages, number of animals, number of terminal bronchioles, and number of Clara cells evaluated by morphometry

Age	No. of animals	No. of terminal bronchioles	No. of cells
24 DGA	4	8	120
27 DGA	3	6	104
30 DGA	3	16	93
1–2 DPN	3	15	115
7 DPN	3	13	94
14 DPN	3	13	88
28 DPN	3	13	102
12–25 weeks	17	56	170

Transmission electron microscopy and morphometry. Fixed right caudal lobes were sliced into 2–4 mm-thick pieces and examined under a Wild M-8 dissecting microscope. Slices containing terminal bronchioles were postfixed for 2 hr in 1% osmium tetroxide and embedded in Araldite-502 following a process that allows selection of specific areas from large tissue faces (22). One-micron-thick sections were cut with glass knives on a Sorval JB4 microtome and were stained with toluidine blue. Areas of the block faces containing terminal bronchiole/alveolar duct junctions were excised, remounted, and thin-sectioned (30–50 nm) with diamond knives on a Sorval MT5000 ultra microtome. The sections were then stained with uranyl acetate/lead citrate and examined with a Zeiss EM10 transmission electron microscope at 60 kV. Morphometric data were collected from transmission electron micrographs (magnification, 15,750×) of Clara cells whose profiles included the basal lamina, nucleus, and luminal apex. Every cell that met these criteria was evaluated. Table 1 summarizes the number of terminal bronchioles examined per group and the number of cells counted per age group. At least two terminal bronchioles were sampled and a minimum of 30 cells were evaluated from each animal. The volume densities of SER and RER were estimated by point-count stereology using the method of Weibel (23). A Weibel 168-point multipurpose test grid was laid over each micrograph; all points falling on cell cytoplasm, SER, or RER (cisterna and membrane) were counted. The volume density of the nucleus was also determined by point counting. Volume densities of SER and RER were calculated as a percentage of cell volume. Estimates were calculated on a per-animal basis and summarized as mean ± 1 SD. We then determined the population distribution of SER within each age group by arranging the cells into six classes based on their volume percentages of SER. All the cells from each age were then summed, and the percentage of cells falling into each class was determined.

Immunohistochemistry. Fixed lungs were sliced into 1–4-mm-thick slices and stored in phosphate buffer before embedding. Slices containing terminal bronchioles and/or lobar bronchi were embedded in paraffin and stained for components of the P450 monooxygenase system using antibodies produced against purified rabbit pulmonary P450 isozymes 2B and 4B and P450 reductase. The antibodies were produced in goats and have been characterized previously (24). The avidin-biotin peroxidase reagents used to detect the location of components of the P450 monooxygenase system were obtained in kit form from Vector Laboratories (Burlingame, CA). Hydrated sections were treated with 3% H_2O_2 to block endogenous peroxidase and were then incubated for 24 hr at 10° with antibodies to the P450 monooxygenase proteins. Dilutions of these antibodies ranged from 1/500 to 1/5000. The avidin-biotin procedure followed the dilutions outlined by the supplier of the reagents. Controls included the substitution of primary antibody with sera from nonimmunized goats or with phosphate-buffered saline. Various other reagents were deleted to examine the tissues for endogenase peroxidase activity and other nonspecific reactivity.

Gel electrophoresis and immunoblotting. Fresh lungs were homogenized in Tris-buffered saline and centrifuged at 9000 × *g*. Supernatant proteins (25 μg/lane) were electrophoresed on 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate, transferred to nitrocellulose, and immunoblotted with the anti-P450 isozyme antibodies described above. Binding of the primary antibody was revealed using rabbit anti-goat peroxidase-antiperoxidase, as described previously (25). Immunoreactivity of blots from 9000 × *g* supernatant fractions prepared from fetal and postnatal animals was compared with that of microsomal fractions obtained from adult lung homogenates by centrifugation at 100,000 × *g*.

Assessment of P450 monooxygenase activity. P450 monooxy-

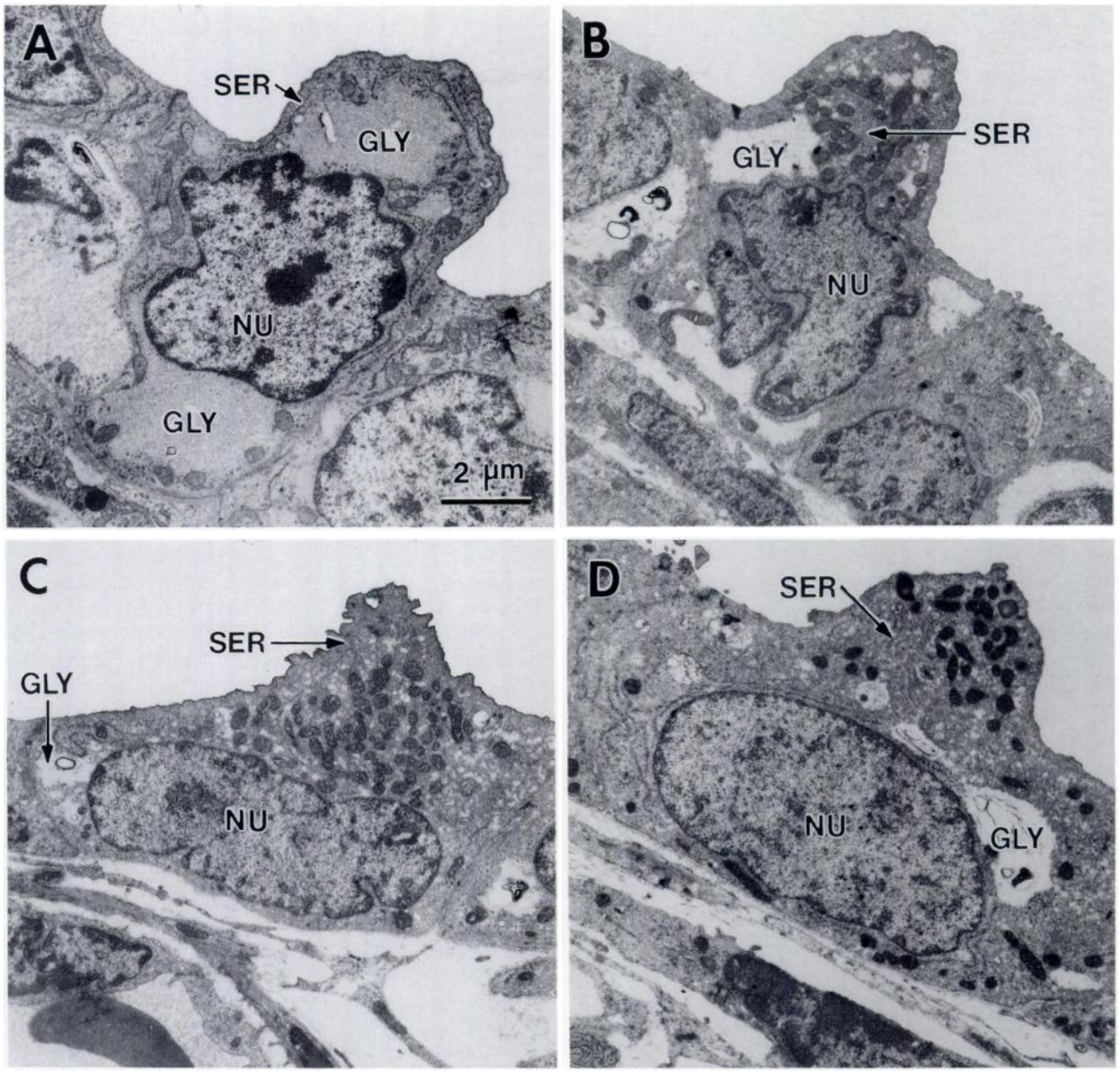

Fig. 1. Ultrastructural comparisons of changes in organelle abundance in Clara cells of terminal bronchioles of pre- and postnatal rabbits. A, 27 DGA. The majority of the cell cytoplasm is filled with glycogen (*GLY*). The nucleus (*NU*) is centrally located and the few organelles present are found predominantly at the apical edges of the cytoplasm. B, 1 DPN. In perinatal animals, SER proliferates in the apical portion of the cytoplasm and appears to displace cytoplasmic glycogen. C, 14 DPN. SER and other organelles fill the majority of the apical cytoplasm; small amounts of glycogen are present. D, Adult. In animals older than 28 DPN, SER fills a significant portion of the cytoplasm on the apical and lateral portions of the nucleus. Glycogen occupies a significantly smaller portion of the cell cytoplasm.

genase activity was measured in lung microsomes prepared by differential ultracentrifugation. Microsomal pellets were washed once. P450 was assayed in dithionite-reduced microsomal suspensions bubbled with CO. An extinction coefficient of 105 mM^{-1} cm^{-1} was used for the calculations (26). Protein was measured according to the method of Bradford (27).

The dealkylation of ethoxy- and pentoxyresorufin was measured in microsomal incubations containing an NADPH-generating system, microsomal protein (125 μg), and substrate (1 μM), in a total volume of 1 ml. Preliminary studies demonstrated that under these conditions activity was linear with respect to time and microsomal protein concentration. Incubations were conducted for 6 min at 37° and reactions were quenched with 2 volumes of ice-cold methanol. Protein was removed by centrifugation and aliquots of the supernatant were injected onto a C_{18} Novapak column (0.8 × 10 cm). Resorufin, eluting at 13–15 min, was measured by fluorescence (λ_{ex}, 535 nm; λ_{em}, 585 nm). Standard curves, prepared by adding resorufin to hepatic microsomal protein, were linear from 2 to 200 pmol/incubation.

Results

Cellular abundance of endoplasmic reticulum. As shown in Fig. 1A, glycogen was the principal cytoplasmic constituent in fetal nonciliated bronchiolar cells. In 24-DGA fetuses, the nucleus occupied a prominent place in the center of these cells and little SER could be detected (Fig. 2). About 7% of the cell volume was composed of RER, most of which was located in the apex. By 27 DGA, nonciliated cell apices contained small profiles of SER, which were continuous with RER.

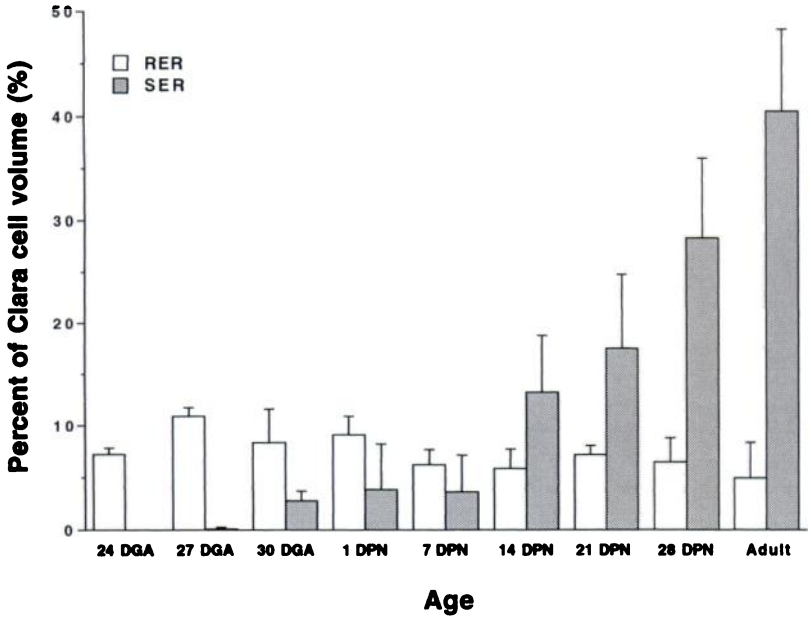

Fig. 2. Morphometric comparison of the abundance of SER and RER in nonciliated bronchiolar epithelial (Clara) cells of terminal bronchioles of rabbits. In fetal animals (24–30 DGA), SER averages <5% of cell volume. The percentage of SER increases from birth to adulthood. In contrast, the percentage of RER decreases with age. All values are mean ± 1 SD.

The average proportion of cell volume occupied by SER at this age was 0.2% (Fig. 2). In all of the cells counted at 24 and 27 DGA, SER occupied <10% of cell volume (Fig. 3A), whereas RER occupied about 10% of cell volume at 27 DGA (Fig. 2).

Nonciliated cells in perinatal animals (30 DGA, 1 DPN, and 7 DPN) contained similar amounts of SER (Fig. 1B). SER was restricted to the apical portions of the cytoplasm and averaged <5% of cell volume (Fig. 2), although the amount of SER present varied substantially within the cell population (Fig. 3, B and C). In this age range, SER occupied <10% of cell volume in >80% of the cells counted. Less than 10% of the cells contained between 20% and 30% SER. In 1-DPN and 7-DPN animals, a small percentage of the cells (<5%) contained SER in an abundance similar to that observed in adults (>30% of cell volume) (Fig. 3F). RER was observed in the cytoplasm lateral to the nucleus and averaged <10% of cell volume.

In 14–21-DPN animals, the proportion of the cell volume occupied by SER nearly doubled (Fig. 2) and the percentage of the cell population in which SER occupied >20% of cell volume increased markedly (Fig. 3D). Furthermore, the abundance of SER in the nonciliated cell population was more heterogeneous (compare Fig. 3, C and D). The ultrastructural features of individual cells also differed from those observed in animals younger than 7 DPN (Fig. 1C).

In animals 28 DPN and older, the predominant cytoplasmic organelle was SER (Fig. 1D). SER averaged 28% (28 DPN) or more (40% in adult) of cell volume (Fig. 2). In >70% of the cells, SER occupied >30% of cytoplasmic volume (Fig. 3, E and F). In a small portion of the cell population (<10%) SER occupied <10% of cytoplasmic volume.

Distribution of immunoreactive P450 protein. In late gestational age fetuses (28 DGA), P450 protein for isozymes 2B and 4B could not be detected by immunohistochemistry in bronchiolar cells located at the junction of the terminal bronchiole and the alveolar duct (Fig. 4, A and G). There was reaction product in the endothelial cells of capillaries in adjacent interalveolar septa. Immunoreactive P450 reductase was detected in the apices of some bronchiolar epithelial cells in 28-DGA fetuses (Fig. 4M). The reactions, although not strong, were more intense than those observed in substitution controls (Fig. 4S). In contrast, epithelial cells of the lobar bronchi in the same lungs contained abundant amounts of reductase antigen and antigen for isozymes 2B and 4B (Fig. 5, A, G, and M).

In the terminal bronchioles of 1–2-DPN animals, protein for P450 isozyme 2B was detected in the epithelial cells lining terminal bronchioles (Fig. 4B). Reaction product was present only in the apices of some nonciliated cells. On serial sections from the same bronchiole, isozyme 4B was detected in most nonciliated cells (Fig. 4H). In some cells reaction product appeared throughout the cytoplasm surrounding the nucleus; in others it was restricted to the apex. By comparison, more cells at this age contained antigen for reductase (Fig. 4N), which was detected in the cytoplasm of most nonciliated cells. All three components of the P450 system were detected in abundance in epithelial cells of the lobar bronchus (Fig. 5, B, H, and N).

Late postnatal animals (7–14 DPN) showed an increase in the abundance and distribution of P450 protein within nonciliated bronchiolar cells in terminal bronchioles. The reaction product was strongest for isozyme 4B (Fig. 4, I and J) and reductase (Fig. 4, O and P). Fewer cells contained reaction product for isozyme 2B (Fig. 4, C and D). Animals at 14 DPN showed more positive cells and heavier labeling than did the younger 7-DPN animals. All three components of the P450 system were present in abundance in epithelial cells of the lobar bronchus at 7 DPN (Fig. 5, C, I, and O) and were little changed at 14 DPN (Fig. 5, D, J, and P).

In animals 28 DPN and older, virtually all nonciliated cells contained reaction product for isozymes 2B and 4B and reductase. The product was distributed in the cell apices and on the lateral portions of the nucleus. The primary difference between 28-DPN animals (Fig. 4, E, K, and Q) and adults (Fig. 4, F, L, and R) was the size of the nonciliated cells. In all animals 28 DPN and older, reaction product in the lobar bronchi resembled that in adults for all three isozymes (Fig. 5, E-F and K-R).

Immunoreactive P450 protein in lung homogenates. Reductase was barely detectable by Western blotting in 9000

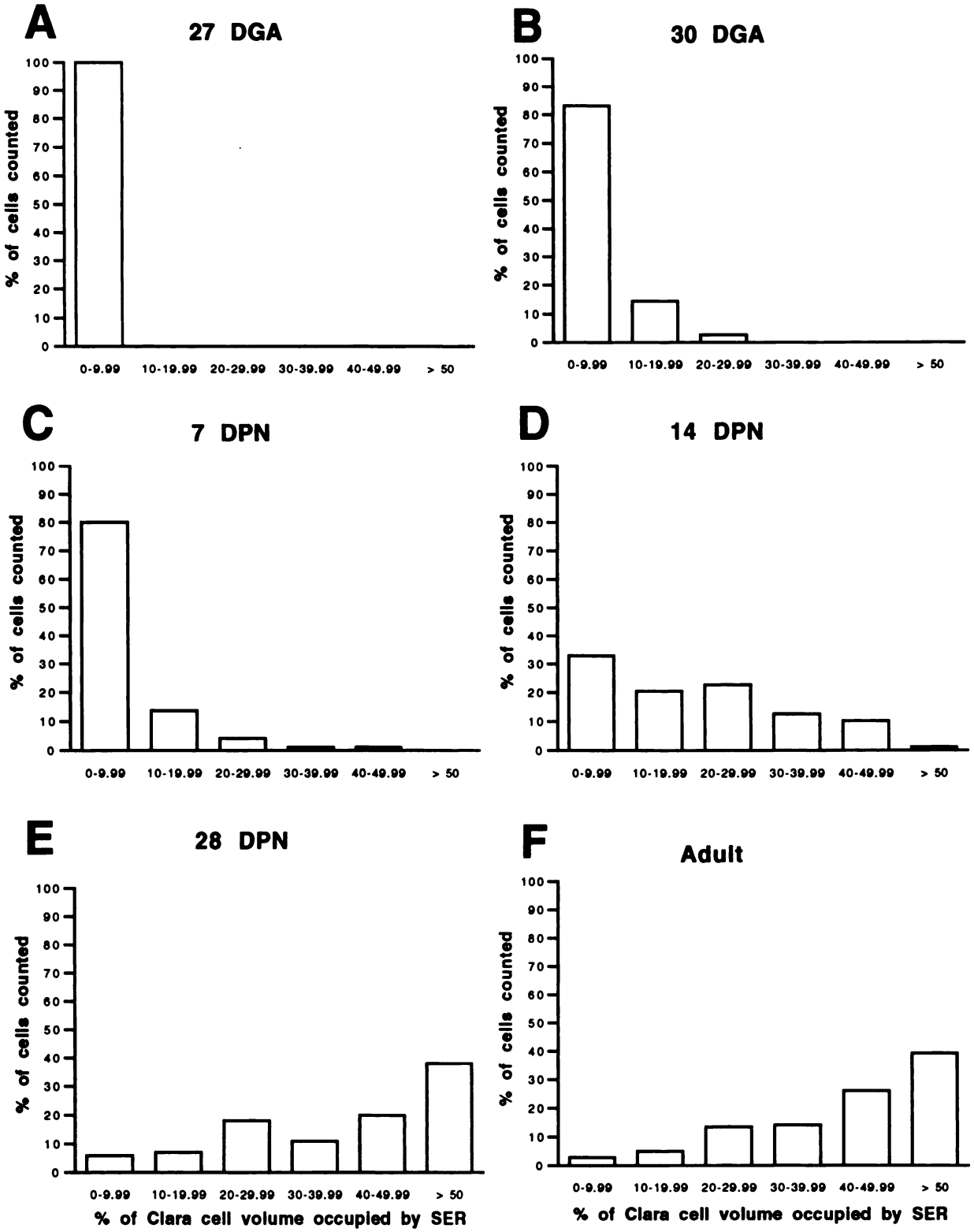

Fig. 3. Moprhometric comparison of the differing abundance of SER among Clara cells in pre- and postnatal rabbit lungs. The cells were grouped into six classes based on the cell volume occupied by SER. Each class represents an increase of 10%. The largest class included all cells in which SER occupied >50% of the cytoplasm. In fetal animals of 24–30 DGA, SER occupied <10% of the cytoplasmic volume in 80% or more of the cells. All postnatal animals, including neonates (1–2 DPN), showed a wide heterogeneity in the fraction of cytoplasmic volume occupied by SER. The number of cells included in the graph for each age was as follows: 24 DGA, 130; 27 DGA, 117; 30 DGA, 99; 7 DPN, 94; 14 DPN, 91; 28 DPN, 102; adult, 141.

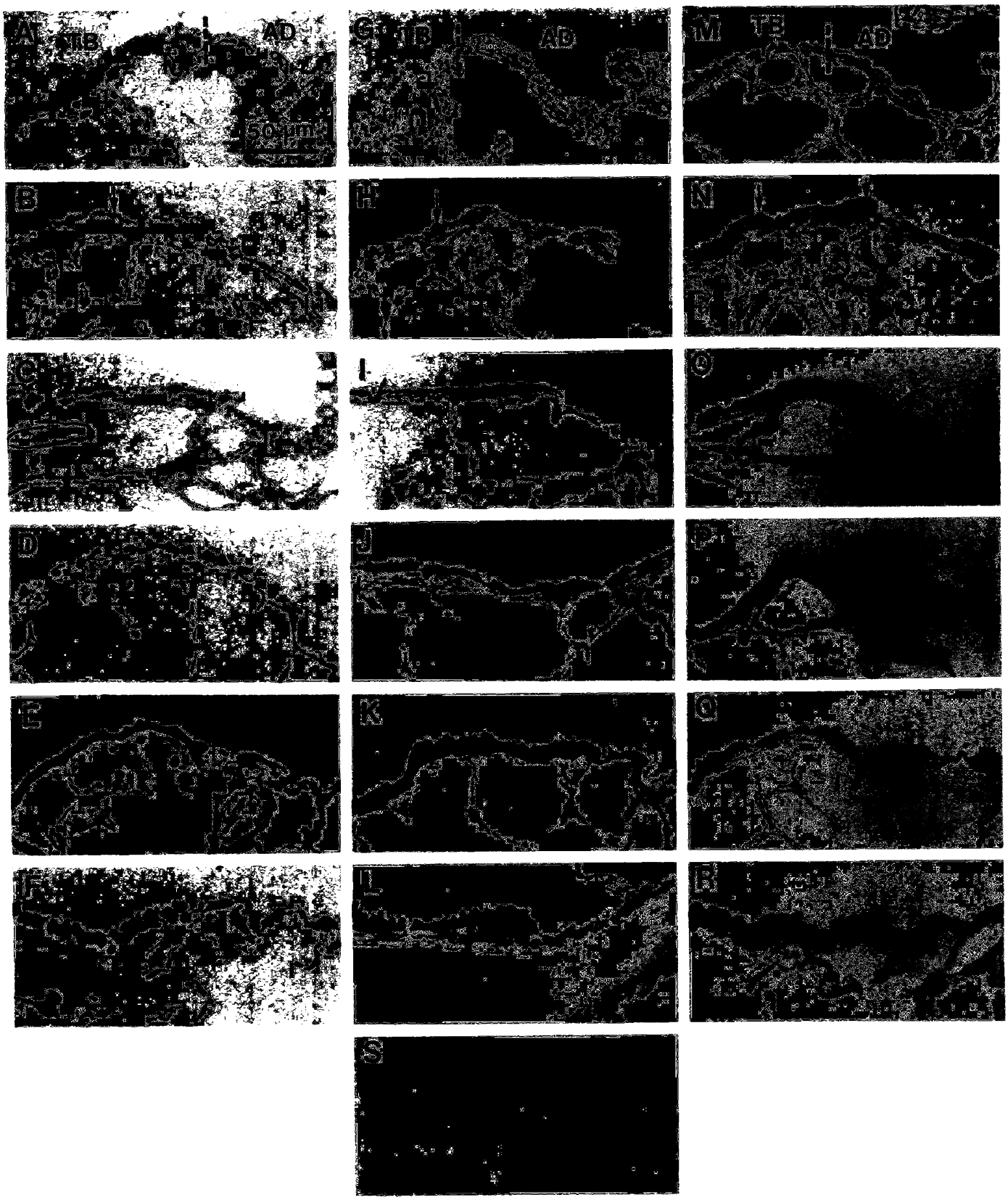

Fig. 4. Immunohistochemical comparison of expression of proteins for P450 monooxygenase isozymes 2B and 4B and reductase in terminal bronchiole epithelium. A-F, Distribution of P450 isozyme 2B. G-L, Distribution of P450 isozyme 4B. M-R, Distribution of reductase. A, G, and M, 28 DGA. *Line*, boundary between terminal bronchiole (*TB*) and alveolar duct (*AD*). B, H, and N, 2 DPN. *Arrow in B*, apical distribution of P450 2B; *arrows in H and N*, cells with P450 distribution in both apical and lateral portions of the cells. C, I, and O, 7 DPN. D, J, and P, 14 DPN. E, K, and Q, 28 DPN. F, L, and R, adult. S, Substitution control from 14-DPN animal.

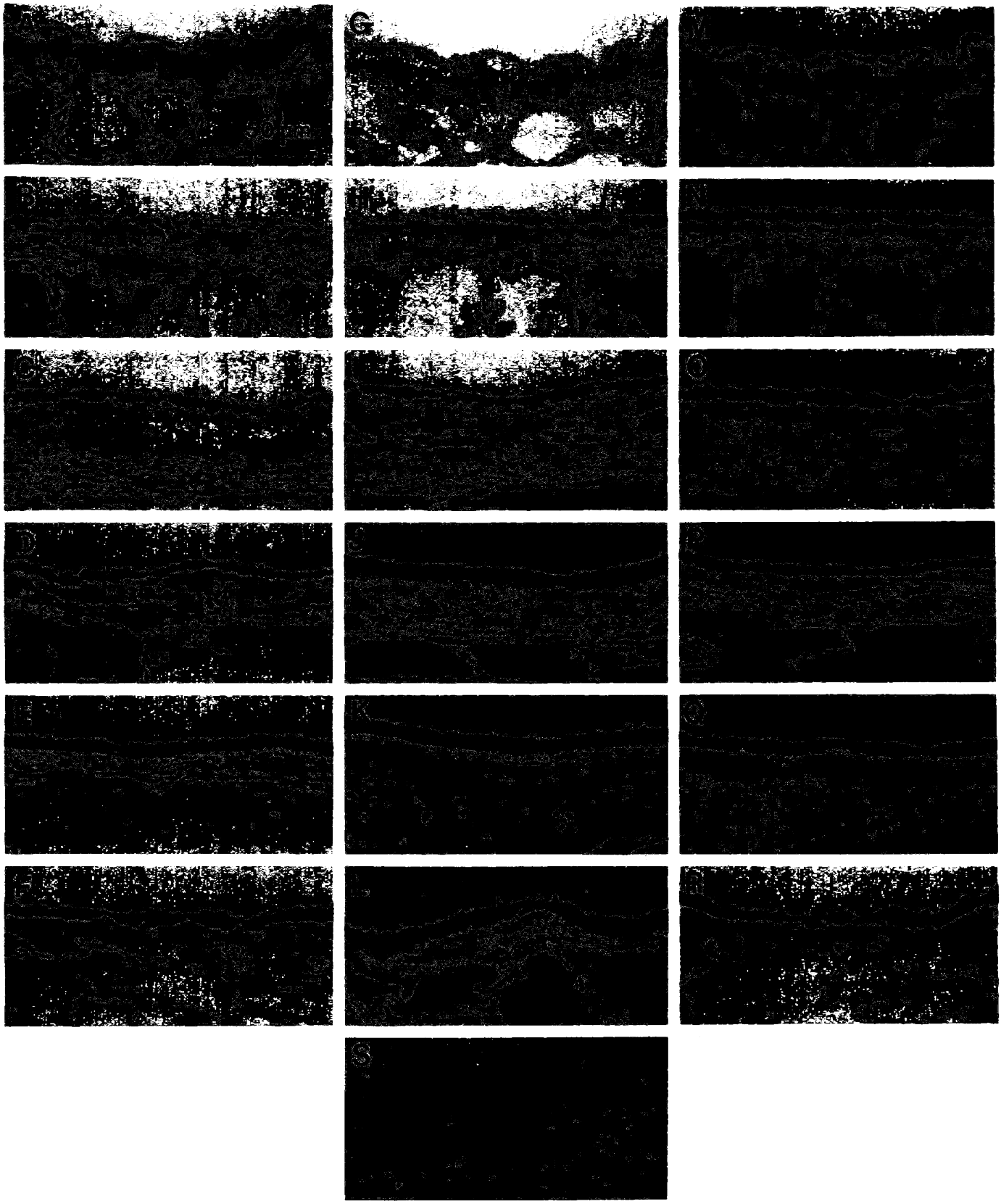

Fig. 5. Immunohistochemical distribution of P450 monooxygenase isozymes 2B and 4B and reductase in the lobar bronchus of animals illustrated in Fig. 4. A-F, Distribution of P450 isozyme 2B. G-L, Distribution of P450 isozyme 4B. M-R, Distribution of reductase. S, Deletion control. A, G, and M, 28 DGA. B, H, and N, 2 DPN. C, I, and O, 7 DPN. D, J, and P, 14 DPN. E, K, and Q, 28 DPN. F, L, and R, Adult.

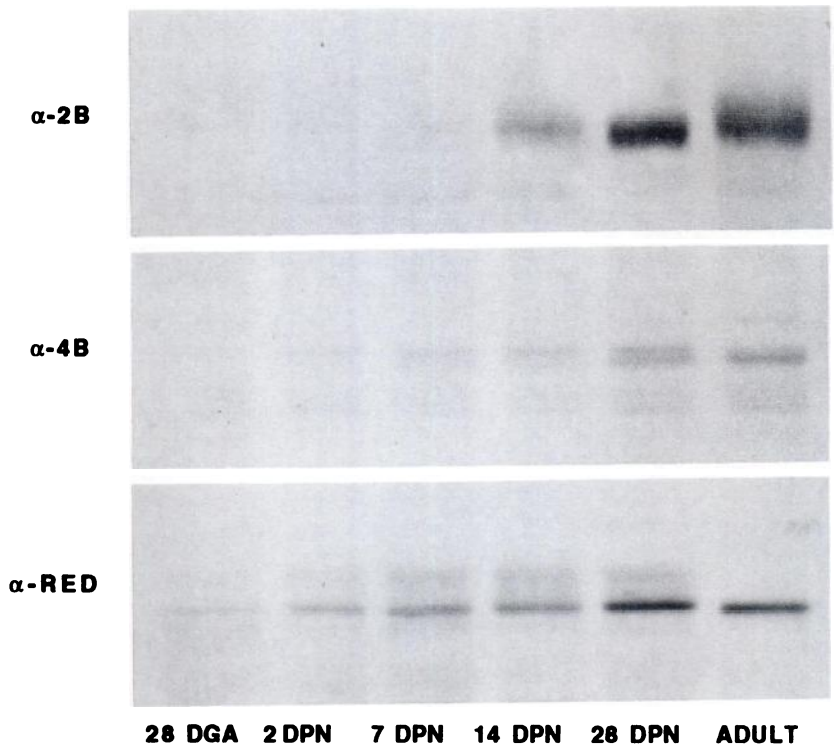

Fig. 6. Detection of immunoreactive protein for components of the P450 monooxygenase system in 9000 × *g* supernatants derived from whole-lung homogenates, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis/Western blots. P450 isozyme 2B was not detected in Western blots before 7 DPN. Isozyme 4B was detectable at 2 DPN and reached adult density at 28 DPN. Reductase (*RED*) was detected at 28 DGA and reached adult intensity at approximately 28 DPN. Twenty-five micrograms of protein were run in each lane.

× *g* supernatant from the 28-DGA animals (Fig. 6). It was clearly present in lungs of 2-DPN animals and reached a density comparable to that in adults by 28 DPN. Isozyme 4B was detectable at 2 DPN, whereas isozyme 2B was undetectable in lungs of animals younger than 7 DPN; both reached adult density by 28 DPN.

P450 monooxygenase in lung homogenates. P450 heme protein was not detected spectrophotometrically in lung microsomes from 28-DGA fetuses or neonates (1 and 2 DPN) (Fig. 7A); however, it was detected in animals 3 DPN and older. The abundance of P450 was still below adult levels at 28 DPN. Ethoxyresorufin *O*-dealkylation activity was not detected in prenatal or 1-DPN animals (Fig. 7B). In animals 14 DPN and younger, activity was <20% of that observed in adults. At 28 DPN, activity doubled and was closer to adult levels. Pentoxyresorufin *O*-dealkylation activity was not detected in the lungs of animals 1 DPN and younger (Fig. 7C). In 14-DPN animals, activity was approximately one third of adult levels. By 28 DPN, activity had doubled but was still less than that observed in adults. For both ethoxy- and pentoxyresorufin *O*-dealkylation, activity was higher in lungs of 3-DPN animals than in 7-DPN animals.

Discussion

Our study defines cytodifferentiation of the nonciliated bronchiolar epithelial, or Clara, cell in terms of expression of the P450 monooxygenase system. The changes are summarized in Table 2. We have shown, in rabbit lung, that Clara cell cytodifferentiation and the expression of the P450 system are entirely postnatal events. Biogenesis of SER, the principal organellar site of the P450 system, is initiated in the apex of the cell immediately before birth, with most of the biogenesis occurring in the first 4 weeks of life. We also noted significant heterogeneity in the abundance of SER among Clara cells even in adult animals. This heterogeneity implies that the biogenesis of SER is not uniform even for nonciliated cells sharing the same basal lamina. Protein components for the P450 system (isozymes 2B and 4B and reductase) first appear in Clara cells of perinatal animals. These three enzymes are expressed at different times during development, with reductase appearing first, followed by isozyme 4B and then isozyme 2B. The heterogeneity in cellular distribution of the endoplasmic reticulum is also reflected in the distribution of antigenic protein during Clara cell differentiation. Clearly, there are subpopulations of Clara cells sharing the same basal lamina that differentiate more rapidly than their neighbors, in terms of protein expression and SER biogenesis. The antigenic protein within Clara cells reaches the adult configuration at approximately the same time (4 weeks) as does the endoplasmic reticulum. P450 monooxygenase activity is not detectable in lung homogenates until several days after the proteins are detectable in bronchiolar epithelial cells. The specific activity of the protein is very low in young postnatal animals and increases with age. This pattern suggests that the endoplasmic reticulum synthesized early in Clara cell cytodifferentiation has a much lower density of P450 proteins than does the endoplasmic reticulum present in adults. We found a close correlation between the postnatal biogenesis of endoplasmic reticulum, the expression of P450 proteins, and the activity of these proteins during the postnatal cytodifferentiation of Clara cells in rabbit lung.

The pattern in which these changes occur clearly indicates that there are a variety of different processes involved in the cytodifferentiation of this particular system in Clara cells. One process is the increase in the density of P450 proteins in endoplasmic reticulum and the increasing functionality of these proteins as Clara cells differentiate. Molecular studies measuring gene products for polycyclic aromatic hydrocarbon-inducible forms of the P450 system in rat lung also indicate that

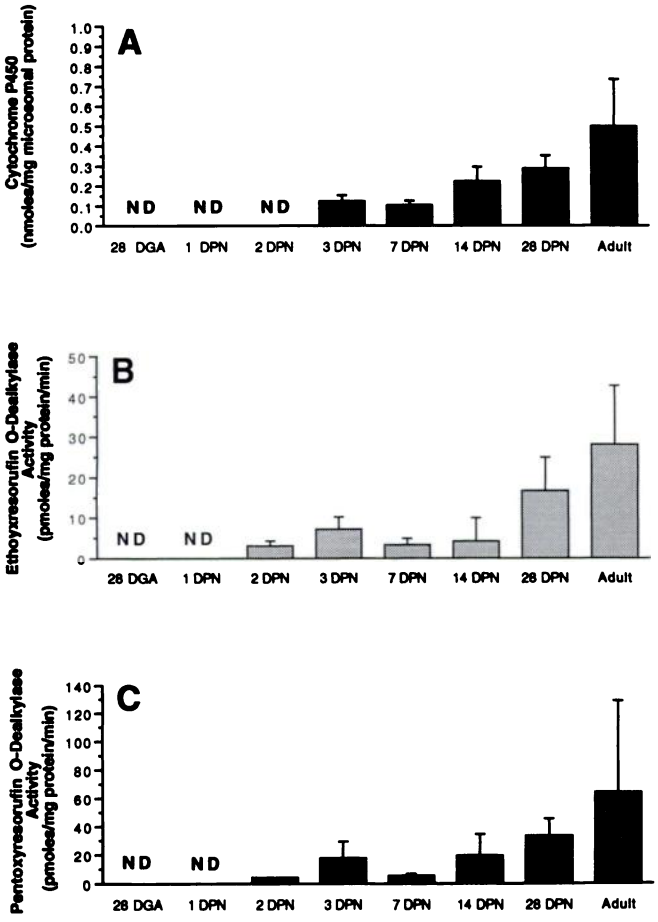

Fig. 7. Changes in pulmonary P450 activity for ethoxyresorufin *O*-dealkylase and pentoxyresorufin *O*-dealkylase during postnatal lung development in rabbits. All values are mean ± 1 SD. *ND*, not detected. A, P450 heme protein could not be detected by spectrophotometry in animals younger than 3 DPN. The specific density (n mol/mg of microsomal protein) increased with age. B, Activity for ethoxyresorufin *O*-dealkylase was not detected before 2 DPN and remained at very low levels for the first 2 weeks of postnatal life. Activity more than doubled between 14 and 28 DPN and doubled again in adults. C, Pentoxyresorufin *O*-dealkylase activity was not detected before 2 DPN and remained at low levels between 2 and 14 DPN. Activity doubled between 14 and 28 DPN and increased again in adult animals.

gene expression is a perinatal phenomenon (19). Another study, also in rats (14), indicates that both protein and gene expression for certain isozymes of the P450 system appear well after birth. An additional study in rats showed detectable levels of P450 mRNAs prenatally, which dropped at birth and increased to adult amounts by 30 DPN (18); however, this observation could not be confirmed using the polymerase chain reaction (17). Unfortunately, a study of P450 gene expression in rabbit lung during development is not available.

The results from this work and our previous studies on Clara cell differentiation in rabbits, together with studies in other species, indicate that the pattern and timing of Clara cell cytodifferentiation is species specific. Alterations in the cellular composition of SER, as measured by ultrastructural characteristics, occur postnatally in rabbit Clara cells. Qualitative studies in hamsters (20) indicate that this process occurs primarily prenatally. In rats, RER and secretory granules, cellular constituents that are characteristic of biosynthesis and secretion of uterglobin-like protein, differentiate very rapidly (28). Whether this is the case for SER has not been studied (28, 29). In adult rats, SER occupies 30–40% of the cell cytoplasmic volume, as it does in rabbits (29). There are species, such as nonhuman primates, in which the nonciliated bronchiolar cells in adults have minimal amounts of SER but substantial amounts of RER (30, 31). Cells in this population undergo cytodifferentiation both pre- and postnatally (32). The relationship between the appearance of SER and cell-specific expression of P450 proteins has been evaluated in only one other species, the hamster (20). Our study shows that when SER is undergoing rapid biogenesis there is a small lag before the appearance of immunochemically detectable P450 protein. The apical distribution of protein in the early developmental stages matches the distribution of endoplasmic reticulum at those stages. In hamsters, there appears to be a 2-day lag before the appearance of immunodetectable protein (20). As in rabbits, Clara cells in adult hamsters have approximately 40% of their cytoplasm filled with SER (30). Quantitative data on Clara cell differentiation in hamsters are not available.

In rabbits, there is a substantial increase in SER before P450s are detected in bronchiolar Clara cells. The assumption from our study is that the cells in which more protein is detected are the ones in which endoplasmic reticulum is expressed in larger amounts. The delay between formation of endoplasmic reticulum and the detection of protein within cells could be the result of at least two factors. The first of these is that the abundance of proteins is too low to be immunologically detectable when the endoplasmic reticulum is <10% of cell volume. However, the apical distribution of endoplasmic reticulum matches the distribution of immunoreactive product in those cells, suggesting that this is not the case. The second factor is that the endoplasmic reticulum formed initially does not contain P450s. This pattern was not reported for hamsters (20).

Our study shows a clear distinction in the expression of different proteins that make up the P450 monooxygenase system in rabbits. Reductase appears in more cells earlier than does either isozyme 2B or 4B. This difference in appearance is also detectable by Western blot in whole-lung microsomes. The timing of expression in hamsters differs from that in rabbits, with reductase and isozyme 2B appearing at the same time prenatally and isozyme 4B appearing 1 day later (20).

The development of P450 monooxygenase activity in rabbit lung closely matches the pattern of appearance of immunoreactive protein within Clara cells. Previous developmental studies of the activities of pulmonary P450s are not extensive. Our finding that P450 activity increases with postnatal age agrees with results from a previous study in rabbits, using different substrates (15), which demonstrated a 3-fold increase in the specific activity of the protein during the first 12 weeks after birth. Our study extends this observation into both the prenatal and the immediate postnatal periods and shows that P450 protein is not detectable by spectrophotometric assay earlier than 3 DPN. Further, the specific activity of isozyme 2B, measured by the *O*-dealkylation of pentoxyresorufin, is very low during the first week of life and is not detectable immediately pre- or postnatally. Previous studies showed no detectable isozyme 2B activity before 14 DPN (15), which may be due to differences in the relative rates of substrate turnover in the two studies or to a lack of sensitivity of the analytical methods used to detect product.

One of the most surprising findings from our study was the

TABLE 2

Development of SER, P450 reductase, and monooxygenase enzymes in rabbit lung

	Assay	Amount or activity					
		27–28 DGA	1–2 DPN	7 DPN	14 DPN	28 DPN	Adult
		% of adult value					
SER[a,b]		0.2	8.2	8.2	30.1	64.5	100
P450 reductase	Immunohistochemistry[b,c]	±	+	++	++	++++	++++
	Western blot	+	+	++	++	++++	++++
P450 isozyme 2B	Immunohistochemistry[b]	0	±	+	++	++++	++++
	Western blot	0	0	±	+	++++	++++
P450 isozyme 4B	Immunohistochemistry[b]	0	+	++	++	++++	++++
	Western blot	0	±	+	++	+++	++++
Microsomal P450[d]		0	0	20.9	44.5	56.0	100
P450 activity	Ethoxyresorufin[e]	0	10.8	11.7	14.9	59.3	100
	Pentoxyresorufin[f]	0	6.5	8.1	29.9	51.8	100

[a] Average adult cell volume for SER is 43.9 ± 3.5%.
[b] Bronchiolar epithelium.
[c] Symbols indicate staining intensities in relation to adult (++++).
[d] Average adult level of microsomal P450 is 0.575 ± 0.238 nmol/mg of microsomal protein.
[e] Average adult ethoxyresorufin *O*-dealkylase activity is 47.99 ± 14.69 pmol/mg of protein/min.
[f] Average adult pentoxyresorufin *O*-dealkylase activity is 64.07 ± 64.51 pmol/mg of protein/min.

lack of uniform differentiation between individual cells, even in the same bronchiole. Our previous immunocytochemical study in rabbits (5) and others in adult rats (33) suggested that there is a small population of cells that lack large amounts of SER and immunoreactive P450. Our cell-by-cell evaluation of the proportion of Clara cells containing SER substantiates that there is indeed a portion (approximately 10%) of the population in adults that has virtually no SER. Applying this analytical approach to developmental studies illustrates how heterogeneous the differentiation process actually is and provides some perspective on the rate at which Clara cells differentiate. As the animals age, there is a substantial increase in Clara cells in which SER occupies a large (>30%) portion of the cytoplasm. This increase suggests that the biogenesis of SER in the Clara cells takes as long as 4 weeks to reach adult configuration. This pattern is also true for protein expression within the cells. The nature of the heterogeneity is not understood. Whether it reflects different rates or stages of differentiation or different parts of the cell cycle within a population of cells with the same potential for phenotypic expression is an unanswered question. The distribution and abundance of protein on a cell-by-cell basis become much more homogeneous as animals reach adulthood. The variability in P450 content among Clara cells in adults may be the basis for the intercellular differences in susceptibility of Clara cells to P450-mediated toxicants. We have observed differences in susceptibility to injury by naphthalene in Clara cells of adult mice (3). It is not known how this heterogeneity and lack of P450 monooxygenase activity affect fetal and postnatal animals, but our findings indicate that there could be altered susceptibility to cytotoxicants in peri- and postnatal animals.

In conclusion, as summarized in Table 2, quantitative assessment of endoplasmic reticulum, in conjunction with a definition of the cellular distribution of P450 protein and measurements of P450 activity, reveals that the differentiation of the P450 monooxygenase system in Clara cells lags behind the rate at which SER is generated. Specifically, biogenesis of endoplasmic reticulum precedes the expression of P450 proteins. Our results also indicate that endoplasmic reticulum formed early in development lacks the same density of P450 proteins as that in adults and that the specific activity of these proteins is well below the levels found in the endoplasmic reticulum of adults.

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References

1. Dutcher, J. S., and M. R. Boyd. Species and strain differences in target organ alkylation and toxicity by 4-ipomeanol: predictive value of covalent binding in studies of target organ toxicities by reactive metabolites. *Biochem. Pharmacol.* **28**:3367–3372 (1979).
2. Doster, A. R., R. L. Farrell, and B. J. Wilson. An ultrastructural study of bronchiolar lesions in rats induced by 4-ipomeanol, a product from mold-damaged sweet potatoes. *Am. J. Pathol.* **111**:56–61 (1983).
3. Plopper, C., C. Suverkropp, D. Morin, S. Nishio, and A. Buckpitt. Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. I. Histopathologic comparison of the respiratory tract of mice, rats and hamsters after parenteral administration of naphthalene. *J. Pharmacol. Exp. Ther.* **261**:353–363 (1992).
4. Boyd, M. R., C. N. Statham, and W. S. Longo. The pulmonary Clara cell as a target for toxic chemicals requiring metabolic activation: studies with carbon tetrachloride. *J. Pharmacol. Exp. Ther.* **212**:109–114 (1980).
5. Serabjit-Singh, C. J., S. J. Nishio, R. M. Philpot, and C. G. Plopper. The distribution of cytochrome P-450 monooxygenase in cells of the rabbit lung: an ultrastructural immunocytochemical characterization. *Mol. Pharmacol.* **33**:279–289 (1988).
6. Baron, J., J. P. Burke, F. P. Guengerich, W. B. Jakoby, and J. M. Voigt. Sites for xenobiotic activation and detoxication within the respiratory tract: implications for chemically induced toxicity. *Toxicol. Appl. Pharmacol.* **93**:493–505 (1988).
7. Forkert, P. G., M. L. Vessey, J. S. Elce, S. S. Park, H. V. Gelboin, and S. P. C. Cole. Localization of phenobarbital- and 3-methylcholanthrene-inducible cytochromes P-450 in mouse lung with monoclonal antibodies. *Res. Commun. Chem. Pathol. Pharmacol.* **53**:147–157 (1986).
8. Devereux, T. R., B. A. Domin, and R. M. Philpot. Xenobiotic metabolism by isolated pulmonary cells. *Pharmacol. Ther.* **41**:243–256 (1989).
9. Chichester, C. H., R. M. Philpot, A. J. Weir, A. R. Buckpitt, and C. G. Plopper. Characterization of the cytochrome P-450 monooxygenase system in nonciliated bronchiolar epithelial (Clara) cells isolated from mouse lung. *Am. J. Respir. Cell. Mol. Biol.* **4**:179–186 (1991).
10. Buckpitt, A., M. Buonarati, L. Avey, A. Chang, D. Morin, and C. Plopper. Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. II. Comparison of stereoselectivity of naphthalene epoxidation in lung and nasal mucosa of mouse, hamster, rat and rhesus monkey. *J. Pharmacol. Exp. Ther.* **261**:364–372 (1992).
11. Devereux, T. R., and J. R. Fouts. Effect of dexamethasone treatment on *N,N*-dimethylaniline demethylation and *N*-oxidation in pulmonary microsomes from pregnant and fetal rabbits. *Biochem. Pharmacol.* **27**:1007–1008 (1977).
12. Fouts, J. R., and T. R. Devereux. Developmental aspects of hepatic and extrahepatic drug-metabolizing enzyme systems: microsomal enzymes and components in rabbit liver and lung during the first month of life. *J. Pharmacol. Exp. Ther.* **183**:458–468 (1972).
13. Melikian, A. A., K. Bagheri, S. S. Hecht, and D. Hoffmann. Metabolism of benzo(*a*)pyrene and 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-

benzo(*a*)pyrene in lung and liver of newborn mice. *Chem. Biol. Interact.* **69**:245–257 (1989).
14. Tornquist, S., M. Sundin, L. Moller, J. Gustafsson, and R. Toftgard. Age dependent expression of cytochrome P-450b and the metabolism of the potent carcinogen 2-nitrofluorene in the rat lung. *Carcinogenesis (Lond.)* **9**:2209–2214 (1988).
15. Parandoosh, Z., and M. R. Franklin. Developmental changes in rabbit pulmonary cytochrome P-450 subpopulations. *Life Sci.* **33**:1255–1260 (1983).
16. Miller, M. S., A. B. Jones, D. P. Chauhan, S. S. Park, and L. M. Anderson. Differential induction of fetal mouse liver and lung cytochromes P-450 by *beta*-naphthoflavone and 3-methylcholanthrene. *Carcinogenesis (Lond.)* **10**:875–883 (1989).
17. Giachelli, C. M., and C. J. Omiecinski. Developmental regulation of cytochrome P-450 genes in the rat. *Mol. Pharmacol.* **31**:477–484 (1987).
18. Simmons, D. L., and C. B. Kasper. Quantitation of mRNAs specific for the mixed-function oxidase system in rat liver and extrahepatic tissues during development. *Arch. Biochem. Biophys.* **271**:10–20 (1989).
19. Omiecinski, C. J., C. A. Redlich, and P. Costa. Induction and developmental expression of cytochrome P450IA1 messenger RNA in rat and human tissues: detection by the polymerase chain reaction. *Cancer Res.* **50**:4315–4321 (1990).
20. Strum, J. M., T. Ito, R. M. Philpot, A. M. DeSanti, and E. M. McDowell. The immunocytochemical detection of cytochrome P-450 monooxygenase in the lungs of fetal, neonatal, and adult hamsters. *Am. J. Respir. Cell. Mol. Biol.* **2**:493–501 (1990).
21. Plopper, C. G., J. L. Alley, C. J. Serabjit-Singh, and R. M. Philpot. Cytodifferentiation of the nonciliated bronchiolar epithelial (Clara) cell during rabbit lung maturation: an ultrastructural and morphometric study. *Am. J. Anat.* **167**:329–357 (1983).
22. Plopper, C. G. Structural methods for studying bronchiolar epithelial cells, in *Models of Lung Disease: Microscopy and Structural Methods* (J. Gil, ed.). Marcel Dekker, Inc., New York, 537–559 (1990).
23. Weibel, E. R. *Stereological Methods*. Academic Press, New York, 1–415 (1979).
24. Serabjit-Singh, C. J., C. R. Wolf, and R. M. Philpot. The rabbit pulmonary monooxygenase system: immunochemical and biochemical characterization of enzyme components. *J. Biol. Chem.* **254**:9901–9907 (1979).
25. Domin, B. A., C. J. Serabjit-Singh, and R. M. Philpot. Quantitation of rabbit cytochrome P-450, form 2, in microsomal preparations bound directly to nitrocellulose paper using a modified peroxidase-immunostaining procedure. *Anal. Biochem.* **136**:390–396 (1984).
26. Matsubara, T., M. Koike, A. Touchi, Y. Tochino, and K. Sugeno. Quantitative determination of P450 in rat liver homogenate. *Anal. Biochem.* **75**:596–603 (1976).
27. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**:248–254 (1976).
28. Massaro, G. D., L. Davis, and D. Massaro. Postnatal development of the bronchiolar Clara cell in rats. *Am. J. Physiol.* **247**:C197-C203 (1984).
29. Massaro, G., and D. Massaro. Development of bronchiolar epithelium in rats. *Am. J. Physiol.* **250**:R783-R788 (1986).
30. Plopper, C. G., D. M. Hyde, and A. R. Buckpitt. Clara cells, in *The Lung: Scientific Foundations* (R. G. Crystal and J. B. West, eds.). Raven Press, New York, 215–226 (1991).
31. Tyler, N. K., and C. G. Plopper. Morphology of the distal conducting airways in rhesus monkey lungs. *Anat. Rec.* **211**:295–303 (1985).
32. Tyler, N. K., D. M. Hyde, A. G. Hendrickx, and C. G. Plopper. Cytodifferentiation of two epithelial populations of the respiratory bronchiole during fetal lung development in the rhesus monkey. *Anat. Rec.* **225**:297–309 (1989).
33. Keith, I. M., E. B. Olson, Jr., N. M. Wilson, and C. R. Jefcoate. Immunological identification and effects of 3-methylcholanthrene and phenobarbital on rat pulmonary cytochrome P-450. *Cancer Res.* **47**:1878–1882 (1987).

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