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Development of Phase II Xenobiotic Metabolizing Enzymes in Differentiating Murine Clara Cells

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Glutathione *S*-transferases (GSTs) and epoxide hydrolases (EHs) protect cells from exogenous insult by detoxifying electrophilic compounds. Little is known about these enzyme systems during postnatal lung development. This study was designed to help establish whether the heightened neonatal susceptibility of the lung to bioactivated cytotoxicants is the result of inadequate ability to detoxify reactive intermediates. We compared the distribution of immunoreactive protein and enzymatic activity of GSTs and EHs in isolated distal airways during pre- and postnatal development in lungs of mice from 16 days gestation to 9 weeks postnatal age (adult). GST alpha, mu, and pi class protein expression in fetal and postnatal lung varied by isozyme and age. Isozymes alpha and mu are expressed at low levels before birth, high levels on postnatal day 7, low levels between postnatal days 14 and 21, high levels at postnatal day 28, and slightly lower levels in adults. Immunoreactive protein of isozyme pi has a peak expression on gestational day 18 and again on postnatal day 4, is undetectable at postnatal day 21, and is at peak levels in the adult mouse lung. GST activity in distal airways increased with age. Microsomal EH protein expression increased in intensity with age, while activity was similar in airways from all ages. We conclude that in the mouse lung (1) cellular expression of glutathione *S*-transferase varies by age and isozyme and does not increase with increasing age, (2) airway glutathione *S*-transferase activity increases with increasing age and does not correlate with immunoreactive protein expression, and (3) airway microsomal epoxide hydrolase activity does not increase, even though immunoreactive protein expression does increase with age. © 2000 Academic Press

Key Words: **glutathione *S*-transferase; epoxide hydrolase; lung development.**

Lung disease is *the* leading cause of death in infants under 1 year of age (American Lung Association, 1999). Epidemiological studies implicate exposure of infants to cigarette smoking a cause in the increase of childhood respiratory diseases (Ware *et al.*, 1984); in the decrease in pulmonary function, which persists into adulthood (Berkey *et al.*, 1986; Cunningham *et al.*, 1994, 1995, 1996; Hanrahan and Halonen, 1998; Wang *et al.*, 1994); and in an increased risk for sudden infant death syndrome (Elliot *et al.*, 1998; Klonoff-Cohen *et al.*, 1995). Cigarette smoke contains many compounds, many of which are bioactivated toxicants (Witschi *et al.*, 1997). However, there have been no definitive studies as yet that identify which compounds or what mechanism may be the cause of these childhood pulmonary problems.

Recent studies with laboratory animals have established that neonates are much more susceptible to pulmonary injury from bioactivated environmental toxicants than are adults. Neonatal rabbits are much more vulnerable to pulmonary injury by the P450-activated furan 4-ipomeanol than are adult rabbits in a dose- and age-dependent manner (Plopper *et al.*, 1994; Smiley-Jewell *et al.*, 2000). This single acute injury to bronchiolar epithelial cells early during neonatal differentiation results in abnormal small airway structure that is still evident in adult rabbits (Smiley-Jewell *et al.*, 1998). There is also an age-related increase in susceptibility to pulmonary injury by the P450-activated polyaromatic hydrocarbon naphthalene in mice (Fanucchi *et al.*, 1997a). Seven-day-old mice are more susceptible to pulmonary injury than are 14-day-old mice, which in turn are more susceptible than are adult mice. Heightened neonatal pulmonary susceptibility occurs, even though levels of P450 activity are low in the developing neonatal lung when compared to the high P450 activity of the mature adult lung (Fanucchi *et al.*, 1997b; Ji *et al.*, 1995; Plopper *et al.*, 1993). This suggests that the increased susceptibility of neonatal animals could be the result of an imbalance of activating and detoxifying enzymes, resulting in a decreased ability to detoxify even small amounts of electrophilic intermediates.

One cellular mechanism to detoxify electrophilic intermediates is conjugation to glutathione. There are many instances

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where conjugation is nonenzymatic (e.g., 4-ipomeanol), but in some cases, such as for naphthalene, this conjugation is enzymatically mediated by glutathione *S*-transferases, which are a family of dimeric cytosolic enzymes grouped into three classes based on isoelectric points: alpha (basic), mu (near-neutral), and pi (acidic) (for reviews see Mannervik, 1985; Mannervik and Danielson, 1988). A small number of studies describe increases in the catalytic activity of glutathione *S*-transferases in whole lung perinatally in both humans and mice (Fryer *et al.*, 1986; Rouet *et al.*, 1984), but whether these increases occur in sites where reactive intermediates are likely to be formed has not been addressed.

Another critical enzyme involved in the detoxification of electrophilic metabolites is epoxide hydrolase. There are three general forms of epoxide hydrolase, each with a different substrate specificity and tissue distribution (Wixtrom and Hammock, 1985), but all produce 1,2-dihydrodiols from epoxides. The microsomal form is involved in the conversion of cyclic epoxides and is found in high levels in the smooth endoplasmic reticulum and in lower levels in other membranous organelles. Cytosolic epoxide hydrolase hydrates aliphatic epoxides and is found in the cytosol and peroxisomes. Cholesterol epoxide hydrolase is in the microsomal fraction and specifically catalyzes the hydration of cholesterol epoxides. As with glutathione *S*-transferase, data from humans and mice suggest that fetal lung contains much less epoxide hydrolase potential than adult lungs (Kaplowitz *et al.*, 1985), although where this expression occurs has not been evaluated.

This study was designed to help establish whether the heightened neonatal susceptibility to bioactivated cytotoxicants in the lung may be the result of an inadequate ability to detoxify reactive intermediates. To determine the pattern of phase II xenobiotic metabolizing enzyme expression, we examined the following during pre- and postnatal development: (1) the intracellular expression of pulmonary glutathione *S*-transferase isozymes alpha, mu, and pi; (2) the intracellular expression of microsomal and cytosolic epoxide hydrolases; and (3) the activities of these enzymes in isolated airway explants.

MATERIALS AND METHODS

Animals and lung preparation. Female timed-pregnant and male Swiss Webster mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). All animals were housed at least 7 days in laminar flow hoods in AAALAC-approved animal facilities at the University of California after receipt from the suppliers before being used in experiments. Free access to food and water was provided. Animals were anesthetized with pentobarbital sodium (60 mg/kg) and killed by exsanguination. Gender was determined by grossly examining gonads from all animals younger than 1 month. For immunohistochemical studies, a cannula was inserted in the trachea and the lungs were inflation-fixed for 1 h with 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1 M phosphate buffer at 30 cm pressure. The lungs were removed, sliced, and embedded in paraffin within 24 h of harvesting the tissue. For enzymatic assays, the lungs were removed from the chest cavity, inflated with 1% Compatigel agarose (FMC BioProducts, Rockland, ME) in Waymouth's MB/752/1 medium (Life Technologies, Grand Island, NY) at 37°C, and plunged into ice-cold Ham's F12 nutrient mixture (Life Technologies, Grand Island, NY) for 30 min. The terminal bronchioles were isolated by blunt dissection under a Wild M-8 stereomicroscope and placed in fresh F12.

Immunocytochemistry. Paraffin sections (5–6 microns thick) from three mice per age group were labeled for immunoreactive proteins of glutathione *S*-transferase using antibodies produced in rabbits against purified human alpha, mu, and pi class isozymes (Novocastra Laboratories, UK). Antibodies to recombinant rat microsomal epoxide hydrolase (a gift from Franz Oesch) and recombinant mouse cytosolic epoxide hydrolase were produced in rabbit. Sections from each age group were run together to eliminate variability. Hydrated sections were treated with 3% H_2O_2 to block endogenous peroxidase and were then incubated for 24 h at 4°C with the above-mentioned antibodies. Dilutions of these antibodies ranged from 1:500 to 1:1250. Immunoreactive protein was visualized with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) using nickel-enhanced 3′,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO) as a chromagen. Controls included the substitution of primary antibody with sera from nonimmunized rabbits, or with phosphate-buffered saline. Fields were recorded on an Olympus Provis A052 microscope with a Sony digital photo camera attached to a Power Macintosh. Images were composed in Adobe Photoshop and printed on a Codonics NP-1600 printer.

Gel electrophoresis and immunoblotting. Fresh lungs and livers were homogenized in 4 volumes of Tris-buffered saline suspension buffer containing 1 mg/ml aprotinin and centrifuged at 9000*g*. The protein concentration for the liver supernatant was 34 μg per lane, and lung supernatant was run in three lanes: 17, 28, and 57 μg per lane. Supernatant proteins were electrophoresed on 15% Instacryl gels (Eastman Kodak, Rochester, NY) in the presence of sodium dodecyl sulfate, transferred to Immobilon (BioRad, Hercules, CA), and immunoblotted with anti-GST and anti-EH antibodies described earlier. Binding of the primary antibody was revealed using rabbit anti-goat peroxidase-antiperoxidase (Cappel, Durham, NC), as described previously (Domin *et al.*, 1984).

Glutathione S-transferase assay. Glutathione *S*-transferase activity was measured in 9000*g* supernatant fractions from microdissected distal airways ($n = 5$ per age group). Conjugation of glutathione to 2,4-chlorodinitrobenzene (CDNB) was measured by the method of Habig *et al.* (1974) and normalized to total protein content using bovine serum albumin as a standard (Lowry *et al.*, 1951). One unit of CDNB activity equals 1 μmol of glutathione adduct formed per minute.

Epoxide hydrolase assay. Microsomal epoxide hydrolase was measured in 9000*g* supernatant fractions from microdissected distal airways ($n = 3$ per age group). Hydrolysis of *cis*-stilbene oxide (CSO) was determined by radiometric partition assays (Gill *et al.*, 1983) with an incubation time of 60 min. Data were normalized to total protein content measured with NanoOrange (Molecular Probes, Eugene, OR) using bovine serum albumin as a standard.

Statistics. Quantitative metabolism data were evaluated for potential age effects using a one-way analysis of variance (ANOVA). Significant differences were determined by using a post hoc multiple comparison test (Bonnferoni–Dunn) to identify the source of variance. Statistical analyses were performed using the SigmaStat software program (Jandel Scientific, San Rafael, CA). The data are expressed as the group means $\pm$ SD.

RESULTS

Glutathione S-Transferase Isozyme mu

In the pseudoglandular stage of lung development (16 days gestational age [DGA]), there is light labeling over every cell in the lung, with a few airway cells more heavily labeled (Fig. 1A). In the canalicular (18 DGA) and saccular (19 DGA)

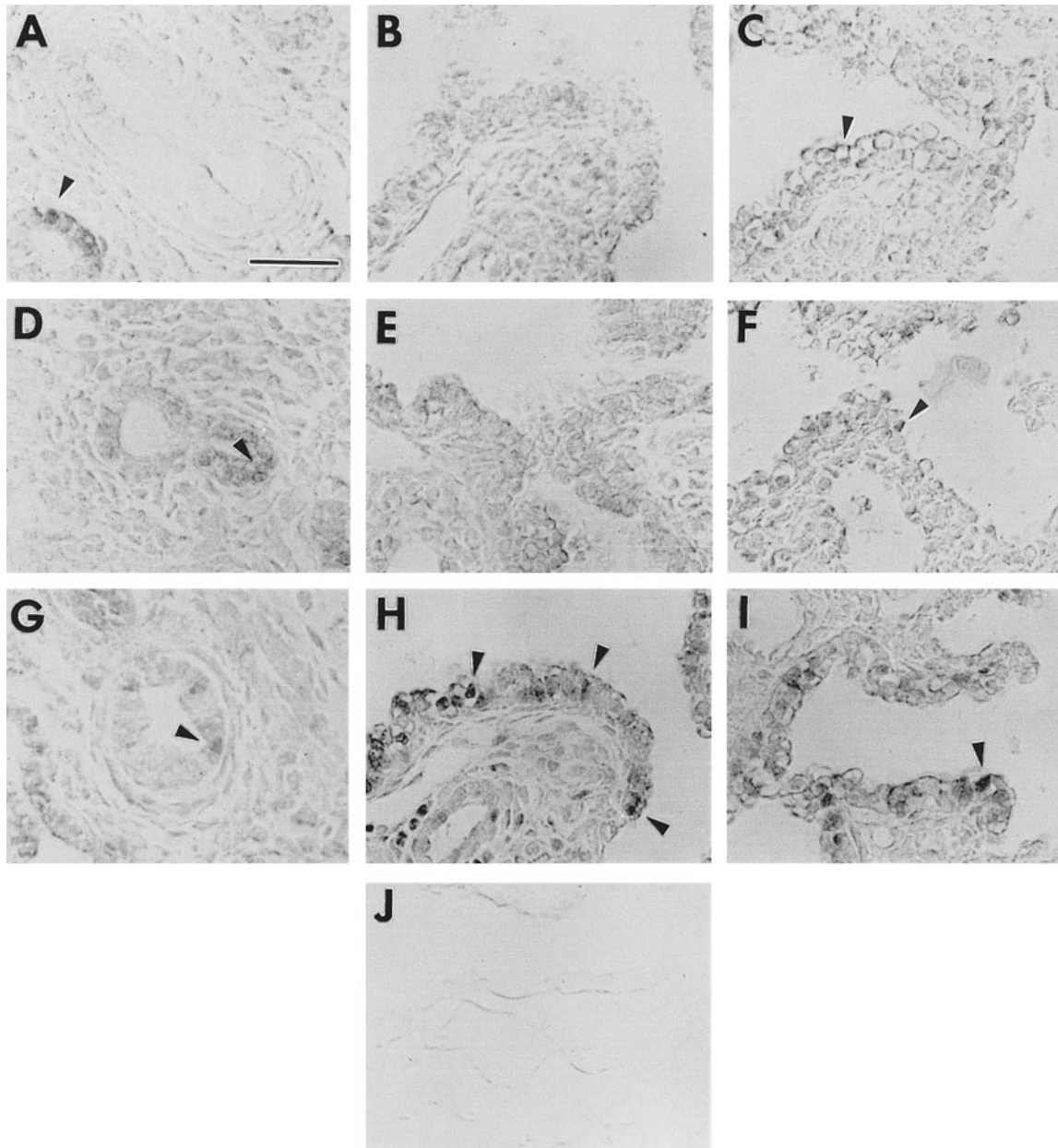

FIG. 1. Immunocytochemical localization of glutathione *S*-transferase (GST) isozyme proteins mu (A, B, C), alpha (D, E, F), and pi (G, H, I) in lungs of fetal mice at three stages of lung development: pseudoglandular (A, D, G), canalicular (B, E, H), and saccular (C, F, I). Paraffin sections from all ages were incubated at the same time with GST antibodies (1:500). Immunoreactive protein was detected earliest in nonciliated cuboidal airway cells in 16 DGA lungs (arrowheads). Fetal cells labeled positively for all GST isozymes. No labeled cells were detected in the phosphate-buffered saline control (J). Magnification bar represents 35 μm.

stages, the mesenchyme is also lightly labeled, whereas the nuclei and apical cytoplasm of the airway epithelial cells label slightly more intensely (Figs. 1B and 1C). In the early postnatal period (1–7 days), there is positive labeling in both ciliated and nonciliated cells (Figs. 2A–2C, 3A–3C). Most of the cells have heavily labeled nuclei and lighter labeled cytoplasm, but at 7 days some of the cells have cytoplasm that is darker than that in the nuclei. At 10 days postnatal the labeling is predominantly on the basal side of the cells and the nuclei in proximal airways (Fig. 4D). In distal airways, cells have less labeling than the early postnatal lungs (Fig. 2D). At 2 weeks postnatal there is very little positive staining in the distal cells (Fig. 2E) and very light labeling in the proximal airways (Fig. 4E). At 3 weeks the distal airways are lightly positive (Fig. 2F) and the proximal cells have more labeling on the luminal sides (Fig. 4F). Nonciliated cells are more heavily labeled than ciliated cells. At 4 weeks the proximal airways cells are very heavily labeled (Fig. 4G). The distal nonciliated cells are darker than

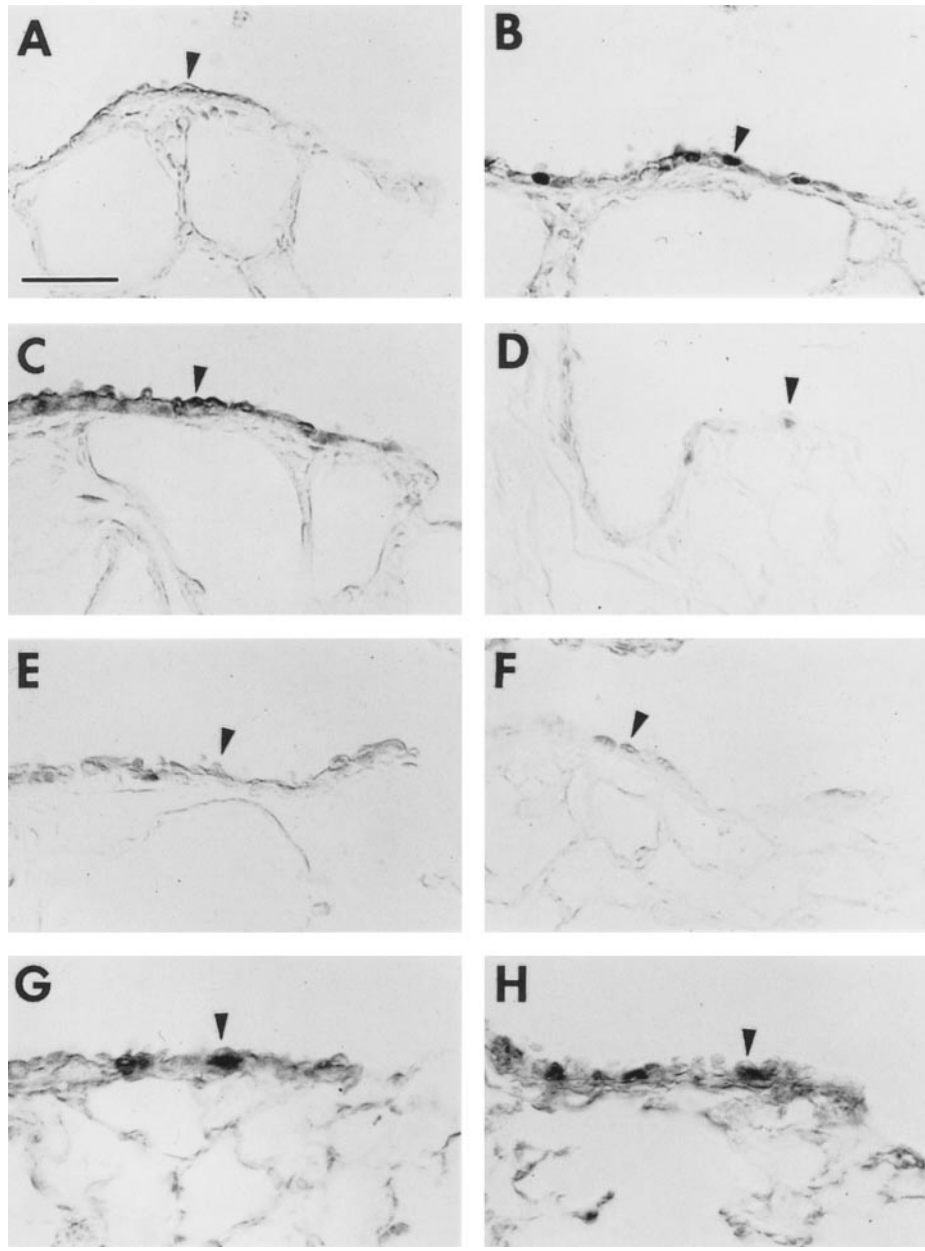

FIG. 2. Immunocytochemical localization of glutathione *S*-transferase (GST) isozyme proteins mu in terminal bronchioles of mice at the following ages: 1 (A), 4 (B), 7 (C), 10 (D), 14 (E), 21 (F), and 28 (G) days postnatal age, and adults (H). Paraffin sections from all ages were incubated at the same time with GST mu antibodies (1:500). Immunoreactive protein was detected in nonciliated cells (arrowheads). Magnification bar represents 35 μm.

the ciliated cells (Fig. 2G). In the adult the proximal ciliated cells are darker than the lightly labeled nonciliated cells (Fig. 4H). Distal airway cells are moderately positive (Fig. 2H).

Glutathione S-Transferase Isozyme alpha

During fetal lung development, there is light labeling in every cell in the lung (Figs. 1D–1F). In the early postnatal period the airway epithelial cells changed from having light positive labeling over the cells in the distal and proximal regions to having some dark labeling over apical regions in distal airways (Figs. 3A–3C) and all cells positive in the proximal airways (Figs. 5A–5C). At 10 days postnatal most distal and proximal airway cells are positive and have darkly labeled nuclei (Figs. 3D, 5D). At 2 weeks the distal airways are moderately positive (Fig. 3E). In the proximal airways the cilia are very dark (Fig. 3E). At 3 weeks the cells of the distal airway are lightly positive (Fig. 3F). The proximal cells are also lightly positive in the apical region (Fig. 5F). By 4 weeks

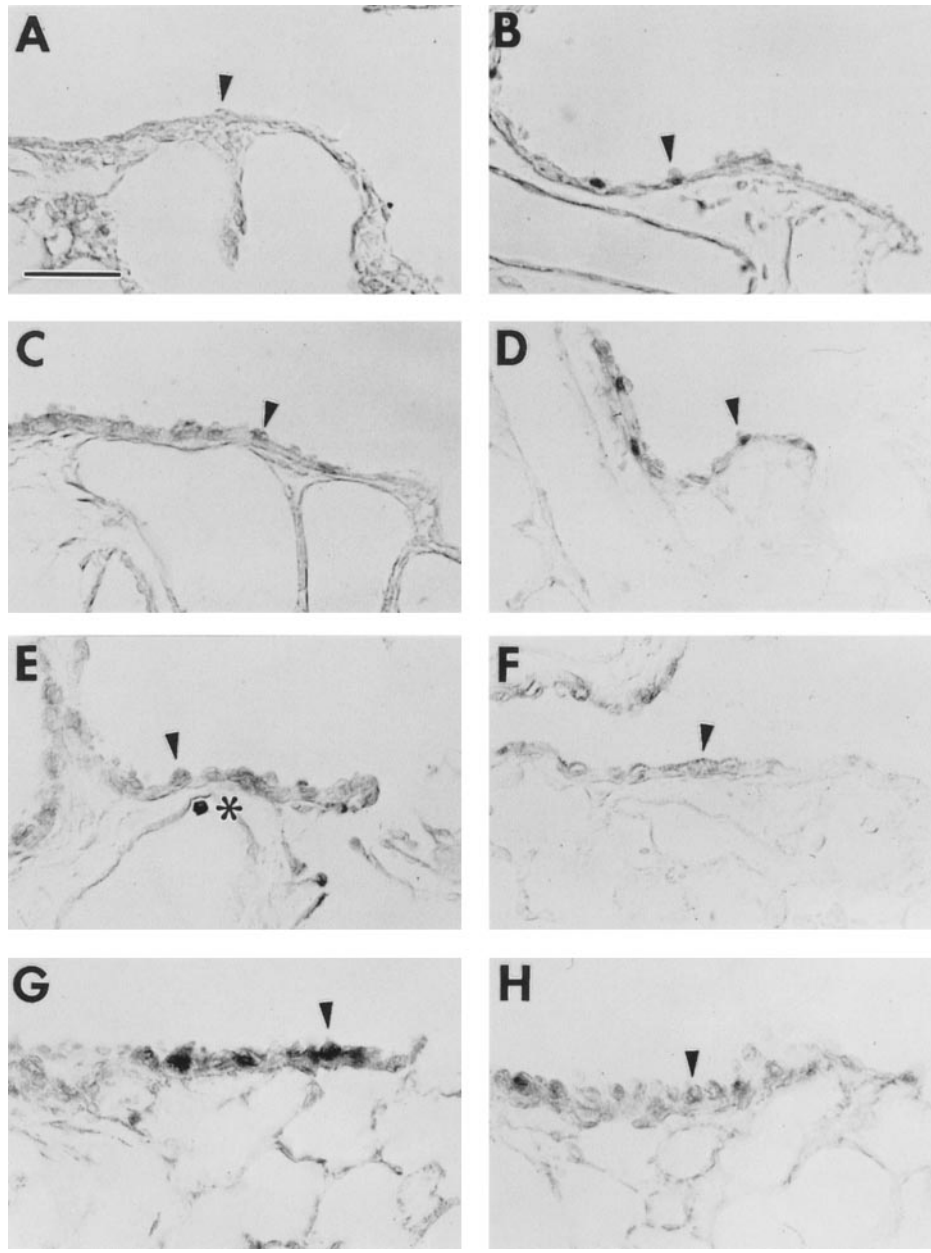

FIG. 3. Immunocytochemical localization of glutathione *S*-transferase (GST) isozyme proteins alpha in terminal bronchioles of mice at the following ages: 1 (A), 4 (B), 7 (C), 10 (D), 14 (E), 21 (F), and 28 (G) days postnatal age, and adults (H). Paraffin sections from all ages were incubated at the same time with GST mu antibodies (1:500). Immunoreactive protein was detected in nonciliated cells (arrowheads) and in an occasional alveolar macrophage (*). Magnification bar represents 35 μm.

the labeling increased again in both the distal and proximal airways (Figs. 3G, 5G).

Glutathione S-Transferase Isozyme pi

In the pseudoglandular stage there is light labeling over every cell in the lung, while the airway cells labeled slightly darker (Fig. 1G). Through the canalicular and saccular stages, the mesenchymal labeling decreases, whereas the nuclei and apical cytoplasm of the airway epithelial cells start to label more intensely (Figs. 1H and 1I). In the early postnatal period (1–7 days) there is positive labeling in type 2 alveolar epithelial cells (Figs. 6A–6C, 7A–7C). Ciliated and nonciliated epithelial cells in both distal and proximal airways are labeled positively. At 10 days postnatal, the proximal airway epithelium is moderately positive (Fig. 7D) and the distal airway epithelium is lightly positive (Fig. 6D). At 2 weeks the labeling decreases in both distal and proximal airways (Figs. 6E, 7E). The lowest amount of labeling occurs at 3 weeks. The distal airways have

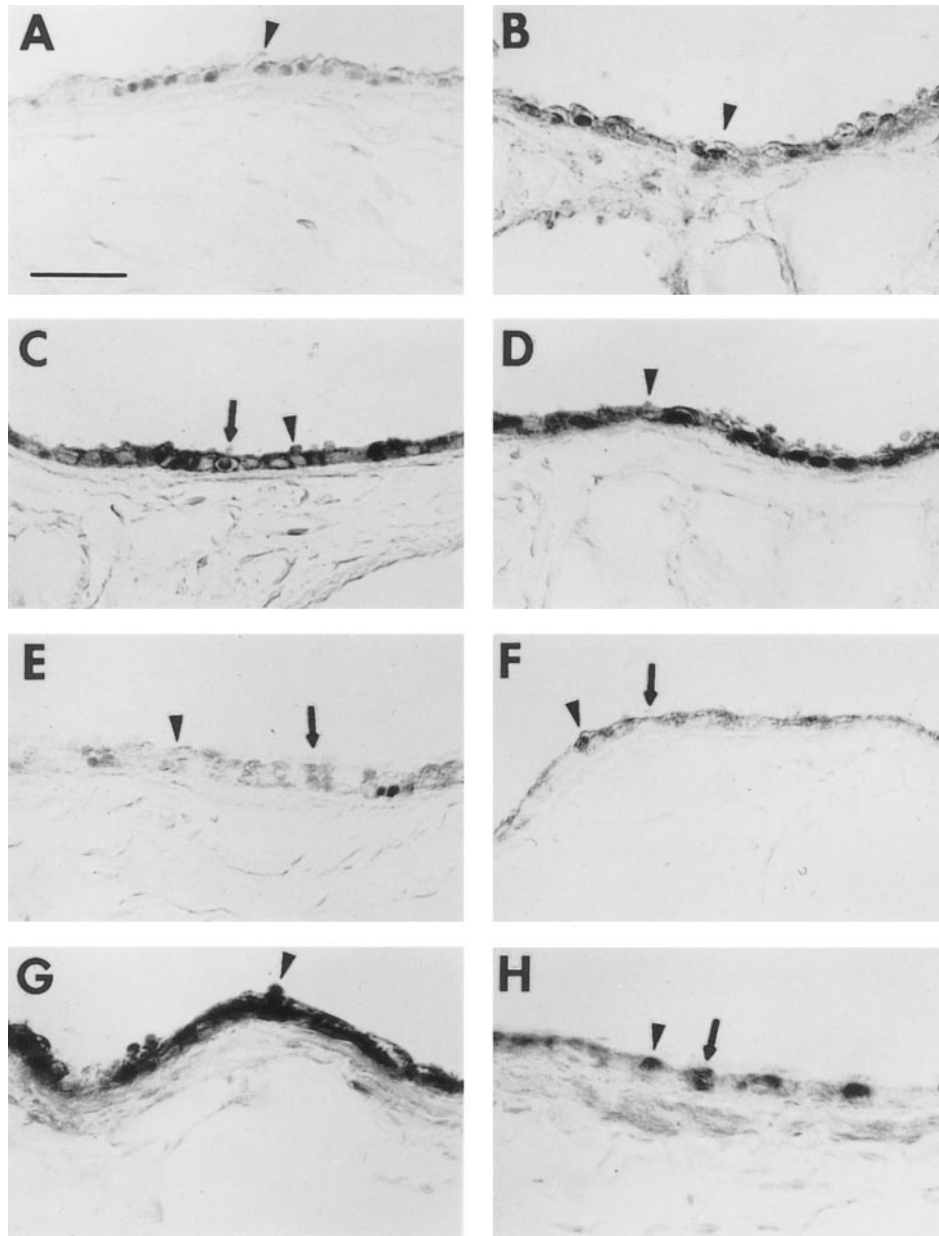

FIG. 4. Immunocytochemical localization of glutathione *S*-transferase (GST) isozyme proteins mu in lobar bronchi of mice at the following ages: 1 (A), 4 (B), 7 (C), 10 (D), 14 (E), 21 (F), and 28 (G) days postnatal age, and adults (H). Paraffin sections from all ages were incubated at the same time with GST mu antibodies (1:500). Immunoreactive protein was detected in nonciliated cells (arrowheads) and in some ciliated cells (arrow). Magnification bar represents 35 μm.

no labeling (Fig. 6F) and the proximal airways have very light labeling, mostly in the apical regions (Fig. 7F). The amount of immunoreactive pi protein starts to increase at 4 weeks and is present in even greater abundance in the adult (Figs. 6G and 6H, 7G and 7H).

Cytosolic Epoxide Hydrolase

Cytosolic epoxide hydrolase protein was detected only in the smooth muscle of pulmonary veins. There was no detectable protein found in pulmonary epithelial cells. Liver tissue was used as a positive control, and there was positive staining in liver cells (data not shown).

Microsomal Epoxide Hydrolase

In general there is always more microsomal epoxide hydrolase protein expressed in the proximal airways than is expressed in the distal airways. Unlike the glutathione *S*-transferase, microsomal epoxide hydrolase protein is not detectable

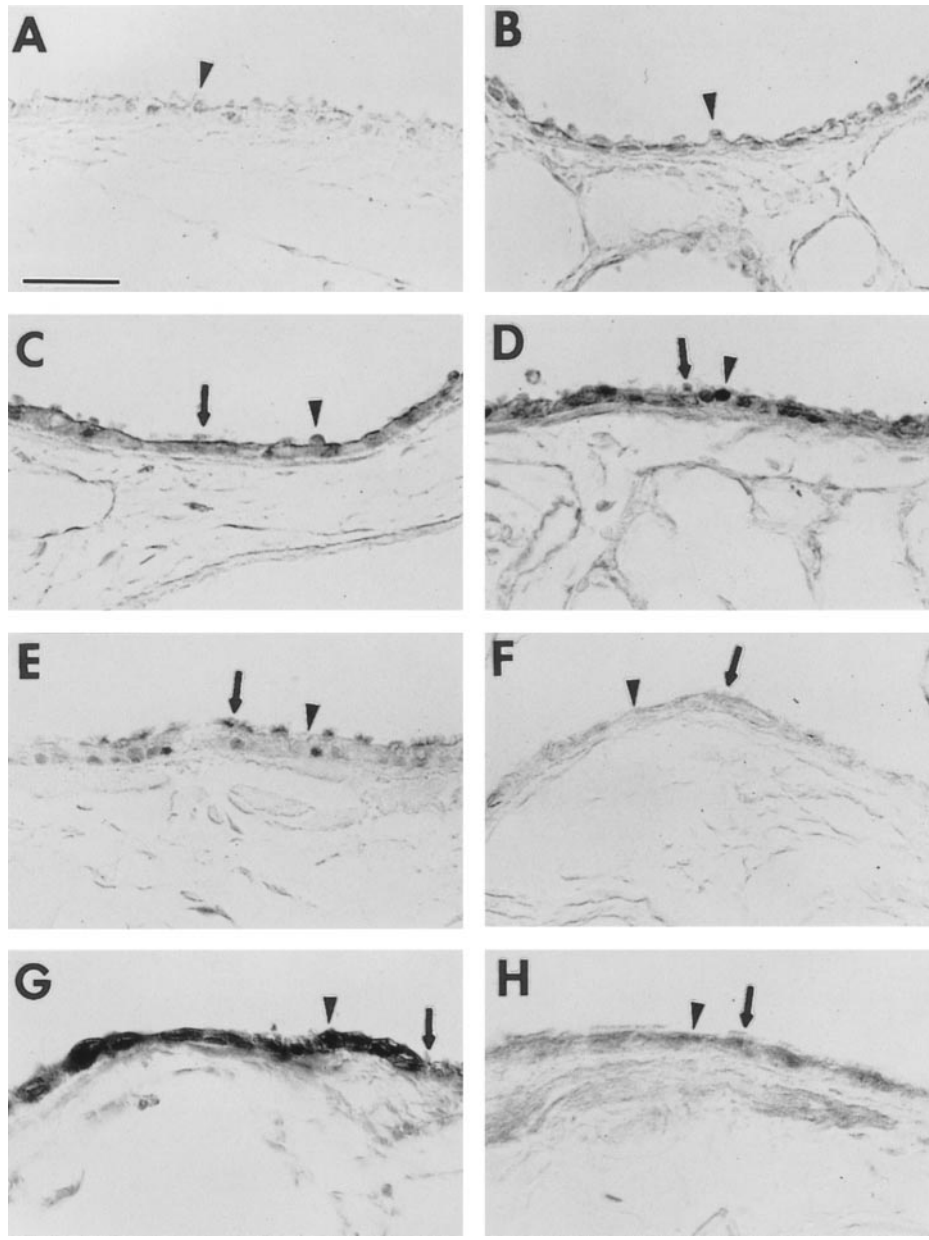

FIG. 5. Immunocytochemical localization of glutathione *S*-transferase (GST) isozyme proteins alpha in lobar bronchi of mice at the following ages: 1 (A), 4 (B), 7 (C), 10 (D), 14 (E), 21 (F), and 28 (G) days postnatal age, and adults (H). Paraffin sections from all ages were incubated at the same time with GST mu antibodies (1:500). Immunoreactive protein was detected in nonciliated cells (arrowheads) and in some ciliated cells (arrow). Magnification bar represents 35 μm.

in fetal lung (Fig. 8A). It is detected earliest at 1 day postnatal. In proximal airways, airway epithelial cells are lightly labeled, with the edges of the cells slightly darker (Fig. 8B). In the terminal bronchioles, airway epithelial cells are lightly labeled (Fig. 9A). Pulmonary alveolar macrophages are intensely labeled. At 4 days postnatal the airway cells in the proximal airways are still only lightly labeled, but labeling is more diffuse than that at 1 day (Fig. 8C). Labeling in cells of the terminal bronchioles increases slightly at 4 days (Fig. 9B). Labeling for the microsomal epoxide hydrolase protein increases dramatically at 7 days postnatal. In proximal airways, the apex of nonciliated cells labeled intensely. Ciliated cells are lightly labeled (Fig. 8D). In terminal bronchioles, there is similar staining, but it is less intense than that in the proximal airways (Fig. 9C). At 14 days, the labeling in the proximal airways is so intense that it covers all of the airway cells (Fig. 8E). Labeling in the terminal bronchioles, however, remains constant (Fig. 9D). At 21 days postnatal labeling in the proximal airways reaches adult levels and patterns of expression (Figs. 8F–8H). In terminal bronchioles adult patterns and ex-

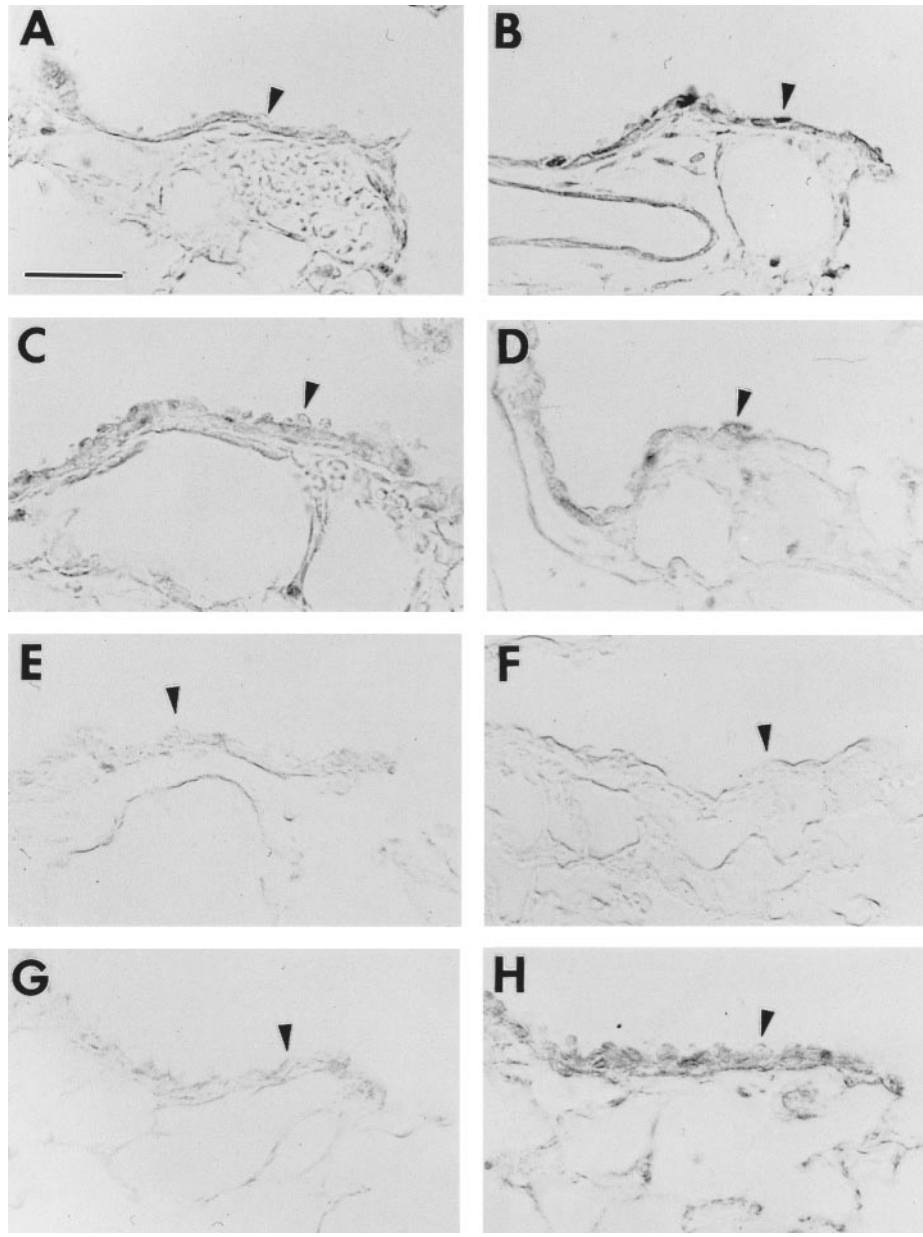

FIG. 6. Immunocytochemical localization of glutathione *S*-transferase (GST) isozyme proteins pi in terminal bronchioles of mice at the following ages: 1 (A), 4 (B), 7 (C), 10 (D), 14 (E), 21 (F), and 28 (G) days postnatal age, and adults (H). Paraffin sections from all ages were incubated at the same time with GST mu antibodies (1:500). Immunoreactive protein was detected in nonciliated cells (arrowheads). Magnification bar represents 35 μm.

pression are not reached until after 28 days postnatal (Figs. 9F–9H).

Specificity of Antibodies

Immunoblots of the glutathione *S*-transferase isozymes (Fig. 10) and epoxide hydrolases show only one band present for each antibody, and the intensity of the band increased with increasing protein. Liver 9000*g* supernatants contain much more glutathione *S*-transferase protein than does the lung. Bands were observed at 25 kDa (GST alpha), 26 kDa (GST mu), and 22.5 kDa (GST pi). The antibodies were class-specific as shown in Fig. 10. The immunoblot of microsomal epoxide hydrolase showed a single band at 49 kDa, which increased with increasing protein. Unlike glutathione *S*-transferase, the liver contained only slightly more microsomal epoxide hydrolase per mg protein than did the lung. The immunoblot of cytosolic epoxide hydrolase showed a single band at 59 kDa in the lane containing liver, but no bands were detected in any of the lung lanes.

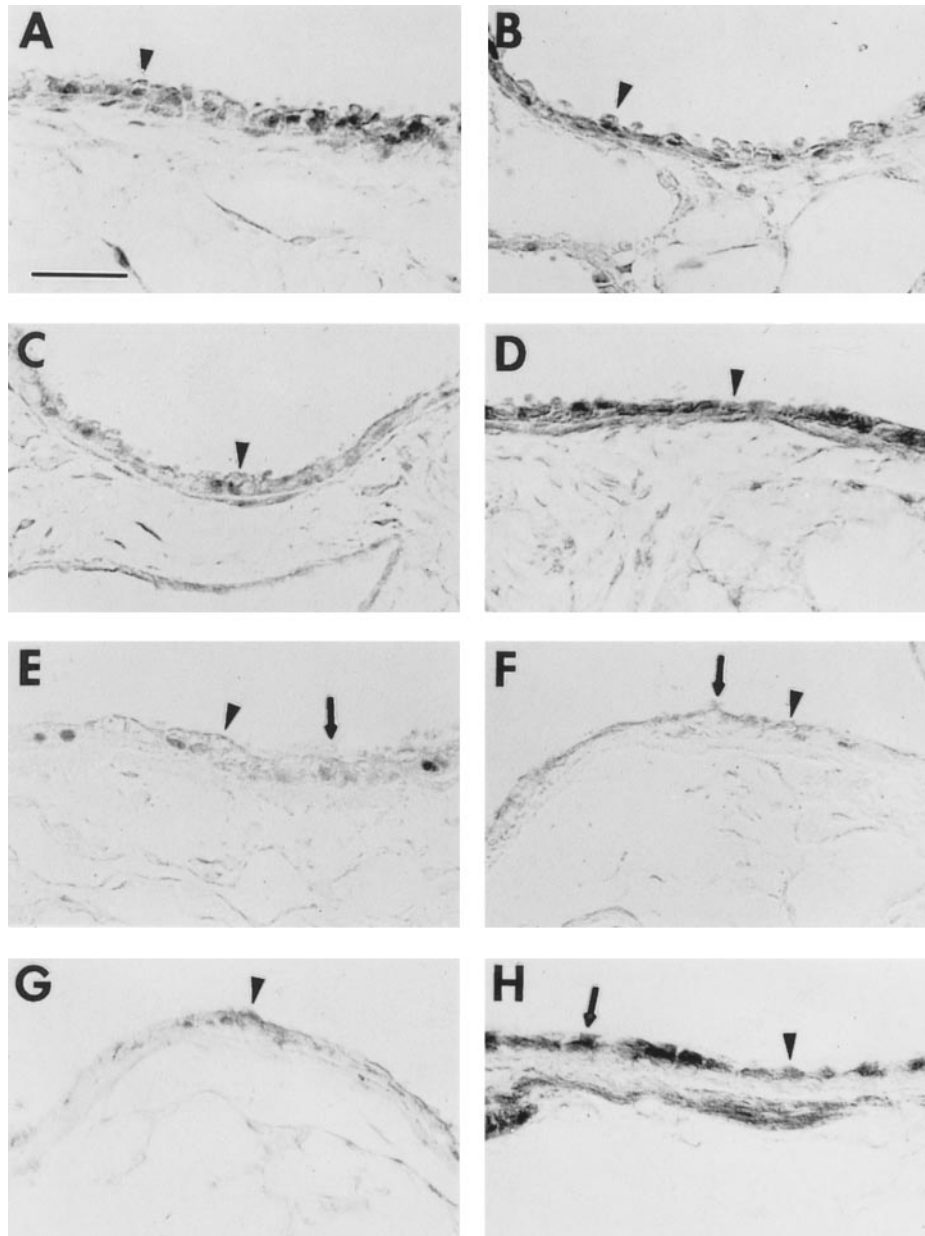

FIG. 7. Immunocytochemical localization of glutathione *S*-transferase (GST) isozyme proteins pi in lobar bronchi of mice at the following ages: 1 (A), 4 (B), 7 (C), 10 (D), 14 (E), 21 (F), and 28 (G) days postnatal age, and adults (H). Paraffin sections from all ages were incubated at the same time with GST mu antibodies (1:500). Immunoreactive protein was detected in nonciliated cells (arrowheads) and in some ciliated cells (arrow). Magnification bar represents 35 μm.

Glutathione S-Transferase Activity

The activity of GST as measured by the conjugation of CDNB in microdissected distal airways from 7- to 14-day postnatal and adult mice increased with increasing age (Fig. 11A).

Epoxide Hydrolase Activity

Bronchiolar microsomal epoxide hydrolase (as measured by the hydrolysis of *cis*-stilbene oxide) did not increase with increasing age. There was no significant difference in microsomal epoxide hydrolase activity in airways isolated from postnatal mice compared to airways from adult mice (Fig. 11B).

DISCUSSION

The purpose of this study was to establish whether the heightened neonatal susceptibility to bioactivated cytotoxi-

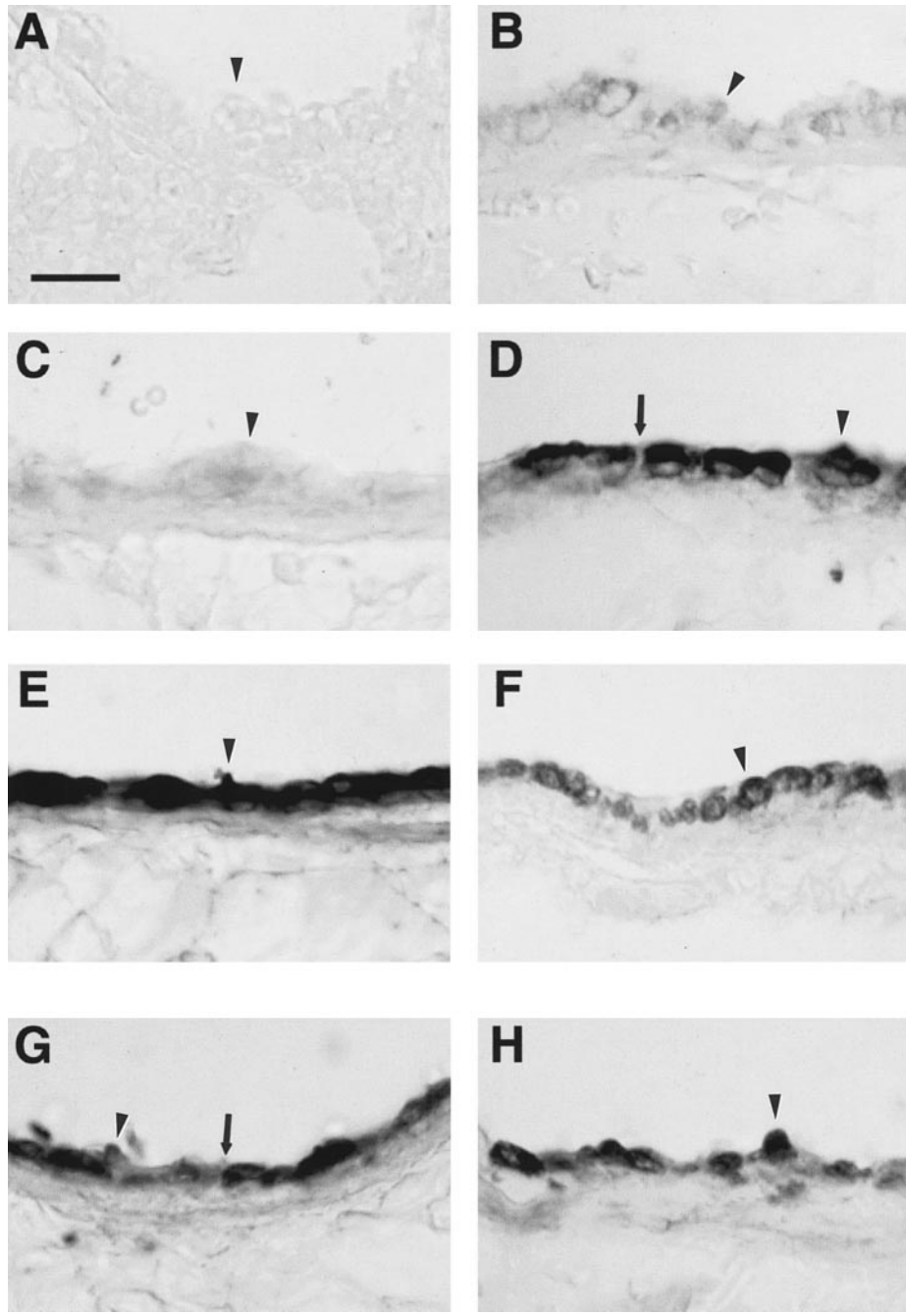

FIG. 8. Immunocytochemical localization of microsomal epoxide hydrolase (mEH) protein in lobar bronchi of mice at the following ages: fetal (A), 1 (B), 4 (C), 7 (D), 14 (E), 21 (F), and 28 (G) days postnatal age, and adults (H). Paraffin sections from all ages were incubated at the same time with mEH antibody (1:1250). Immunoreactive protein was detected in nonciliated cells (arrowheads) and in ciliated cells (arrows). Magnification bar represents 20 μm.

cants in the lung is the result of an inadequate ability to detoxify reactive intermediates by defining the pattern of phase II xenobiotic metabolizing enzyme expression during pre- and postnatal development. We found that the cellular expression of immunoreactive protein and enzymatic activity for glutathione *S*-transferases increase during the postnatal period of lung development in mice. The expression of immunoreactive microsomal epoxide hydrolase protein also increases during this time, although the level of microsomal epoxide hydrolase activity does not increase. Immunoreactive protein for cytosolic epoxide hydrolase was not detected in airway epithelium in mice of any age studied, but was detected in the smooth muscle of pulmonary veins.

In the few mammalian species that have been studied, bio-

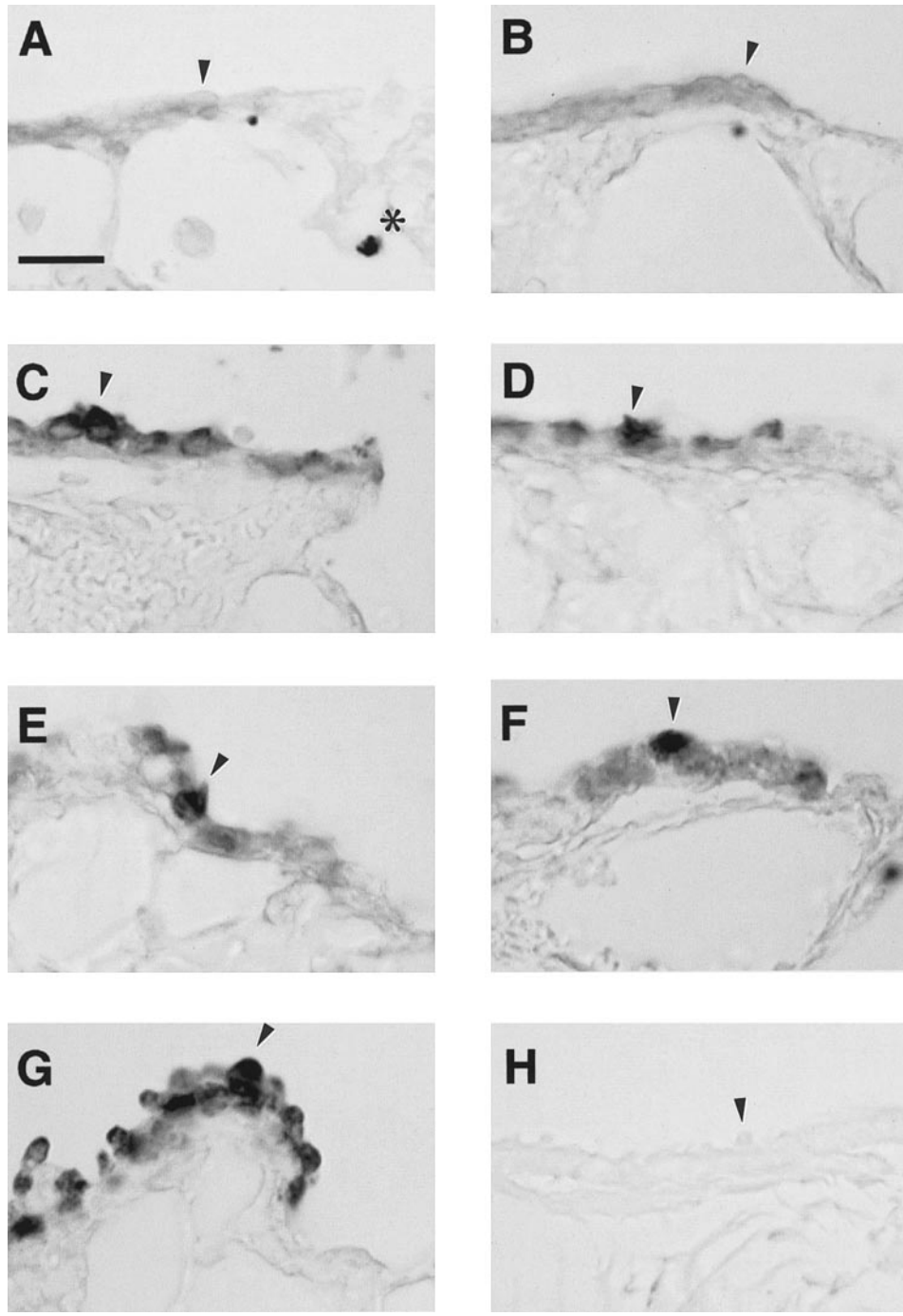

FIG. 9. Immunocytochemical localization of microsomal epoxide hydrolase (mEH) protein in terminal bronchioles of mice at the following ages: 1 (A), 4 (B), 7 (C), 10 (D), 14 (E), 21 (F), and 28 (G) days postnatal age, and adults (H). Paraffin sections from all ages were incubated at the same time with mEH antibody (1:1250). Immunoreactive protein was detected in nonciliated cells (arrowheads) and in an occasional alveolar macrophage (*). Magnification bar represents 20 μm.

transformation activity tends to be low in embryonic tissues and then increases postnatally to a level that is maintained in adult life (reviewed in Fanucchi and Plopper, 1997). It is well recognized that the Clara cell is the primary target in the adult lung for a large number of environmental contaminants. However, little information exists regarding the susceptibility of Clara cells during differentiation. Clara cell susceptibility to CYP-activated toxicants is accounted for by the abundance of immunoreactive CYP protein found in this cell type (Serabjit-Singh *et al.*, 1988) and by the high level of CYP activity in

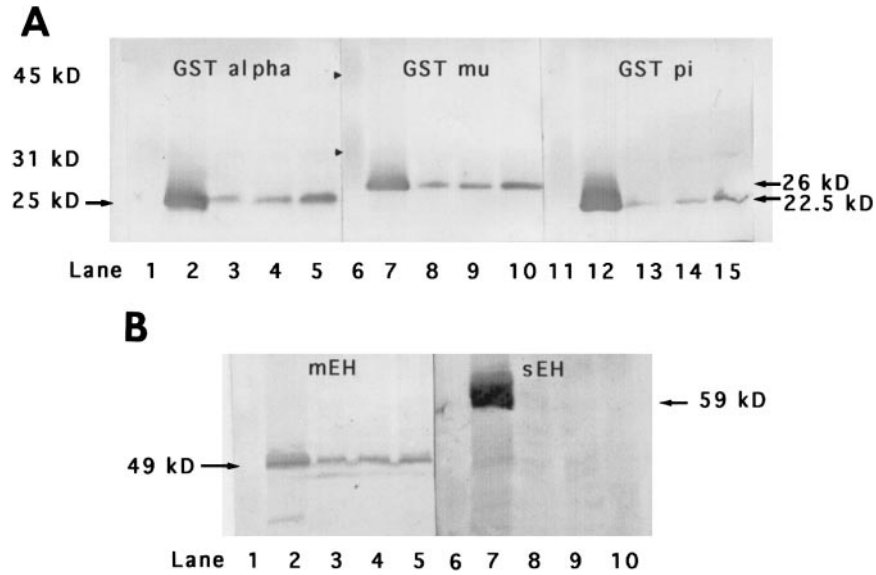

FIG. 10. Western blots of glutathione *S*-transferase isozymes alpha, mu, pi (A) and epoxide hydrolase (B). (A) Lanes 1, 6, and 11 are molecular weight standards. Lanes 2, 7, and 12 contain 34 μg protein of mouse liver homogenate. The remaining lanes contain 17 μg (3, 8, 13), 28 μg (4, 9, 14), or 57 μg (5, 10, 15) protein of mouse lung homogenate. (B) Lanes 1 and 6 are molecular weight standards. Lanes 2 and 7 contain 34 μg protein of mouse liver homogenate. The remaining lanes contain 17 μg (3, 8), 28 μg (4, 9), or 57 μg (5, 10) protein of mouse lung homogenate.

isolated Clara cell populations (Chichester *et al.,* 1991; Devereux *et al.,* 1985). In addition, distinct species-specific and site-specific differences in acute toxicity to naphthalene (a CYP-activated toxicant) have been reported (Buckpitt *et al.,* 1992, 1995; Plopper *et al.,* 1992a,b). The species and regional differences correlate closely with the rate and stereoselectivity of naphthalene epoxidation (Buckpitt *et al.,* 1992). The positive correlation between metabolic activation and toxicity, however, does not hold true in neonatal rabbits exposed to 4-ipomeanol (Plopper *et al.,* 1994) or in neonatal mice exposed to naphthalene (Fanucchi *et al.,* 1997a). Although differentiating Clara cells in neonates have low levels of CYP monooxygenase activity, they are much more susceptible to CYP-activated compounds than are mature Clara cells of adults.

One explanation for this apparent mismatch between the level of P450 activity and toxicity of bioactivated compounds is that toxic cell injury can occur whenever there is a higher level of activating versus detoxifying enzyme activity present. Our current study suggests that this may indeed be the case. The phase II metabolizing enzyme glutathione *S*-transferase activity is lower in distal airways of postnatal mice compared to the activity in adult mice. This decreased potential to detoxify reactive intermediates may contribute to the increased susceptibility of differentiating Clara cells to CYP-activated toxicants. In contrast, the microsomal epoxide hydrolase activity of distal airways is similar in the distal airways of postnatal and adult mice. This would suggest that the balance of activation versus detoxification in the neonates favors detoxification and should result in neonates being less susceptible to bioactivated compounds. This, however, is not the case, a discrepancy which may be addressed by two possible expla-

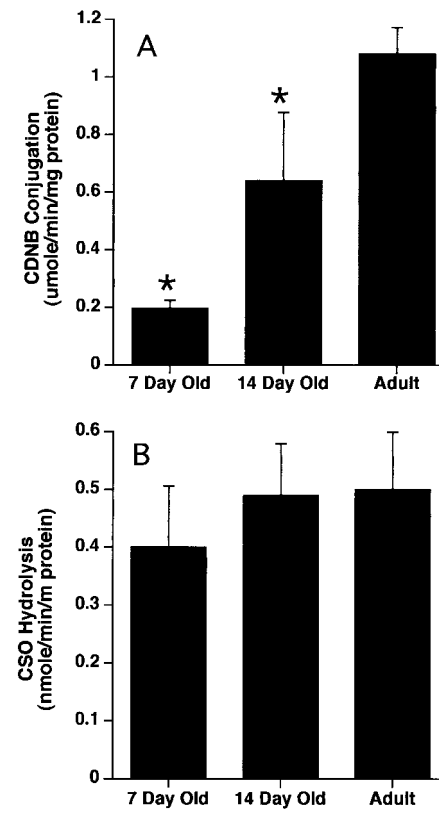

FIG. 11. Enzyme activity of glutathione *S*-transferase isozymes (A) and microsomal epoxide hydrolase (B) in microdissected distal airways from postnatal and adult mice. Results are means ± SD of at least three mice at each time point. *, indicates activity is significantly lower than that of adult ($p < 0.05$).

nations: (1) epoxide hydrolase may not play an important role in deactivating reactive intermediates in neonatal mice, or (2) the areas of highest epoxide hydrolase activity may not correlate with the areas of highest P450 activity.

In this study, glutathione *S*-transferase activity (as measured by the conjugation of CDNB to glutathione) in distal airways from lungs of neonatal mice increases progressively with increasing age. This is different from what has been described for humans, in whom the total activity of pulmonary glutathione *S*-transferase decreases fivefold between 13 weeks gestation and birth and then remains constant postnatally (Fryer *et al.,* 1986). This decrease is associated with a loss in detectable protein (Cossar *et al.,* 1990). More than 90% of glutathione *S*-transferase activity in lungs of humans results from the acidic (pi) isoenzyme throughout development. The remaining glutathione *S*-transferase activity is made up of the basic (alpha) and near-neutral (mu) isoenzymes. The decline in the total glutathione *S*-transferase activity results from a loss in the amount of acidic isoenzyme present (Beckett *et al.,* 1990; Fryer *et al.,* 1986; Strange *et al.,* 1985).

There is a mismatch between specific activity for glutathione *S*-transferases evaluated by conjugation to CDNB in bronchioles of postnatal mice protein expression detected in this study. Catalytic activity toward CDNB is only one assessment of the detoxification potential of the glutathione *S*-transferases and may not be totally representative (CDNB is a general substrate that allows evaluation of total glutathione *S*-transferase activity, but may over- or underrepresent specific isozymes). However, because of the small sample size of distal airways that can be obtained from neonatal mice, we were able to evaluate catalytic activity with CDNB only.

In the present study, we found that cellular protein expression for isozymes alpha and mu varies throughout development. Compared to steady-state levels in adults, protein levels are low before birth, high on postnatal day 7, low between postnatal days 14 and 21, and high at postnatal day 28. Immunoreactive protein of isozyme pi has a peak expression on gestational day 18 and declines to a low at postnatal day 21 (little to no detectable protein) and is again at peak levels in the adult mouse lung. Postnatal variation in the expression of individual isozymes of glutathione *S*-transferases has also been previously observed in the liver. The mRNA levels of various subunits of glutathione *S*-transferase (Yb_1, Yb_2, Yb_3, Ya, and Yp) in the liver of postnatal rats vary throughout postnatal development (Abramovitz and Listowsky, 1988). A sudden transient decrease in glutathione *S*-transferase activity during the fifth postnatal week of liver development in rats has also been observed (Baars *et al.,* 1980).

The subcellular distribution of glutathione *S*-transferase isozymes has been described in the lungs of adult rats and mice (Coursin *et al.,* 1992; Forkert *et al.,* 1999; Lee and Dinsdale, 1994). Labeling of immunoreactive protein was documented in both cytoplasmic and nuclear compartments of Clara and ciliated cells. A similar labeling pattern was reported in developing as well as adult kidneys in hamsters (Oberley *et al.,* 1991). In the present study, we also observed labeling of the glutathione *S*-transferase isozymes' immunoreactive protein in both the nuclear and cytoplasmic compartments. The compartmentalization did vary with postnatal age, however. We observed nuclear labeling of all three isozymes at most postnatal ages, but at 7 days postnatal the labeling for isozymes alpha and mu was shifted to the cytoplasmic compartment. Nuclear labeling was again present at 10 days postnatal. The consequence of protein expression in the cytoplasmic versus the nuclear compartment is not clear.

Although glutathione *S*-transferase activity increases with increasing age, epoxide hydrolase activity does not. Data from humans suggest that fetal lung contains more cytosolic than microsomal epoxide hydrolase activity (Pacifici and Rane, 1982) and that, compared with adult lungs, fetal lung contains much less epoxide hydrolase potential (Kaplowitz *et al.,* 1985). In the lung, epoxide hydrolase mRNA expression gradually increases to adult levels by 65 days (Simmons and Kasper, 1989). Increases in fetal human pulmonary microsomal epoxide hydrolase activity, however, do not correlate with increases in gestational age (Omiecinski *et al.,* 1994). In this study, we found that mice have greater expression of microsomal than cytosolic epoxide hydrolase in airway epithelium. Cytosolic epoxide hydrolase protein is detected only in pulmonary veins, not in airway epithelium. This may not be in contrast to the human data, though, because the site-specific expression of epoxide hydrolases has not been evaluated in humans. We measured epoxide hydrolase activity in isolated airways and in isolated blood vessels. The blood vessels contained at least 10 times more cytosolic epoxide hydrolase activity than did the airways (data not shown). This may indicate that, by evaluating whole lung, the contribution of epoxide hydrolase activity from nonairway tissue can profoundly influence the assay.

In addition to the balance between activation and detoxification enzymes such as glutathione *S*-transferases and epoxide hydrolases, inadequate or reduced glutathione levels or the inability to generate additional glutathione may be important in Clara cell toxicity, particularly in differentiating cells. Striking species and airway-level differences in the steady-state levels and rates of resynthesis of glutathione have been reported (Duan *et al.,* 1996). Without an adequate amount of reduced glutathione available, no amount of glutathione transferase activity will protect cells from injury. Steady-state levels and rates of glutathione synthesis have not been examined in the differentiating Clara cells of neonates.

Although steady-state levels of the activating and detoxification enzymes may appear to be in balance in airway epithelium of distal lung in neonatal mice, the detoxification enzymes may be depleted before all of the toxicant is removed. Prolonged exposure to low concentrations of toxicant may more effectively deplete glutathione levels as well as phase II enzymes in target cell populations. The putative reactive intermediate of naphthalene metabolism, naphthalene oxide, is not

cytotoxic when added as a bolus to isolated Clara cells, but it is cytotoxic when infused slowly to the cells (Chichester *et al.*, 1994). The same may be true for differentiating Clara cells in lungs of neonatal mice. There may be sufficient activation by CYP monooxygenases to sustain a low concentration of toxic intermediate within the target cell that may eventually overwhelm the phase II detoxification system.

In summary, the present study demonstrates age-dependent patterns in microsomal epoxide hydrolase and glutathione *S*-transferase expression and activity in mouse lung. This pattern is different from what has been described for humans, although these differences may exist because very little human tissue is available to study in the postnatal period of lung development. The discordance between cellular expression of protein and detectable glutathione *S*-transferase activity, as opposed to the close correlation observed for microsomal epoxide hydrolase in the mouse, emphasizes the need to carefully characterize the activity of an enzyme within the subcompartment in which the cellular expression is being defined.

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