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In Situ Localization and Distribution of Xenobiotic-Activating Enzymes and Aryl Hydrocarbon Hydroxylase Activity in Lungs of Untreated Rats

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SUMMARY

The present investigation was undertaken to more precisely establish where xenobiotics can be oxidatively metabolized and bioactivated within the lung. To accomplish this, antibodies raised against NADPH-cytochrome P-450 reductase (EC 1.6.2.4) and cytochromes P-450 BNF-B, PB-B, and PCN-E (the major forms of cytochrome P-450 induced by β-naphthoflavone, phenobarbital, and pregnenolone-16α-carbonitrile, respectively) that had been purified to apparent homogeneity from rat liver microsomes were used to determine the localizations and distributions of these enzymes immunohistochemically at the light microscopic level within lungs of untreated rats. Additionally, the intrapulmonary sites at which benzo(α)pyrene undergoes hydroxylation were identified *in situ* by means of fluorescence histochemistry. Immunohistochemical staining for NADPH-cytochrome P-450 reductase and cytochromes P-450 BNF-B, PB-B, and PCN-E was detected in bronchial epithelial cells, both ciliated and nonciliated (Clara) bronchiolar epithelial cells, and type II pneumocytes as well as other cells in the alveolar wall. Results of microfluorometric analyses of the immunofluorescence staining intensities of bronchial epithelial cells, Clara cells, and type II pneumocytes demonstrated further that Clara cells bound the antibodies raised to NADPH-cytochrome P-450 reductase and cytochrome P-450 PB-B to significantly greater extents than did bronchial epithelial cells and type II pneumocytes. Thus, in lungs of untreated rats, Clara cells contain the greatest amounts of these two enzymes. In marked contrast, the antibodies directed against cytochromes P-450 BNF-B and PCN-E were each bound to similar extents by bronchial epithelial cells, Clara cells, and type II pneumocytes. In agreement with immunohistochemical observations on the intrapulmonary localizations of NADPH-cytochrome P-450 reductase and cytochromes P-450 BNF-B, PB-B, and PCN-E in untreated rats, benzo(α)pyrene was hydroxylated *in situ* by bronchial and bronchiolar epithelial cells and alveolar wall cells, especially type II pneumocytes. These immunohistochemical and histochemical findings, thus, demonstrate that bronchial epithelial cells, Clara and ciliated bronchiolar epithelial cells, and type II pneumocytes as well as other alveolar wall cells represent sites for the *in vivo* oxidative metabolism and bioactivation of xenobiotics in lungs of untreated rats.

The lung, being a major portal of entry of xenobiotics into the body, is continuously exposed to airborne environmental chemicals and represents a major target for neoplasia and other toxicities induced by these as well as many other xenobiotics. Most carcinogenic chemicals and many pulmonary toxins such as 4-ipomeanol are, however, relatively inert substances that must be bioactivated in order to exert their cytotoxic and/or tumorigenic actions (1–3). The activation of these chemicals usually involves their transformation into electrophilically reactive metabolites that, presumably by attacking and binding to nucleophilic sites on DNA and other cellular macromolecules, induce genomic alterations and various cytotoxicities (1–4). The biotransformation of procarcinogens and other xenobiotics into reactive electrophiles such as arene oxides is primarily catalyzed by microsomal and nuclear monooxygenase enzyme systems consisting of NADPH-cytochrome P-450 reductase and different forms of cytochrome P-450 (1–3, 5–7). Although arene oxides that are produced during the cytochromes P-450-mediated monooxygenations of aromatic chemicals can be detoxicated by hydration to their corresponding (*trans*)-dihydrodiols under the influence of epoxide hydrolase (8), certain (*trans*)-dihydrodiols generated from polycyclic aromatic hydrocarbons can be further monooxygenated by polycyclic aromatic hydrocarbon-inducible forms of cytochrome P-

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450 (e.g., P-450 BNF-B[4]) to yield (*trans*)-dihydrodiol-epoxides, which are extremely reactive electrophiles generally considered to represent the ultimate carcinogenic metabolites derived from polycyclic aromatic hydrocarbons (1, 9).

Differences in the abilities of the numerous diverse cell types present in the lung to both activate and detoxicate xenobiotics might be critical for determining which pulmonary cells would be most susceptible to the geno- and cytoxicities that result as a consequence of the oxidative metabolism of various procarcinogens and pulmonary toxins. Indeed, autoradiographic findings have revealed that 4-ipomeanol is activated to the greatest extent by the Clara cell, the pulmonary cell most readily damaged following *in vivo* exposure to this chemical (2, 10). Results of studies on isolated rodent Clara cells and type II pneumocytes (11, 12) and on cultured rodent and human tracheobronchial epithelial cells (13) have also demonstrated that these cells are capable of oxidatively metabolizing and activating various xenobiotics, including a procarcinogen such as benzo(α)pyrene. Except for the alveolar macrophage, however, little is known regarding the participation of other cells, such as the ciliated bronchiolar epithelial cell, in the oxidative metabolism of xenobiotics in the lung (14). Furthermore, it should be appreciated that the metabolism of xenobiotics by isolated or cultured cells might not truly reflect the metabolic capabilities of these cells *in situ*, because the abilities of isolated cells to metabolize xenobiotics could be altered as a consequence of exposure to proteases during the preparation of cell suspensions (14), while cells maintained in either primary or continuous culture may exhibit depressed monooxygenase activities (15).

The use of immunohistochemistry together with histochemistry represents a powerful experimental approach for determining the precise intratissue localizations and distributions of xenobiotic-metabolizing enzymes. This approach has been successfully employed by this laboratory to establish where xenobiotics can be oxidatively metabolized *in situ* within the liver and in a number of extrahepatic tissues (16–18). In the present study, the localizations and distributions of NADPH-cytochrome P-450 reductase and the major forms of cytochrome P-450 induced by β-naphthoflavone (P-450 BNF-B), phenobarbital (P-450 PB-B), and pregnenolone-16α-carbonitrile (P-450 PCN-E) were determined immunohistochemically within lungs of untreated rats. In conjunction with these determinations, a fluorescence histochemical method was utilized to identify the intrapulmonary sites at which benzo(α)pyrene is hydroxylated; this allowed immunohistochemical findings on NADPH-cytochrome P-450 reductase and cytochromes P-450 BNF-B, PB-B, and PCN-E to be correlated with xenobiotic monooxygenase activity.

[4] The nomenclature of the individual forms of cytochrome P-450 discussed in this article has been described elsewhere (5, 6). In the classification given by Nebert *et al.* (6), P-450 BNF-B is the CYPIA1 gene product, P-450 BNF/ISF-G the CYPIA2 gene product, P-450 PB-B the CYPIIB1 gene product, P-450 PB-D the CYPIIB2 gene product, and P-450 PCN-E appears to be the CYPIIIA2 gene product. No other genes appear to exist in the CYPIA family. Because the existence of other CYPIIB gene products in rat lung has not been carefully addressed, the possibility exists that some other cross-reactive forms might be present. The CYPIIIA family appears to encode at least four forms of cytochrome P-450 in rat liver: PCNb, the CYPIIIA2 gene product, which appears to be identical to P-450 PCN-E (7); PCNa which is the CYPIIIA1 gene product (7), PCNc (7); and a fourth protein recently isolated by Kato and Yamazoe (personal communication). Neither PCNc nor the protein described by Kato and Yamazoe have been assigned to gene sequences.

Experimental Procedures

Materials. 3,3′-Diaminobenzidine tetrahydrochloride was obtained from Hach Chemical Co. (Loveland, CO), and 4-chloro-1-naphthol was purchased from Aldrich Chemical Co. (Milwaukee, WI). Normal (nonimmune) rabbit and sheep sera were obtained from Cooper Biomedical, Inc. (Malvern, PA). Biotinylated rabbit and sheep immunoglobulins directed against sheep and rabbit immunoglobulins, respectively, biotinylated horseradish peroxidase, avidin, and fluorescein isothiocyanate-conjugated avidin were purchased from Vector Laboratories, Inc. (Burlingame, CA). NADH (grade III), NADPH (Type I), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose sheets were purchased from Millipore Corp. (Bedford, MA), and reagents for gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA). Benzo(α)pyrene, purchased from Pfaltz and Bauer, Inc. (Stamford, CT), was purified by reverse phase high pressure liquid chromatography on an Ultrasphere ODS column (Beckman Instruments, Inc., St. Louis, MO), using a solvent consisting of 90% methanol/10% water and monitoring absorbance at 296.5 nm. All other chemicals were of the highest purity available.

Antigens and antibodies. Procedures for the isolation and purification to apparent homogeneity of NADPH-cytochrome P-450 reductase (19) and cytochromes P-450 BNF-B (20), PB-B (21, 22), and PCN-E (20) from rat liver microsomes, the preparation of polyclonal sheep or rabbit antibodies against each enzyme, and the specificities of these antibodies have been described previously. Coupled sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunoblotting was performed as described by Guengerich *et al.* (20, 23). Immunoreactive protein bands on the blots were visualized following immunoperoxidase staining using 4-chloro-1-naphthol as the chromogen and then were quantitated by means of reflectance scanning densitometry, as described by Butler *et al.* (24).

In view of similarities among the sequences of some forms of cytochrome P-450, the matter of cross-reactivity needs to be considered. Cytochrome P-450 BNF-B, the major form of cytochrome P-450 induced in rats by β-naphthoflavone, shares common epitopes with P-450 BNF/ISF-G, a minor constitutive form of rat hepatic microsomal cytochrome P-450, while antibody to cytochrome P-450 PB-B, the major phenobarbital-inducible cytochrome P-450, also recognizes P-450 PB-D, another minor constitutive form of rat hepatic microsomal cytochrome P-450 (20). In rat lung, cytochrome P-450 PB-B appears to be the major constitutive form of the hemeprotein present, with cytochrome P-450 BNF-B being found at much lower levels in untreated rats (23). Cytochromes P-450 BNF/ISF-G and PB-D, however, have not been detected in pulmonary microsomes of untreated rats (25, 26). Because information is not available regarding which of the proteins that are encoded by the CYPIIIA gene family are expressed in lungs of untreated rats, it is possible that the antibody raised against cytochrome P-450 PCN-E might recognize more than one of these hemeproteins. Thus, although antibodies to rat hepatic microsomal cytochromes P-450 (20, 23, 27–29) and NADPH-cytochrome P-450 reductase (30–32) are capable of cross-reacting with the corresponding enzymes in rat pulmonary preparations, it must be noted that immunohistochemical staining produced by these antibodies within rat lung may be indicative of the presence of antigens that are only immunochemically similar, rather than identical, to the rat liver enzymes.

Immunohistochemical procedures. Male Sprague-Dawley rats weighing 160–180 g (Harlan Sprague-Dawley Inc., Indianapolis, IN) were killed by decapitation, and their lungs were immediately perfused through the pulmonary artery with ice-cold 0.9% NaCl. After the trachea was cannulated, the lungs were inflated with ice-cold fixative solution consisting of 1% acetic acid in 95% ethanol. Following tracheal ligation, the lungs and heart were removed *en bloc*, and 1- to 2-mm-thick slices of tissue obtained from each pulmonary lobe were fixed at 4° for 4 hr by constant shaking in fixative. The fixed tissue specimens were then dehydrated, cleared, and embedded in paraffin, and 4-μm-thick serial sections were prepared. NADPH-cytochrome P-450 reduc-

tase and cytochromes P-450 BNF-B, PB-B, and PCN-E were localized at the light microscopic level by means of both immunoperoxidase (avidin-biotin-peroxidase) (33, 34) and immunofluorescence (avidin-fluorescein isothiocyanate) (34) staining.

For immunoperoxidase staining, sections were initially exposed to aqueous 10% dimethyl sulfoxide for 40 min at 24°, to enhance the uniform penetration of antibodies into the tissue (34). To block the endogenous peroxidase activity, sections were then exposed for 1 hr at 37° to 0.05% phenylhydrazine in 50 mM Tris·HCl buffer, pH 7.75, containing 0.154 M NaCl (35). Following this, sections were sequentially exposed to 10% normal serum (10 min at 24°) in order to reduce nonspecific antibody binding, antiserum to the enzyme being studied (2 hr at 37°), biotinylated anti-sheep or anti-rabbit immunoglobulins (30 min at 24°), avidin complexed with biotinylated horseradish peroxidase (45 min at 24°), 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2, and, finally, aqueous 2% OsO_4 to intensify and stabilize the immunoperoxidase stain. For immunofluorescence staining, fluorescein isothiocyanate-conjugated avidin was substituted for the avidin-biotin-peroxidase complex, and sections were not exposed to phenylhydrazine, 3,3′-diaminobenzidine tetrahydrochloride, H_2O_2, or OsO_4. To determine the intrapulmonary distributions of NADPH-cytochrome P-450 reductase and cytochromes P-450, the intensities with which bronchial epithelial cells, Clara cells, and type II pneumocytes were stained by each antibody were determined microfluorometrically after completion of immunofluorescence staining, as described previously (19, 34, 36, 37). The identification of specific cell types in immunohistochemically stained sections was confirmed by examining serial sections that had been stained with toluidine blue.

In order to assess the presence and degree of nonspecific immunoperoxidase staining resulting from endogenous peroxidase activity and/or the nonspecific binding of antibodies, serial sections were exposed to equivalent dilutions of either the appropriate normal serum or adsorbed antiserum. Little if any staining for NADPH-cytochrome P-450 reductase and cytochromes P-450 was evident in sections exposed to normal sera or adsorbed antisera and when the biotinylated rabbit and sheep antibodies were replaced with normal sera (data not shown). Similarly, only minimal immunofluorescence staining was noted when sections were exposed to normal or adsorbed antisera as well as when biotinylated antibodies were replaced with normal sera.

Histochemical demonstration of benzo(α)pyrene hydroxylase activity. Sites for the *in situ* hydroxylation of benzo(α)pyrene within lungs of untreated rats were identified using modifications of the fluorescence histochemical procedure for visualizing phenolic benzo(α)pyrene metabolites described by Wattenberg and Leong (38) and Bresnick *et al.* (39). Unfixed cryostat sections, 6 μm in thickness, were prepared from lungs that had been inflated with 0.9% NaCl containing 4% gelatin and then frozen by immersion in a 2-methylbutane/dry ice slurry. The sections were adhered to coverslips, air dried, and dipped into acetone containing 5 μg of benzo(α)pyrene/ml. Following evaporation of the acetone, sections were incubated for 1 hr at 37° in a medium consisting of 0.05 M potassium phosphate buffer, pH 7.4, 0.58 mM NADPH, 0.11 mM NADH, 1.6 mM glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase. After the medium was replaced with ice-cold phosphate buffer, the sections were mounted with alkaline glycerol, pH 8.2, containing 10 mM sodium phosphate and were kept at 0–4°. Phenolic benzo(α)pyrene metabolites having a greenish-yellow fluorescence (excitation, 400 nm; emission, 522 nm) were visualized by means of incident-light fluorescence microscopy using an Orthoplan microscope (E. Leitz Inc., Wetzlar, West Germany) with a Ploemopak 2.1 fluorescence illuminator containing an H2 filter cube (excitation, 390–490 nm; RKP 510 beam-splitting mirror; LP 515 suppression filter). To maintain the sections at 0–4° and thereby prevent the diffusion of metabolites and minimize fluorescence fading during observation and photography, slides were placed on a cold plate attached to the microscope stage. The microscope was enclosed in a transparent Plexiglass chamber that was continuously purged with dry nitrogen gas to prevent water condensation on both the slides and microscope objectives. In control experiments, sections were incubated in the absence of benzo(α)pyrene (data not shown) and/or reduced pyridine nucleotides.

Results

Localization of NADPH-cytochrome P-450 reductase and cytochromes P-450 within lungs of untreated rats. When sections prepared from fixed paraffin-embedded lungs of untreated, male Sprague-Dawley rats were exposed to antisera raised against rat hepatic microsomal NADPH-cytochrome P-450 reductase (Fig. 1) and cytochromes P-450 BNF-B (Fig. 2), PB-B (Fig. 3), and PCN-E (Fig. 4), immunoperoxidase staining for each enzyme was detected in bronchial and bronchiolar epithelial cells and cells of the alveolar wall. Specific staining for NADPH-cytochrome P-450 reductase and the cytochromes P-450 was not evident in smooth muscle, connective tissue, or blood vessels. Although photomicrographs are not presented, immunofluorescence staining yielded identical results. Additionally, little if any immunohistochemical staining was observed when lung sections were exposed to normal sera rather than to the antisera to NADPH-cytochrome P-450 reductase and cytochromes P-450 BNF-B, PB-B, and PCN-E (Figs. 1–4, B, E, and H), as well as when adsorbed antisera were used (data not shown).

Immunoperoxidase staining for NADPH-cytochrome P-450 reductase was readily apparent in bronchial epithelial cells (Fig. 1A), in Clara as well as ciliated bronchiolar epithelial cells (Fig. 1D), and in alveolar wall cells (Fig. 1G). Despite the fact that alveolar wall cells other than type II pneumocytes cannot be unequivocally identified at the light microscopic level, the photomicrograph in Fig. 1G reveals that immunohistochemical staining for NADPH-cytochrome P-450 reductase was produced throughout the alveolar wall, although it was especially prominent in type II pneumocytes.

Visually, Clara cells appeared to be stained much more intensely by the antibody to NADPH-cytochrome P-450 reductase than were other pulmonary cells. This observation was confirmed by the results of microfluorometric analyses of immunofluorescence staining intensities (Table 1), which revealed that, whereas the anti-cytochrome P-450 reductase bound equally to bronchial epithelial cells and type II pneumocytes, approximately 90% more antireductase bound to Clara cells. When immunohistochemical staining procedures are optimized as they were in the present study, the intensity of immunohistochemical staining, and, hence, the extent of antibody binding are directly related to the antigen's content (34). Thus, these findings demonstrate that bronchial epithelial cells and type II pneumocytes in untreated rats containing similar levels of NADPH-cytochrome P-450 reductase whereas Clara cells contain almost twice as much enzyme.

Consistent with the fact that pulmonary microsomes of untreated rats only contain extremely low levels of cytochrome P-450 BNF-B (23), the antibody to this form of cytochrome P-450 produced very weak staining in lungs of untreated rats. Despite this, the presence of cytochrome P-450 BNF-B was detectable in bronchial epithelial cells (Fig. 2A), both Clara and ciliated bronchiolar epithelial cells (Fig. 2D), and alveolar wall cells (Fig. 2G). In contrast to findings on NADPH-cytochrome P-450 reductase, however, these cells all appeared to be stained with equal intensity for cytochrome P-450 BNF-B. Consonant with these observations, the data presented in Table 1 demon-

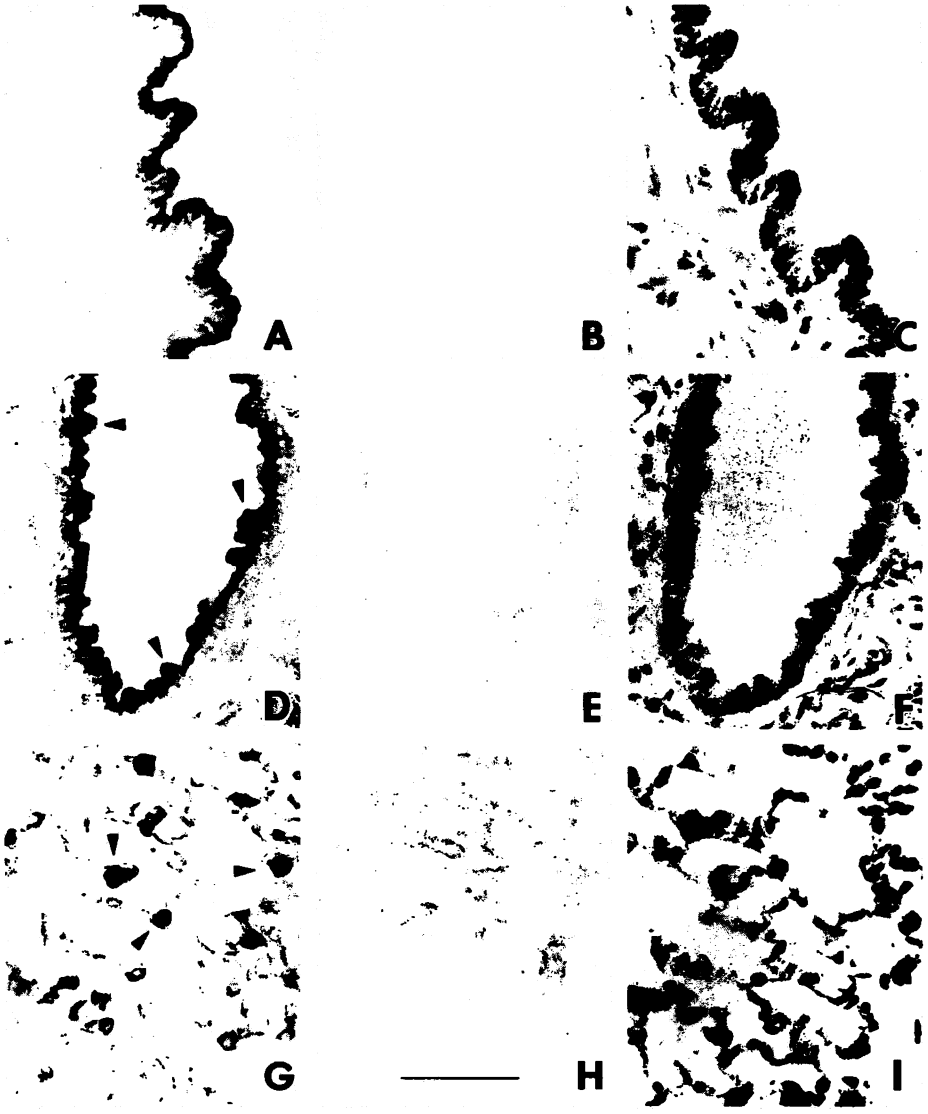

Fig. 1. Immunoperoxidase staining for NADPH-cytochrome P-450 reductase within rat lung. A–C, Serial sections of a bronchus; D–F, serial sections of a bronchiole (*arrowheads* in D point to Clara cells); G–I, the same alveolar area in serial sections (*arrowheads* in G point to type II pneumocytes). A, D, and G, sections exposed to sheep antiserum to rat hepatic microsomal NADPH-cytochrome P-450 reductase; B, E, and H, sections exposed to normal sheep serum; C, F, and I, sections stained with toluidine blue. The *bar* in H = 50 μm.

strate that the anti-cytochrome P-450 BNF-B bound to similar, albeit extremely low but statistically significant ($p < 0.05$), extents to bronchial epithelial cells, Clara cells, and type II pneumocytes.

Immunohistochemical findings on cytochrome P-450 PB-B in lungs of untreated rats differed considerably from those on cytochrome P-450 BNF-B. For instance, the antibody to cytochrome P-450 BNF-B produced much more intense staining of bronchial epithelial cells (Fig. 3A), Clara and ciliated bronchiolar epithelial cells (Fig. 3D), and alveolar wall cells (Fig. 3G) than did the antibody to cytochrome P-450 BNF-B. Furthermore, analogous to results with the antibody to NADPH-cytochrome P-450 reductase, Clara cells appeared to be stained much more intensely by the anti-cytochrome P-450 PB-B than were other pulmonary cells. In agreement with these observations, the data in Table 1 reveal that, whereas the antibody to cytochrome P-450 PB-B bound to equivalent extents to bronchial epithelial cells and type II pneumocytes, approximately 50% more anti-cytochrome P-450 PB-B bound to Clara cells.

The photomicrographs in Fig. 4 illustrate that the antibody raised against rat hepatic microsomal cytochrome P-450 PCN-E stained bronchial epithelial cells (Fig. 4A), Clara and ciliated bronchiolar epithelial cells (Fig. 4D), and type II pneumocytes as well as other alveolar wall cells (Fig. 4G) in lungs of untreated rats. In contrast to the results obtained with antibodies to cytochrome P-450 PB-B and NADPH-cytochrome P-450 reductase, however, the anti-cytochrome P-450 PCN-E bound to very similar extents to bronchial epithelial cells, Clara cells, and type II pneumocytes (Table 1). These findings, thus, indicate that lungs of untreated rats contain either cytochrome P-450 PCN-E or a related protein in the CYPIIIA family and, further, that similar levels of the protein are present in bronchial epithelial cells, Clara cells, and type II pneumocytes. The presence of this cytochrome P-450 in lungs of untreated male Sprague-Dawley rats was further substantiated by means of coupled sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunoblotting (Fig. 5). Using the same antiserum that was employed for immunohistochemical staining, a protein band was detected in pulmonary microsomes of untreated rats that exhibited the same mobility as the major band found in hepatic microsomes of male rats pretreated with pregnenolone-16α-carbonitrile, a chemical known to induce cytochrome P-450 PCN-E in rat liver (7, 20). Comparable findings were obtained with other anti-cytochrome P-450 PCN-E prepara-

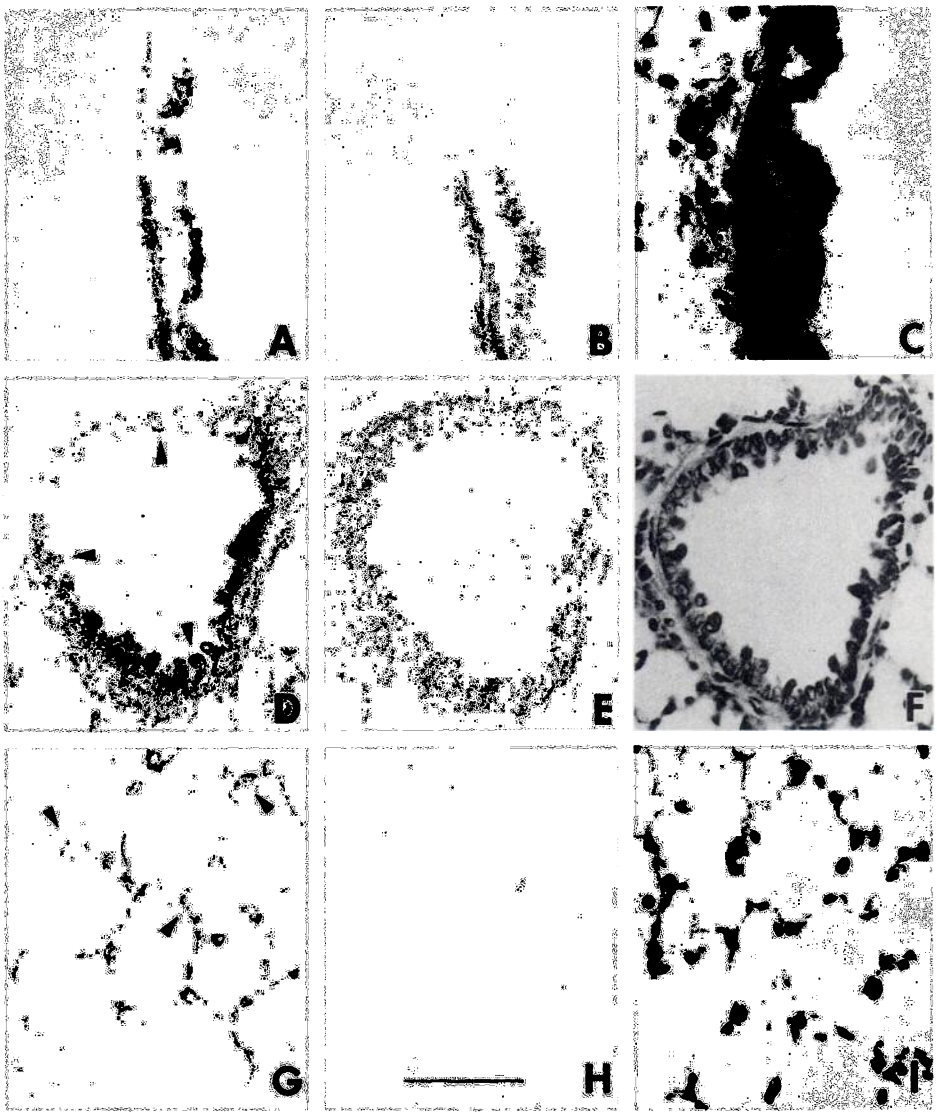

Fig. 2. Immunoperoxidase staining for cytochrome P-450 BNF-B within rat lung. A–C, Serial sections of a bronchus; D–F, serial sections of a bronchiole (*arrowheads* in D point to Clara cells); G–I, the same alveolar area in serial sections (*arrowheads* in G point to type II pneumocytes). A, D, and G, sections exposed to rabbit antiserum to rat hepatic microsomal cytochrome P-450 BNF-B; B, E, and H, sections exposed to normal rabbit serum; C, F, and I sections stained with toluidine blue. The *bar* in H = 50 μm.

tions that had been cross-adsorbed against hepatic microsomes of rats pretreated with various xenobiotics in order to improve specificity. A minor band was also seen in hepatic microsomes of pregnenolone-16α-carbonitrile-pretreated rats and is probably due to the presence of another form of cytochrome P-450 in the CYPIIIA family (7). This minor band was not detected in pulmonary microsomes of untreated rats, however. In other immunoblotting experiments in which purified cytochrome P-450 PCN-E was used as a standard, the levels of immunoreactive protein were estimated to be 0.2 nmol/mg of liver microsomal protein and 4 pmol/mg of lung microsomal protein. Thus, as evidenced by the immunoblot shown in Fig. 5, the overall level of the enzyme in lungs of untreated rats is approximately 2% of that found in liver.

Localization of benzo(α)pyrene hydroxylase activity within lungs of untreated rats. The incubation of unfixed cryostat sections prepared from lungs of untreated rats with benzo(α)pyrene and reduced pyridine nucleotides resulted in the generation of fluorescent phenolic metabolites from the polycyclic aromatic hydrocarbon in bronchial epithelial cells (Fig. 6A), Clara ciliated bronchiolar epithelial cells (Fig. 6B), and type II pneumocytes and other cells in the alveolar wall (Fig. 6C). Although some degree of variability was evident, benzo(α)pyrene visually appeared to be hydroxylated to similar extents by bronchial and bronchiolar epithelial cells and type II pneumocytes. Only minimal hydroxylase activity was detected in the absence of reduced pyridine nucleotides (Fig. 6, D–F). The appearance of connective tissue in the region of the basement membrane of the bronchus and bronchiole in sections incubated in either the presence (Fig. 6, A and B) or absence (Fig. 6, D and E) of reduced pyridine nucleotides resulted from nonspecific autofluorescence, which differed in color from the greenish-yellow fluorescence of the phenolic metabolites of benzo(α)pyrene. This brownish-yellow autofluorescence was also observed when sections were incubated in the absence of both benzo(α)pyrene and reduced pyridine nucleotides (data not shown).

Discussion

The complexity and cellular diversity of the lung have severely impeded the identification of the specific pulmonary cells within which xenobiotics can be metabolized *in vivo*. To overcome these difficulties and more precisely determine which

Fig. 3. Immunoperoxidase staining for cytochrome P-450 PB-B within rat lung. A–C, Serial sections of a bronchus; D–F, serial sections of a bronchiole (*arrowheads* in D point to Clara cells); G–I, the same alveolar area in serial sections (*arrowheads* in G point to type II pneumocytes). A, D, and G, sections exposed to rabbit antiserum to rat hepatic microsomal cytochrome P-450 PB-B; B, E, and H, sections exposed to normal rabbit serum; C, F, and I, sections stained with toluidine blue. The *bar* in H = 50 μm.

cells in lungs of untreated rats are capable of oxidatively metabolizing and bioactivating procarcinogens and other xenobiotics, immunohistochemistry was utilized in conjunction with histochemistry in the present investigation. The results of this study demonstrated that benzo(α)pyrene is monooxygenated *in situ* by several different pulmonary cell types, including bronchial epithelial cells, Clara and ciliated bronchiolar epithelial cells, and type II pneumocytes as well as other cells in the alveolar wall that cannot be unequivocally identified at the light microscopic level. Because the biotransformation of benzo(α)pyrene and other xenobiotics into cytotoxic, mutagenic, and carcinogenic metabolites is usually mediated by the cytochromes P-450-containing monooxygenase enzyme systems (1–3, 5, 9), xenobiotic activation would be most likely to occur to the greatest extent within those cells that contain both cytochrome(s) P-450 and NADPH-cytochrome P-450 reductase. The fact that the cytochrome P-450 reductase was immunohistochemically detected within each pulmonary cell type that was stained for one or more of the forms of cytochrome P-450 studied, therefore, indicates that these cells represent major sites for the *in vivo* oxidative metabolism and bioactivation of xenobiotics in lungs of untreated rats.

Immunohistochemical findings on the localization of NADPH-cytochrome P-450 reductase within the bronchial and bronchiolar epithelia and the alveolar wall in lungs of untreated rats described in the present communication agree with those reported by Dees *et al.* (40). They are also in agreement with recent findings on the localization of this flavoprotein in human lung (41). Furthermore, in the present study, microfluorometric determinations of the extents to which the anti-NADPH-cytochrome P-450 reductase bound to pulmonary cells of untreated rats demonstrated that the level of NADPH-cytochrome P-450 reductase in Clara cells is approximately twice as great as that found in either bronchial epithelial cells or type II pneumocytes.

The cellular localizations in several forms of cytochrome P-450 in lungs of untreated animals have been the subject of a number of recent studies (26, 29, 42–46). Some inconsistencies have been reported, however, in the intrapulmonary localizations of certain of these hemeproteins, especially polycyclic aromatic hydrocarbon-inducible forms. For instance, Foster *et al.* (44) were only able to immunohistochemically detect a 3-methylcholanthrene-inducible cytochrome P-450 in Clara cells of untreated rats, whereas Keith *et al.* (26) found that both type II pneumocytes and Clara cells of untreated rats contain methylcholanthrene- as well as phenobarbital-inducible forms

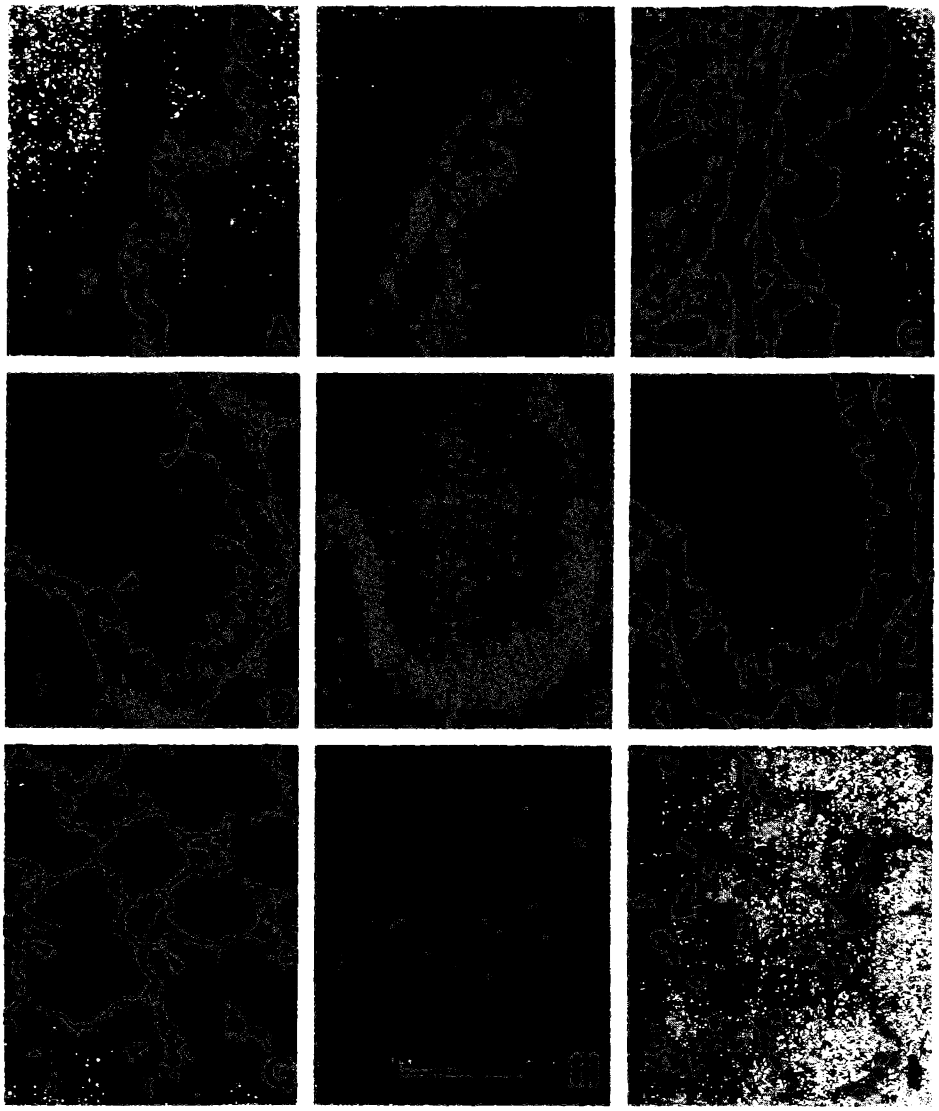

Fig. 4. Immunoperoxidase staining for cytochrome P-450 PCN-E within rat lung. A–C, Serial sections of a bronchus; D–F, serial sections of a bronchiole (*arrowheads* in D point to Clara cells); G–I, the same alveolar area in serial sections (*arrowheads* in G point to type II pneumocytes). A, D, and G, sections exposed to rabbit antiserum to rat hepatic microsomal cytochrome P-450 PCN-E; B, E, and H, sections exposed to normal rabbit serum; C, F, and I, sections stained with toluidine blue. The *bar* in H = 50 μm.

TABLE 1

Binding of antibodies raised against rat hepatic microsomal NADPH-cytochrome P-450 reductase and cytochromes P-450 BNF-B, PB-B, and PCN-E to pulmonary cells of untreated rats

Antibody binding values given represent the mean ± standard error of at least 20 separate microfluorometric determinations made after completion of immunofluorescence staining, using sections obtained from 10 untreated rats. The extent of binding of an antibody to a specific cell type was calculated by subtracting the mean intensity of fluorescence emitted at 525 nm from within 7-μm^2 circular areas in the cytoplasm of cells in a section exposed to normal serum from that emitted from within corresponding cells in a serial section exposed to an equal dilution of antiserum. The data are expressed in terms of 1 − absorbance (×100) so that there is a positive relationship between the intensity of immunofluorescence staining and the extent of antibody binding.

Antibody to	Antibody binding to		
	Bronchial epithelial cells	Clara cells	Type II pneumocytes
NADPH-cytochrome P-450 reductase	58.9 ± 2.3	109.1 ± 2.2[a]	58.4 ± 2.3
Cytochrome P-450 BNF-B	9.6 ± 2.0	5.8 ± 1.3	4.0 ± 0.8
Cytochrome P-450 PB-B	37.0 ± 2.4	55.1 ± 2.1[a]	38.6 ± 1.5
Cytochrome P-450 PCN-E	23.4 ± 1.8	26.6 ± 1.5	21.9 ± 1.2

[a] The extent of antibody binding is significantly greater ($p < 0.05$) than that to the other cell types, as determined by one-way analysis of variance and Tukey's *w* procedure.

of cytochrome P-450. Although the basis for such conflicting immunohistochemical findings remains to be determined, it must be appreciated that differences in staining for the same or closely related forms of cytochrome P-450 as well as the inability to immunohistochemically detect a specific cytochrome P-450 in fixed tissues could result from fixation-induced modifications in the three-dimensional structure and/or antigenic determinants of the hemeprotein, either of which could significantly alter the interaction between the cytochrome P-450 and its antibody. The use of unfixed tissues or different fixatives for preparing tissue specimens, as well as different preparations of polyclonal and monoclonal antibodies, could also account, at least in part, for the discrepancies in immunohistochemical findings on certain cytochromes P-450

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunoblotting of rat liver and lung microsomes. The indicated amounts of microsomal protein isolated from livers of pregnenolone-16α-carbonitrile-pretreated male rats (20) and lungs of untreated male rats (23) were applied to the indicated lanes of the gel. Electrophoresis, transfer of the resolved proteins to nitrocellulose sheets, and immunoperoxidase staining following exposure to rabbit antiserum to rat hepatic microsomal cytochrome P-450 PCN-E were performed as described previously (20, 23).

in lungs of untreated animals that have been reported by different laboratories. It is also possible that some of these inconsistencies might be due to species differences in the intrapulmonary localizations of certain cytochromes P-450.

The qualitative and semiquantitative immunohistochemical findings of this investigation demonstrated that the major forms of cytochrome P-450 induced by β-naphthoflavone, phenobarbital, and pregnenolone-16α-carbonitrile are each present in bronchial epithelial cells, Clara and ciliated bronchiolar epithelial cells, and type II pneumocytes as well as other alveolar wall cells. The fact that very weak immunohistochemical staining for cytochrome P-450 BNF-B was observed in these cells is consistent with the finding that only extremely low levels of this protein are detectable in pulmonary microsomes of untreated rats (23). Although visual observations of immunohistochemical staining are clearly subjective, especially when the intensity of staining is quite weak and only slightly greater than any nonspecific background staining in the tissue section, the presence of the β-naphthoflavone-inducible form of cytochrome P-450 in lungs of untreated rats was verified by microfluorometric determinations of anti-cytochrome P-450 BNF-B binding to pulmonary cells. Semiquantitative immunohistochemical findings further documented the existence of significant differences in the intrapulmonary distributions of different forms of cytochrome P-450. That is, cytochrome P-450 PB-B is expressed to the greatest extent in Clara cells, whereas cytochromes P-450 BNF-B and PCN-E are each present at similar levels in bronchial epithelial cells, Clara cells, and type II pneumocytes.

The observation that immunohistochemical staining for NADPH-cytochrome P-450 reductase and cytochromes P-450 BNF-B, PB-B, and PCN-E was detected in bronchial and bronchiolar epithelial cells and alveolar wall cells in lungs of untreated rats indicates that each of these cells can oxidatively metabolize xenobiotics and, thus, would be susceptible to the geno- and cytotoxicities that result as a consequence of the *in vivo* exposure of the lung to procarcinogens and other toxic chemicals. In this respect, the finding that Clara cells contain the greatest amounts of both NADPH-cytochrome P-450 reductase and cytochrome P-450 PB-B, the form of P-450 that appears to be primarily responsible for catalyzing the activation of 4-ipomeanol (20), is clearly consistent with the fact that the Clara cell is the pulmonary cell most susceptible to damage following *in vivo* exposure to 4-ipomeanol (2, 10). On the other hand, because cytochromes P-450 BNF-B and PCN-E are each present at similar levels in bronchial epithelial cells, Clara cells, and type II pneumocytes, the oxidative metabolism and bioactivation of xenobiotics catalyzed by these two forms of cytochrome P-450 would be likely to occur to similar extents in these cells.

Histochemical results demonstrating that benzo(α)pyrene is monooxygenated by bronchial and bronchiolar epithelial cells and alveolar wall cells in lungs of untreated rats correlated extremely well with immunohistochemical findings on the intrapulmonary localizations of NADPH-cytochrome P-450 reductase and cytochromes P-450. Indeed, the observation that benzo(α)pyrene appeared to be hydroxylated to similar extents by bronchial and bronchiolar epithelial cells and type II pneumocytes is entirely consistent with findings on the intrapulmonary distribution of cytochrome P-450 BNF-B, the cytochrome P-450 most active in catalyzing aryl hydrocarbon hydroxylase activity (20). It should be noted that the use of high pressure liquid chromatography-purified benzo(α)pyrene together with a cold stage to reduce fluorescence fading as well as the diffusion of polar metabolites in tissue sections resulted in significant increases in both the sensitivity and accuracy of the fluorescence histochemical assay for aryl hydrocarbon hydroxylase activity. The need for these modifications might explain why the hydroxylation of benzo(α)pyrene was originally not detected in the bronchial and bronchiolar epithelia (47).

The results of the present investigation are consistent with observations on the accumulation of O^6-ethylguanine (48) and O^6-methylguanine (49) in DNA of Clara cells and type II pneumocytes following *in vitro* exposure to diethylnitrosamine and the tobacco-specific carcinogen 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone, respectively. The finding that bronchial epithelial cells contain NADPH-cytochrome P-450 reductase and different forms of cytochrome P-450 and are capable of monooxygenating benzo(α)pyrene is also consonant with and provides support for the suggestion (50) that secretory

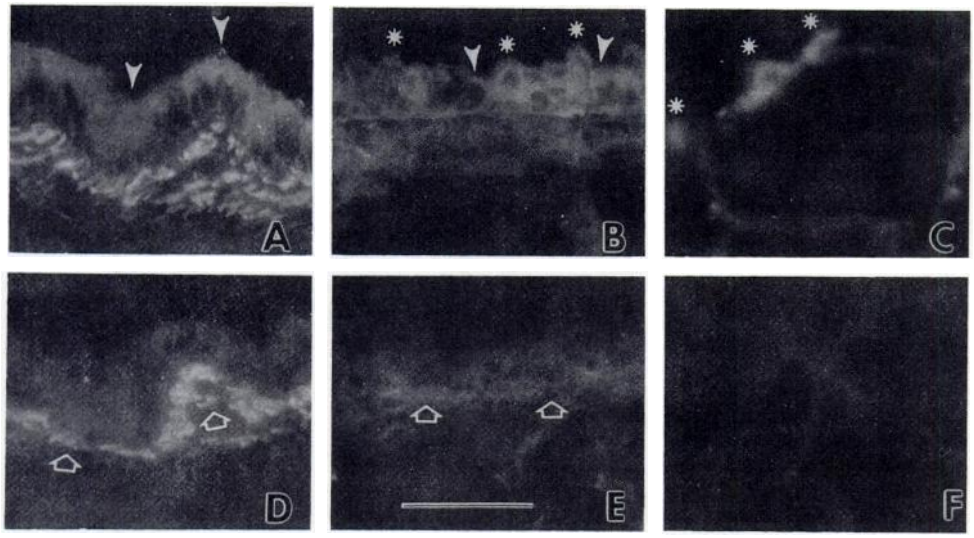

Fig. 6. Fluorescence histochemical localization of benzo(α)pyrene hydroxylase activity within rat lung. A and D, sections of a bronchus (*arrowheads* in A point to epithelial cells and those in D point to the basement membrane); B and E, sections of a bronchiole (*arrowheads* and *asterisks* in B indicate ciliated bronchiolar epithelial cells and Clara cells, respectively, and *arrowheads* in E point to the basement membrane); C and F, the same alveolar area in adjacent sections (*asterisks* in C indicate type II pneumocytes). A–C, Sections incubated with benzo(α)pyrene, NADPH, NADH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase; D–F, sections incubated with benzo(α)pyrene only. The greenish-yellow fluorescence resulting from the generation of phenolic metabolites from benzo(α)pyrene in the sections shown in A–C and the nonspecific brownish-yellow autofluorescence in all sections were visualized as described in Experimental Procedures. The *bar* in E = 25 μm.

cells in the bronchial epithelium are the cells of origin of the bronchogenic carcinomas that arise following the intratracheal administration of polycyclic aromatic hydrocarbons (51). On the other hand, although the presence of xenobiotic-activating enzymes is clearly one important determinant of a cell's susceptibility to the toxic actions of procarcinogens and other xenobiotics, it must be emphasized that the balance between xenobiotic activation and detoxication processes would be expected to be of utmost importance for determining within which cells reactive metabolites would accumulate and induce geno- and/or cytotoxicities. However, only limited information is currently available regarding where xenobiotics can be detoxicated *in situ* within the lung.

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