0026-895X/92/061039-08$03.00/0

MOLECULAR PHARMACOLOGY, 41:1039-1046

Distribution of Cytochrome P450 1A1 and NADPH-Cytochrome P450 Reductase in Lungs of Rabbits Treated with 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: Ultrastructural Immunolocalization and *In Situ* Hybridization

LILA H. OVERBY, SUSAN NISHIO, ALISON WEIR, GERALDINE T. CARVER, CHARLES G. PLOPPER, and RICHARD M. PHILPOT

Laboratory of Cellular and Molecular Pharmacology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 (L.H.O., G.T.C., R.M.P.), and Department of Anatomy, School of Veterinary Medicine, University of California, Davis, California 95616 (S.N., A.W., C.G.P.)

Received February 4, 1992; Accepted March 12, 1992

SUMMARY

Induction of cytochrome P450 1A1 (P450 1A1) in a variety of tissues is a well established consequence of exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. Although localization of the induced protein within the lung has been described, the precise intracellular distribution of the enzyme is not clear. Analysis of tissue sections, microsomal proteins, and mRNA from lungs of treated and untreated rabbits established that P450 1A1 had been induced by treatment with TCDD. Rabbit lungs from animals treated with TCDD were examined with immunocytochemistry and *in situ* hybridization, to identify the cell types that contain P450 1A1 and those that contain mRNA encoding P450 1A1. Endothelial cells of the entire vascular bed of rabbit lung reacted markedly with anti-P450 1A1. Likewise, cells lining both arteries and veins, as well as capillary endothelial cells, reacted strongly with the cDNA probe for mRNA encoding P450 1A1. Clara cells at all levels of airway labeled prominently for both P-450 1A1 and P450 1A1 mRNA. In addition, type 2 cells, alveolar macrophages, and, to a lesser degree, ciliated cells reacted with the cDNA probe. P450 reductase, which is required for P450 activity, has previously been identified in Clara cells, type 2 cells, and alveolar macrophages, but not in endothelium of rabbit lung. We have now obtained similar results for the localization of mRNA encoding P-450 reductase. This finding brings into question the function of P450 1A1 in endothelium.

P450 1A1, the first entry in the extensive list of enzymes belonging to the P450 superfamily of monooxygenases (1), is expressed at very low levels in tissues of untreated animals. However, its synthesis can be induced markedly by any of a number of naturally occurring and synthetic chemicals, the most potent of which is TCDD. Most interest in P450 1A1 stems from its ability to catalyze the monooxygenation of polycyclic aromatic hydrocarbons, an activity that can lead to the formation of carcinogenic products (2). Because of this, the participation of P450 1A1 in the metabolism of xenobiotics has been well studied. Nevertheless, it is not certain that biotransformation of exogenous chemicals constitutes the primary physiological function of P450 1A1.

Induction of P450 1A1 has been studied in numerous species and has been documented for tissues as varied as human placenta (3, 4) and rabbit striated muscle (5). The precise localization of the induction within a given tissue, particularly those with multiple cell types, has been investigated less thoroughly. The distribution of P450 1A1 is perhaps best described for rabbit lung. The amount of P450 1A1 in lungs of untreated rabbits is 2–3% of the total P450, but in isolated nonciliated bronchiolar (Clara) and alveolar type II cells P450 1A1 accounts for only about 0.5% of the total (6). In contrast, the relative concentrations of P450 2B and 4B (65–70% and 30–35% of the total, respectively) are the same for all three preparations. The distribution pattern of P450 1A1 is maintained after treatment of rabbits with TCDD, inasmuch as the amount in the cell fractions increases to about 8% and in the whole lung to about 26%. Results of immunochemical localization studies suggest that P450 1A1 (7), like P450 2B and 4B (8, 9), is expressed in the airway epithelium and interalveolar septum but may be prominent in the endothelium of the vasculature of lungs, at least in rabbits treated with TCDD (7). A similar distribution for P450 1A1 has been described for mouse lung (10).

ABBREVIATIONS: P450, cytochrome P450; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; reductase, cytochrome P450 reductase; PBS, 0.01 M sodium phosphate, pH 7.2, 0.85% sodium chloride solution; SSC, sodium chloride/sodium citrate (1×=0.15 M sodium chloride, 15 mM sodium citrate; FMO, flavin-containing monooxygenase; TEM, transmission electron microscopy; SDS, sodium dodecyl sulfate.

Significant expression of P450 1A1 in endothelium as an explanation for the difference in distribution between P450 1A1 and other forms of P450 in lung is particularly intriguing because no immunochemical evidence of reductase in these cells could be obtained at the ultrastructural level (9). Because reductase is likely required for P450 catalytic function, P450 1A1 expressed in endothelium may actually act as a binding protein or in some other capacity.

In the present study, we have examined the cellular and subcellular localization of P450 1A1 in lungs of untreated and TCDD-treated rabbits and compared this with the distribution of mRNA encoding P450 1A1. The cell types that contained P450 1A1 and its mRNA were the same in untreated and treated animals. Endothelial cells exhibited the largest increases in P450 1A1 content after treatment with TCDD. Lesser increases were observed for Clara and type II cells and alveolar macrophages. No mRNA for the reductase was detected in endothelial cells, with the distribution being the same as reported previously for the enzyme (9).

Materials and Methods

Animals, treatments, and preparation of tissues. Adult, male, New Zealand White rabbits (Dutchland Farms, Denver, PA) were not treated or were administered TCDD (10 μg/kg) 96 hr before killing. Both groups were allowed free access to food and water.

Rabbits were anesthetized with Nembutal (50 mg/kg), and the lungs were removed. For immunocytochemistry, lungs from six rabbits (three untreated and three treated) were perfused with paraformaldehyde (1%) in phosphate buffer (0.1 M, pH 7.4), via a tracheal cannula. Fixation was for 1 hr at a fluid pressure of 30 cm. The lungs were held in phosphate buffer (0.2 M, pH 7.4) until processed for light microscopy or TEM. Lungs from six additional animals (three from each group) provided sections for *in situ* hybridization and samples for isolation of microsomal fractions and mRNA. The right apical lobe of each lung was cannulated and infused with a cryoprotectant composed of polyvinyl pyrolidone (20%, M_r 10,000), sucrose (1.5 M), and $CaCl_2$ (3 mM), to which dithiothreitol (3 mg/ml) and RNase A inhibitor (550 units/ml) were added immediately before use. The lobe was then sliced (3 mm), cut into blocks (5 × 8 mm), and frozen in hexane chilled in an ethanol/dry ice bath (−56°). After 1 min the frozen blocks were transferred to dry tubes and held at −56° until placed in storage at −70°. These blocks were sectioned for examination by *in situ* hybridization. Simultaneously with the start of infusion, the remaining lobes of the right lungs were placed in ice-cold buffer and used for the preparation of microsomal fractions. The left lung from each rabbit was immersed in liquid nitrogen within 3–5 min of thoracotomy, stored at −70°, and used for isolation of mRNA.

Preparation of microsomal fractions and immunoblotting. Pulmonary microsomal fractions were prepared as described previously (11). Immunoblotting of microsomal proteins that had been separated by electrophoresis in the presence of SDS (12) was carried out by the method of Towbin *et al.* (13), as modified by Domin *et al.* (14). Goat polyclonal antibodies to rabbit P450 1A1 were a gift from Dr. E. F. Johnson (Scripps Clinic and Research Institute, La Jolla, CA). This antibody preparation recognizes two forms of P450, 1A1 and IA2, only one of which (1A1) is present in lung (15).

Preparation of mRNA and Northern blotting. Polyadenylated RNA was purified by the methods of Chirgwin *et al.* (16) and Glisin *et al.* (17), using the procedure developed by Gasser *et al.* (18). Samples of mRNA were analyzed by hybridization with a ^{32}P-labeled cDNA probe for P450 1A1 (19), after electrophoresis on agarose gels (20) and transfer to a nylon (Nytran) membrane (Schleicher and Schuell, Keene, NH). The full-length cDNA for rabbit P450 1A1 was provided by Dr. E. F. Johnson.

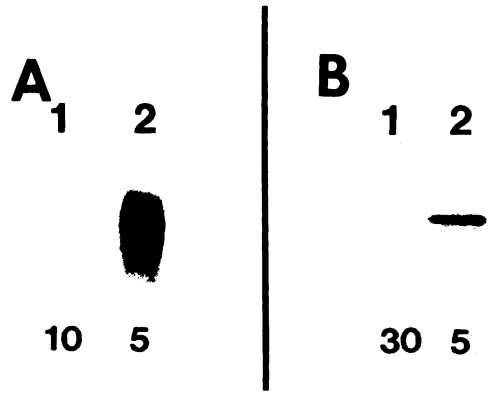

Fig. 1. Induction of P450 1A1 in rabbit lung after treatment with TCDD. A, Northern analysis of pulmonary mRNA from lungs of untreated and TCDD-treated rabbits. mRNA samples from an untreated rabbit (*lane 1*) (10 μg of mRNA) and a rabbit treated with TCDD (*lane 2*) (5 μg of mRNA) were analyzed for mRNA encoding P450 1A1, by hybridization with ^{32}P-labeled cDNA for P450 1A1. B, Immunoblot of pulmonary microsomal proteins from untreated and TCDD-treated rabbits. Microsomal samples from the lung of an untreated rabbit (*lane 1*) (30 μg of protein) and the lung of a rabbit treated with TCDD (*lane 2*) (5 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunostained with anti-P450 1A1.

Preparation of tissue sections. Paraffin sections were prepared by standard procedures (21). For TEM, blocks of tissue with cross-sections and longitudinal sections of airways containing terminal bronchiole/alveolar duct junctions were selected by microdissection and stored in phosphate buffer at 4°. The blocks were washed in buffer, dehydrated in *N,N*-dimethylformamide (50–90%), and infiltrated with Lowicryl K4Ms (Ted Pella, Inc., Redding, CA), at 4°. The tissues were transferred to polyethylene capsules filled with fresh Lowicryl, which were then sealed and polymerized with UV irradiation at 4°, in a foil-lined box, for 1 hr. Blocks were sectioned on an ultramicrotome (Sorvall MT 5000) with a diamond knife, and the 0.5-μm sections were placed on nickel grids. Frozen sections for *in situ* hybridization were cut from blocks of tissue with a glass knife, in a Bright Cryocut (Hacker Bright Instrument Co., Fairfield, NJ). The sections were thawed onto subbed slides (degreased and coated with 1% gelatin, followed by 0.25 mg/ml poly-L-lysine) and air-dried at −33° for 30 min.

Immunohistochemistry. Routine histological sections were made from tissue blocks selected to contain terminal bronchioles. Hydrated sections were treated with hydrogen peroxide (3%) in methanol, to block endogenous peroxidase activity, and were incubated for 2 hr in 1/2500, 1/5000, and 1/7500 dilutions of primary antibody. Control treatments included preimmune serum and substitution of buffer for sera. The avidin-biotin peroxidase procedure (Vector Laboratories, Burlingame, CA) was used for light microscopic detection of antigens in paraffin sections. The avidin-biotin peroxidase protocol followed the dilution and staining procedures outlined by the supplier of the reagents. Sections were photographed with a Zeiss photomicroscope. In order to visualize the control sections, photographic conditions that enhanced the edge effect were required. TEM immunocytochemistry was carried out with a modification of the procedure described by Roth *et al.* (22), in which rabbit anti-goat IgG-gold or Protein G-gold were substituted for Protein A-gold. Grids were floated in droplets of ovalbumin (1%) in PBS and then incubated on drops of primary antiserum for 3 hr at room temperature. The antiserum was diluted with ovalbumin (1%) in PBS and used at concentrations ranging from 1/1000 to 1/10,000. Monodispersion of immuno-gold was verified, using Formvar-coated (Ted Pella, Redding, CA) grids treated with poly-L-lysine (1 mg/ml), before incubation with the diluted gold preparation. The treated sections were rinsed and then incubated for 1 hr, at room temperature, in drops of 5-nm Protein G-gold (Jannsen Life Sciences, Beerse, Belgium) diluted (1/20) in PBS. Sections were poststained in

Fig. 2. Light microscopic immunocytochemistry of the distribution of P450 1A1 in lungs of TCDD-treated and untreated rabbits. [Please note that the sections were not counterstained and that enhanced edge effect was needed in order to visualize the control tissues (A and D). In contrast, visualization of sections B, C, E, and F was due entirely to antibody staining]. A and B, Terminal bronchiole (*TB*) of untreated rabbit incubated with normal goat serum (A) or anti-P450 1A1 (B). Minimal labeling was observed with anti-P450 1A1 in bronchiolar epithelium (*arrowheads*) and surrounding parenchymal tissue. No labeling was observed with normal goat serum. C, Terminal bronchiole (*TB*) of TCDD-treated rabbit incubated with anti-P450 1A1, showing labeling in the apices of bronchiolar epithelial cells (*arrowheads*) and in the endothelial compartments of interalveolar septa (*arrows*). D, Pulmonary vein (*PV*), larger pulmonary artery (*PA*), and parenchyma from TCDD-treated rabbit incubated with normal goat serum in place of primary antibody. No labeling was observed in any area of the endothelium (*arrows*) or interalveolar septa. E and F, Large pulmonary artery (*PA*) and parenchyma from untreated (E) or TCDD-treated (F) rabbit incubated with anti-P450 1A1. Endothelium (*arrows*) contained minimal label in either capillaries or larger vessels of untreated animal (E), whereas obvious staining of endothelial cells lining the large vessels (*arrows*) and in the interstitium was observed with sections from treated animals (F). Labeling in septa and large vessels was primarily in the perinuclear areas of endothelial cells and cuboidal cells at alveolar junctions (*arrowheads* in *inset*). Original magnification, 60×, inset magnification, 100×. The primary antibody was used at a dilution of 1/10,000.

4% aqueous uranyl acetate and examined in a Zeiss EM10 transmission electron microscope.

***In situ* hybridization.** Air-dried frozen sections were permeabilized and acetylated before *in situ* hybridization, according to the method of Phelps and Floros (23), with the exceptions that glutaraldehyde (0.1%) was added to the paraformaldehyde (4%) fixative solution and Triton X-100 (0.01%) was added to the PBS rinses. Sections selected for one set of negative controls were treated with RNase A (10 mg/ml, 50 μl/section) for 30 min immediately before acetylation. Sections were incubated with radiolabeled cDNA probe (1 μl; 10^6 cpm for ^{35}S and 10^5 cpm for ^{3}H) in hybridization buffer (9 μl) composed of formamide diluted (1:1) with NaCl (1.2 M), Tris (20 mM, pH 7.5), EDTA (2 mM), salmon sperm DNA (0.2 mg/ml), Denhardt's (24) solution (2×). The slides were placed in sealed chambers with hybridization buffer and were incubated for 16 hr at 42°. After hybridization, slides were flushed with SSC/SDS (2×/0.1%), washed with SSC/SDS (1×/0.1%) for 1 hr while being rocked at 6–8 oscillations/min, soaked in SSC/SDS (0.1×/0.1%) for 15 min, and rinsed briefly with H_2O. After drying and dehydration, the slides were coated with 50% Ilford K-2 autoradiographic emulsion (Polysciences, Fort Washington, PA) at 42° and were exposed for 4–7 days (^{35}S-labeled probes) or 3 weeks (^{3}H-labeled probes), at 4°. Development was for 2 min at 17° with D-19 developer (Kodak, Rochester, NY) and fixing (Kodak fixer) for 15 min at 17°. Sections were counterstained lightly with eosin Y, followed by methylene blue-azure A (LeukoStat Stain Kit, Fisher Scientific), and were photographed with a Zeiss photomicroscope.

Probes for reductase, liver FMO, and actin, and labeling of cDNAs. Antibodies to rabbit reductase have been described previously (9, 25). These antibodies were used to screen a cDNA library prepared from rabbit pulmonary mRNA. A clone containing the complete reductase coding region was isolated and purified. The identity of the 2.2-kb clone was confirmed by comparing end sequences with the sequence

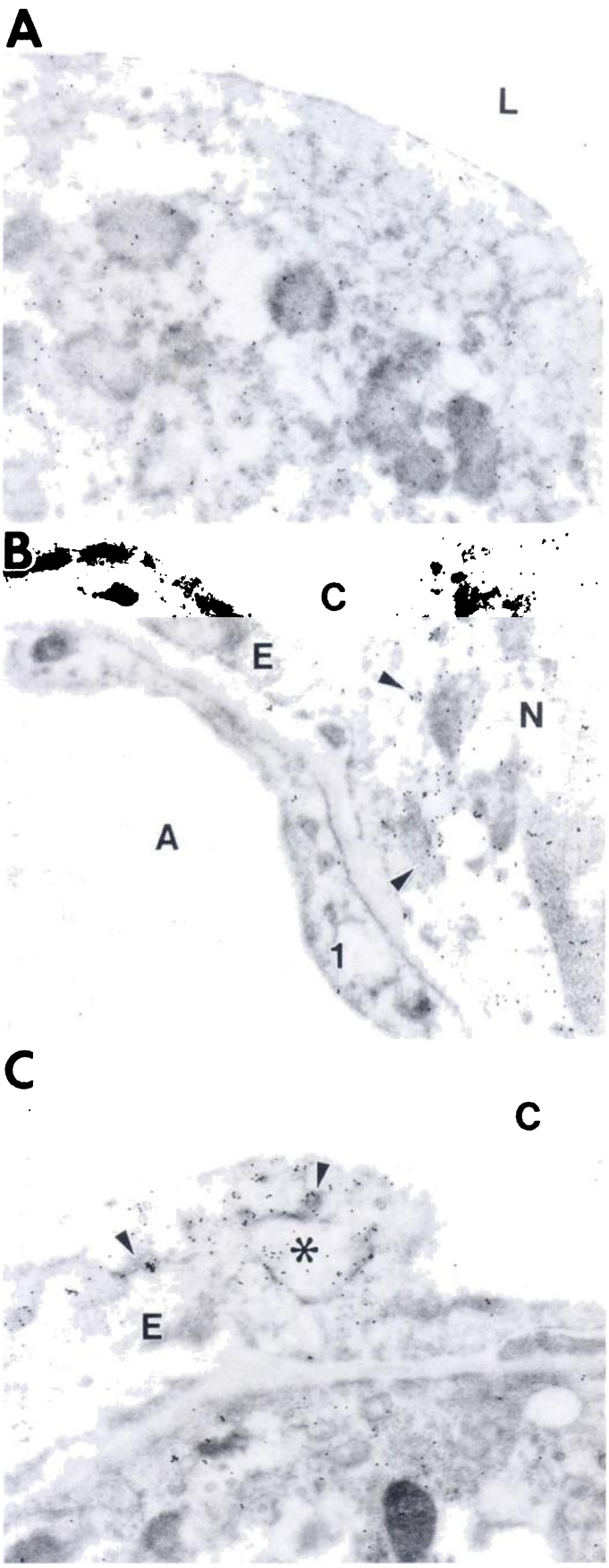

Fig. 3. Electron microscopic immunocytochemical localization of P450 1A1 within pneumocytes of rabbits treated with TCDD. A, Apex of nonciliated bronchiolar epithelial cell, showing labeling of areas rich in smooth endoplasmic reticulum. No labeling of plasma membrane surfaces facing the airway lumen (*L*) was observed. B, Anti-P450 1A1 labeled the thick perinuclear portions of the endothelial cell (*E*) cytoplasm (*arrowhead*) and, to a much lesser extent, the cytoplasm of type I cells (*1*). C, Labeling of capillary endothelial cells (*E*) of TCDD-treated rabbits by anti-P450 1A1. The majority of the label was observed in the thicker portions of the cytoplasm and appeared to be closely associated with internal membranous structures (*) that contained large cavities or with what appeared to be endocytotic vesicles (*arrowheads*). *N*, Endothelial nucleus; *C*, capillary lumen; *A*, alveolar space. Original magnification for each micrograph, 28,000×.

published for cDNA encoding the rabbit reductase (26). Human β-actin cDNA, which cross-reacts with actin mRNA from most species, was purchased from Boehringer Mannheim (La Jolla, CA). The cDNA for the rabbit liver FMO, which is not expressed in lung, has been described (27). For *in situ* hybridization, cDNA probes (P450 1A1, reductase, liver FMO, and actin) were labeled with ^{35}S or ^{3}H by means of a random-primed DNA labeling kit (Boehringer Mannheim). P450 1A1 cDNA was also labeled with ^{32}P for Northern blot experiments. Only radiolabeled dCTP (Amersham) was used for incorporating ^{32}P or ^{35}S into the cDNAs, whereas both radiolabeled dCTP and dATP (Amersham) were used to incorporate ^{3}H.

Results

Induction of P450 1A1 by TCDD. Induction of P450 1A1 in lungs used for *in situ* hybridization studies is shown clearly by results of both immunoblots of microsomal proteins and Northern analysis of mRNA samples from untreated and TCDD-treated rabbits (Fig. 1, A and B). These results are entirely consistent with those obtained previously, showing a 15–20-fold increase in pulmonary P450 1A1 after treatment of rabbits with TCDD (6). Induction in lungs used for TEM immunocytochemical studies is shown by results of light microscopic studies. Although immunocytochemical labeling, with anti-P450 1A1, on paraffin sections of tissue from untreated rabbits was virtually undetected, labeling of tissue from treated rabbits was prominent (Fig. 2). With treated tissue, most, but not all, Clara cells contained reaction product. In addition, the endothelium of the vasculature and areas of the interalveolar septa were labeled (Fig. 2F). At higher magnification, the positive areas of the interalveolar septa appeared to be associated with capillary endothelium (Fig. 2F, *inset*). Focal staining of cuboidal cells located on the edges of septa (likely type II cells) was also noted.

Ultrastructural distribution of P450 1A1 in lungs from rabbits treated with TCDD. Immunocytochemistry by TEM confirmed the reactivity of anti-P450 1A1 in Clara cells of the distal bronchioles and in endothelial cells of the vasculature of lungs from rabbits treated with TCDD (Fig. 3). Within the Clara cells, the majority of the gold label was found in the apical cytoplasm, in conjunction with smooth endoplasmic reticulum (Fig. 3A). The quantity of label per Clara cell was highly variable, and a small proportion of the Clara cells were not labeled. In the interalveolar septa, all endothelial cells examined were labeled. This label was found primarily in the perinuclear region associated with endoplasmic reticulum (Fig. 3B), and no labeling of plasma membrane was observed (Fig. 3, A and C). There was minimal labeling of type I and type II cells and no labeling of smooth muscle cells or cells of the immune system. No macrophages were identified in the TEM sections.

***In situ* hybridization of mRNA encoding P450 1A1 in**

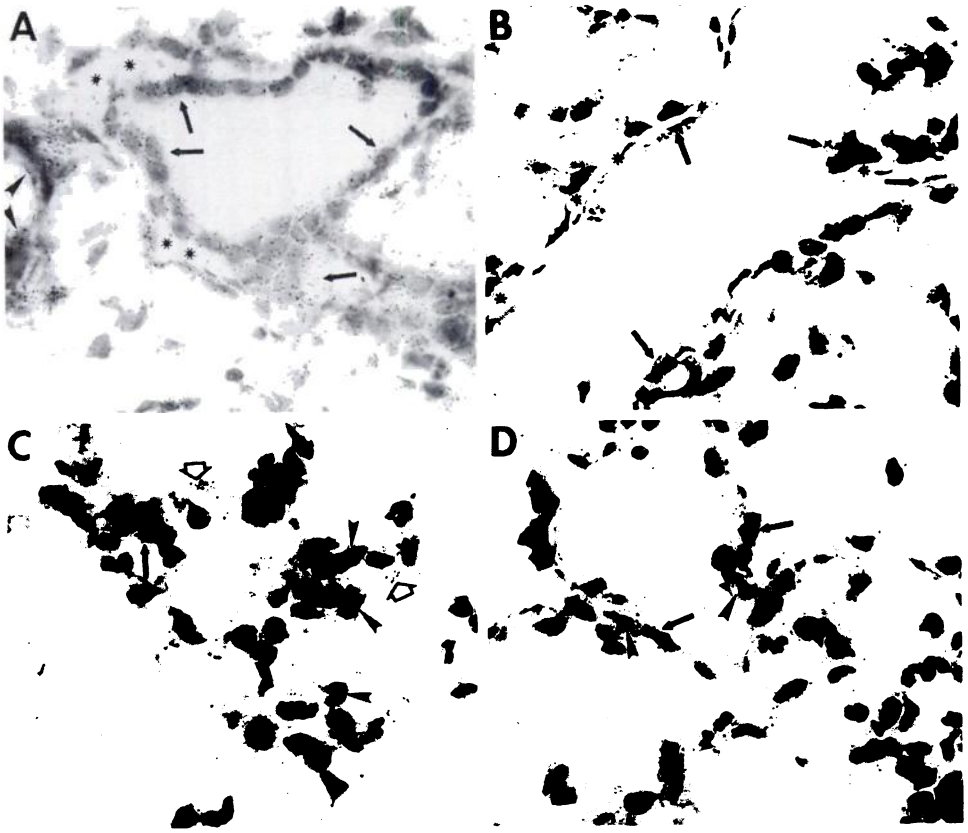

Fig. 4. *In situ* hybridization of ^{35}S- and ^{3}H-labeled probes for P450 1A1 to sections of lung from rabbits treated with TCDD. A, Cells of the airway epithelium (*arrows*) and endothelium of an artery (*arrowheads*) labeled with the ^{35}S-labeled probe. Label was also observed in parenchymal cells, but not in cells of the connective tissue plane (*). B, Labeling of the endothelium of a pulmonary vein (*arrows*) and cells in the interalveolar septa with the ^{35}S-labeled probe. Again, the connective tissue plane (*) is negative. C and D, Precise labeling of cells in the interalveolar septa with the ^{3}H-labeled probe. More than 9 grains/cell were observed over parenchymal cells (40% of the total) with a variety of nuclear profiles (*solid arrows*) and over thickened portions of the septa not associated with a nucleus (*open arrows*). Approximately 10% of the parenchymal cells contained 6–10 grains/cell (*arrowheads*), and the remainder contained 1–5 (30%) or no (20%) grains/cell. Original magnification of A and B, 256× C and D, 380×.

lungs from rabbits treated with TCDD. Hybridization of mRNA for P450 1A1 with ^{35}S-labeled (Fig. 4, A and B) or ^{3}H-labeled probe (Fig. 4, C and D) gave results consistent with the immunochemical localization of P450 1A1 protein. Sections treated with ^{35}S-labeled probe showed many more grains than those treated with ^{3}H-labeled probe. However, use of the ^{35}S-labeled probe produced much higher background levels, and the pattern of localization was actually much clearer with the ^{3}H-labeled probe. The majority of the airway epithelial cells were positive for P450 1A1 mRNA (6–10 grains/cell), as were the endothelial cells of both large and small blood vessels (Fig. 4B). In addition, focal labeling appeared in the interalveolar septa. One cell type associated with thickened portions of the septa, containing a large, pale, oval nucleus, labeled prominently (Fig. 4, C and D). We believe these cells to be capillary endothelial cells sectioned *en face*. Additional positive cells had nuclear profiles typically seen with endothelial cells sectioned in other planes. Labeled cells (10–14 grains/cell) with endothelial nuclear profiles accounted for about 40% of the cells in the interalveolar septa that had visible nuclei. A second focal pattern of labeling, not associated with nuclei, was observed in the cytoplasm of cells in the thick portions of septa (Fig. 4C). Cells at the intersections of septa were also labeled (6–9 grains/cell). This population, which has darkly stained nuclei and which projected into the air space, accounted for approximately 10% of the cells in the interalveolar septa and is likely made up of alveolar type II cells and macrophages. The few alveolar macrophages that could be identified by their location and morphology were all labeled. Of the remaining 50% of the cells in the interalveolar septa, about 30% labeled with <5 grains/cell and 20% contained no grains at all. Identified immune system cells invariably contained no grains. The validity of the *in situ* hybridization results obtained with the cDNA probe to P450 1A1 was tested with a series of control experiments. A cDNA for human actin was used as a positive control and was found to bind to nearly every cell (4–12 grains/cell) in every section examined (Fig. 5A). In contrast, the grains observed with the negative controls (RNase treatment followed by the ^{35}S-P450 1A1 cDNA, as well as ^{35}S-cDNA encoding the liver FMO) were distributed randomly, at a density of about 0.2/cell (Fig. 5, B and C). Also, approximately equal grain densities were observed over tissue and glass with the negative controls. The probe for liver FMO was used because it is similar in size to the P450 1A1 cDNA (2.0 versus 2.2 kilobases) and because mRNA for liver FMO is not expressed in lung (25). Also, the relationship between liver FMO and the FMO expressed in lung (about 55% identity in the coding region) provided a good negative control.

Localization of mRNA encoding reductase. The pattern of labeling observed with the ^{3}H-labeled cDNA probe for reductase (Fig. 6) was similar to that seen previously with TEM immunocytochemical localization of the protein (8). The ma-

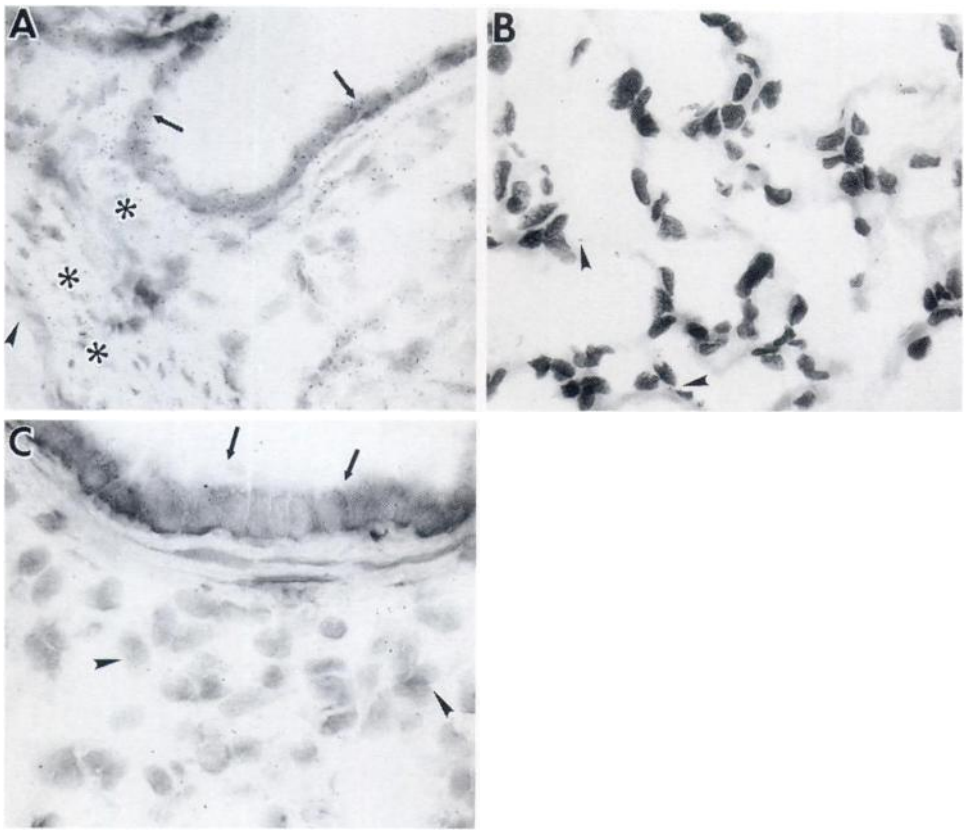

Fig. 5. Results of positive and negative control experiments for *in situ* hybridization to lung tissue from rabbits treated with TCDD. A, Labeling of all cells (4–12 grains/nucleus), including those in airways (*arrows*), endothelium (*arrowheads*), and connective tissue (*), with ^{35}S-labeled probe for actin. B, Hybridization of ^{35}S-labeled probe for P450 1A1 to a section treated with RNase A. Only a few randomly distributed grains (*arrowheads*) developed over this section. C, Hybridization of ^{35}S-labeled probe for the hepatic FMO 1A1. The figure shows little or no cross-hybridization with the pulmonary FMO (56% sequence identity with the hepatic enzyme) known to be present in airway (*arrows*) and parenchymal (*arrowheads*) cells. Original magnification of A and B, 380×; C, 640×.

Fig. 6. Tritiated probe for reductase hybridized to lung tissue from a rabbit treated with TCDD. A, Hybridization of reductase probe to nonciliated bronchiolar epithelial cells in airways (*large arrows*). An alveolar macrophage in the airway and one in the parenchyma (*small arrows*) were also positive for reductase mRNA. B, Prominent hybridization of reductase probe to a few cells (*small arrow*) in the interalveolar septa. The location and appearance of these cells are consistent with alveolar macrophages; however, identification of these cells at the light level is difficult. Many cells of the parenchyma contain 1–4 grains/cell (*arrowheads*). Original magnification of A, 640×; B, 380×.

jority of the grains were associated with the epithelium of the airways. Large cells protruding into the airways and accounting for approximately 50% of the epithelial cells (undoubtedly Clara cells) were labeled (Fig. 6A). A significant but low level of label was also present over tissue in the parenchyma, and more than half the cells in the interalveolar septa contained 1–4 grains/cell after treatment with the tritiated probe (Fig. 6B). Although this density of label is much less than associated with Clara cells, it is well above the background established with the negative controls. The identities of the labeled cells present in the interalveolar septa are uncertain, although the general pattern was similar to that observed with the cDNA probe for P450 1A1 mRNA. In particular, some labeling was perinuclear and some was associated with cytoplasmic extensions along

alveolar walls. A few cells contained more than 4 grains/cell and displayed large pale nuclei that protruded from alveolar walls, characteristics typical of alveolar macrophages.

Discussion

The major site for induction of P450 1A1 in rabbit lung appears to be the endothelium of the vasculature. Clearly, induction also occurs in the Clara cell and type II cell, but not to the same extent. This pattern is consistent with our previous conclusion that the distribution of P450 1A1 in lungs of rabbits treated with TCDD differs from that of P450 2B and P450 4B (6). Also, P450 1A1 can be detected in all cells of the endothelium, but not in all Clara cells. In the pulmonary vasculature the thickened perinuclear portion of endothelial cells, which is the area containing organelles, including endoplasmic reticulum, labeled for P450 1A1, and in Clara cells detection of P450 1A1 was confined to the apical portion, consistent with localization to smooth endoplasmic reticulum. No binding of anti-P450 1A1 to the plasmalemma of any cell type was observed. This contrasts with previous observations that both P450 2B and P450 4B are associated with the plasma membrane as well as the endoplasmic reticulum of Clara cells and the plasma membrane of endothelial cells (9).

The distribution of mRNA for P450 1A1 agrees with the distribution of P450 1A1. As was the case for protein, there was wide variation in the amount of P450 1A1 mRNA in the Clara cells, with the levels in some being actually less than detectable. It has been suggested that variability in P450 content in Clara cells of the rat is indicative of different subsets of Clara cells, each with distinctive patterns of expression (28). However, we believe that this variability is just as likely a function of cyclic expression in every Clara cell, rather than evidence of distinct populations. Some mRNA was also detected in ciliated cells of the airways. Evaluation of mRNA localization in the interalveolar septa was not as clear, because cell identification in that region at the light microscopic level is difficult. Patterns of labeling for both protein and mRNA were the same in serial frozen sections. The variety of nuclear profiles seen with the most heavily labeled cells was consistent with the cross-sections that can be obtained with the discoid nucleus of an endothelial cell. Also, the number of heavily labeled cells accounted for about 40% of the total, the expected proportion of endothelial cells in the interalveolar septum. A second population of cells, present at intersections of septa and accounting for about 10% of the total, was labeled to a lesser extent. These are thought to be type II cells.

The distribution of reductase in rabbit lung differs in several respects from the distribution of P450 1A1. For example, detection of mRNA for reductase in Clara cells shows little variability from cell to cell. This even distribution has been described for the reductase protein in rat (28, 29), rabbit (9, 30), and human (31) lung, in direct contrast to the variability in detection of P450 1A1 protein and mRNA discussed above. This difference between the patterns of distribution of reductase and P450 1A1 is even more marked with the endothelium of the vasculature, where mRNA for reductase is not detected in any cells. This negative finding is entirely consistent with results of studies in which reductase has not been detected in the vasculature endothelium of rabbits (9), rats (28, 29), minipigs (28), hamsters (32), or humans (31). In contrast, localization of P450 1A1 to the endothelium of the pulmonary vasculature of humans (33), rabbits (7, 9), rats (28), and mice (10) has been observed. In addition, endothelial cells of gill and heart of two species of fish (scup and trout) have also been identified as sites of expression of P450 1A1 (34).

Although it is relatively easy to speculate about possible roles for P450-mediated metabolism in endothelium, it is not clear that these cells actually contain a P450 monooxygenase system. The lack of positive identification of the reductase (mRNA or protein) in endothelium suggests several possibilities. First, oxidative metabolism occurs in endothelium but is limited by low levels of reductase. This would be consistent with observations that the specific activity of P450 1A1 in pulmonary microsomes decreases after induction, a decrease that is not due to the presence of apoenzyme (15). Second, oxidative metabolism catalyzed by P450 1A1 in endothelium is mediated by electron flow via cytochrome b_5, a substrate-specific pathway in lung (35, 36) that can be initiated by NADH and cytochrome b_5 reductase or NADPH and very low concentrations of reductase (35). Third, P450 1A1-catalyzed metabolism in endothelium is mediated by hypervalent oxygen substrates such as hydroperoxides (37). Fourth, the function of P450 1A1 in endothelium is not associated with oxidative metabolism but, rather, with its exceptionally high affinity for binding exogenous ligands such as TCDD (38). These possibilities are presently under investigation in our laboratory.

References

1. Nebert, D. W., D. R. Nelson, M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. C. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. Phillips, R. Sato, and M. R. Waterman. The P450 superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8**:1–13 (1989).
2. Bend, J. R., and C. J. Serabjit-Singh. Xenobiotic metabolism by extrahepatic tissues: relationship to target organ and cell toxicity, in *Drug Metabolism and Drug Toxicity* (J. R. Mitchell and M. G. Horning, eds.). Raven Press, New York, 99–136 (1984).
3. Wong, T. K., B. A. Domin, P. E. Bent, T. E. Blanton, M. W. Anderson, and R. M. Philpot. Correlation of placental microsomal activities with protein detected by antibodies to rabbit cytochrome P-450 isozyme 6 in preparations from humans exposed to polychlorinated biphenyls, quaterphenyls, and dibenzofurans. *Cancer Res.* **46**:999–1004 (1986).
4. Fujino, T., S. S. Park, D. West, H. L. Gurtoo, R. E. Tarone, and H. V. Gelboin. Monoclonal antibody phenotyping of interindividual differences in cytochrome P-450-dependent reactions of single and twin human placenta. *Cancer Res.* **44**:3916–3923 (1984).
5. Serabjit-Singh, C. J., J. R. Bend, and R. M. Philpot. Cytochrome P-450 monooxygenase system: localization in smooth muscle of rabbit aorta. *Mol. Pharmacol.* **28**:72–79 (1985).
6. Domin, B. A., T. R. Devereux, and R. M. Philpot. The cytochrome P-450 monooxygenase system of rabbit lung: enzyme components, activities, and induction in the nonciliated bronchiolar epithelial (Clara) cell, alveolar type II cell, and alveolar macrophage. *Mol. Pharmacol.* **30**:296–303 (1986).
7. Dees, J. H., B. S. Masters, U. Muller-Eberhard, and E. F. Johnson. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and phenobarbital on the occurrence and distribution of four cytochrome P-450 isozymes in rabbit kidney, lung and liver. *Cancer Res.* **42**:1423–1432 (1982).
8. Serabjit-Singh, C. J., C. R. Wolf, R. M. Philpot, and C. G. Plopper. Cytochrome P-450: localization in rabbit lung. *Science (Washington D. C.)* **207**:1469–1470 (1980).
9. Serabjit-Singh, C. J., S. J. Nishio, R. M. Philpot, and C. G. Plopper. The distribution of cytochrome P-450 monooxygenase in cells of rabbit lung: an ultrastructural immunocytochemical characterization. *Mol. Pharmacol.* **33**:279–289 (1988).
10. Anderson, L. M., J. M. Ward, S. S. Park, A. B. Jones, J. L. Junker, H. V. Gelboin, and J. M. Rice. Immunohistochemical determination of inducibility phenotype with a monoclonal antibody to a methylcholanthrene-inducible isozyme of cytochrome P-450. *Cancer Res.* **47**:6079–6085 (1988).
11. Philpot, R. M., E. Arinc, and J. R. Fouts. Reconstitution of the rabbit pulmonary microsomal mixed-function oxidase system from solubilized components. *Drug Metab. Dispos.* **3**:118–126 (1975).
12. Laemmli, U. K., and M. Favre. Maturation of the head of bacteriophage T4. I. Packaging events. *J. Mol. Biol.* **80**:575–599 (1973).
13. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).

14. 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).
15. Domin, B. A., and R. M. Philpot. The effect of substrate on the expression of activity catalyzed by cytochrome P-450: metabolism mediated by rabbit isozyme 6 in pulmonary microsomal and reconstituted monooxygenase systems. *Arch. Biochem. Biophys.* **246**:128–142 (1986).
16. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299 (1979).
17. Glisin, V., R. Crkvenjakov, and C. Byus. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* **13**:2633–2637 (1974).
18. Gasser, R., M. Negishi, and R. M. Philpot. Primary structures of multiple forms of cytochrome P-450 isozyme 2 derived from rabbit pulmonary and hepatic cDNAs. *Mol. Pharmacol.* **32**:22–30 (1988).
19. Okino, S. T., L. C. Quattrochi, H. J. Barnes, K. J. Griffin, E. F. Johnson, and R. H. Tukey. Cloning and characterization of cDNAs encoding 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible rabbit mRNAs for cytochrome P-450 isozymes 4 and 6. *Proc. Natl. Acad. Sci. USA* **82**:5310–5314 (1985).
20. Bailey, J. M., and N. Davidson. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. *Anal. Biochem.* **70**:75–85 (1976).
21. Carleton, H. M., R. A. B. Drury, and E. A. Wallingtron. *Carleton's Histological Technique*, Ed. 5. Oxford University Press, Oxford, UK, 57–106 (1980).
22. Roth, J., M. Bendayan, and L. Orci. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. *J. Histochem. Cytochem.* **26**:1074–1081 (1978).
23. Phelps, D. S., and J. Floros. Localization of surfactant protein synthesis in human lung by *in situ* hybridization. *Am. Rev. Respir. Dis.* **137**:939–942 (1988).
24. Denhardt, D. T. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* **23**:641–646 (1966).
25. Serabjit-Singh, C. J., C. R. Wolf, and R. M. Philpot. The rabbit pulmonary monooxygenase system: immunochemical and biochemical characterization of the enzyme components. *J. Biol. Chem.* **254**:9901–9907 (1979).
26. Katagiri, M., H. Murakami, Y. Yabusaki, T. Sugiyama, M. Okamoto, T. Yamamoto, and H. Ohkawa. Molecular cloning and sequence analysis of full-length cDNA for rabbit liver NADPH-cytochrome P-450 reductase mRNA. *J. Biochem. (Tokyo)* **100**:945–954 (1986).
27. Lawton, M. P., R. Gasser, R. E. Tynes, E. Hodgson, and R. M. Philpot. The flavin-containing monooxygenase enzymes expressed in rabbit liver and lung are products of related but distinctly different genes. *J. Biol. Chem.* **265**:5855–5861 (1990).
28. Keith, I. M., E. B. Olson, N. M. Wilson, and C. R. Jefcoate. Immunological identification of and effects of 3-methylcholanthrene and phenobarbital on rat pulmonary cytochrome P-450. *Cancer Res.* **47**:1878–1882 (1987).
29. Dees, J. H., L. D. Coe, Y. Yasukochi, and B. S. Masters. Immunofluorescence of NADPH-cytochrome *c* (P-450) reductase in rat and minipig tissues injected with phenobarbital. *Science (Washington D. C.)* **208**:1473–1475 (1980).
30. Plopper, C. G., D. L. Cranz, L. Kemp, C. J. Serabjit-Singh, and R. M. Philpot. Immunohistochemical demonstration of cytochrome P-450 monooxygenase in Clara cells throughout the tracheobronchial airways of the rabbit. *Exp. Lung Res.* **13**:59–69 (1987).
31. Hall, P., I. Stupans, W. Burgess, D. J. Burkett, and M. E. McManus. Immunohistochemical localization of NADPH-cytochrome P-450 reductase in human tissues. *Carcinogenesis (Lond.)* **10**:521–530 (1989).
32. Strum, J. M., T. Ito, R. M. Philpot, A. M. DeSanti, and E. M. McDowell. The immunochemical detection of cytochrome P-450 monooxygenase in lungs of fetal, neonatal and adult hamsters. *Am. J. Respir. Cell Mol. Biol.* **2**:493–501 (1990).
33. Anttila, S., E. Hietanen, H. Vainio, A.-M. Camus, H. V. Gelboin, S. S. Park, L. Heikkila, A. Karjalainen, and H. Bartsch. Smoking and peripheral type of cancer are related to high levels of pulmonary cytochrome P-450IAI in lung cancer patients. *Int. J. Cancer* **47**:681–685 (1991).
34. Miller, M. R., D. E. Hinton, J. J. Blair, and J. J. Stegeman. Immunohistochemical localization of cytochrome P-450E in liver, gill, and heart of scup (*Stenotomus chrysops*) and rainbow trout (*Salmo gairdneri*). *Mar. Environ. Res.* **24**:37–39 (1988).
35. Croft, J. E., W. G. Harrelson, Z. Parandoosh, and R. M. Philpot. Unique properties of NADPH- and NADH-dependent metabolism of *p*-nitroanisole catalyzed by cytochrome P-450 isozyme 2 in pulmonary and hepatic microsomal preparations from rabbits. *Chem. Biol. Interact.* **57**:143–160 (1986).
36. Waskell, L., E. Canova-Davis, R. Philpot, Z. Parandoosh, and J. Y. L. Chaing. Identification of the enzymes catalyzing metabolism of methoxyflurane. *Drug Metab. Dispos.* **14**:643–648 (1986).
37. Nordblum, G. D., R. E. White, and M. J. Coon. Studies on hydroperoxide-dependent substrate hydroxylation by purified liver microsomal cytochrome P-450. *Arch. Biochem. Biophys.* **175**:524–533 (1976).
38. Poland, A., P. Teitelbaum, and E. Glover. [^{125}I]2-Iodo-3,7,8-trichlorodibenzo-*p*-dioxin-binding species in mouse liver induced by agonists for the Ah receptor: characterization and identification. *Mol. Pharmacol.* **36**:113–120 (1989).

Send reprint requests to: Richard M. Philpot, NIEHS, P.O. Box 12233, MD 19–08, Research Triangle Park, NC 27709.