0026-895X/86/030296-08$00.00/0

MOLECULAR PHARMACOLOGY, 30:296-303

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

BARBARA A. DOMIN, THEODORA R. DEVEREUX, and RICHARD M. PHILPOT

Laboratory of Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

Received March 25, 1986; Accepted June 17, 1986

SUMMARY

Enzyme components and activities of the cytochrome P-450 monooxygenase system in microsomal preparations from the Clara cell, alveolar type II cell, and alveolar macrophage fractions isolated from lungs of untreated rabbits and rabbits treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin were examined. Results are compared to those obtained with microsomal preparations from whole lung. Concentrations of cytochrome P-450 isozymes 2 and 5 and NADPH-cytochrome P-450 reductase activities were higher in preparations from Clara cell fractions than in preparations from type II cell fractions or whole lung. For the most part, however, differences among these preparations were 2-fold or less. Microsomal preparations from the macrophage fraction contained low or undetectable levels of cytochrome P-450 isozymes but relatively high levels of cytochrome P-450 reductase activity. The concentration of cytochrome P-450 isozyme 6, in contrast to those of isozymes 2 and 5, was found to be highest in microsomal preparations from whole lung. Treatment of rabbits with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin increased the concentrations of isozyme 6 in preparations from the Clara and type II cell fractions and from whole lung about 20-fold. In contrast, the content of isozyme 6 in preparations from the macrophage fraction increased greater than 90-fold. In all cases, induction of isozyme 6 resulted in substantial increases in the *O*-deethylation of 7-ethoxyresorufin and only minor increases in the hydroxylation of benzo(*a*)pyrene. Activities per unit of isozyme 6, following induction, were similar in all preparations, and we estimate that less than 20% of the potential activity of isozyme 6 is expressed with benzo(*a*)pyrene and greater than 40% with 7-ethoxyresorufin. These similarities exist in spite of significant differences among the preparations from different fractions in the ratios of isozyme 6 to NADPH-cytochrome P-450 reductase.

In several mammalian species, the toxicity of a number of chemical toxicants that are formed *in vivo* by cytochrome P-450-mediated metabolism of relatively unreactive parent compounds is highly selective for lung (1, 2). In addition to exhibiting a high degree of tissue selectivity, the toxic effects of these compounds are also confined to a limited number of pulmonary cell types. It may be that this specificity results from differences in the cellular distribution and properties of pulmonary P-450 systems. This possibility is best illustrated with 4-ipomeanol, a substituted furan whose preferential site of action is the nonciliated bronchiolar epithelial (Clara) cell (3). A direct relationship between cytochrome P-450-mediated metabolism and the Clara cell-specific toxicity of 4-ipomeanol has been suggested by a number of findings. Immunochemical analysis of tissue sections from rabbit lung suggests that cytochrome P-450 isozymes 2 and 5, which catalyze the pulmonary metabolism of 4-ipomeanol (4, 5), are present in the Clara cell at relatively high concentrations (6, 7). The presence of these isozymes in isolated Clara cells and the ability of the Clara cell to metabolize 4-ipomeanol have also been established (7, 8). High cytochrome P-450 monooxygenase activity in the Clara cell has also been suggested to be important in some types of pulmonary carcinogenesis (9).

In addition to the Clara cell, cytochrome P-450 monooxygenase activities have been observed with alveolar type II cells and alveolar macrophages isolated from various species. Cytochrome P-450 has been detected (10) and P-450 isozymes 2 and 5 identified (7) in type II cells isolated from rabbit lung. The activity of the cytochrome P-450 system of the rabbit type II cell has been determined with several substrates, including BP (8, 10–12). Cytochrome P-450-mediated metabolism of BP is also observed with type II cells and Clara cells isolated from rats (13). Higher activities were observed with both cell types following treatment of rats with β-naphthoflavone (13).

ABBREVIATIONS: P-450 system(s), cytochrome P-450 monooxygenase system(s); BP, benzo(*a*)pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 7-ERF, 7-ethoxyresorufin; 7-EC, 7-ethoxycoumarin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Attempts to characterize the P-450 system of the alveolar macrophage have produced conflicting results. The rabbit alveolar macrophage has been reported to contain detectable cytochrome P-450 and to metabolize *p*-nitroanisole (14); to have low monooxygenase activities with several substrates (15), including BP (15, 16), but no detectable cytochrome P-450 (15); or not to have detectable activity with BP (17). Metabolism of BP in alveolar macrophages from humans, with higher activities in cells from smokers, has been reported (18–20).

The P-450 systems of Clara cells, type II cells, and alveolar macrophages isolated from rabbit lung are examined in the present study. Specific enzymes of the P-450 systems, induction of cytochrome P-450 isozyme 6 by TCDD, and the metabolism of BP, 7-ERF, and 7-EC are described.

Materials and Methods

Animals and treatments. Adult, male New Zealand White rabbits were obtained from Dutchland Farms (Denver, PA). The rabbits were either not treated or were administered TCDD (10 μg/kg, intraperitoneally) 72–96 hr prior to killing. Both groups were given free access to food and water.

Isolation of pulmonary cell fractions. For isolation of alveolar macrophages, lungs were removed intact and the macrophages were removed by lavage with cold (4°) balanced salts solution buffered with HEPES (21). Cells in the lavage were isolated by centrifugation (800 × *g* for 10 min). Blood cells and cell debris were removed from suspensions of 10^8–10^9 cells by elutriation (Beckman JE-6 rotor, Beckman Instruments, Palo Alto, CA). The fraction obtained at 1200 rpm (flow rate = 22 ml/min) contained predominantly (>95%) macrophages. No Clara or type II cells could be observed in this fraction. Preparation of Clara and type II cell fractions was as described previously (10, 21) except for some modification of the initial protease treatment procedure. The protease concentration was increased to 0.15%, the time of incubation was shortened to 10 min, and the tissue was degassed for 30 sec. These modifications, which will be described in detail elsewhere, markedly reduced the problem of proteolysis, as determined by detection of multiple peptides on Western blots, and resulted in the detection of higher and more uniform enzyme contents. Estimates of purity (21) were 40–60% for the Clara cell fractions and >80% for the type II cell fractions. The Clara cell fractions contained 5–10% type II cells, 20–30% macrophages, and <5% ciliated and unidentified small cells. The type II cell fractions contained <2% Clara cells, <10% macrophages, and <5% lymphocytes.

Electrophoresis, Western blotting, and immunochemical procedures. Polyacrylamide gel electrophoresis (7.5% gels) in the presence of sodium dodecyl sulfate is described by Laemmli and Favre (22) and Western blotting, by Towbin *et al.* (23). The conditions used for immunochemical staining were those of Domin *et al.* (24). Isozyme levels were determined by comparing slope values of staining intensities for three concentrations of samples with slope values for three concentrations of standards. The standards used were pulmonary microsomes from untreated (isozymes 2 and 5) or TCDD-treated (isozyme 6) rabbits. The isozyme contents of these samples were ascertained by comparison with purified isozymes 2, 5, and 6. The antibodies to isozymes 2 and 5 have been described (25). Antibodies to isozyme 6 were generously provided by Dr. E. F. Johnson (Scripps Clinic and Research Foundation, La Jolla, CA).

Assays for monooxygenase activities. The *O*-deethylation of 7-ERF was determined by the methods of Burke and Mayer (26) and Norman *et al.* (27), and the hydroxylation of BP was by the method of Dehnen *et al.* (28) as modified by Domin and Philpot (29). The *O*-deethylation of 7-EC was determined by the method of Ullrich and Weber (30). The activity of NADPH-cytochrome P-450 reductase was determined by the method of Masters *et al.* (31), with 1 unit being equal to the amount of enzyme required for the reduction of 1 nmol of cytochrome *c*/min. Incubations to which purified NADPH-cytochrome P-450 reductase was added (20,000 units/mg of protein) were carried out as described previously (29).

Analysis of metabolites formed from BP. Metabolites formed from BP (100 μM, 92 μCi of ^{14}C) in incubations (30 min, 37°) containing sonicated type II or Clara cells (1.5–2.0 mg of protein), NADPH (2 mM), $MgCl_2$ (5 mM), bovine serum albumin (0.06% in 30 mM tris buffer, pH 7.4) were extracted and analyzed by high pressure liquid chromatography (32). The solvent system (water/methanol) was a linear gradient from 50 to 70% methanol (35 min), 70% methanol (1 min), a linear gradient from 72 to 100% methanol (10 min), and 100% methanol (10 min).

Materials. Chemicals for electrophoresis were from Bio-Rad (Richmond, CA), nitrocellulose paper was from Schleicher and Schuell (Keene, NH), immunochemical reagents were from Cappel Laboratories (Malvern, PA), and 7-ERF was from Molecular Probes (Junction City, OR). Radiolabeled BP (7,10-^{14}C, 58.5 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, IL) and BP metabolite standards were obtained from the National Cancer Institute Carcinogen Reference Standard Repository (Bethesda, MD). All other chemicals were purchased from commercial sources at the highest purity available.

Results

Cytochrome P-450 isozyme 6 in the Clara cell, type II cell, and alveolar macrophage: Induction by TCDD. Cytochrome P-450 isozyme 6 was detected on Western blots of microsomal samples from the Clara cell and type II cell fractions of untreated or TCDD-treated rabbits (Fig. 1, A and B). When the differences between the amounts of sample used are considered, it is clear that the concentration of isozyme 6 in both cell types was markedly increased by TCDD. In contrast to what was found with the Clara and type II cell fractions, isozyme 6 was not detected in microsomal samples (up to 100 μg of protein) from alveolar macrophages of untreated rabbits (Fig. 1C, *lanes 1–3*). However, isozyme 6 was detected in the microsomal fraction of macrophages from rabbits treated with TCDD (Fig. 1C, *lanes 4–6*).

Cytochrome P-450 isozyme 2 in the Clara cell, type II cell, and alveolar macrophage. Cytochrome P-450 isozyme 2 was detected on Western blots of microsomal samples from the Clara cell and type II cell fractions of untreated rabbits (Fig. 2A). Well defined bands of staining, in addition to that for isozyme 2, were also observed. These bands, which were more evident with the Clara cell preparations (three different preparations are shown in Fig. 2A) were not observed with microsomal samples from whole lung (Fig. 2A, *lanes 14–16*). Unlike the nonspecific staining observed with excessive protein (see results for fractions from untreated rabbits in Fig. 1), these bands were detected clearly with as little as 1–2 μg of protein, and their intensities varied among different preparations. With low levels of microsomal protein from the Clara cell (0.8 μg) and type II cell (1 μg) fractions of rabbits treated with TCDD, isozyme 2 was detected (Fig. 2B), but no additional bands were observed. Although the lower molecular weight peptides were observed with larger amounts of protein (not shown), their apparent concentrations were less than those observed with samples from untreated rabbits. Isozyme 2 was also detected in microsomal samples from alveolar macrophages (Fig. 3), but at much lower concentrations than with samples from Clara or type II cells. Treatment of rabbits with TCDD decreased the content of isozyme 2 in the macrophage (Fig. 3).

Cytochrome P-450 isozyme 5 in the Clara and type II

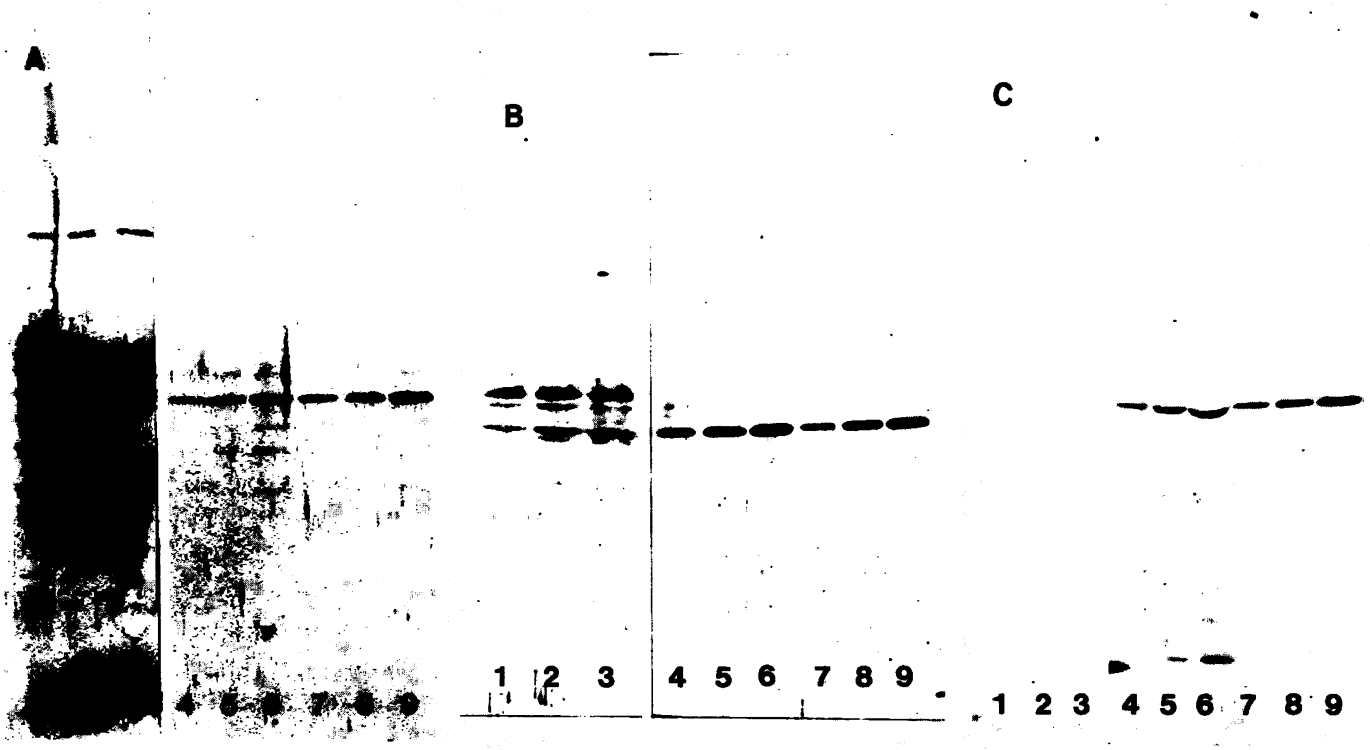

Fig. 1. Western blots of cytochrome P-450 isozyme 6 in microsomal preparations from the Clara, type II, and macrophage fractions from lungs of untreated and TCDD-treated rabbits. A. Results obtained with the Clara cell fraction of untreated rabbits (*lanes 1–3*; 20, 40, and 80 μg of protein) and TCDD-treated rabbits (*lanes 4–6*; 0.5, 1, and 2 μg of protein). Results with microsomes from whole lung of TCDD-treated rabbits are shown in *lanes 7–9* (0.5, 1, and 2 μg of protein). B. Results obtained with the type II cell fraction of untreated rabbits (*lanes 1–3*; 20, 40, and 80 μg of protein) and TCDD-treated rabbits (*lanes 4–6*; 1, 2, and 4 μg of protein). Results obtained with microsomes from whole lungs of TCDD-treated rabbits are shown in *lanes 7–9* (0.7, 1.3, and 2.7 μg of protein). C. Results obtained with the macrophage fraction from untreated rabbits (*lanes 1–3*; 25, 50, and 100 μg of protein) and TCDD-treated rabbits (*lanes 4–6*; 5, 10, and 20 μg of protein). Results obtained with microsomes from whole lungs of TCDD-treated rabbits are shown in *lanes 7–9* (1.7, 3.3, and 6.6 μg of protein).

cell. Cytochrome P-450 isozyme 5 was detected on Western blots of microsomal samples from Clara and type II cell fractions of untreated (Fig. 4A) and TCDD-treated (Fig. 4B) rabbits. The content of isozyme 5 in either cell type did not appear to be affected significantly by TCDD. Isozyme 5 was also detected in microsomal samples (up to 80 μg of protein) from alveolar macrophages but could not be quantitated due to high levels of nonspecific staining.

Quantitation of enzyme components of P-450 systems in microsomes from Clara cells, type II cells, alveolar macrophages, and whole lung. The concentrations of isozymes 2, 5, and 6 and NADPH-cytochrome P-450 reductase activities were determined for three sets of microsomal preparations from untreated and TCDD-treated rabbits (Table 1). Each set was prepared from the lungs of six rabbits. Higher concentrations of isozymes 2 and 5 were found in microsomes from Clara cells (about 2-fold) and type II cells (about 1.5-fold) than in microsomes from whole lung. Analysis of P-450 reductase activities gave similar results. In contrast, the concentration of isozyme 6 was 3 times higher in microsomes from whole lung than in microsomes from Clara or type II cells. In microsomes from isolated alveolar macrophages, cytochrome P-450 isozyme concentrations were either very low (14 pmol/mg, isozyme 2; trace, isozyme 5) or undetectable (<0.1 pmol/mg, isozyme 6), whereas the activity of cytochrome P-450 reductase was greater than half that observed with microsomes from whole lung (Tables 1 and 2).

Treatment of rabbits with TCDD resulted in marked increases in cytochrome P-450 isozyme 6 content in microsomal samples from all three cell types and intact lung (Table 1). Increases in isozyme 6 content were far greater in the macrophage (>90-fold) than in the Clara cell, type II cell, or whole lung (22- to 25-fold). Effects of TCDD on the concentrations of isozymes 2 and 5 and NADPH-cytochrome P-450 reductase were minor with the exception of a marked decrease (80%) in isozyme 2 content in the macrophage. The relative differences between enzyme concentrations in the cell fractions and whole lung, and between preparations from untreated and TCDD-treated rabbits, are shown in Table 2. The concentrations of total cytochrome P-450, determined by difference spectroscopy, in microsomes from intact lungs were 0.28 ± 0.1 nmol/mg of protein for samples from untreated rabbits and 0.51 ± 0.1 nmol/mg of protein for samples from TCDD-treated rabbits. The sums of the concentrations of isozymes 2, 5, and 6, determined by Western blotting were 85% (untreated) and 95% (treated) of the concentrations based on spectral determinations. Observed variability in total cytochrome P-450 concentrations among samples was similar with either method of quantitation.

Metabolism of 7-EC in microsomal preparations from untreated rabbits. Rates of *O*-deethylation of 7-EC were higher in microsomes from Clara and type II cells than in microsomes from whole lung (Table 3). However, rates calculated on the basis of isozyme 2 concentrations were within 10%

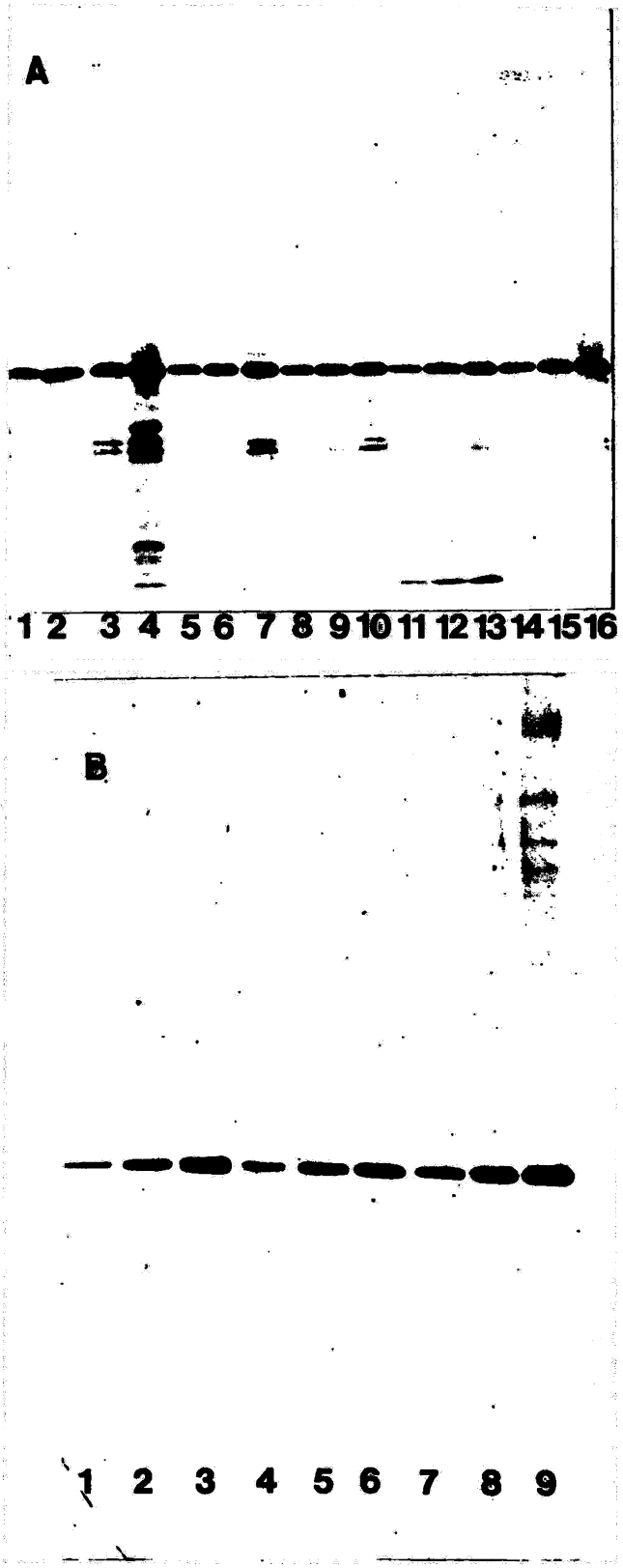

Fig. 2. Western blots of cytochrome P-450 isozyme 2 in microsomal preparations of the Clara and type II cell fractions from lungs of untreated and TCDD-treated rabbits. A. Results obtained with microsomes from three Clara cell fractions (*lanes 1–4*, *5–7*, and *8–10*), a type II cell fraction (*lanes 11–13*), and whole lung (*lanes 14–16*) of untreated rabbits. The amounts of protein used were: 0.5 μg (*lanes 1*, *5*, *8*, *11*, and *14*); 1.0 μg (*lanes 2*, *6*, *9*, *12*, and *15*); 2.0 μg (*lanes 3*, *7*, *10*, *13*, and *16*); and 10 μg (*lane 4*). B. Results obtained with microsomes from the Clara cell fraction (*lanes 1–3*; 0.2, 0.4, and 0.8 μg of protein) and type II cell fraction (*lanes 4–6*; 0.25, 0.5, and 1.0 μg of protein) of TCDD-treated rabbits. Results with microsomes from whole lungs of untreated rabbits are shown in *lanes 7–9* (0.5, 1, and 2 μg of protein).

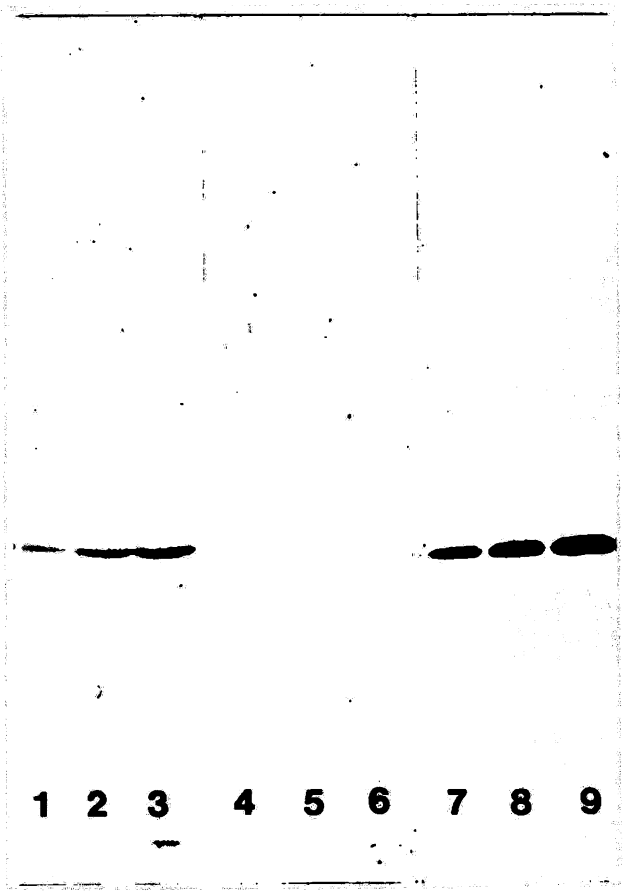

Fig. 3. Western blots of cytochrome P-450 isozyme 2 in microsomal preparations of alveolar macrophages from untreated and TCDD-treated rabbits. Samples from untreated rabbits are shown in *lanes 1–3* (20, 40, and 80 μg of protein) and those from TCDD-treated rabbits are shown in *lanes 4–6* (10, 20, and 40 μg of protein). Results with microsomes from whole lungs of untreated rabbits are shown in *lanes 7–9* (0.54, 1.1, and 2.2 μg of protein).

of each other (Table 3). Marginal rates of metabolism were obtained with microsomes from alveolar macrophages; the mean rate (per mg of protein) was more than 50 times lower than with preparations from Clara cells.

The effects of TCDD on metabolism catalyzed by isozyme 6 in microsomal preparations from rabbit lung. The *O*-deethylation of 7-ERF and the hydroxylation of BP were greater in microsomal preparations from rabbits treated with TCDD than in those from untreated rabbits (Table 4). In all cases, increases in activities with BP were much less than increases with 7-ERF or increases in isozyme 6 content. (Rates of metabolism in macrophage preparations from untreated rabbits were consistent with isozyme 6 concentrations near the limit of detection.) Rates of metabolism per unit of isozyme 6 in microsomes from isolated cells or whole lung of rabbits treated with TCDD were similar (Table 5). *O*-deethylation of 7-ERF was about one-half the potential rate based on isozyme 6 content and the activity of the purified isozyme (Table 5). In contrast, hydroxylation of BP was less than 20% of the poten-

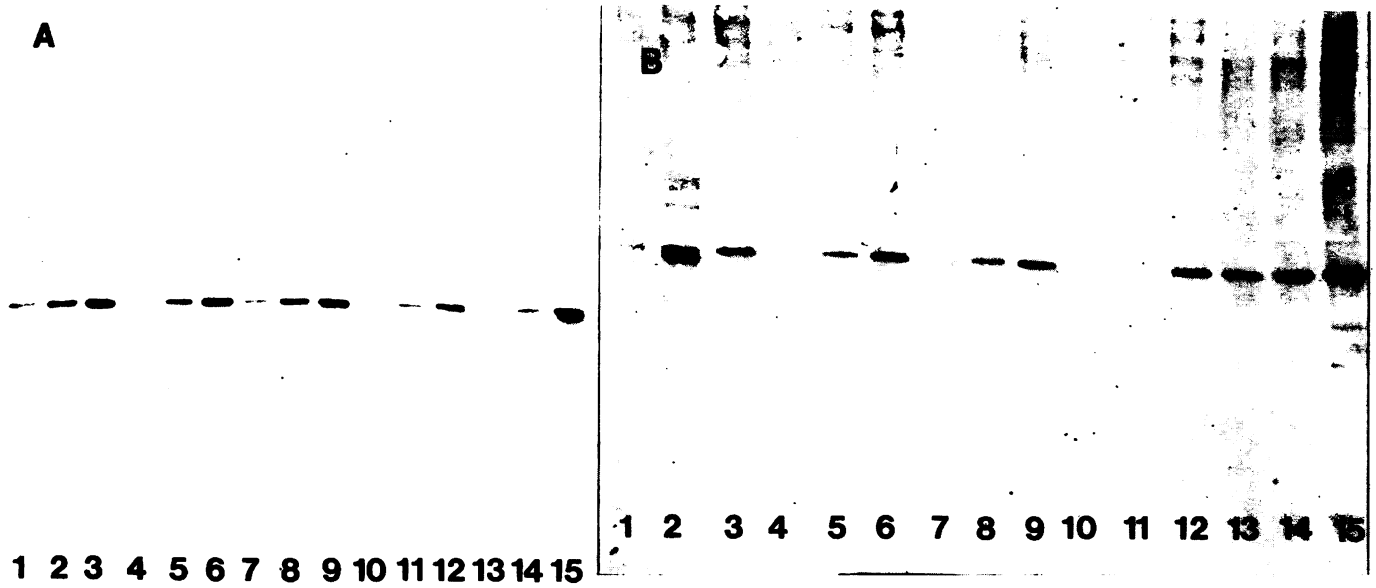

Fig. 4. Western blots of cytochrome P-450 isozyme 5 in microsomal preparations of the Clara and type II cell fractions from untreated and TCDD-treated rabbits. A. Results obtained with microsomes from three Clara cell fractions (*lanes 1–3*, *4–6*, and *7–9*), and two type II cell fractions (*lanes 10–12* and *13–15*) of untreated rabbits. The amounts of protein used were: 1 μg (*lanes 1*, *4*, *7*, *10*, and *13*); 2 μg (*lanes 2*, *5*, *8*, *11*, and *14*); and 4 μg (*lanes 3*, *6*, *9*, *12*, and *15*). B. Results obtained with microsomes from two Clara cell fractions (*lanes 1–3* and *7–9*), and two type II cell fractions (*lanes 4–6* and *10–12*) of TCDD-treated rabbits. The amounts of protein used were: 0.5 μg (*lanes 1*, *4*, *7*, and *10*); 1 μg (*lanes 2*, *5*, *8*, and 11); and 2 μg (*lanes 3*, *6*, *9*, and *12*). Results with microsomes from whole lungs of untreated rabbits are shown in *lanes 13–15* (1.4, 2.7, and 5.4 μg of protein).

TABLE 1

Microsomal concentrations of cytochrome P-450 isozymes and NADPH-cytochrome P-450 reductase activities in samples from Clara cells, type II cells, alveolar macrophages, and whole lungs of untreated and TCDD-treated rabbits

Microsomal sample	TCDD	Cytochrome P-450 isozymes[a]			Reductase[b]
		2	5	6	
		pmol/mg of protein			*units/mg*
Clara cell	–	318 ± 40	156 ± 17	1.9 ± 0.8	441 ± 73
Clara cell	+	442 ± 17	101 ± 46	47 ± 8	656 ± 190
Type II cell	–	244 ± 44	125 ± 35	1.7 ± 0.8	248 ± 73
Type II cell	+	264 ± 109	127 ± 97	40 ± 11	405 ± 66
Macrophage	–	14 ± 3	Trace	<0.1	80 ± 20
Macrophage	+	3 ± 1	Trace	9.3 ± 2.9	92 ± 13
Whole lung	–	157 ± 45	78 ± 7	5.7 ± 2.7	131 ± 41
Whole lung	+	243 ± 18	119 ± 82	125 ± 50	220 ± 82

[a] Results from Western blotting are given as the mean ± standard deviation (n = 3).
[b] Reductase activities are given as the mean ± standard deviation (n = 3).

TABLE 2

Relative contents of cytochrome P-450 isozymes and NADPH-cytochrome P-450 reductase activities in microsomes from Clara cells, type II cells, alveolar macrophages, and whole lungs of untreated and TCDD-treated rabbits

Sample comparisons	TCDD	Concentration ratios			
		P-450 isozymes			Reductase
		2	5	6	
Clara/Clara	+/–	1.4	0.6	25	1.5
Type II/Type II	+/–	1.1	1.0	24	1.6
Macrophage/Macrophage	+/–	0.2		>90	1.1
Whole lung/Whole lung	+/–	1.5	1.5	22	1.7
Clara/Whole lung	–/–	2.0	2.0	0.3	3.4
Type II/Whole lung	–/–	1.6	1.6	0.3	1.9
Macrophage/Whole lung	–/–	0.09	<0.002	<0.02	0.6
Clara/Whole lung	+/+	1.8	0.8	0.4	3.0
Type II/Whole lung	+/+	1.1	1.1	0.3	1.8
Macrophage/Whole lung	+/+	0.01	<0.001	0.07	0.4

tial activity based on the respective contents and turnover numbers of isozymes 6 and 2. These results are consistent with our previous findings that expression of isozyme 6 activity in microsomes from whole lung of rabbits treated with TCDD is greater with 7-ERF than with BP (29). The addition of NADPH-cytochrome P-450 reductase to incubations increased BP hydroxylation by 6.4- to 10-fold and 7-ERF *O*-deethylation by only 1.4- to 2.0-fold in single microsomal preparations of cells isolated from rabbits treated with TCDD (not shown).

Formation of various metabolites of BP in Clara and type II cell fractions isolated from untreated and TCDD-treated rabbits. Preparations of isolated Clara and type II cells were sonicated and incubated with BP. The rates of formation of organic-extractable products are reported in Table 6. Similar patterns of metabolism were observed with both cell types. With cells from untreated rabbits, about 40% of the products eluted with the 9,10-diol and 9-hydroxy standards, 25–30% with the 3-hydroxy standard, 20% with the quinine standards, and 10% with the 4,5-diol standard. Rates of formation of products eluting with the 7,8-diol standard were low. Treatment of rabbits with TCDD increased the percentage of metabolites eluting with the 7,8-diol and the 9,10-diol and 9-hydroxy standards, and decreased the relative formation of metabolites eluting with the 3-hydroxy standard and the quinone standards. Treatment with TCDD increased overall metabolism by less than 2-fold. Total metabolism was slightly greater (about 1.5-fold) with the Clara cell than with the type II cell preparations.

Discussion

Although the components of the rabbit pulmonary P-450 system have been examined in some detail, most of the infor-

TABLE 3

Metabolism of 7-EC in microsomes of Clara cells, type II cells, alveolar macrophages, and whole lungs from untreated rabbits

Microsomal sample	7-EC O-deethylation[a]	
	(mg of Protein)$^{-1}$	(pmol of Isozyme 2)$^{-1}$
	(pmol product) (min)$^{-1}$	
Clara cells	1794 ± 241	5.8 ± 1.4
Type II cells	1552 ± 103	6.4 ± 0.7
Macrophages	32 ± 11	2.2 ± 0.3
Whole lung	910 ± 240	6.0 ± 1.8

[a] Results are given as the mean ± standard error (n = 3).

mation about these enzymes has been derived from studies of the microsomal fraction from whole lung or of proteins purified from this fraction. Whereas this has provided a general picture of the pulmonary system, the results are "averages" from a complex tissue and cannot be related to individual cell types. However, the cellular distribution of P-450 systems in the lung is beginning to be understood. Components of the P-450 system have been detected in the Clara cell in tissue sections (6, 7, 33) and in isolated Clara and type II cells (7), and monooxygenase activities have been observed with isolated Clara, type II, and alveolar macrophage fractions (10–16). We have now examined these cell fractions for the presence of cytochrome P-450 isozymes 2, 5, and 6, and for evidence of the induction of isozyme 6 by TCDD.

The relative concentrations of isozymes 2 and 5 and of NADPH-cytochrome P-450 reductase appear to be similar in microsomal preparations from the Clara and type II cell fractions and from whole lung. In addition, expression of isozyme 2-catalyzed activity is the same in all three preparations. Preparations from the Clara cell fraction contain the highest concentrations of these enzymes, but differences among the three preparations are generally less than 3-fold. Potentially, the isozyme concentrations per unit of endoplasmic reticulum are the same, and the differences noted result from unequal contributions of other membrane components to the total microsomal protein. Based on the amounts of endoplasmic reticulum present in various cell types, a higher proportion of microsomal protein should be derived from this organelle in preparations from the Clara cell fraction than from the type II cell fraction or whole lung. In the case of microsomal preparations from whole lung, we estimate that less than 30% of the total protein is from endoplasmic reticulum. Fractions in which the concentrations of cytochrome P-450 isozymes and NADPH-cytochrome P-450 reductase are 3- to 4-fold greater than in the starting material are recovered when the microsomal pellet from whole lung is subjected to centrifugation on sucrose density gradients and chromatography on Sepharose 4B.[1] Precise quantitation of enzyme levels is also compromised to some extent by the proteolysis observed with preparations from the Clara and type II cell fractions. However, changes in the isolation procedure have decreased this problem to the extent that sample variability in enzyme content cannot be related to variability in the extent of proteolysis.

Estimates from our results (microsomal enzyme contents and microsomal protein yield per cell) indicate that concentrations of isozymes 2 and 5 and reductase in the Clara cell are only 2 to 3 times those in the type II cell. This is consistent with the finding that the rate of metabolism of 4-ipomeanol, which is catalyzed by isozymes 2 and 5 (4), is about 3 times greater in the Clara than in the type II cell fraction from rabbit lung (8). The magnitude of these differences does not provide convincing evidence that the selective effects of 4-ipomeanol and several other pulmonary toxins are a consequence of higher rates of cytochrome P-450-mediated metabolism in the Clara cell. The role of metabolic capacity in cell-specific pulmonary toxicity is also brought into question by the results of several other studies: first, the toxic effects of 3,5-di-*tert*-butyl-4-hydroxytoluene, which appear to be dependent upon cytochrome P-450-mediated metabolism, are localized in the type I alveolar epithelial cell and not in the Clara cell (34); second, methylcyclopentadienyl manganese tricarbonyl, which does cause selective necrosis of the Clara cell in mice and rats, appears to be inactivated, rather than activated, by cytochrome P-450-mediated metabolism (34).

Cytochrome P-450 isozymes 2 and 5 and NADPH-cytochrome P-450 reductase are also detected in microsomes from alveolar macrophages. Although the concentration of isozyme 5 could not be determined accurately, the content of isozyme 2 in macrophage microsomes was found to be 10- to 20-times lower than in preparations from the Clara cells, type II cells, or whole lung. In contrast to the low levels of cytochrome P-450 isozymes, the specific activity of NADPH-cytochrome P-450 reductase in the macrophage is 60% that of whole lung. The level of reductase activity in the macrophage preparations (80 units/mg) is in good agreement with the concentration of the enzyme determined by Western blotting (equivalent to 91 units/mg, data not shown). These results are consistent with reports of low monooxygenase activities and substantial reductase activities in the alveolar macrophage (15).

Although the relative concentrations of cytochrome P-450 isozymes 2 and 5 and NADPH-cytochrome P-450 reductase are similar in microsomal preparations from the Clara and type II

[1] R. Vanderslice and R. M. Philpot, unpublished result.

TABLE 4

The metabolism of 7-ERF and BP in microsomal samples from Clara cells, type II cells, alveolar macrophages, and whole lung of untreated and TCDD-treated rabbits

Microsomal sample	Rates of metabolism[a]					
	7-ERF O-deethylation			BP hydroxylation		
	Untreated	TCDD-treated	Increase (-fold)	Untreated	TCDD-treated	Increase (-fold)
	(pmol product)/(mg protein × min)					
Clara cell	2.5 ± 3.8	46 ± 11	18	26 ± 16	35 ± 21	1.3
Type II cell	1.5 ± 0.3	35 ± 16	23	9.7 ± 3.8	32 ± 9	3.3
Macrophage	0.13 ± 0.09	8.6 ± 5.3	66	1.9 ± 1.3	5.7 ± 0.8	3.0
Whole lung	8.2 ± 2.8	109 ± 30	13	30 ± 16	52 ± 10	1.7

[a] Results are given as the mean ± standard deviation (n = 3).

TABLE 5

Extent of expression of 7-ERF and BP metabolism in microsomal samples from Clara cells, type II cells, alveolar macrophages, and whole lungs from rabbits treated with TCDD

Microsomal sample	Rates of metabolism				Relative activity (% potential)[b]	
	(nmol Isozyme 6)$^{-1}$		(nmol Total P-450)$^{-1}$[a]		7-ERF	BP
	7-ERF	BP	7-ERF	BP		
	(nmol product) (min)$^{-1}$					
Clara cells	0.98 ± 0.10	0.79 ± 0.54	0.08 ± 0.01	0.06 ± 0.04	46 ± 5	17 ± 11
Type II cells	0.87 ± 0.30	0.80 ± 0.07	0.08 ± 0.02	0.08 ± 0.03	41 ± 14	19 ± 2
Macrophages	0.86 ± 0.38	0.65 ± 0.19	0.59 ± 0.04	0.42 ± 0.13	41 ± 18	17 ± 5
Whole lung	0.91 ± 0.15	0.50 ± 0.31	0.22 ± 0.03	0.12 ± 0.06	43 ± 7	13 ± 8

[a] Total P-450 was taken as the sum of isozymes 2, 5, and 6 as determined by immunoquantitation.
[b] Potential activity was calculated on the basis of isozyme 6 content and a turnover number of 2.1 for 7-ERF metabolism, and on the basis of isozymes 6 and 2 contents and turnover numbers of 3.7 and 0.1, respectively, for BP metabolism (29). Isozyme 6 was calculated to contribute 80 ± 3% (Clara), 85 ± 3% (type II), 99 ± 1% (macrophage), and 94 ± 2% (whole lung) of the potential BP hydroxylation activity.

TABLE 6

Metabolism of BP to various products in isolated Clara and type II cell fractions from untreated and TCDD-treated rabbits

Standard	Rates of formation			
	Clara cell fraction		Type II cell fraction	
	Untreated	TCDD-treated	Untreated	TCDD-treated
	(pmol)/(mg protein × min)			
9,10-diol	0.1	7.1	0.4	6.0
4,5-diol	4.6	5.7	2.4	3.1
7,8-diol	0.1	1.8	0.1	1.5
Quinones	7.4	9.1	4.9	7.3
9-hydroxy	14.0	23.5	9.3	16.3
3-hydroxy	11.0	12.0	5.9	11.4
Total	37.2 ± 13.1	59.2 ± 16.5	23.0 ± 7.2	45.6 ± 17.2

cell fractions and whole lung, the same does not hold for isozyme 6. The relative content of isozyme 6 in the cell fractions (<0.5% of the total cytochrome P-450 detected) is 5 times lower than in whole lung (2–3% of the total). This differential is maintained following treatment of rabbits with TCDD as similar increases in isozyme 6 content (20- to 25-fold) are observed with all three preparations. These results indicate that the distribution of isozyme 6 in the lung differs significantly from that of isozymes 2 and 5, and that the majority of the pulmonary isozyme 6 resides in pneumocytes other than the Clara cell, type II cell, or the alveolar macrophage, which contains relatively little isozyme 6. Results obtained with macrophages from TCDD-treated rabbits provide further evidence that the relative distribution of isozyme 6 differs from that of isozymes 2 and 5, and that the extent of induction of isozyme 6 can vary significantly as a function of cell type. A unique pattern of distribution for isozyme 6, including the possibility of its presence in endothelial cells, has also been observed in sections of lung from TCDD-treated rabbits (33). It is noteworthy that the responses of the rabbit and human alveolar macrophages to 3-methylcholanthrene-type inducers appear to be similar. Macrophages from smokers metabolize BP at higher rates than those from non-smokers (19), and the profile of metabolites formed resembles that produced by isozyme 6 (18, 29, 35). This is consistent with the detection of a homologue of isozyme 6 in microsomal preparations from placentas of women who were either smokers or were exposed to other chemical mixtures that contained 3-methylcholanthrene-type inducers (36).

Increases in isozyme 6 content in microsomal preparations from Clara, type II, and macrophage cell fractions and from whole lung are accompanied by much greater increases in the *O*-deethylation of 7-ERF than in the hydroxylation of BP. Minimal increases in the metabolism of BP following treatment of rabbits with TCDD are also observed with sonicated Clara or type II cell fractions. These increases are observed primarily with products derived from metabolism at the 9,10- and 7,8-positions of BP, which is indicative of isozyme 6 catalysis (29). We have shown previously with microsomal preparations from whole lung that the difference between increases in the metabolism of 7-ERF and BP is a function of the extent to which the catalytic potential of isozyme 6 is expressed with these substrates (29). Analysis of the present results indicates that expression of isozyme 6 activity in the three cell fractions from TCDD-treated rabbits is similar to that observed with the whole lung preparation. Consistent rates of metabolism per unit of isozyme 6 are observed with all four preparations, and the percentage of potential activity expressed is 2 to 3 times greater with 7-ERF than with BP. As seen with microsomal preparations from whole lung (29), the addition of purified NADPH-cytochrome P-450 reductase to incubations of microsomes from the cell fractions results in a greater increase in the metabolism of BP than of 7-ERF. Although reductase is involved in the differential expression of isozyme 6 activity, its role is clearly not a function of the relative amounts of reductase and cytochrome present. Ratios of reductase to isozyme 6 are 5 to 8 times higher in the cell fractions than in whole lung, and ratios of reductase to total cytochrome P-450 vary by nearly 40-fold.

In conclusion, our results indicate that the concentrations of the major components of the rabbit pulmonary P-450 system are similar in microsomal preparations from the Clara cell and type II cell fractions and from whole lung. In contrast, the macrophage contains very low levels of cytochrome P-450 isozymes and relatively high levels of NADPH-cytochrome P-450 reductase. Results with preparations from either untreated or TCDD-treated rabbits show that the distribution of cytochrome P-450 isozyme 6 differs from that of isozymes 2 and 5. Increases in isozyme 6 concentrations following treatment of rabbits with TCDD are similar in the Clara cell, type II cell, and whole lung, but are significantly greater in the macrophage. In the induced state the macrophage may play a major role in the metabolism of some inhaled aromatic hydrocarbons, particularly those for which a high proportion of the potential activity is expressed.

Acknowledgments

The authors thank Peter Bent, Janet Diliberto, and Blair Hoyle for their excellent technical assistance, and Debbie Garner for preparing the manuscript.

References

1. Minchin, R. F., and M. R. Boyd. Localization of metabolic activation and deactivation systems in the lung: significance to the pulmonary toxicity of xenobiotics. *Annu. Rev. Pharmacol. Toxicol.* **23**:217–238 (1983).
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. Boyd, M. R. Evidence for the Clara cell as a site of cytochrome P-450-dependent mixed-function oxidase activity in lung. *Nature (Lond.)* **269**:713–715 (1977).
4. Wolf, C. R., C. N. Statham, M. G. McMenamin, J. R. Bend, M. R. Boyd, and R. M. Philpot. The relationship between the catalytic activities of rabbit pulmonary cytochrome P-450 isozymes and the lung-specific toxicity of the furan derivative, 4-ipomeanol. *Mol. Pharmacol.* **22**:738–744 (1982).
5. Slaughter, S. R., C. N. Statham, R. M. Philpot, and M. R. Boyd. Covalent binding of metabolites of 4-ipomeanol to rabbit pulmonary and hepatic microsomal proteins and to the enzymes of the pulmonary cytochrome P-450-dependent monooxygenase system. *J. Pharmacol. Exp. Ther.* **224**:252–257 (1983).
6. Serabjit-Singh, C. J., C. R. Wolf, R. M. Philpot, and C. G. Plopper. Cytochrome P-450: localization in rabbit lung. *Science (Wash. D. C.)* **207**:1469–1470 (1980).
7. Devereux, T. R., C. J. Serabjit-Singh, S. R. Slaughter, C. R. Wolf, R. M. Philpot, and J. R. Fouts. Identification of cytochrome P-450 isozymes in nonciliated bronchiolar epithelial (Clara) and alveolar type II cells isolated from rabbit lung. *Exp. Lung Res.* **2**:221–230 (1981).
8. Devereux, T. R., K. G. Jones, J. R. Bend, J. R. Fouts, C. N. Statham, and M. R. Boyd. *In vitro* metabolic activation of the pulmonary toxin, 4-ipomeanol, in nonciliated bronchiolar epithelial (Clara) and alveolar type II cells isolated from rabbit lung. *J. Pharmacol. Exp. Ther.* **220**:223–227 (1981).
9. Boyd, M. R., and H. M. Reznik-Schuller. Metabolic basis for the pulmonary Clara cell as a target for pulmonary carcinogenesis. *Toxicol. Pathol.* **12**:56–61 (1984).
10. Devereux, T. R., J. J. Diliberto, and J. R. Fouts. Cytochrome P-450 monooxygenase, epoxide hydrolase and flavin monooxygenase activities in Clara cells and alveolar type II cells isolated from rabbit. *Cell Biol. Toxicol.* **1**:57–65 (1985).
11. Devereux, T. R., G. E. R. Hook, and J. R. Fouts. Compound metabolism by isolated cells from rabbit lung. *Drug Metab. Dispos.* **7**:70–75 (1979).
12. Devereux, T. R., and J. R. Fouts. Xenobiotic metabolism by alveolar type II cells isolated from rabbit lung. *Biochem. Pharmacol.* **30**:1231–1237 (1981).
13. Jones, K. G., J. F. Holland, and J. R. Fouts. Benzo(a)pyrene hydroxylase activity in enriched populations of Clara cells and alveolar type II cells from control and β-napthoflavone-pretreated rats. *Cancer Res.* **42**:4658–4661 (1982).
14. Fisher, A. B., G. A. Huber, and L. Furia. Cytochrome P-450 content and mixed-function oxidation by microsomes from rabbit alveolar macrophages. *J. Lab. Clin. Med.* **90**:101–108 (1977).
15. Hook, G. E. R., J. R. Bend, and J. R. Fouts. Mixed-function oxidases of the alveolar macrophage. *Biochem. Pharmacol.* **21**:3267–3277 (1972).
16. Roment, L., and D. Jenssen. Rabbit alveolar macrophage-mediated mutagenesis of polycyclic aromatic hydrocarbons in V79 Chinese hamster cells. *Mutat. Res.* **111**:245–252 (1983).
17. Reid, W. D., J. M. Glick, and G. Krishna. Metabolism of foreign compounds by alveolar macrophages of rabbits. *Biochem. Biophys. Res. Commun.* **49**:626–634 (1972).
18. Harris, C. C., I. C. Hsu, G. D. Stoner, B. F. Trump, and J. K. Selkirk. Human pulmonary alveolar macrophages metabolise benzo(a)pyrene to proximate and ultimate mutagens. *Nature (Lond.)* **272**:633–634 (1978).
19. Cantrell, E. T., G. A. Warr, D. L. Bisbee, and R. R. Martin. Induction of aryl hydrocarbon hydroxylase in human pulmonary alveolar macrophages by cigarette smoking. *J. Clin. Invest.* **52**:1881–1884 (1973).
20. McLemore, T. L., G. A. Warr, and R. R. Martin. Induction of aryl hydrocarbon hydroxylase in human pulmonary alveolar macrophages and peripheral lymphocytes by cigarette tars. *Cancer Lett.* **2**:161–168 (1977).
21. Devereux, T. R., and J. R. Fouts. Isolation of pulmonary cells and use in studies of xenobiotic metabolism. *Methods Enzymol.* **77**:147–154 (1982).
22. Laemmli, U. K., and M. Favre. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* **80**:575–599 (1973).
23. 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).
24. 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 peroxide-immunostaining procedure. *Anal. Biochem.* **136**:390–396 (1984).
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. Burke, M. D., and R. T. Mayer. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.* **2**:583–588 (1974).
27. Norman, R. L., E. F. Johnson, and U. Muller-Eberhard. Identification of the major cytochrome P-450 form transplacentally induced in neonatal rabbits by 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin. *J. Biol. Chem.* **253**:8640–8647 (1978).
28. Dehnen, W., R. Tomingas, and J. Roos. A modified method for the assay of benzo(a)pyrene hydroxylase. *Anal. Biochem.* **53**:373–383 (1973).
29. 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).
30. Ullrich, V., and P. Weber. The O-dealkylation of 7-ethoxycoumarin by liver microsomes. A direct fluorometric test. *Hoppe Seyler's Z. Physiol. Chem.* **353**:1173–1177 (1972).
31. Masters, B. S. S., C. H. Williams, and H. Kamin. The preparation and properties of microsomal TPNH-cytochrome *c* reductase from pig liver. *Methods Enzymol.* **10**:565–573 (1967).
32. Holder, G., H. Yagi, P. Dansette, D. M. Jerina, W. Levin, A. Y. H. Lu, and A. H. Conney. Effects of inducers and epoxide hydrolase on the metabolism of benzo(a)pyrene by liver microsomes and a reconstituted system: analysis by high pressure liquid chromatography. *Proc. Natl. Acad. Sci. USA* **71**:4356–4360 (1974).
33. Dees, J. H., B. S. 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).
34. Haschek, W. M., P. J. Hakkinen, H. P. Witschi, R. P. Hanzlik, and G. J. Traiger. Non-ciliated bronchiolar epithelial (Clara) cell necrosis induced by organometallic carbonyl compounds. *Toxicol. Lett.* **14**:85–92 (1982).
35. Autrup, H., C. C. Harris, G. D. Stoner, J. K. Selkirk, P. W. Schafer, and B. F. Trump. Metabolism of [H^3]benzo(a)pyrene by cultured bronchus and cultured human pulmonary alveolar macrophages. *Lab. Invest.* **38**:217–224 (1978).
36. 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).

Send reprint requests to: Dr. Richard M. Philpot, Laboratory of Pharmacology (MD 19-08), NIEHS/NIH, P. O. Box 12233, Research Triangle Park, NC 27709.