CYTOCHROMES P-450 IN MURINE LUNG
An Immunohistochemical Study with Monoclonal Antibodies

P. G. FORKERT,1 M. L. VESSEY, S. S. PARK, H. V. GELBOIN, AND S. P. C. COLE2

Departments of Anatomy and Medicine (P.G.F., M.L.V.) and Departments of Oncology and Microbiology & Immunology (S.P.C.C.), Queen’s University; and Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health

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ABSTRACT:
We have used a panel of monoclonal antibodies (Mabs) and immunofluorescence to investigate the distribution of species of cytochrome P-450 inducible by 3-methylcholanthrene (3-MC) and phenobarbital (PB) in the lungs of “responsive” C57BL/6 and “nonresponsive” DBA/2 mice. A Mab (1–7–1) specific for an epitope common to rat cytochromes P-450c (P450iA1) and P-450d (P450iA2) detected the corresponding murine species (P1-450/P3-450) in cells of the alveolar septa, including type II cells, as well as endothelial cells lining blood vessels. Cytochrome P1-450 (Mabs 1–31–2, 1–36–1) is localized in type II cells of the alveolar septa but is not found in endothelial cells. Patterns of immunoreactivity with the 3-MC-specific Mabs indicate that alveolar septal cells possess P1-450 and P3-450, whereas endothelial cells of the vasculature are not the sites of P1-450, but rather of P2-450. Similar immunoreactivities for the 3-MC-defined P-450s are demonstrated in the lungs of C57BL/6 and DBA/2 mice. A Mab (2–66–3) which recognizes P-450b/P-450e (P450iB1/P450iB2) is reactive with P-450s localized in type II alveolar and bronchiolar epithelial cells, including nonciliated Clara cells. The epitope for cytochromes P-450b/P-450e is present in constitutive form within the lung and does not appear to be responsive to induction by PB. In contrast, epitopes for P1-450/P3-450 and P2-450 are not expressed constitutively but are only detected when induced by 3-MC. The regional and cellular selectivities exhibited by lung tissues for the major 3-MC- and PB-inducible forms of P-450 emphasize the prevalence of tissue specificities with regard to the expression of certain forms of P-450.

Cytochromes P-450 are hemoproteins which exist in multiple forms and constitute a group of isozymes with differing but overlapping specificities and inducibilities for a variety of endogenous and exogenous substrates, including steroids, prostaglandins, chemical carcinogens, drugs, and numerous other xenobiotics (1–3). Determinations of the total P-450 content of tissues usually accomplished by a spectral method possess limited utility for detection of P-450 isozymic forms. At present, immunohistochemical techniques are widely used due to the specificity and sensitivity that can be achieved for identification and characterization of P-450 species. Moreover, the preparation of Mabs1 directed to epitopes specific for a number of P-450 proteins has provided useful probes for determination of isozymic profiles of various tissues from different animal species (4–8). To date, different forms of P-450 have been identified in a number of organ systems including both hepatic and nonhepatic tissues.

Cytochromes P-450 have traditionally been classified into species defined by their inducing agents, such as 3-MC, PB, isosafrole, ethanol, and numerous other chemicals. Individual forms of P-450 may be present constitutively in tissues of untreated animals, and increase significantly or remain at the same levels following treatment with specific inducers. Alternatively, a P-450 species may not be detectable in tissues of untreated animals but is only expressed when the inducer is administered. Thus, isozymic content and the presence and extent of inducibilities are unique to specific systems and vary from tissue to tissue. Moreover, in complex heterogeneous tissues such as the lung, different forms of P-450 may reside in specific cell populations, thus rendering characterization particularly complex.

Our previous studies have used immunohistochemistry in conjunction with Mabs to detect and localize P-450 species in lungs of CD-1 mice (9). More recently, we have investigated the distribution and induction sites of P-450s in livers of “responsive” CD-1 and C57BL/6 and “nonresponsive” DBA/2 mice (10). In the present study, we have used immunohistochemistry and an extensive panel of Mabs to conduct a more detailed investigation of the cellular distribution of 3-MC- and PB-inducible P-450s in lung tissues. We have applied Mabs specific for related P-450 proteins encoded by the gene subfamilies, P450IA and P450IIIB, to titrate the cellular distribution of individual P-450s. We have probed for the cellular sites of epitopes for the 3-MC-inducible P-450s, P-450/P1-450 and P2-450, and for the major PB-inducible P-450s, P-450b/P-450e.

In the present study, we have applied immunohistochemistry on lung tissues of genetically inbred strains of mice (C57BL/6 and DBA/2). C57BL/6 is the prototypic murine strain which, in the liver, is inducible or responsive for aryl hydrocarbon hydroxylase, a major catalytic activity associated with P-450, while DBA/2 mice represent a strain which is relatively noninducible for the same enzyme activity (11). Furthermore, we have compared the responses of “responsive” C57BL/6 and “nonresponsive” DBA/2 strains of mice with regard to their inducibilities by 3-MC and PB within the lung.
Materials and Methods

Male mice of C57BL/6 and DBA/2 strains (25–28 g) were obtained from Charles River Canada (St. Constant, Quebec, Canada). Animals were housed over hardwood bedding (Beta Chip, Northeastern Products Corp., Warrensburg, NY) and kept in a sound-proof, temperature (23 ± 1°C) and light (6:00 a.m.–8:00 p.m.)-controlled environment.

Mice belonging to each strain were divided into four groups consisting of four animals each. Group 1 was administered 3-MC (80 mg/kg) (Sigma Chemical Co., St. Louis, MO) in corn oil at 72 and 48 h before death. Group 2 was administered sodium phenobarbital (40 mg/kg) (BDH Chemicals, Toronto, Ontario, Canada) in saline twice daily for four consecutive days, and the animals were killed 24 h after the last dose. Groups 3 and 4 served as controls and were given identical volumes of the respective vehicles. All agents were administered by ip injection.

The following Mabs (12–15) were used for immunostaining:

<table>
<thead>
<tr>
<th>Mab</th>
<th>Inducer</th>
<th>P-450 species</th>
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<tbody>
<tr>
<td>1-7-1</td>
<td>3-MC</td>
<td>P-450/P-450e</td>
</tr>
<tr>
<td>1-31-2</td>
<td>3-MC</td>
<td>P-450/P-450e</td>
</tr>
<tr>
<td>1-36-1</td>
<td>3-MC</td>
<td>P-450/P-450e</td>
</tr>
<tr>
<td>2-66-3</td>
<td>PB</td>
<td>P-450/P-450e</td>
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Lungs were inflated and prepared for immunohistochemical staining using the technique described previously (9). Frozen sections (5 μm) of lung tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After two rinses with PBS, the sections were incubated in 10% normal sheep serum and 1% bovine serum albumin diluted in PBS for 20 min to block nonspecific antibody binding. Following two additional rinses in PBS, lung sections were reacted with primary antibody (1:400) for 2 h. The sections were rinsed with PBS and incubated with fluorescein-conjugated sheep anti-mouse IgG (1:100) for 1 h. The primary and secondary antibodies were diluted in 10% normal sheep serum, 5% normal sheep serum, and 0.1% saponin in PBS. All incubations were performed at room temperature in a humidified atmosphere. Simultaneous incubations were conducted on tissue sections from the same lungs for appropriate comparisons of treatment regimens with different Mabs.

The control sections were reacted with control Mabs, HyHel-5 or HyHel-9, that are specific only to the egg white lysozyme (16). Additional controls were performed by incubations in which the primary antibodies were omitted and by incubations in which normal sera were used instead of specific antisera. The sections were mounted with glycerol (50%) containing phenylenediamine (2%) and viewed under epifluorescence with a Zeiss Photomicroscope II.

For reference to lung morphology, lung tissues were prepared, fixed, and embedded in Epon according to procedures we have used in previous studies (17). Lungs were fixed by tracheal instillation of 1% glutaraldehyde in 0.1 M PIPES buffer. Lung slices were postfixed in 1% osmium tetroxide in 0.1 M PIPES buffer and stained en bloc with uranyl acetate. Following dehydration with ethanol, the tissues were infiltrated and embedded in Epon. Sections (0.5 μm) were stained with toluidine blue for morphologic observations.

Results

Lung tissues are composed primarily of conducting airways and parenchyma, which forms the respiratory component. The respiratory portion of the lung consists predominantly of interstitial cells, blood vessels, and alveolar lining cells. We examined the tissues for the morphological sites of cytochromes P-450 by light microscopy and focused on parenchymal cellular elements and bronchioles of the lung. While type I and type II cells line the alveolar septa, ciliated and nonciliated Clara cells form the epithelium of the bronchioles. In contrast to the low cuboidal shape of the ciliated cell, the Clara cell is columnar and possesses a prominent apex which protrudes into the airway lumen, thus providing for easy identification.

Table 1 summarizes the effects of the administration of 3-MC, PB, or the vehicles, corn oil and saline, on 3-MC- and PB-inducible cytochromes P-450 within the lung. Table 2 depicts the regional distribution of specific cytochromes P-450 within the bronchiolar and parenchymal areas of lung tissues.

| Table 1: Cytochromes P-450 in the lungs of C57BL/6 and DBA/2 mice as detected by immunofluorescence |
|-------------------------------------------------|------------------|-----------------|-----------------|-----------------|
| Cytochromes P-450 species                        | 3-MC              | Corn oil        | PB              | Saline          |
| P-450                                           | ++               | ND              | ND              | ND              |
| P-450/P-450                                     | +++              | ND              | ND              | ND              |
| P-450b/P-450e                                   | +++              | +++             | +++             | +++             |

* 3-MC (80 mg/kg) was administered ip to animals at 48 and 72 h before death.

** Corn oil was used as vehicle for 3-MC administration.

*** PB (40 mg/kg) was administered ip twice daily, and the animals were killed 24 h after the last dose.

** Saline was used as vehicle for PB administration.

Regional distribution of cytochromes P-450 in the lungs of C57BL/6 and DBA/2 mice as detected by immunofluorescence

| Table 2: Regional distribution of cytochromes P-450 in the lungs of C57BL/6 and DBA/2 mice as detected by immunofluorescence |
|-------------------------------------------------|------------------|-----------------|-----------------|
| Cytochromes P-450 species                       | Alveolar Septa   | Endothelium     | Bronchioles     |
| P-450                                           | ++               | ND              | ND              |
| P-450/P-450                                     | +++              | ++              | ND              |
| P-450b/P-450e                                   | ++               | ND              | ND              |

*++* denotes increasing intensities of immunofluorescence; ND, not detectable. Strain-related differences for detection and localization of P-450 species are not observed in C57BL/6 and DBA/2 mice.
Type II cells of the alveolar septa are labeled for cytochrome P-450 by reaction with Mab 1-36-1 (arrows). Magnification × 435.

Distribution of PB-inducible Cytochromes P-450. As with 3-MC-inducible cytochromes P-450, a Mab (2-66-3) raised against PB-induced rat hepatic cytochromes P-450 cross-reacted with proteins present in lung cells of C57BL/6 and DBA/2 mice. In contrast to the lack of expression of 3-MC-inducible P-450s in untreated mice, P-450b/P-450e were present in lungs of untreated mice as well as in the tissues of mice treated with 3-MC and PB.

The PB-inducible cytochromes P-450 were located in the bronchiolar epithelium and were highly concentrated in the nonciliated Clara cells (table 2, fig. 5). These P-450 species were also found in the parenchymal region of the lung in type II cells (table 2; fig. 6) but were not discerned in the endothelial cells.

As with all controls which were performed with tissue sections from the same lung in identical incubations with experimental sections, the labeling was negative.

Discussion

Pulmonary tissues exhibit unique properties with respect to their content of cytochromes P-450, isozymic content, and sub-
The use of additional Mabs (1-31-2, 1-36-1) which are specific for only P1-450, have produced observations which demonstrate the presence of this isozyme in lungs of both C57BL/6 and DBA/2 mice treated with 3-MC. Livers of DBA/2 mice, however, differ with respect to content of these P-450 species. Whereas P1-450 is present in microsomes from livers of 3-MC-treated DBA/2 mice, P1-450 could not be detected (14). Recent immunohistochemical studies in this laboratory have shown that P1-450 is also present in the livers of untreated DBA/2 mice (10).

Although analogous 3-MC-inducible P-450s are present in both lung and liver tissues of inbred strains of mice, the conditions under which they are expressed differ in the two tissues. These P-450s are present constitutively in livers of untreated C57BL/6 and DBA/2 mice (10); in contrast, they are not detected in liver tissues of untreated mice. Whereas 3-MC-specific P-450s are highly inducible in livers of “responsive” C57BL/6 mice treated with 3-MC, they are relatively noninducible in livers of “nonresponsive” DBA/2 mice. On the other hand, lung tissues of both “responsive” C57BL/6 and “nonresponsive” DBA/2 mice exhibit good inducibility when treated with 3-MC.

As expected, pulmonary reactivity to the PB-specific Mab is similar in tissues from C57BL/6 and DBA/2 mice. Regardless of whether the animals are untreated or treated with PB or 3-MC, lung tissues of both strains of mice contain cytochromes P-450 with the epitopes recognized by the PB-defined Mabs (Table 1). These findings demonstrate that P-450b/P-450e (2-66-3) are constitutive to the lung; the administration of either PB or 3-MC does not alter the pattern or intensity of labeling.

The localization of various forms of P-450 within the lung is of substantial interest due to the cellular heterogeneity which exists and the cell-specific manifestations of chemically induced toxicities. The patterns of immunoreactivities obtained in the present study emphasize that the cellular sites of different forms of P-450 are well defined and are restricted to specific cell populations. Although lung tissues are composed of a large number of cell types, P-450s appear to reside primarily in only the type II, Clara, and endothelial cells. The results of this investigation show that P450 and P450, as detected by Mabs 1-31-2 and 1-36-1, is localized in the alveolar septa and endothelial cells lining blood vessels (figs. 1 and 2, table 2). P450, as detected by Mabs 1-31-2 and 1-36-1, is localized in the alveolar septa in the type II cells and is not seen in the endothelium (figs. 3 and 4; table 2). These findings suggest that endothelial cells are not the sites of P-450 but rather of P-450. Studies in rat lung using polyclonal antibodies have identified 3-MC-inducible P-450s in endothelial, Clara, and type II cells (23). None of the 3-MC-specific Mabs used in this study detected P-450s in the Clara cells in murine lungs, suggesting that the cellular distribution of P-450s differs in the same tissues from different animal species.

It is of interest that type II cells contain both PB- and 3-MC-inducible P-450s. It remains to be elucidated whether a heterogeneous population of type II cells exists for these major forms of P-450 or whether individual alveolar cells possess regulatory mechanisms that permit the expression of different P-450 proteins encoded by separate gene families. Nevertheless, the localization of the major 3-MC- and PB-inducible cytochromes P-450 within particular cell populations of the lung emphasizes the prevalence of tissue specificities with regard to the expression of individual forms of P-450.
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References