Monoclonal antibodies against human cytochrome *P*-450 recognizing different pregnenolone 16 α -carbonitrile-inducible rat cytochromes *P*-450

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Six murine monoclonal antibodies against human hepatic cytochrome *P*-450 have been raised, using human liver microsomes (microsomal fractions) or semi-purified human cytochrome *P*-450 as immunogen. All six antibodies recognized the same highly purified of human liver cytochrome *P*-450 of molecular mass 53 kDa and gave rise to a single band at 53 kDa on immunoblots of human liver microsomes from 11 individuals. The antibodies also recognized proteins at 52 kDa and 54 kDa on immunoblots of control and induced male-rat liver microsomes, showing four different banding patterns. Antibodies HL4 and HP16 recognized a 52 kDa protein that was only weakly expressed in untreated rats and which was strongly induced by pregnenolone 16 α -carbonitrile (PCN) but not by phenobarbitone (PB), 3-methylcholanthrene (3MC), isosafrole (ISF), Aroclor 1254 (ARO), clofibrate or imidazole. HP10 and HL5 recognized a constitutive 52 kDa protein that was weakly induced by PCN but not by the other agents and was suppressed by 3MC and ARO. HP3 recognized a 54 kDa protein that was undetectable in control rats but was strongly induced by PB, PCN, ISF and ARO. HL3 appeared to recognize a combination of the proteins recognized by the other antibodies plus a 54 kDa protein that was weakly expressed in control rats. The constitutive proteins recognized were male-specific.

INTRODUCTION

Cytochrome *P*-450 (*P*-450) is an extensive multi-genically coded family of haemoproteins that are responsible for the oxidative metabolism of a wide variety of endogenous and exogenous compounds, including steroids, prostaglandins, fatty acids, vitamins, drugs and chemical carcinogens (Adesnik & Atchison, 1985; Black & Coon, 1986; Waxman, 1986; Kalow, 1987; Nebert *et al.*, 1987). The existence of very many structurally and functionally diverse forms of *P*-450 has necessitated the development of biochemical probes to characterize and distinguish the various forms. Polyclonal antibodies have been used for many years as probes that are either specific for a single form of *P*-450 or selective for a small group of structurally related forms (e.g. Dannan *et al.*, 1983; Wolf *et al.*, 1986). More recently, monoclonal antibodies have been produced against *P*-450 from animals (Reubi *et al.*, 1984; Thomas *et al.*, 1984a; Gelboin & Friedman, 1985; Reik *et al.*, 1985; Sesardic *et al.*, 1986; Park *et al.*, 1986) and humans (Beaune *et al.*, 1985; Mendelson *et al.*, 1985; Barnes *et al.*, 1986), with the intention of providing immunological probes of the highest specificity. An important use of antibodies, either poly- or mono-clonal, is to assess relationships among different forms of *P*-450 and among orthologous forms in different species (Thomas *et al.*, 1984b; Song *et al.*, 1985; Wrighton *et al.*, 1985), in particular comparing man and animals (Adams *et al.*, 1985; Watkins *et al.*, 1985; Beaune *et al.*, 1986; Pelkonen

et al., 1986; Sesardic *et al.*, 1986; Wong *et al.*, 1986; Wrighton *et al.*, 1986). The published immunological comparisons between human and animal *P*-450 have mainly involved the use of antibodies raised against purified animal *P*-450 forms to probe human tissues. We report here the generation of a panel of monoclonal antibodies against human *P*-450 and its use in a comparison of human and rat *P*-450.

MATERIALS AND METHODS

Generation of hybridoma lines

Monoclonal antibodies were generated essentially as described by Kohler & Milstein (1975). Female BALB/c mice, 8 weeks old, were immunized by intraperitoneal injection with either 100 μ g of human liver microsomal proteins or 35 μ g of semi-purified human hepatic microsomal *P*-450 (2.7 nmol/mg of protein, showing three proteins bands on SDS/PAGE), in 50 % complete Freund's adjuvant in 0.9 % NaCl. The mice were boosted on weeks 1, 2 and 25 as described above, but incomplete adjuvant was used. At 3 days after the final boost, spleen cells were fused with mouse myeloma Ag8.653 cells. Colonies which were positive by e.l.i.s.a. and immunoblotting were cloned twice by limiting dilution to ensure line monoclonality and stability. Monoclonal antibodies were prepared for use by 45 % satd.-(NH₄)₂SO₄ precipitation of either cell-culture supernatant or ascitic fluid from mice injected with the relevant hybridoma lines. All

Abbreviations used: PCN, pregnenolone 16 α -carbonitrile; PB, phenobarbitone; 3MC, 3-methylcholanthrene; ISF, isosafrole; ARO, Aroclor 1254; CLO, clofibrate; IMI, imidazole; *P*-450, cytochrome *P*-450; PAGE, polyacrylamide-gel electrophoresis; e.l.i.s.a., enzyme-linked immunoadsorbent assay; PBS, phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/8 mM-Na₂HPO₄, pH 7.2); i.p., intraperitoneal.

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the antibodies described here were isotypized as IgG_k by using an e.l.i.s.a.-based 'sandwich' assay and a commercial kit.

Immunoblotting

Human and rat liver microsomal proteins were resolved by discontinuous SDS/PAGE (Laemmli, 1970) by using a 7–10%-(w/v)-polyacrylamide linear-gradient resolving gel. Electroblothing of the resolved proteins on to nitrocellulose paper (Schleicher and Schuell; BA 85, 0.45 µm pore size) was performed as described by Towbin *et al.* (1979). The nitrocellulose paper was incubated successively in: 'blotwash' [10% (v/v) newborn-calf serum/0.2% Triton X-100 in PBS] for 30 min; 'blotwash' containing monoclonal antibody for 90 min; 'blotwash', for 10 min, three times; rabbit anti-mouse IgG-horseradish peroxidase conjugate for 60 min; 'blotwash' for 10 min, three times. The nitrocellulose was then rinsed briefly in PBS before staining with diaminobenzidine (0.5 mg/ml)/0.1 M-Tris/HCl (pH 7.6)/0.03% (v/v) H₂O₂. Once staining was complete, the nitrocellulose was immersed in a solution of 7% (v/v) acetic acid/5% methanol (final concns.) to prevent further development of background.

Animal treatments and preparation of liver microsomal fractions

Adult (200–250 g) male Sprague-Dawley rats (Charles River, Margate, Kent, U.K.) were used either untreated or after one of the following treatments (i.p. injections and gavage administrations were once daily; all percentages are w/v): phenobarbitone (PB; BDH, Poole, Dorset, U.K.), 0.1% in drinking water for 7 days; pregnenolone 16α-carbonitrile [PCN; generously given by Dr. J. Pike, The Upjohn Co., Kalamazoo, MI, U.S.A.; 5% (w/v) in 2.5% (v/v) Tween 80], 50 mg/kg by gavage for 4 days; 3-methylcholanthrene (3MC; Sigma Chemical Co., Poole, Dorset, U.K.; 1% in olive oil), 80 mg/kg i.p. once 3 days before death; isosafrole (ISF; generously given by Dr. C. Elcombe, ICI, Alderley Park, Macclesfield, Cheshire, U.K.; 10% in olive oil), 200 mg/kg i.p. for 3 days; Aroclor 1254 (ARO; also from Dr. C. Elcombe; 20% in olive oil), 25 mg/kg i.p. for 6 days; clofibrate acid (CLO; from Sigma), 0.6% in powdered rat chow for 14 days; imidazole (IMI; from Sigma; 120 mg/ml), 200 mg/kg i.p. for 5 days. Appropriate treatments with drug vehicle alone were also carried out. Except for 3MC, all treatments were stopped 24 h before death. Samples of human liver were obtained from renal-transplant donors within 30 min of circulatory arrest, placed immediately into ice-cold iso-osmotic NaCl and frozen within 1 h as approx. 1 cm cubes at –80 °C until use. Rat and human liver microsomal suspensions were prepared by differential ultracentrifugation involving KCl washing as described previously (Burke *et al.*, 1983).

RESULTS

Characterization of the monoclonal antibodies

Six monoclonal antibodies were identified by the dual criteria of the recognition of a human liver microsomal protein on e.l.i.s.a. and the recognition of a protein of *M_r* between 45 000 and 60 000 on immunoblots of human liver microsomes. Antibodies HL3, HL4 and HL5 were raised by inoculation with human liver microsomes

from the same individual and came from different initial culture wells from the same fusion. Antibodies HP3, HP10 and HP16 were raised by inoculation with semi-purified human hepatic microsomal *P*-450 from another individual. HP3 and HP16 came from different culture wells after the same initial fusion, whereas HP10 came from a different initial fusion. A seventh monoclonal antibody, HP15, which came from the same initial fusion and same initial culture well as HP16 but was derived from a different clone, gave a positive result on e.l.i.s.a., but did not give rise to a stained band on immunoblots. HP15 is not considered further here.

Each of the antibodies recognized (results not shown) a highly purified human hepatic microsomal *P*-450 of *M_r* 53 000, which was purified to electrophoretic homogeneity and a specific content of 15.5 nmol/mg of protein (P. M. Shaw & M. D. Burke, unpublished work). Each of the six antibodies also gave rise to a single stained band at a location of 53 kDa on immunoblots of liver microsomes prepared from 11 different individuals, and although there was considerable inter-individual variation in the intensity of this band, the same rank order of intensities was shown with each antibody (Fig. 1). None of the antibodies recognized human epoxide hydrolase (either purified in our own laboratories or generously provided by Dr. A. R. Boobis, Royal Postgraduate Medical School, London W.12, U.K.) or rat UDP-glucuronyltransferases (generously provided by Dr. B. Burchell, University of Dundee, Dundee, Scotland, U.K.) on immunoblots. The recognition of a single band of the same *M_r* as pure human *P*-450 on immunoblots of liver microsomes from 11 different individuals strongly suggests that the antibodies recognize the same, single form of human *P*-450, or possibly several forms sharing extensive structural homology and with very similar *M_r*.

Fig. 1. Inter-individual differences in the level of cytochrome P450hA7 in adult human liver microsomes

The microsomal fractions of 11 adult human livers (lanes 1–11, 20 µg of protein/lane) were submitted to SDS/PAGE and electroblotted on to nitrocellulose paper. The nitrocellulose was immunochemically stained by incubating with antibody HL3 as described in the Materials and methods section.

Recognition of rat liver microsomal proteins

All the antibodies recognized proteins of M_r 52000 or 54000 on immunoblots of liver microsomes from control and drug-induced male rats, but there were four different recognition patterns (based on the relative intensities of immunostained bands). HP10 recognized a 52 kDa protein that was constitutive (i.e. expressed strongly in untreated rats), was induced weakly by PCN, and was not induced by PB and was suppressed by 3MC and ARO (Fig. 2a). HP16 also recognized a 52 kDa protein, but this was expressed only weakly in untreated rats and was induced strongly by PCN; it was not induced by PB and was suppressed by 3MC and ARO (Fig. 2b). HP3 recognized a 54 kDa protein that was undetectable in

untreated rats but was strongly induced by PCN, PB, ISF and ARO (Fig. 2c). HL3 recognized both a 52 kDa and a 54 kDa protein (Fig. 2d). The 52 kDa protein (lower band) recognized by HL3 was constitutive, induced by PCN, but not PB, and suppressed by 3MC. The 54 kDa protein (upper band) recognized by HL3 was weakly expressed in untreated rats and strongly induced by PCN, PB, ISF and ARO. The 54 kDa protein recognized by HL3 and HP3 and the 52 kDa protein recognized by HL3, HP10 and HP16 appeared to be slightly induced by CLO but not affected by IMI. HL4 and HL5 gave results (not shown) similar to those given by HP16 and HP10 respectively.

In liver microsomes of untreated female rats, HL5 and HP10 gave a single faint band of the same M_r (52000) as the strong band shown with untreated male rats (results not shown). HL3, HL4, HP3 and HP10 detected no proteins in liver microsomes of untreated female rats.

Fig. 2. Cross-reactivity of monoclonal antibodies with the proteins of induced rat liver microsomes

Rat liver microsomes submitted to SDS/PAGE and electroblotted on to nitrocellulose as described in the Materials and methods section were: untreated control (lane 1); PB-induced (lane 2); PCN vehicle control (lane 3); PCN-induced (lane 4); 3MC/ISF/ARO vehicle control (lane 5); 3MC-induced (lane 6); ISF-induced (lane 7); ARO-induced (lane 8); CLO-induced (lane 9); and IMI-induced (lane 10). The nitrocellulose sheets were immunochemically stained with the following monoclonal antibodies: (a) HP10, (b) HP16, (c) HP3, (d) HL3.

DISCUSSION

The human P-450 (P450hA7) purified by us and recognized by each of the antibodies tested here shows N-terminal sequence homology for 18 amino acids (P. M. Shaw & M. D. Burke, unpublished work) with human P-450 HLp (Watkins *et al.*, 1985) and P-450_{NF} (Beaune *et al.*, 1986). The apparent monomeric molecular mass on SDS/PAGE, 53 kDa, of P-450hA7 and the human hepatic microsomal protein recognized by the antibodies is greater than that reported for P-450 HLp (51 kDa) or P-450_{NF} (52 kDa), but M_r values for P-450 are notoriously variable between different laboratories (Black & Coon, 1986). Alternatively, since human P-450 HLp and P-450_{NF} are members of a multigene family (Molowa *et al.*, 1986; Beaune *et al.*, 1986), P-450hA7 and the human hepatic microsomal protein recognized by the antibodies might be further members of the same gene family.

Different forms of human hepatic microsomal P-450 with M_r values ranging between 48000 and 54000 have been identified in the same laboratory (Wang *et al.*, 1983; Distlerath *et al.*, 1985; Guengerich *et al.*, 1986; Shimada *et al.*, 1986). Since our antibodies were raised against either crude human liver microsomes (HL3, HL4 and HL5) or semi-purified human P-450 (HP3, HP10 and HP16) and were not selected by using a highly purified individual form of P-450, it is perplexing why they recognized only a single protein of the same M_r as P450hA7 on immunoblots of hepatic microsomes from 11 different individuals. Possibly P450hA7 was more abundant and more antigenic than other P-450 forms present in the microsomes. Nevertheless, until we have isolated and tested more forms of human P-450, we cannot dismiss the possibility that some of the antibodies may recognize additional forms of human P-450. Our results suggest a considerable inter-individual variation in the expression of P450hA7 and possibly of other immunologically related forms of P-450 of the same M_r .

Each of our monoclonal antibodies to human P-450 recognized one or more PCN-induced rat liver microsomal proteins of M_r 52000 and 54000. We have attempted to identify these proteins by comparing their induction patterns and sex specificities with those reported in the literature. Three PCN-inducible P-450 forms have been purified from rat liver: P-450 PB-2a and P-450_{PCN-E}, which are probably identical (Waxman *et*

al., 1985), and P-450p (Elshourbagy & Guzelian, 1980). In addition, P450PCN1 and P450PCN2 have been identified by sequencing of their cDNAs (Gonzalez *et al.*, 1986). P450PCN1 is not normally expressed in male or female rats, but is induced by either PCN or PB, although not by 3MC (Gonzalez *et al.*, 1986); this pattern of expression corresponds to the 54 kDa PB- and PCN-induced protein recognized by our antibody HP3. P-450 PB-2a/PCN-E also shows a similar induction pattern, but differs in being a major constitutive form in male rats (Waxman *et al.*, 1985).

There was no pattern of recognition in our immunoblots corresponding to the pattern of expression of P450PCN2, which is normally higher in adult male than female rats, induced by PB but not by PCN and suppressed by 3MC (Gonzalez *et al.*, 1986).

The induction patterns represented in Figs. 2(a) and 2(b) indicate the presence of at least two rat liver P-450 forms that have not previously been reported. The antibodies HP10 and HL5, which recognize a constitutive protein that is induced marginally by PCN but not by PB, and antibodies HP16 and HL4, which recognize a protein that is weakly expressed in adult male rats and strongly induced by PCN but not by PB, will prove useful in the purification, characterization and molecular cloning of the DNA for these P-450 forms.

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