Inter-relatedness of some isoenzymes of cytochrome *P*-450 from rat, rabbit and human, determined with monoclonal antibodies

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1. Monoclonal antibodies have been raised to rat liver cytochromes *P*-450 b and c, and rabbit liver cytochrome *P*-450 form 4. A total of six antibodies have been studied. 2. Each antibody reacted strongly both with its homologous antigen and with microsomal fractions selectively enriched with that antigen by treatment of animals with inducing compounds. However, several of the antibodies showed cross-reactivity, either within or between species. 3. A combination of enzyme-linked immunosorbent assay, immuno-adsorption, Western blotting and competitive radioimmunoassay revealed that each of the antibodies reacted with a different epitope. 4. Proteolytic digestion of antigen followed by Western blotting of the peptide fragments enabled antibodies, otherwise identical in their reactivity, to be distinguished. 5. It is concluded that complex structural relationships exist amongst the different isoenzymes of cytochrome *P*-450 and that epitope mapping will help in characterizing both animal and human cytochromes *P*-450.

INTRODUCTION

In the last few years, major advances have been made in elucidating the structure of several isoenzymes of cytochrome *P*-450. In some instances most of the amino acid sequence of the haemoprotein has been obtained (Yuan *et al.*, 1983; Haniu *et al.*, 1984), whereas in other studies the entire nucleotide sequence of the structural gene has been determined (Fujii-Kuriyama *et al.*, 1982; Sogawa *et al.*, 1984). Where both approaches have been applied, there has been good agreement between them. Such studies have revealed that the isoenzymes of cytochrome *P*-450 appear to belong to a number of different classes, with one or more members of each class in each species (Fujii-Kuriyama *et al.*, 1984). The members of a class of cytochrome *P*-450 show a considerable degree of structural homology, and respond to inducers in a similar way. The members of one class of cytochrome *P*-450 sometimes differ markedly in structure from the members of another class of cytochrome *P*-450 (Fujii-Kuriyama *et al.*, 1984). As yet, there is no accepted nomenclature for the different isoenzymes of cytochrome *P*-450. The term 'cytochrome *P*-450' is used here in a generic sense to denote any or all forms of the haemoprotein. Specific forms of cytochrome *P*-450 from rat liver have been designated according to the convention of Ryan *et al.* (1984) and those from rabbit liver according to the convention of Johnson *et al.* (1984).

Because of the regions of homology of cytochrome *P*-450 amongst different species, it has been possible to hybridize cDNA probes generated to an isoenzyme of cytochrome *P*-450 from one species to the DNA of

another species (Phillips *et al.*, 1985). Although this can provide valuable information, it is sometimes possible that the DNA to which such a cDNA probe hybridizes is not part of its structural gene (Atchison & Adesnik, 1983). An alternative approach to investigate the structural and functional relationships between forms of cytochrome *P*-450 is the application of monoclonal antibodies (MAbs). By their nature, MAbs react with a single epitope which may or may not be unique to the homologous antigen (Kohler & Milstein, 1975). In either case, well-characterized MAbs should prove invaluable in a study of the structure and function of cytochromes *P*-450. In particular, MAbs raised to a form of cytochrome *P*-450 from one species are proving extremely useful in the analysis of cytochromes *P*-450 in man (e.g. see Fujino *et al.*, 1982).

The present paper describes results of a study with MAbs raised to three different forms of cytochrome *P*-450, and the relationships between these and other forms of the enzyme.

EXPERIMENTAL

Materials

Chemicals and their sources were as previously reported (Boobis *et al.*, 1985b) with the following additions. Bio-Gel HTP hydroxyapatite was purchased from Bio-Rad Laboratories. 1,8-Diamino-octane, rabbit muscle aldolase, ovalbumin from chicken egg, bovine liver L-glutamate dehydrogenase, bovine liver catalase, α -chymotrypsin and papain were all obtained from Sigma Chemical Co. Miles Laboratory provided V8 protease

Abbreviations used: BNF, β -naphthoflavone; DTT, dithiothreitol; e.l.i.s.a., enzyme-linked immunosorbent assay; MAb, monoclonal antibody; 3-MC, 3-methylcholanthrene; PB, phenobarbitone; PBS, phosphate-buffered saline (10 mM-potassium phosphate buffer, pH 7.4, containing 0.15 M-NaCl).

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(from *Staphylococcus aureus*). DE-51, DE-52 and DE-53 anion exchange resins were purchased from Whatman. Cooke 96-well polystyrene microtitre plates for use in solid phase e.l.i.s.a. and individual removable microtitre wells were obtained from Dynatech. PM30 ultrafiltration membranes were from Amicon. L-[U-¹⁴C]leucine, > 300 mCi/mmol in sterile aqueous solution, was obtained from Amersham International. Renex 690 was a generous gift from Atlas Chemical Industries (U.K.). Isosafrole (*cis* and *trans*) was purchased from Eastman-Kodak.

Treatment of animals with inducing compounds

Male Wistar (Olac) and Alderley Park (Central Toxicology Laboratory, ICI plc) rats weighing between 180 and 250 g were maintained under constant heating and lighting cycles. Groups of animals were treated with inducing compounds as follows: 3-methylcholanthrene (3-MC), 80 mg/kg by intraperitoneal injection 48 h before death; phenobarbitone sodium (PB), 80 mg/kg by intraperitoneal injection daily for 4 days, the last dose being administered 24 h before death; and isosafrole, 160 mg/kg in corn oil by intraperitoneal injection daily for 4 days, the last dose being administered 24 h before death. Further details of the treatments can be found in a previous paper (Boobis *et al.*, 1985b). Animals were permitted free access to food (PRD diet; Labsure Animal Products) until 18 h before death, and tap water *ad libitum*. Animals were killed by stunning and exsanguination and the microsomal fractions were isolated as previously described (Boobis *et al.*, 1985b).

Preparation of antigens

Treatment of rats with either BNF or 3-MC causes increases in predominantly cytochromes *P*-450 c and d (Guengerich *et al.*, 1982), whereas treatment with PB results in substantial elevation in the levels of forms b and e (Ryan *et al.*, 1982). Treatment of rabbits with either BNF or 3-MC causes an increase in predominantly cytochrome *P*-450 forms 4 and 6 (Atlas *et al.*, 1977).

Rabbit cytochrome *P*-450 form 4 (Boobis *et al.*, 1981b) and partially purified cytochrome *P*-450 c from rat (Boobis *et al.*, 1985b) were prepared as previously described. A cytochrome *P*-450 preparation from rat enriched in the form(s) inducible by PB (forms b and e) was prepared from the livers of male Alderley Park rats injected daily with PB (80 mg/kg) in 0.9% saline by intraperitoneal injection. The first dose was administered as 2 × 40 mg/kg and animals were killed 24 h after the last dose. The cytochrome *P*-450 fraction was partially purified, to a specific content of 5.0 nmol/mg, as previously described (Boobis *et al.*, 1985b).

Purification of rat cytochrome *P*-450 c to apparent homogeneity

Cytochrome *P*-450 c was purified to apparent homogeneity from the livers of rats treated with 3-MC by methods based on those of Guengerich & Martin (1980) and Guengerich *et al.* (1982). Male Wistar rats weighing 150–220 g (Olac) were housed as previously described (Boobis *et al.*, 1981a) and treated with 3-MC as described above. Microsomal fraction was isolated (Boobis *et al.*, 1980) and resuspended in 0.1 M-potassium pyrophosphate buffer, pH 7.5, containing 1 mM-EDTA and 23 μM-DTT.

The microsomal suspension, in 100 mM-phosphate buffer, pH 7.5, containing 20% (v/v) glycerol and

1 mM-EDTA, was solubilized with sodium cholate and the cytochrome *P*-450 concentrated by precipitation with 16% (v/v) poly(ethylene glycol). The pellet was resuspended in 100 mM-phosphate buffer, pH 7.5, containing 0.5% cholate, 20% (v/v) glycerol and 1 mM-EDTA and applied to an ω-octylamino-Sepharose 4B column; the adsorbent was synthesized as described by Guengerich & Martin (1980). Cytochrome *P*-450 was eluted from the column with 100 mM-phosphate buffer, pH 7.5, containing 20% (v/v) glycerol, 1 mM-EDTA, 0.4% cholate and 0.2% Renex 690.

The cytochrome *P*-450-containing fractions were pooled, diluted 4.5-fold in 20% (v/v) glycerol and applied to a column of Bio-Gel HTP previously equilibrated with 10 mM-phosphate buffer, pH 7.5, containing 20% (v/v) glycerol, 0.1 mM-EDTA, 0.2% cholate and 0.2% Renex 690. The column was washed with the same buffer, the phosphate was increased to 40 mM, and the cytochrome *P*-450 was eluted by increasing the ionic strength of the buffer to 90 mM.

The cytochrome *P*-450 fractions eluted with 90 mM-phosphate were pooled and concentrated with an Amicon ultrafiltration cell and a PM30 membrane. The concentrated solution was dialysed against 5 mM-phosphate buffer, pH 7.6, containing 20% (v/v) glycerol, 0.1 mM-EDTA, 0.1% Renex 690 and 0.2% cholate.

The concentrated, dialysed cytochrome *P*-450 fraction was applied to a set of three DEAE-cellulose columns, DE-51, DE-52 and DE-53, arranged in series as described by Guengerich *et al.* (1982). The fraction containing cytochrome *P*-450 c was eluted by a linear gradient of NaCl from 0 to 150 mM. Fractions were analysed by SDS/polyacrylamide-gel electrophoresis to identify those containing cytochrome *P*-450 c. These were then pooled, concentrated with a PM30 membrane in an Amicon ultrafiltration cell and then dialysed against 5 mM-phosphate buffer, pH 7.5, containing 0.1 mM-EDTA, 0.1 mM-DTT, 0.1% Renex 690, 0.2% cholate and 20% (v/v) glycerol. The non-ionic detergent, Renex 690, was removed from the sample by chromatography on Bio-Gel HTP, the column being eluted with 10 mM-phosphate buffer pH 7.45, containing 20% (v/v) glycerol, 0.1 mM-EDTA, 0.1 mM-DTT and 0.2% cholate, until no more material absorbing at 276 nm was eluted from the column. The cytochrome *P*-450 fraction was removed by increasing the phosphate to 400 mM. The cytochrome *P*-450-containing fractions were finally pooled, concentrated by ultrafiltration and dialysed against 50 mM-phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, 0.1 mM-EDTA and 0.1 mM-DTT. The fraction was apparently homogeneous on SDS/polyacrylamide (10% acrylamide) gel electrophoresis, with a specific content of 14 nmol/mg of protein.

Production of antibodies

MAbs 12/2/3/2 (IgG_{2a}), 9/20/11 (IgG₁) and 3/4/2 (IgG₁) against rat cytochrome *P*-450 c were produced in separate fusions, as previously described (Boobis *et al.*, 1985b). MAbs 105 (IgG₁) and 106 (IgG₁), against rabbit liver cytochrome *P*-450 form 4 were prepared as previously reported (Boobis *et al.*, 1981b). MAb 1/4 (IgG_{2a}) against rat PB-inducible cytochrome(s) *P*-450 was prepared in the same manner as that described previously for the production of MAb 3/4/2 (Boobis *et al.*, 1985b). In the present study, two control antibodies have been used, B3A3 (IgG₁) raised against bovine growth

hormone, and NBS1-48-5p26, the result of fusing splenic lymphocytes from a non-immunized mouse with myeloma cells (generously given by Dr. H. V. Gelboin, Bethesda, MD, U.S.A.). Neither antibody reacted with cytochrome P-450 from control, PB- or 3-MC-treated rats, rabbits or human liver.

MAbs were prepared in quantity by innoculating mice with the appropriate hybridoma cells as previously described (Boobis *et al.*, 1985b) and collecting the ascites fluid. The Ig fraction was purified by DEAE Affi-Gel Blue chromatography as previously reported (Boobis *et al.*, 1985b).

Solid phase e.l.i.s.a., SDS/polyacrylamide-gel electrophoresis, Western blotting, labelling of MAb 9/20/11 with [¹⁴C]leucine and the assay of protein were all performed as described in detail elsewhere (Boobis *et al.*, 1985b). Relative molecular masses have been assigned on the relative mobility of the proteins on SDS/polyacrylamide-gel electrophoresis, by comparison with the relative mobilities of a series of standard proteins. The exact mobility of a given protein is notoriously unreliable, so that an assignment of precise relative molecular mass is not possible. However, comparison of mobilities of authentic forms of cytochrome P-450 with those of the proteins increasing in intensity in the microsomal fractions of animals treated with the different inducing compounds enables the identity of the major isoenzymes of cytochrome P-450 to be made with relative confidence.

Competitive radioimmunoassay

MAb 9/20/11 was internally labelled by growing the hybridoma cells in [¹⁴C]leucine-supplemented medium as previously described (Boobis *et al.*, 1985b). The specific radioactivity of the purified Ig fraction was 3000 d.p.m./μg of protein. This labelled antibody preparation was then used in a solid phase competitive binding assay based on a modification of the method of Song *et al.* (1984).

Individual polystyrene wells were coated with microsomal fraction from the livers of BNF-treated rats (Boobis *et al.*, 1981b). Serial, 2-fold dilutions of MAbs 3/4/2, 12/2/3/2, 9/20/11 and NBS-1-48-5p26, included as a negative control, were added to the wells, the highest concentration being 1 mg/ml. To each well was added 3.3 μg (equivalent to 10000 d.p.m.) of the labelled 9/20/11 preparation. Following incubation and washing of the wells, they were dried and radioactivity was determined in a model 2660 liquid-scintillation counter (Packard Instrument Co.) after the addition of 10 ml of liquid scintillation cocktail (Packard Instagel). Specific binding of labelled 9/20/11 was calculated by subtracting radioactivity obtained from wells incubated with PBS alone from that of the test wells. Results have been expressed as percentage of maximum observed binding, i.e., the binding obtained in the absence of additional unlabelled MAb preparation.

Epitope mapping

Limited proteolysis of homogeneous rat cytochrome P-450 c was performed by a modification of the method of Cleveland *et al.* (1977).

Samples of rat cytochrome P-450 c were diluted in 250 mM-Tris/HCl, pH 6.8, containing 0.1% SDS, 10% (v/v) glycerol and 0.001% Bromphenol Blue. Proteolytic digestion was performed on 15 μg of purified cytochrome P-450 c by α-chymotrypsin (1.0 μg), V8 protease (1.0 μg)

or papain (0.05 μg) at 37 °C for 1, 5, 10, 30, and 60 min. Proteolysis was stopped by the addition of 20 μl of 50% (v/v) 2-mercaptoethanol containing 10% (w/v) SDS.

The digested samples were incubated at 100 °C for 5 min and were then subjected to SDS/polyacrylamide-gel electrophoresis in the presence of 12.5% (w/v) acrylamide as described by Laemmli (1970). Gels were stained for protein with Coomassie Brilliant Blue R, as previously described (Boobis *et al.*, 1977). Examination revealed that the samples digested for 10 min provided optimum proteolysis. Such fragments were therefore subjected to Western blotting by the procedure of Towbin *et al.* (1979). Between 2 and 3 μg of digested protein or 0.7 μg of intact rat cytochrome P-450 c were applied to the polyacrylamide gel. Following blotting onto nitrocellulose membranes and incubation with MAbs to rat cytochrome P-450 c, immunoreactive peptides were visualized by incubating with horseradish peroxidase-conjugated rabbit anti-(mouse IgG) and developing with 4-chloro-α-naphthol reagent, as previously described (Boobis *et al.*, 1985b).

RESULTS

All of the antibodies reacted strongly with the homologous form of cytochrome P-450 in e.l.i.s.a. (Table 1). The MAbs also reacted strongly with the homologous microsomal fraction, but in some instances showed cross-reactivity with other microsomal fractions (Table 2). However, most of the MAbs were specific for the homologous microsomal preparation, the most notable exception being 3/4/2.

Three of the MAbs investigated reacted strongly with microsomal fractions from BNF-treated rats, not surprisingly as the antibodies were raised against a partially purified preparation of rat cytochrome P-450 c. The difference in the pattern of reactivity between MAb 3/4/2, on the one hand, and MAbs 9/20/11 and 12/2/3/2, on the other, strongly suggested that these two groups of MAbs reacted with different epitopes. To investigate further whether the MAbs reacted with the same or different epitopes, MAb 9/20/11 was labelled with [¹⁴C]leucine by growing cells in culture medium fortified with the labelled amino acid. This antibody was used in a competitive solid phase assay against microsomal fraction from BNF-treated rats. As would be predicted, unlabelled 9/20/11 competed with the labelled

Table 1. Reactivity of MAbs with their homologous antigens determined by e.l.i.s.a.

MAb	Cytochrome P-450	A ₄₉₂ *
3/4/2	Rat form c	0.633 ± 0.057
12/2/3/2	Rat form c	0.720 ± 0.042
9/20/11	Rat form c	0.685 ± 0.038
105	Rabbit form 4	0.566 ± 0.054
106	Rabbit form 4	0.642 ± 0.061
1/4	Rat form b	0.851 ± 0.039
B3A3, NBS 1-48-5	Rat form c	< 0.05
B3A3, NBS 1-48-5	Rabbit form 4	< 0.05
B3A3, NBS 1-48-5	Rat form b	< 0.05
None	Rabbit form 4	< 0.05

* Values are means ± S.E.M. (n = 3) and have been corrected for background

Table 2. Reactivity of MAbs with hepatic microsomal fractions from various species determined by e.l.i.s.a

EC₅₀ is the concentration (mg/ml) of MAb producing 50% maximum response against the indicated preparation. Serial, 2-fold dilutions of the MAbs were prepared, starting at 0.5 mg/ml, and assayed by solid phase e.l.i.s.a.

Species	Inducer	MAb...	-log[EC ₅₀ value (mg/ml)]						
			B3A3	1/4	3/4/2	12/2/3/2	9/20/11	105	106
Wistar rat	None		< 1	< 1	1.20	< 1	< 1	< 1	< 1
	PB		< 1	3.22	1.33	< 1	< 1	< 1	< 1
	3-MC		< 1	< 1	2.40	3.40	4.40	< 1	< 1
New Zealand white rabbit	3-MC		< 1	< 1	1.52	< 1	< 1	4.05	3.80
Human	None		< 1	< 1	1.30	< 1	< 1	< 1	< 1

antibody for binding (Fig. 1). In this situation, the two preparations of the antibody are obviously binding to the same epitope. Similarly, 12/2/3/2 competed with labelled 9/20/11 for binding, indicating that these two antibodies bound to the same or very close epitopes. In contrast, neither the control antibody, NBS-1-48-5p26, nor 3/4/2 had any effect on the binding of labelled 9/20/11. In the case of NBS-1-48-5p26, this was presumably because this antibody did not react at all with the preparation of antigen. However, as shown in Table 1, MAb 3/4/2 did bind to rat cytochrome *P*-450 c, and presumably this was to a spatially distinct epitope from that with which MAbs 9/20/11 and 12/2/3/2 reacted.

The binding of the MAbs to microsomal fractions was investigated further by the technique of Western blotting. All of the anti-(rat cytochrome *P*-450) MAbs reacted with protein in the homologous microsomal preparation (Fig. 2). With 3/4/2 and 1/4 only one band was detected, with microsomal fraction from 3-MC and PB-treated rats respectively. The relative molecular masses of the two bands corresponded to those of rat cytochrome *P*-450 c (57000) and rat cytochrome *P*-450 b and/or e (52500) respectively. Both 9/20/11 and 12/2/3/2 recognized two proteins in microsomal fraction from 3-MC-treated rats, corresponding in relative molecular masses to rat forms c (57000) and d (54000). Blotting of these two MAbs against microsomal fraction from isosafrole-treated rats produced much more intense staining of the band with *M_r* 54000, corresponding to form d, than of the heavier band, most probably form c.

MAb 3/4/2 reacted with the heavier protein, form c, in microsomes from isosafrole-treated rats but was negative against form d in this preparation. A consistent observation with 3/4/2 in such fractions was the presence of a much lighter immunoreactive protein, *M_r* 48500.

MAbs 12/2/3/2, 9/20/11 and 1/4 did not produce any positive reaction with microsomal fraction from control animals. MAbs 12/2/3/2 and 9/20/11 were negative with microsomal fraction from PB-treated rat and MAb 1/4 was negative with microsomal fraction from 3-MC-treated rat. However, MAb 3/4/2 recognised a single protein (*M_r* 57000) in microsomal fraction from PB-treated rats and this differed in relative molecular mass from that protein recognised by MAb 1/4 in this preparation (Fig. 2).

MAbs 12/2/3/2, 9/20/11 and 1/4 were negative with microsomal fraction from 3-MC-treated rabbits, whereas MAb 3/4/2 recognised two proteins in this preparation, corresponding in relative molecular masses to rabbit forms 4 and 6, the reaction being stronger with form 6.

Fig. 1. Solid phase competition assay of binding of [¹⁴C]leucine labelled-9/20/11 to microsomal proteins from BNF-treated rat

Serial, 2-fold dilutions of MAbs 3/4/2 (■), 9/20/11 (●), 12/2/3/2 (□) and NBS 1-48-5 (▲) were added to a fixed amount of [¹⁴C] MAb 9/20/11 and the mixture was incubated in individual microtitre wells coated with microsomal fraction from BNF-treated rats. Binding was then determined as described in the text.

This was consistent with the reactivity of the MAbs with purified forms of rabbit cytochrome *P*-450 in e.l.i.s.a. Whereas MAb 3/4/2 reacted positively with both forms 4 and 6 (Table 3), the other three MAbs, 12/2/3/2, 9/20/11 and 1/4, were all negative against these two isoenzymes (results not shown).

MAbs 105 and 106, despite a strong positive reaction in e.l.i.s.a. with both hepatic cytochrome *P*-450 form 4 and hepatic microsomal fraction from 3-MC-treated rabbit, were negative in Western blotting with all of the microsomal preparations (results not shown). This was because these antibodies reacted with epitopes that were distorted during linearization with detergent (Table 3). Treatment of either microsomal fraction or purified cytochrome *P*-450 with 8 M-urea reduced the binding of MAbs 105 and 106 by 1000–10000-fold compared with that with the native antigen preparations. In marked contrast, urea treatment had virtually no effect on the binding of MAb 3/4/2 to either its purified antigen (rat

Fig. 2. Western blotting of microsomal fractions from rat, rabbit and human liver with MAbs 3/4/2, 12/2/3/2, 9/20/11 and 1/4

Microsomal proteins were separated by SDS/polyacrylamide-gel electrophoresis and then subjected to Western blotting with: (a) MAb 3/4/2, (b) MAb 12/2/3/2, (c) MAb 9/20/11 and (d) MAb 1/4, as described in the Materials and methods section. Hepatic microsomal fractions (20 μ g of protein/well) were as follows: lane 1, 3-MC-treated rat; lane 2, isosafrole-treated rat; lane 3, PB-treated rat; lane 4, control rat; lane 5, 3-MC-treated rabbit; lane 6, human. The relative molecular masses of the immunoreactive bands were determined by comparison of their relative mobilities with those of standard proteins as described in the text.

form c) or the homologous microsomal fraction. The reactivity of MAbs 105 and 106 was therefore investigated in e.l.i.s.a. with homogeneous preparations of cytochrome *P*-450. Both MAbs 105 and 106 reacted specifically with rabbit cytochrome *P*-450 form 4 (A_{492} in e.l.i.s.a., 0.566 ± 0.054 and 0.642 ± 0.061 , respectively) and were negative with rabbit cytochrome *P*-450 forms 2, 3b, 3c, and 6, and with rat cytochrome *P*-450 c ($A_{492} < 0.05$).

All of the antibodies were tested for reactivity with microsomal fractions from human liver, but only 3/4/2 produced any positive reaction, recognising a single band at M_r 55000 (Fig. 2).

To determine whether 3/4/2, 12/2/3/2 and 9/20/11

did indeed all react with the same protein, with relative molecular mass corresponding to that of rat form c, in the microsomal fraction from BNF-treated animals, immunoadsorption studies were performed with 3/4/2 and 12/2/3/2 immobilized on CNBr-activated Sepharose. Following incubation of solubilized microsomal fraction from BNF-treated rat liver, the supernatants were subjected to Western blotting with MAbs 3/4/2, 12/2/3/2 and 9/20/11 alone and in combination. When the entire microsomal fraction was blotted, 9/20/11 and 12/2/3/2 reacted with two proteins strongly and with almost equal intensity (Fig. 3a). MAb 3/4/2 reacted with only one protein, with the same relative molecular mass

Table 3. Effect of urea treatment on the reactivity of MAbs determined by e.l.i.s.a.

EC₅₀ is the concentration (mg/ml) of MAb producing 50% maximum response against the indicated antigen preparation. Serial, 2-fold dilutions of the MAbs were prepared, starting at 0.5 mg/ml, and assayed by solid phase e.l.i.s.a. 'Linearized' antigen preparations were treated by boiling in 8 M-urea prior to coating the microtitre plates for e.l.i.s.a.

Antigen preparation	MAb	-log [EC ₅₀ value (mg/ml)]	
		Native protein	Linearized protein
Rabbit microsomes (3-MC-treated)	105	4.05	< 0.3
Rabbit microsomes (3-MC-treated)	106	3.80	1.05
Rat microsomes (3-MC-treated)	3/4/2	2.49	2.40
Rat form c	3/4/2	3.80	3.96
Rabbit form 4	3/4/2	2.60	1.70
Rabbit form 6	3/4/2	2.30	1.57

Fig. 3. Western blotting of supernatants following immunoadsorption of solubilized microsomal proteins from BNF-treated rats by immobilized MAbs

MAbs were covalently coupled to CNBr-activated Sepharose 4B as described in the text. Microsomal fraction from BNF-treated rats was solubilized with cholate and the solubilized proteins were then incubated with the immobilized MAbs. The unbound material remaining in the supernatant was subjected to SDS/polyacrylamide-gel electrophoresis followed by Western blotting. (a) Microsomal fraction prior to adsorption; (b) supernatant remaining after adsorption by immobilized 3/4/2; (c) supernatant remaining after adsorption by immobilized 12/2/3/2. Blotting was performed as follows: lane 1, 12/2/3/2; lane 2, 9/20/11; lane 3, 3/4/2; lane 4, 3/4/2 + 12/2/3/2.

(57000) as the heavier of the two proteins recognised by 12/2/3/2 and 9/20/11 (Fig. 3a). Following adsorption by immobilized 3/4/2 the intensity of staining of the heavier protein by both 12/2/3/2 and 9/20/11 was substantially reduced, although the protein of smaller relative molecular mass showed a similar intensity of staining to that prior to immunoadsorption. Blotting with 3/4/2 after immunoadsorption with 3/4/2 produced similar

intensity of staining of the heavier band as with 12/2/3/2 or with 9/20/11 (Fig. 3b).

Blotting with the combination of 12/2/3/2 and 3/4/2 produced almost equal intensity of staining of the two bands, presumably because these two MAbs bound additively to the protein of greater molecular mass.

Immunoadsorption by 12/2/3/2 reduced the intensity of staining of both proteins equally, in the presence of

12/2/3/2, and of the protein recognised by 3/4/2 alone. The combination of 3/4/2 and 12/2/3/2 produced more intense staining of the protein of greater relative molecular mass in this preparation than that of smaller relative molecular mass (Fig. 3c).

The conclusion from the foregoing experiments was that MAbs 9/20/11, 12/2/3/2 and 3/4/2 all bound to epitopes on the same isoenzyme of cytochrome *P*-450, almost certainly rat form c, and that the epitope to which 3/4/2 bound was different to that (or those) to which the other two MAbs bound. This was further investigated by preparing epitope maps of homogeneous form c with the three MAbs. Cytochrome *P*-450 form c was subjected to limited proteolysis and the peptide fragments produced were subjected to Western blotting.

The most obvious differences were apparent after digestion with chymotrypsin (Fig. 4). With MAb 3/4/2, the most intense staining band was of very low M_r , whereas with 9/20/11 the most intense staining band was of intermediate M_r and with 12/2/3/2 it was the starting material. Following papain digestion, 3/4/2 reacted with several peptides, the most intensely stained of which was of intermediate M_r . The epitope(s) with which 9/20/11 and 12/2/3/2 reacted was more sensitive to papain digestion, as indicated by very low intensity of staining. Digestion with protease V8 produced a complex pattern of staining with 3/4/2. The most intense staining band, which showed almost equal intensity of staining with the starting material, was a peptide of intermediate M_r . Although a peptide of similar M_r could be detected with

12/2/3/2, its staining intensity was very different from that of the starting material. With 9/20/11, some of the peptides of intermediate M_r detected were of different M_r from those detected with 3/4/2. After digestion with chymotrypsin, almost no immunoreactive peptides could be detected with 9/20/11, although a single faint band was apparent of intermediate M_r (Fig. 4). Similarly, very few bands could be detected with 12/2/3/2, but the most intense staining of these was of different M_r from that detected with 9/20/11. MAb 9/20/11 reacted with several peptides after digestion of the cytochrome *P*-450 with papain, the most intense staining of which was of intermediate M_r . In contrast, 12/2/3/2 reacted with only a few such peptides, the most intense staining of which was the starting material. Following treatment with protease V8, the staining intensity and relative molecular masses of the peptides recognised by 9/20/11 were different from those of the peptides recognised by 12/2/3/2.

Thus, it is clear from the epitope maps produced (Fig. 4) that 3/4/2 bound to a very different epitope from that to which 12/2/3/2 and 9/20/11 bound. In addition, although these two antibodies produced similar epitope maps, they were far from identical, with any of the proteolytic enzymes used.

DISCUSSION

Structural similarities amongst the hydrocarbon-inducible forms of cytochrome *P*-450 in different species have become apparent within the past 2 or 3 years, as amino acid and nucleotide sequencing has been performed on the various isoenzymes. Thus rat form d shows considerable homology with rabbit form 4 (Fujita *et al.*, 1984), and similarly rat forms c and d share considerable homology (Sogawa *et al.*, 1984). The results of the present study provide further insight into these relationships.

MAb 3/4/2 reacts with a single protein in microsomal fraction from BNF-treated rats but with two proteins in microsomal fraction from 3-MC-treated rabbits. In contrast, 12/2/3/2 and 9/20/11 react only with microsomes from BNF-treated rats, but in both cases they react with two proteins. The protein of greater relative molecular mass is similar in relative mass to that recognised by 3/4/2. All three MAbs react with a homogeneous preparation of rat cytochrome *P*-450 c (cytochrome *P*-448) and competitive solid phase assay with [14 C]leucine-labelled 9/20/11 confirmed that MAbs 9/20/11 and 12/2/3/2 bind to the same protein, and indeed to the same or very similar epitopes. Reactivity in solid phase e.l.i.s.a. and competitive solid phase assay demonstrated that 3/4/2 binds to an epitope different from that to which the other two MAbs bind.

Immunoabsorption studies confirmed that all three MAbs bind to the same isoenzyme, most probably rat form c. MAbs 9/20/11 and 12/2/3/2 bind to a second protein, which is most probably rat form d, particularly as intensity of reaction with this protein is increased by prior treatment of the rats with isosafrole, a known inducer of this isoenzyme (Ryan *et al.*, 1980). By all the criteria investigated, 9/20/11 and 12/2/3/2 are identical in reactivity. In addition, these two MAbs both inhibit mono-oxygenase activity of microsomal fraction from BNF-treated rats, whereas 3/4/2 is non-inhibitory (Boobis *et al.*, 1985a,b). The technique of epitope mapping has proved extremely powerful in distinguishing

Fig. 4. Limited proteolysis of rat cytochrome *P*-450 c followed by Western blotting

Homogeneous rat form c was subjected to limited proteolysis as described in the text and the peptides thus produced were separated by SDS/polyacrylamide-gel electrophoresis followed by Western blotting with: (a) MAb 3/4/2, (b) MAb 9/20/11, (c) MAb 12/2/3/2. Lane 1, digestion by papain; lane 2, digestion by chymotrypsin; lane 3, digestion by protease V8; lane 4, undigested cytochrome *P*-450.

between closely related antibodies. The epitope maps obtained with 3/4/2 are different from those obtained with the other two MABs, thus confirming the results of the other techniques. However, although the maps obtained with 12/2/3/2 and 9/20/11 are similar, they are not identical. Thus, these two antibodies must either bind to different epitopes that are very close together, or they bind to overlapping epitopes. Recently Song *et al.* (1985) identified three groups of MABs against 3-MC-inducible cytochrome *P*-450 from the rat, based on competition for their epitopes. However, within a single group of MABs there were subtle differences in their reactivity profiles, suggesting that, as in the present study, competing MABs can react with epitopes that are not identical but which are very close to, or even overlap, each other. Further mapping studies are necessary with the MABs described here to determine which of these possibilities is correct.

Although MABs 12/2/3/2 and 9/20/11 both react with two proteins in microsomal fraction from BNF-treated rats, most probably forms c and d, they do not recognise any protein in microsomal fraction from 3-MC-treated rabbits. These antibodies are thus species-specific but not isoenzyme-specific. In contrast, MAB 3/4/2 reacts with only a single protein in microsomal fraction from BNF-treated rats, most probably form c, but with two proteins in microsomal fraction from 3-MC-treated rabbits, most probably forms 4 and 6. This antibody is thus not species-specific, but is isoenzyme-specific in the rat, though not in the rabbit. However, the relative reactivity of MAB 3/4/2 with purified isoenzymes (Table 3), together with the selective effect that urea treatment has on binding of MAB 3/4/2 to the heterologous antigens (rabbit forms 4 and 6), suggests that there may be subtle differences between the epitope on form c and that on the rabbit isoenzymes. MABs raised to rabbit form 4 react with this isoenzyme in microsomal fraction from 3-MC-treated rabbit, both MAB 105 and 106 showing species- and isoenzyme-specificity. These relationships are summarized in Fig. 5. However, 106 inhibits the *N*-hydroxylation of 2-acetylaminofluorene whereas 105 does not (Boobis *et al.*, 1985a). Thus, these two antibodies must recognise different epitopes.

MAB 3/4/2 recognizes an epitope common to rat form c and rabbit forms 4 and 6 whereas 12/2/3/2 and 9/20/11 recognise epitopes common to rat forms c and d. Both 1/4 and 3/4/2 recognise a single protein in microsomal fraction from PB-treated rat, but the protein recognised by the latter antibody differs in relative molecular mass from that recognised by 1/4, which corresponds to rat form b and/or e, and also from forms a, d, f, g, h, i and j, all of which have relative molecular masses smaller than that of form c (Levin *et al.*, 1984; Ryan *et al.*, 1985).

The form of cytochrome *P*-450 recognised by 3/4/2 in microsomal fraction from PB-treated rats is inducible by PB, since no reaction was observed with microsomal fraction from control rats, analysed at the same protein concentration as that used for the PB microsomes. Neither form c nor d is inducible by PB (Guengerich *et al.*, 1982; Thomas *et al.*, 1983). MABs 12/2/3/2 and 9/20/11, both of which recognise rat forms c and d, do not react with the protein recognised by 3/4/2 in microsomes from PB-treated rats. In addition, Western blotting of proteolytic digests of microsomal fractions from PB- and 3-MC-treated rats revealed major differences in the epitope maps obtained with MAB 3/4/2

Fig. 5. Diagram illustrating the relationships between different forms of cytochrome *P*-450 determined with six monoclonal antibodies

Each circle represents a different monoclonal antibody, binding to a separate epitope from the other antibodies studied. Within each circle are shown the forms of cytochrome *P*-450 possessing the epitope with which the antibody, represented by that circle, reacts. The forms of cytochrome *P*-450 are designated as follows: 4, 6, rabbit liver forms 4 and 6 respectively; b, c, d, e, rat liver forms b, c, d, e, respectively; PB?, human?, forms of cytochrome *P*-450 in liver from PB-treated rat and from human liver respectively, as yet unidentified.

(D. Sesardic, D. S. Davies & A. R. Boobis, unpublished work). Thus, 3/4/2 must recognise a PB-inducible form of cytochrome *P*-450 that is not form a, b, c, d, e, f, g, h, i or j. If this is indeed an isoenzyme of cytochrome *P*-450 then it must represent an as yet unpurified form.

MAB 3/4/2 reacts with a form of cytochrome *P*-450 in the microsomal fraction from human liver, presumably containing the same epitope as that recognised in rat form c, rabbit forms 4 and 6 and an unidentified, PB-inducible form in rat liver. Whether the form recognised in human liver is analogous to the hydrocarbon-inducible form in rat liver, rat form c, has yet to be determined. But, as has been reported by others (e.g. Fujino *et al.*, 1982), MABs raised against rodent cytochromes *P*-450 should prove extremely powerful tools in analysing the structure and function of the human isoenzymes.

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