An anti-peptide antibody targeted to a specific region of rat cytochrome *P*-450IA2 inhibits enzyme activity

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An anti-peptide antibody has been produced which binds to and specifically inhibits the activity of cytochrome *P*-450IA2 in rat hepatic microsomes. This was achieved by raising an antibody against a synthetic peptide (Ser-Glu-Asn-Tyr-Lys-Asp-Asn), the sequence of which occurs in cytochrome *P*-450IA2 at positions 290–296. The selection of this region of cytochrome *P*-450IA2 was based on several criteria, including prediction of surface and loop areas, identification of variable regions between cytochromes *P*-450IA2 and *P*-450IA1, and consideration of a site on cytochrome *P*-450IA1 where chemical modification has been shown to cause substantial enzyme inactivation. The specificity of antibody binding was determined by enzyme-linked immunosorbent assay and by immunoblotting using hepatic microsomal preparations and purified cytochrome *P*-450 isoenzymes. This showed that the antibody binds specifically to rat and mouse cytochrome *P*-450IA2 and to no other cytochrome *P*-450, as was predicted from the amino acid sequences of the peptide and the cytochromes *P*-450. The effect of the antibody upon enzyme activity was studied in hepatic microsomes from rats treated with 3-methylcholanthrene. The antibody was shown to inhibit specifically the activity of reactions catalysed by cytochrome *P*-450IA2 (phenacetin *O*-de-ethylase and 2-acetylaminofluorene activation), but had no effect on aryl hydrocarbon hydroxylase activity, which is catalysed by cytochrome *P*-450IA1, or on aflatoxin B1 activation.

INTRODUCTION

Cytochromes *P*-450 comprise a superfamily of haemoproteins [1] which metabolize a wide range of endogenous and exogenous compounds [2]. Some of the sub-families of isoenzymes are subject to differential induction by such substances as polycyclic aromatic hydrocarbons, phenobarbitone, alcohol, hypolipidaemic agents and steroids [3,4]. Although some reactions are catalysed almost exclusively by one isoenzyme, others are catalysed equally well by a number of different isoenzymes. This overlap makes the determination of isoenzyme specificity extremely difficult. It is not sufficient to demonstrate that a purified isoenzyme will catalyse a given reaction when reconstituted. Rather, it is necessary to determine the contribution of that isoenzyme to the reaction of interest in the presence of the full complement of isoenzymes. Undoubtedly, one of the most effective means of accomplishing this is immunoinhibition by antibodies against specific isoenzymes.

In addition to their use in the study of the substrate specificities of cytochrome *P*-450 isoenzymes, antibodies have been used extensively for the identification, location and quantification of these proteins [5,6]. However, because of the antigenic similarity of related isoenzymes [7], an antiserum raised against one form will frequently cross-react with other isoenzymes, even if a highly purified preparation is used for immunization. Thus, although polyclonal antibodies are frequently inhibitory, they are not necessarily specific. Monoclonal antibodies

are potentially more specific, but their binding is still not directed to a specific site. Consequently, the actual epitope with which they react is usually unknown, so that the extent of cross-reaction with other forms is still not predictable and must be determined experimentally. In addition, the production of monoclonal antibodies that are inhibitory is not assured. Targeting antibodies to a specific binding site on the haemoprotein can be achieved by the use of anti-peptide antibodies provided that adequate sequence information is available and that there is sufficient diversity between the isoenzymes to ensure specific binding. In order to cause inhibition of enzyme activity, these antibodies must be directed to areas of functional importance on the surface of cytochrome *P*-450.

The primary structures of many of the isoenzymes of cytochrome *P*-450, both constitutive and inducible, have now been deduced for the rat, mouse, rabbit and human [1,8]. We [9] and others [10] have shown previously that this information can be used successfully to produce anti-peptide antibodies to defined sites on cytochromes *P*-450. The specificity of binding of such antibodies is determined solely by the primary structure of the cytochrome *P*-450 region selected for use as the immunogen. The selection of suitable regions is of particular importance for discrimination between the isoenzymes within a sub-family, which are closely related antigenically [11].

In the present study, we have concentrated on cytochrome *P*-450IA2, which is inducible in liver by

Abbreviations used: Fmoc, fluoroenylmethoxycarbonyl; KLH, keyhole-limpet haemocyanin; 2-AAF, 2-acetylaminofluorene; Me₂SO, dimethyl sulphoxide; PCN, pregnenolone 16 α -carbonitrile; CFB, clofibrate; PB, sodium phenobarbitone; MC, 3-methylcholanthrene; PBS, phosphate-buffered saline (composition given in the text).

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polycyclic aromatic hydrocarbons both in laboratory animals and in man. This isoenzyme is of toxicological importance as it is involved in the activation of a number of carcinogenic aromatic amines. The choice of the region at residues 290–296 of rat cytochrome *P*-450IA2 as a target for an anti-peptide antibody was based on a number of criteria designed to direct such antibodies to a surface region of rat cytochrome *P*-450IA2 and to inhibit specifically the activity of this isoenzyme. From a consideration of the available amino acid sequences of cytochromes *P*-450 [8], it was predicted that the anti-peptide antibody would bind to a single region on rat and mouse cytochromes *P*-450IA2.

The peptide corresponding to this region was synthesized and an anti-peptide antibody was produced. This antibody has been characterized by e.l.i.s.a., immunoblotting and immunocytochemistry, and its effects on microsomal cytochrome *P*-450-catalysed mono-oxygenase activities have been determined. These properties have been compared with those of a polyclonal antibody raised against purified rat cytochrome *P*-450IA2 (anti-protein antibody).

EXPERIMENTAL

Materials

N- α -9-Fluorenylmethoxycarbonyl (Fmoc)-amino acids were purchased from Millipore (London, U.K.) and *t*-butylthiocysteine was from Novabiochem AG (Läufelfingen, Switzerland). All gel-filtration and ion-exchange resins were from Pharmacia (Milton Keynes, U.K.). Hydroxyapatite was obtained from Bio-Rad Laboratories (Watford, Herts., U.K.). All SDS/polyacrylamide-gel electrophoresis reagents were from National Diagnostics (Aylesbury, Bucks., U.K.), except for pre-stained molecular mass standards which were from Sigma (Poole, Dorset, U.K.). Keyhole-limpet haemocyanin (KLH) was from Cambridge Bioscience (Cambridge, U.K.). Polystyrene 96-well microtitre plates were from Dynatech Laboratories (Billinghurst, W. Sussex, U.K.), and conjugates of immunoglobulins with horseradish peroxidase were from ICN Biomedicals (High Wycombe, Bucks., U.K.). Acetonitrile (grade S) was from Rathburn Chemicals (Walkerburn, Scotland). Benzo[*a*]pyrene, 2-acetylaminofluorene (2-AAF) and Bromocresol Purple were from Aldrich (Gillingham, Dorset, U.K.). Dimethyl sulphoxide (Me₂SO) (> 99.5% pure) was obtained from Fluka Chemicals (Glossop, Derbyshire, U.K.). Pregnenolone 16 α -carbonitrile (PCN) and clofibrate (CFB) were generously given by G. D. Searle (Skokie, IL, U.S.A.) and ICI Pharmaceuticals (Macclesfield, Cheshire, U.K.) respectively. Sodium phenobarbitone (PB) was obtained from Macarthy's (Romford, Essex, U.K.). Tissue culture plates were purchased from Becton Dickinson (Cowley, Oxon, U.K.), and sterile 0.45 μ m filters were from Flow Laboratories (Rickmansworth, Herts, U.K.). Nutrient broth No. 2 was obtained from Oxoid (Basingstoke, Hampshire, U.K.). *Salmonella typhimurium*, strain TA98, was generously provided by Professor Bruce Ames (University of California, Berkeley, CA, U.S.A.) and was maintained in accordance with published recommendations [12]. All other chemicals were purchased from Sigma or from BDH (Dagenham, Essex, U.K.) and were of analytical grade or the best equivalent.

Synthesis of peptides and coupling to carrier protein

Peptides were synthesized manually by a solid-phase method using a polyamide support and Fmoc amino acids as described previously [9], except that the peptides were purified by gel filtration on a Sephadex G-25 column (45 cm $\times$ 2.2 cm) in 1 M-acetic acid to give products of > 90% purity, as determined by h.p.l.c., using a C₁₈ μ Bondapak column (Millipore, Harrow, Middlesex, U.K.), with a gradient of 0–25% (v/v) acetonitrile in the presence of 0.1% (v/v) trifluoroacetic acid over 20 min at a flow rate of 1 ml/min, with constant monitoring of the eluent at 210 nm. The peptides had the correct composition by amino acid analysis, and the correct molecular masses as determined by mass spectrometry ($M+H^+$: m/z = 869 and 743 for the rat and human peptides respectively). To enable conjugation of the peptides to proteins in a specific orientation, these peptides were also synthesized with a cysteine residue at the *N*-terminus. KLH was derivatized with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester [13] and then added to the peptides at a ratio of 2.3 mg/ μ mol of cysteinyl peptide, as determined by the free thiol content of the preparation [14]. The pH of the mixtures was adjusted to 7.3, and they were then stirred for 2 h and dialysed against phosphate-buffered saline (PBS; 1.5 mM-KH₂PO₄, 8.1 mM-Na₂HPO₄, 2.7 mM-KCl, 137 mM-NaCl, pH 7.5). The degree of conjugation was assessed by amino acid analysis and was 0.24 μ mol/mg of KLH for the rat peptide and 0.13 μ mol/mg for the human peptide. A 'null' conjugate, in which free cysteine was substituted for peptide, was produced in the same way.

Immunization

Male New Zealand White rabbits (3 kg body weight, from Froxfield Farms Ltd., Petersfield, Hampshire, U.K.) were immunized by repeated injections of 200 μ g of peptide conjugate, or 100 μ g of rat cytochrome *P*-450IA2 purified as described below, in Freund's adjuvant in a total volume of 1 ml. The first injection was administered subcutaneously, with complete adjuvant, and subsequent injections were with incomplete adjuvant, subcutaneously 14 days later and intraperitoneally after 21 days. Blood samples were taken from a marginal ear vein on day 28. Control (pre-immune) blood samples were collected from untreated rabbits.

Purification of antibodies

The rat peptide was coupled to CNBr-activated Sepharose 4B [15]. Incorporation of peptide, determined by amino acid analysis, was 0.93 μ mol/ml of Sepharose. Affinity purification of anti-peptide antibodies was by the method of Edwards and co-workers [16].

Immunological analyses

The method for e.l.i.s.a. has been described in detail previously [9]. In brief, the e.l.i.s.a. plates were coated with 100 μ l of microsomal fraction (100 μ g/ml), purified cytochrome *P*-450 (5 μ g/ml) or peptide conjugate (5 μ g/ml) diluted in PBS. It was found that in this particular case, free peptide could not be used as the coating antigen as this did not bind to the e.l.i.s.a. plate (microtitre plates from several manufacturers were tested, with the same result). Where indicated, microsomes were treated with either 8 M-urea or 0.05% (w/v) SDS in PBS. Urea-treated microsomes were applied directly to the

microtitre plate. Excess SDS was removed from treated microsomes by gel filtration (Sephadex G-25) using a 45 cm $\times$ 2.2 cm column with detection at 280 nm. The void volume, containing the solubilized microsomal protein, was diluted appropriately in PBS before application to the e.l.i.s.a. plate. The binding titre was that concentration of antibody that gave half-maximal binding to an antigen as determined by e.l.i.s.a.

Immunoblot analysis was carried out essentially as described by Towbin and co-workers [17] incorporating the modifications described previously [9].

Animals, and their treatment with inducing compounds

Male Wistar rats (200–250 g) and juvenile rats (100–150 g) were obtained from Harlan Olac (Bicester, Oxon., U.K.) and male New Zealand White rabbits (3 kg) were from Froxfield Farms Ltd. Male Balb/c mice (6–10 weeks) and Dunkin Hartley guinea pigs (250–300 g) were from A. Tuck & Son (Battlebridge, Essex, U.K.), and male Golden Syrian hamsters (90–110 g) were supplied by Belgrave Trading Ltd (Lambourn, Berks., U.K.). Groups of animals were left untreated or were administered inducing compounds, essentially as described by Rich *et al.* [18]. Animals were killed humanely in accordance with approved Home Office procedures appropriate to the species, the livers were rapidly removed and microsomal fractions were prepared as described previously [19].

Preparation of human microsomes

Human liver samples were obtained from the human tissue bank at the Royal Postgraduate Medical School [19]. Local Ethics Committee approval and Coroner's permission were obtained for their use in these studies. The preparation of the microsomal fraction was as described previously [19].

Purification of rat cytochromes *P*-450IA1 and *P*-450IA2

Cytochromes *P*-450IA1 and *P*-450IA2 were purified from hepatic microsomal fractions from rats treated with 3-methylcholanthrene (MC). The methods used for purification have been described previously [9]. Both isoenzyme preparations were 85–90 % pure as determined by SDS/polyacrylamide-gel electrophoresis, and each was free from contamination by the other form, as judged both by electrophoretic and immunological methods.

Measurement of cytochrome *P*-450-dependent mono-oxygenase activities

Mutagenicity assay. Bacterial mutagenicity was used as an end point to detect the formation of short-lived reactive intermediates produced from 2-AAF and aflatoxin B1. Cytochrome *P*-450-dependent mono-oxygenase activity has been shown to be rate-limiting in the mutagenicity of the compounds used. Mutagenic activation was assessed using an adaptation of the microtitre fluctuation test described by Venitt & Bosworth [20]. For each plate, 0.4 ml of liver microsomal fraction [0.125 mg of protein/ml in 0.25 M-potassium phosphate buffer, pH 7.25, containing 30 % (v/v) glycerol] was mixed with 0.5 ml of serum and then incubated for 5 min at 37 °C. The serum consisted of either anti-peptide or anti-protein serum diluted with pre-immune serum to give a constant amount of protein. An NADPH-generating system, consisting of 4 μ mol of

NADP⁺, 5 μ mol of D-glucose 6-phosphate, 6 units of glucose-6-phosphate dehydrogenase and 8 μ mol of MgCl₂ in 0.1 ml of water was then added, and the mixture was sterilized by passage through a 0.45 μ m filter. Mutagens, 2-AAF (0–2.5 μ g) or aflatoxin B1 (0–5.0 μ g), in 10 μ l of Me₂SO, and 25 μ l of a 10 h culture of *S. typhimurium* TA98 in 2.5 % (w/v) Oxoid No. 2 nutrient broth [containing (2.5–5.0) $\times 10^7$ bacteria] were added and the samples were then incubated for 20 min at 37 °C in a shaking water bath. To each reaction mixture was then added 4 ml of double-strength Vogel–Bonner medium [21] supplemented with 0.8 % (w/v) glucose, 20 μ g of *d*-biotin/ml and 1.5 μ g of L-histidine/ml, and dispensed at 50 μ l/well into 96-well tissue culture plates. The plates were incubated at 37 °C for 24 h, after which 250 μ l of selection/indicator medium [0.8 % (w/v) glucose and 5 μ g of Bromocresol Purple/ml] was added to each well and the plates were returned to the incubator. After a further 24 h of incubation, wells were scored for those containing growing revertant bacteria, identified by the presence of yellow acidic medium.

Aryl hydrocarbon hydroxylase activity. This activity was determined by using the method of Atlas *et al.* [22], except that the hepatic microsomal fraction (5 μ g of protein), from MC-treated rats, was mixed with antiserum for 5 min at 37 °C before addition to the reaction mixture.

Phenacetin *O*-de-ethylase activity. This activity of hepatic microsomal fraction from MC-treated rats was determined by the method of Murray & Boobis [23], using a final phenacetin concentration of 4 μ M. The hepatic microsomal fraction (30 μ g of protein) was incubated for 30 min at 4 °C with various amounts of antiserum in a total volume of 0.2 ml. The mixtures were then warmed to 37 °C, and the reaction was initiated by addition of substrate.

RESULTS

The amino acid sequence of rat cytochrome *P*-450IA2 [24] was examined and a region was selected which was unique to this isoenzyme, based on a comparison with the sequence of cytochrome *P*-450IA1 and indeed with all of the cytochrome *P*-450 sequences available [8], and which was likely to be antigenic in the native protein. This was performed using algorithms to predict surface and loop regions, as these have been shown to be important factors in determining regional antigenicity [25–27]. Surface regions were predicted using the free energy of transfer (from the inside to the surface of a protein) values for each amino acid residue provided by Janin [28]. The mean surface index was calculated at each sequence position (*i*), for residues *i* to *i* + 4. The 50 five-residue sections with the highest mean indices were combined to form 16 non-overlapping regions. The result of this analysis is shown in Fig. 1. Prediction of loop regions was performed in a similar way, using the scale of Leszczynski & Rose [29], which lists the frequency of occurrence of each amino acid residue in protein loop regions. The average loop index was then calculated as above and the 50 sections with the highest values were combined to yield 21 non-overlapping potential loop regions (Fig. 1). Eight regions were common to both predictive methods, and these regions were analysed for

Fig. 1. Analysis of the primary structure of rat cytochrome *P-450IA2*

The results of the algorithms applied to this sequence show the regions with the most likelihood of occurrence in (a) surface regions and (b) loop regions. Those regions common to both are shown (c) and the number of residues different between cytochromes *P-450IA2* and *P-450IA1* within each of these areas is indicated.

similarity between rat cytochrome *P-450IA2* and the closely related member of the same sub-family, cytochrome *P-450IA1* [30]. The region with the least similarity, containing 9 mismatches, was 289–297. Ser-290 in this region of cytochrome *P-450IA2* corresponds to a cysteine in cytochrome *P-450IA1* (Table 1). Modification of this residue by 2-bromo-4'-nitroacetophenone has been shown to result in the loss of more than 80 % of activity of the isoenzyme [36], and thus suggests a functional role for this region in the native protein. The peptide chosen for synthesis consisted of residues 290–296 of rat cytochrome *P-450IA2* (Table 1), these being the seven central residues of the region indicated above. The sequence Ser-Glu-Asn-Tyr-Lys-Asp-Asn is to our knowledge unique to this isoenzyme in the rat.

A second 7-residue peptide (Ser-Lys-Lys-Gly-Pro-Arg-Ala) which occurs in the corresponding region (291–297) in the human cytochrome *P-450IA2* sequence [33] (Table 1) was also synthesized.

Antiserum from a rabbit immunized with the rat peptide conjugated to KLH bound to the immunizing conjugate in e.i.s.a. (Table 2). In the unpurified form, this anti-peptide serum bound almost equally well to the null conjugate as to the immunizing conjugate (1:30000 compared with 1:10000). However, following its

Table 2. Binding titres of anti-peptide and anti-protein antibodies in e.i.s.a.

The binding titre of the antibodies to a series of antigens bound to microtitre plates was determined. Microsomes prepared from rats treated with MC were applied in PBS, 8 M-urea in PBS or 0.05 % (w/v) SDS in PBS, prepared as described in the Experimental section. Peptides, rat cytochrome *P-450IA2* sequence 290–296 and human cytochrome *P-450IA2* sequence 291–297 were coupled to KLH and the binding titre was compared with that for a null conjugate which lacked any peptide. The serum titres shown are those dilutions that gave half-maximal binding. For the purified anti-peptide antibody, the titre is the quantity of antibody that gave half of the maximum binding. n.d., not determined.

Antigen	Binding titre		
	Anti-protein antibody	Anti-peptide antibody	
		Serum	Purified ($\mu\text{g/ml}$)
Native microsomes	1:2200	1:100	3.0
Microsomes + urea	1:800	1:100	3.0
Microsomes + SDS	1:1000	1:100	3.0
Cytochrome <i>P-450IA2</i>	1:1900	1:100	3.0
Cytochrome <i>P-450IA1</i>	1:2200	< 1:30	> 30.0
KLH-Cys-rat peptide	< 1:30	1:30000	0.1
KLH-Cys-human peptide	n.d.	n.d.	10.0
KLH-Cys	< 1:30	1:10000	10.0

purification by affinity chromatography, the anti-peptide antibody showed a much greater binding titre (0.1 $\mu\text{g/ml}$) for the immunogen than for the null conjugate (10 $\mu\text{g/ml}$) (Table 2). Anti-(cytochrome *P-450IA2*) antiserum (anti-protein antibody) did not bind significantly to either of these conjugates.

The purified anti-peptide antibody bound to hepatic microsomes from MC-treated rats and to purified rat

Table 1. Aligned sequences of cytochromes *P-450IA2* and *P-450IA1* from rat, mouse, rabbit and human in the region of the sequence of rat cytochrome *P-450IA2* chosen for synthesis

The amino acid sequences, except for that of rabbit cytochrome *P-450IA2*, are predicted from their cDNA sequences and are numbered from the *N*-terminal methionine.

Gene subfamily	Species	Sequence	Position	Source
<i>P-450IA2</i>	Rat	-Ser-Glu-----Asn-Tyr-Lys-Asp-Asn-	290–296	[24]
	Mouse	-Ser-Glu-----Asn-Tyr-Lys-Asp-Asn-	290–296	[31]
	Rabbit	-Ser-Glu----Lys-Asn-Ser-Lys-Ala-Asn-	290–297	[32]
	Human	-Ser-Lys-----Lys-Gly-Pro-Arg-Ala-Ser-	291–298	[33]
<i>P-450IA1</i>	Rat	-Cys-Gln-Asp-Arg-Arg-Leu-Asp-Glu-Asn-	293–301	[30]
	Mouse	-Cys-Gln-Asp-Arg-Lys-Leu-Asp-Glu-Asn-	290–298	[31]
	Rabbit	-Tyr-Arg-Val-Asp-Arg-Leu-Asp-Glu-Asn-	293–301	[34]
	Human	-Cys-Gln-Glu-Lys-Gln-Leu-Asp-Glu-Asn-	289–297	[35]

Fig. 2. Relative binding of the anti-peptide antibody to hepatic microsomes from rats treated previously with a variety of inducing compounds

Microtitre plates were coated with microsomal preparations from untreated rats (○) and those treated with MC (●), PB (■), PCN (□), CFB (▲) or acetone (△) and compared with bovine serum albumin (◆). To each group a series of dilutions of the anti-peptide antibody was added and the relative binding was determined as described in the text.

cytochrome *P*-450IA2 with equal binding titres (3 μg/ml), but did not bind to rat cytochrome *P*-450IA1 (> 30 μg/ml). Further, this antibody bound equally well to native, urea-treated and SDS-treated microsomes (Table 2). Binding titres of the anti-peptide antibody to microsomes from variously treated rats was in the order MC ≫ untreated = acetone > CFB > PB > PCN (Fig. 2). This is consistent with the known effects of these inducers on the specific content of cytochrome *P*-450IA2 [37]. In the other species examined, the anti-peptide antibody bound to MC-induced and untreated mouse liver microsomes with binding titres of 2 μg/ml and 20 μg/ml respectively, but not to liver microsomes from untreated or MC-treated rabbit, hamster or guinea pig. No binding to human liver microsomes was observed (binding titre > 30 μg/ml), nor was there any binding to the human cytochrome *P*-450IA2 peptide (binding titre > 30 μg/ml).

Immunoblotting of microsomes prepared from the livers of MC-treated rats with the anti-peptide antibody showed binding to a single protein with the same apparent molecular mass (54 kDa) as purified rat cytochrome *P*-450IA2. This is in contrast with the antibody raised against purified cytochrome *P*-450IA2, which bound to both of the hydrocarbon-inducible isoenzymes, cytochromes *P*-450IA1 and *P*-450IA2, in microsomes prepared from MC-treated rats (Fig. 3). The anti-peptide antibody showed no binding to hepatic microsomal preparations from untreated rats, nor from those rats treated with PB, CFB, PCN or acetone. In the other

Fig. 3. Comparison of the results obtained with anti-peptide antiserum (lane a) and the anti-protein antiserum (lane b) in immunoblotting of liver microsomes obtained from rats treated with MC

Fig. 4. Inhibition of microsomal phenacetin *O*-de-ethylase activity by antisera

Liver microsomes from MC-treated rats were incubated for 30 min at 4 °C with a series of volumes of either anti-peptide (●) or anti-protein (○) serum. The incubation mixture was then warmed to 37 °C and the reaction was started by addition of phenacetin and incubated for 5 min. The activities shown are expressed as percentages of that measured in control incubations containing an equal volume of pre-immune serum.

species examined, only mouse cytochrome *P*-450IA2 was recognized by the anti-peptide antibody, there being no binding to microsomes prepared from the livers of MC-treated rabbit, hamster or guinea pig. Nor was there any binding to any of five different human liver microsomal samples. The e.l.i.s.a. and immunoblotting results are consistent with the known primary structures of

cytochromes *P*-450IA1 and *P*-450IA2 in this region (Table 1).

The effect of the anti-peptide antibody upon cytochrome *P*-450-dependent mono-oxygenase activities of liver microsomes from MC-treated rats was determined, using various substrates, and compared with that of the anti-protein antibody. Both antibodies significantly inhibited phenacetin *O*-de-ethylase activity (Fig. 4) and the mutagenicity of 2-AAF, for which *N*-hydroxylation is the rate-limiting step (Fig. 5). The anti-protein antibody inhibited a greater proportion of both phenacetin *O*-de-ethylase and 2-AAF activation than did the anti-peptide antibody. The anti-peptide antibody had no effect upon either aryl hydrocarbon hydroxylase activity or the activation of aflatoxin B1 by microsomes prepared from the livers of MC-treated rats, or upon the activation of 2-AAF by microsomal fractions from human liver (results not shown). However, the anti-protein antibody inhibited

hepatic microsomal aryl hydrocarbon hydroxylase activity of MC-treated rats by 70 % and also inhibited the activation of 2-AAF by microsomal fractions from human liver by > 95 %.

DISCUSSION

Recently, a model for the three-dimensional structure of mammalian cytochromes *P*-450 was proposed [38], based upon sequence similarity between the α -helices in *Pseudomonas putida* cytochrome *P*-450CI (*P*-450cam) [39] and areas of the mammalian cytochromes *P*-450. From this, it was predicted that the tertiary structure of all eukaryotic cytochromes *P*-450 is essentially the same as that of cytochrome *P*-450cam but with three major areas of sequence insertion. Based on this model, the predicted binding site of the anti-peptide antibody is on the surface of cytochrome *P*-450IA2, in a loop region adjacent to the I-helix.

Before determining the effects of the anti-peptide antibody on the catalytic activity of cytochrome *P*-450, its binding specificity was characterized. The antibody recognizes the native protein in hepatic microsomal preparations, confirming the surface location of the region. The binding specificity of the anti-peptide antibody, predicted on the basis of a comparison of the primary sequence of the peptide with those of the isoenzymes of cytochrome *P*-450 currently available [8], was confirmed by e.l.i.s.a. and immunoblotting. The antibody binds only to cytochrome *P*-450IA2 and not to the other member of the cytochrome *P*-450IA sub-family, cytochrome *P*-450IA1, in the rat. In contrast, the anti-protein antibody binds almost equally well to both cytochrome *P*-450IA2 and *P*-450IA1, as others have found with such antibodies [11]. This is obviously due to the considerable similarity in amino acid sequence that exists between these two isoenzymes. The anti-protein antibody does not bind to the rat peptide, probably because the region at 290–296 is not immunogenic in the native protein.

As the immunizing peptide sequence also occurs in mouse cytochrome *P*-450IA2 [31], but in none of the other cytochrome *P*-450 family I isoenzymes of the human, rabbit or mouse (Table 1), it was correctly predicted that the antibody would bind only to this isoenzyme in the species tested. Lack of binding to the equivalent region of human cytochrome *P*-450IA2 was confirmed using the corresponding peptide for the human isoenzyme. In addition, the antibody does not bind to microsomes prepared from untreated or MC-treated hamster or guinea pig. Thus, it is predicted that the primary structures of cytochrome *P*-450IA2 in these species (if present) do not contain the peptide sequence.

Binding of the anti-protein antibody is decreased, but not abolished, by treatment of the microsomes with urea or SDS, indicating that some of the antibodies present in this preparation react with linear sequences, whereas others react with structures of a higher order. This finding was not unexpected, and has been commonly observed with such antibodies. In contrast, the anti-peptide antibody binds with an equal titre to native, urea-treated and SDS-treated rat liver microsomes, confirming that the binding site for this antibody in the intact membrane-bound protein is a linear sequence of residues. This property is advantageous in that such anti-

Fig. 5. Inhibition of the activation of 2-AAF in the fluctuation test by the hepatic microsomal fraction (100 pmol of cytochrome *P*-450/plate) prepared from rats treated with MC

The microsomal fraction was preincubated with anti-peptide serum (a) or anti-protein serum (b) to give concentrations of 0 (●), 25 (○), 50 (△) or 100 mg of immune serum protein/nmol of cytochrome *P*-450 (▲). The effect of the 100 mg/nmol concn. was maximal in both cases, as the addition of higher concentrations did not reduce the activation further. Results are expressed as numbers of positive wells/plate.

peptide antibodies may be used in denaturing systems of analysis, for example immunoblotting and immunocytochemistry. The use of the antibody in immunoblotting has been demonstrated here. The antibody also has excellent properties in immunocytochemistry (K. J. Rich & R. J. Edwards, unpublished work).

The effect of the anti-peptide antibody on activities known to be catalysed by cytochromes *P*-450IA2 and *P*-450IA1 was determined. The substrate concentration employed in the assay of phenacetin *O*-de-ethylase activity was chosen to maximize the contribution of cytochrome *P*-450IA2 to this reaction with hepatic microsomes from MC-treated rats [40]. The anti-peptide reduced this activity by more than 50 %, inhibition being somewhat less than that seen with the anti-protein antibody. However, this difference is most probably due to the lower titre of the anti-peptide antibody, which is partly a consequence of it binding to only a single epitope on the protein. Maximum inhibitable phenacetin *O*-de-ethylase activity by these antibodies was not determined in these experiments.

The *N*-hydroxylation of 2-AAF is a key and rate-limiting step in the generation of the ultimate mutagenic species for this compound [41]. This pathway has been shown to be catalysed predominantly by cytochrome *P*-450IA2 in hepatic microsomes from MC-treated rats. However, cytochrome *P*-450IA1 also contributes to this reaction [42]. The maximum inhibition of 2-AAF activation by the anti-peptide antibody was 50 % at 1 µg of 2-AAF and 70 % at 2.5 µg of 2-AAF. The anti-protein antibody achieved a greater degree of maximal inhibition; this is most probably due to its cross-reactivity with cytochrome *P*-450IA1 (see below).

The inhibition of mono-oxygenase activities by the anti-peptide antibody is as predicted from its binding specificity. Thus this antibody does not inhibit the activation of aflatoxin B1 by rat liver microsomes, an activity catalysed by other than hydrocarbon-inducible isoenzymes of cytochrome *P*-450 [43], nor does it inhibit the activation of 2-AAF by microsomal fractions of human liver, an activity catalysed exclusively by cytochrome *P*-450IA2 in this tissue [44], whereas the anti-protein antibody does inhibit this latter activity. Aryl hydrocarbon hydroxylase activity has been shown previously to be largely catalysed by cytochrome *P*-450IA1 in hepatic microsomes from MC-treated rats [45]. The specificity of the anti-peptide antibody is again apparent from its lack of effect on this activity, which is inhibited by 70 % by the anti-protein antibody.

Thus a region of cytochrome *P*-450IA2 has been identified which was predicted to be involved in the activity of the isoenzyme. This prediction was confirmed in studies on the effects of the antibody on representative mono-oxygenase activities. These demonstrated not only the inhibitory effect of the antibody but also its specificity, as expected from the primary sequence. It is of interest that, amongst a series of anti-peptide antibodies raised against rat cytochrome *P*-450IIB1 by Frey and co-workers [10], one was a potent inhibitor of 7-ethoxycoumarin *O*-de-ethylase activity. The binding site for this antibody, according to our model for mammalian cytochromes *P*-450 [38], lies in the region between the B' and C α -helices. The area lies on the surface of the protein adjacent to the binding site for the anti-peptide antibody studied here.

This general region does not obviously form a direct

part of the active site of cytochrome *P*-450, so an antibody binding to this region should not interfere with substrate accessibility, although until the three-dimensional structures of the eukaryotic cytochromes *P*-450 are determined this cannot be completely excluded. However, inhibition is more likely to be indirectly mediated, for example through interference with the binding of cytochrome *P*-450 reductase or with the passage of electrons from cytochrome *P*-450 reductase to the haem. In support of this hypothesis, Parkinson *et al.* [46] found that modification of a cysteine residue in this region of rat cytochrome *P*-450IA1 with 4,4'-dithiodipyridine was reversible upon addition of cytochrome *P*-450 reductase, with release of thiopyridone.

The approach adopted here for the production of inhibitory antibodies of predefined specificity targeted to particular isoenzymes of cytochrome *P*-450 offers significant advantages over the use of monoclonal antibodies in such studies. Although monoclonal antibodies that inhibit the activity of cytochrome *P*-450 have been produced, both by us and by other groups, and these have proved invaluable in studies of the substrate specificities of isoenzymes of cytochrome *P*-450, the production of such antibodies that are inhibitory remains serendipitous. Further, although methods are available to determine the binding sites of such monoclonal antibodies [47,48], these are often lengthy and technically demanding procedures. Moreover, most of these methods require that the antibody binds to a continuous epitope, a property which can never be guaranteed. Thus the specificity of such antibodies is assured only after their characterization. In contrast, anti-peptide antibodies can be targeted to any surface area on the cytochrome *P*-450 apoprotein and the region of binding will perforce be known in advance.

In conclusion, an antibody which reacts specifically with a region of rat cytochrome *P*-450IA2 has been produced. As this antibody is inhibitory and the region of binding on the cytochrome is known, this has enabled an area of the isoenzyme involved in enzyme activity to be located. It is of interest that within this region there is a high degree of variation of amino acid residues between different isoenzymes of cytochrome *P*-450. Thus, utilizing the model of eukaryotic cytochromes *P*-450 previously elaborated, this provides great potential for the production of specific inhibitory antibodies targeted to any isoenzyme for which the primary sequence is known.

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REFERENCES

1. Nebert, D. W., Adeisnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. & Waterman, M. R. (1987) *DNA* **6**, 1-11
2. Black, S. D. & Coon, M. J. (1987) *Adv. Enzymol.* **60**, 35-87
3. Conney, A. H. (1986) *Life Sci.* **39**, 2493-2518
4. Nebert, D. W. & Gonzalez, F. J. (1987) *Annu. Rev. Biochem.* **56**, 945-993
5. Gelboin, H. V. & Friedman, F. K. (1985) *Biochem. Pharmacol.* **34**, 2225-2234

6. Thomas, P. E., Bandiera, S., Reik, L. M., Maines, S. L., Ryan, D. E. & Levin, W. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 2563–2566
7. Sesardic, D., Boobis, A. R., McQuade, J., Baker, S., Lock, E. A., Elcombe, C. R., Robson, R. T., Hayward, C. & Davies, D. S. (1986) *Biochem. J.* **236**, 569–577
8. Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. & Waterman, M. R. (1989) *DNA* **8**, 1–13
9. Edwards, R. J., Singleton, A. M., Sesardic, D., Boobis, A. R. & Davies, D. S. (1988) *Biochem. Pharmacol.* **37**, 3735–3741
10. Frey, A. B., Waxman, D. J. & Kreibich, G. (1985) *J. Biol. Chem.* **260**, 15253–15265
11. Reik, L. M., Levin, W., Ryan, D. E. & Thomas, P. E. (1982) *J. Biol. Chem.* **257**, 3950–3957
12. Maron, D. M. & Ames, B. N. (1983) *Mutat. Res.* **113**, 173–215
13. Liu, F.-T., Zinnecker, M., Hamaska, T. & Katz, D. H. (1979) *Biochemistry* **18**, 690–697
14. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
15. Axén, R., Porath, J. & Ernback, S. (1967) *Nature (London)* **214**, 1302–1304
16. Edwards, R. J., Singleton, A. M., Boobis, A. R. & Davies, D. S. (1989) *J. Immunol. Methods* **117**, 215–220
17. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
18. Rich, K. J., Sesardic, D., Foster, J. R., Davies, D. S. & Boobis, A. R. (1989) *Biochem. Pharmacol.* **38**, 3305–3322
19. Boobis, A. R., Brodie, M. J., Kahn, G. C., Fletcher, D. R., Saunders, J. H. & Davies, D. S. (1980) *Br. J. Clin. Pharmacol.* **9**, 11–19
20. Venitt, S. & Bosworth, D. (1983) *Carcinogenesis* **4**, 339–345
21. Vogel, H. J. & Bonner, D. M. (1956) *J. Biol. Chem.* **218**, 97–106
22. Atlas, S. A., Boobis, A. R., Felton, J. S., Thorgeirsson, S. S. & Nebert, D. W. (1977) *J. Biol. Chem.* **252**, 4712–4721
23. Murray, S. & Boobis, A. R. (1986) *Biomed. Environ. Mass Spectrom.* **13**, 91–93
24. Sogawa, K., Gotoh, O., Kawajiri, K., Harada, T. & Fujii-Kuriyama, Y. (1985) *J. Biol. Chem.* **260**, 5026–5032
25. Benjamin, D. C., Berzofsky, J. A., East, I. J., Gurd, F. R. N., Hannum, C., Leach, S. J., Margoliash, E., Michael, J. G., Miller, A., Prager, E. M., Reichlin, M., Sercarz, E. E., Smith-Gill, S. J., Todd, P. E. & Wilson, A. C. (1984) *Annu. Rev. Immunol.* **2**, 67–101
26. Novotný, J., Handschumacher, M., Haber, E., Bruccoleri, R. E., Carlson, W. B., Fanning, D. W., Smith, J. A. & Rose, G. D. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 226–230
27. Atassi, M. Z. (1984) *Eur. J. Biochem.* **145**, 1–20
28. Janin, J. (1979) *Nature (London)* **277**, 491–492
29. Leszczynski, J. F. & Rose, G. D. (1986) *Science* **234**, 849–855
30. Sogawa, K., Gotoh, O., Kawajiri, K. & Fujii-Kuriyama, Y. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5066–5070
31. Kimura, S., Gonzalez, F. J. & Nebert, D. W. (1984) *J. Biol. Chem.* **259**, 10705–10713
32. Ozols, J. (1986) *J. Biol. Chem.* **261**, 3965–3979
33. Quattrocchi, L. C., Pendurthi, U. R., Okino, S. T., Potenza, C. & Tukey, R. H. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6731–6735
34. Kagawa, N., Mihara, K. & Sato, R. (1987) *J. Biochem. (Tokyo)* **101**, 1471–1479
35. Jaiswal, A. K., Gonzalez, F. J. & Nebert, D. W. (1985) *Science* **228**, 80–83
36. Parkinson, A., Ryan, D. E., Thomas, P. E., Jerina, D. M., Sayer, J. M., van Bladeren, P. J., Haniu, M., Shively, J. E. & Levin, W. (1986) *J. Biol. Chem.* **261**, 11478–11486
37. Thomas, P. E., Reik, L. M., Ryan, D. E. & Levin, W. (1983) *J. Biol. Chem.* **258**, 4590–4598
38. Edwards, R. J., Murray, B. P., Boobis, A. R. & Davies, D. S. (1989) *Biochemistry* **28**, 3762–3770
39. Poulos, T. L., Finzel, B. C. & Howard, A. J. (1987) *J. Mol. Biol.* **195**, 687–700
40. Sesardic, D., Boobis, A. R., Edwards, R. J. & Davies, D. S. (1988) *Br. J. Clin. Pharmacol.* **26**, 363–372
41. Weisburger, J. H. & Weisburger, E. K. (1973) *Pharmacol. Rev.* **25**, 1–66
42. Åström, A. & DePierre, J. W. (1985) *Carcinogenesis* **6**, 113–120
43. Shimada, T., Nakamura, S., Imaoka, S. & Funae, Y. (1987) *Toxicol. Appl. Pharmacol.* **91**, 13–21
44. Shimada, T., Iwasaki, M., Martin, M. V. & Guengerich, F. P. (1989) *Cancer Res.* **49**, 3218–3228
45. Thomas, P. E., Reik, L. M., Ryan, D. E. & Levin, W. (1984) *J. Biol. Chem.* **259**, 3890–3899
46. Parkinson, A., Thomas, P. E., Ryan, D. E., Grosky, L. D., Shively, J. E., Sayer, J. M., Jerina, D. M. & Levin, W. (1986) *J. Biol. Chem.* **261**, 11487–11496
47. Jemmerson, R. & Paterson, Y. (1986) *Science* **232**, 1001–1004
48. Mehra, V., Sweetser, D. & Young, R. A. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7013–7017

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