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A COMPARATIVE STUDY OF CONSTITUTIVE AND INDUCED ALKOXYRESORUFIN O-DEALKYLATION AND INDIVIDUAL CYTOCHROME P450 FORMS IN CYNOMOLGUS MONKEY (*Macaca fascicularis*), HUMAN, MOUSE, RAT AND HAMSTER LIVER MICROSOMES

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Abstract—The expression of constitutive and inducible cytochrome P450 forms was measured in cynomolgus monkey liver and compared with man, rat, mouse and hamster. Four alkoxyresorufin O-dealkylation (AROD) activities widely used as indicators of P450 induction were measured: methoxyresorufin O-demethylation (MROD), ethoxyresorufin O-deethylation (EROD), pentoxyresorufin O-dealkylation (PROD) and benzyloxyresorufin O-dealkylation (BROD). In monkeys there were no sex-differences in untreated, phenobarbitone (PB)- or β-naphthoflavone (BNF)-treated animals in AROD activities, or in individual P450 proteins detected by immunoblotting. Basal MROD and EROD activities varied by less than 7-fold between the five species, but the comparative pattern of basal MROD, EROD, PROD and BROD activities (the "MEPB profile") was very species-specific, with monkeys being similar to rats but different from man, mouse and hamster. The induction of AROD activities by PB and BNF was also highly species-specific. Monkeys expressed constitutive proteins immunorelated to the CYP1A, CYP2A, CYP2B, CYP2C and CYP3A sub-families (human CYP2A6 cross-reacted with the anti-rat CYP2B1 antibodies used, and so CYP2A and CYP2B forms could not be separately identified in the monkey). Single constitutive immunoblot bands were identified in monkey for CYP1A (54 kDa), CYP2A/CYP2B (51 kDa) and CYP3A (51 kDa), respectively, but two strong (51 and 52 kDa) plus two weak (49 and 49.5 kDa) bands were shown for CYP2C. Human liver expressed CYP1A2 (54 kDa), CYP2A6 (51 kDa), CYP3A4 (50.5 kDa) and three CYP2C9-immunorelated protein bands (48, 50 and 54 kDa). In monkeys BNF induced the 54 kDa CYP1A protein and CYP1A-dependent MROD, EROD and PROD activities (18-, 15- and 6-fold increases in activity, respectively), whereas PB strongly induced the 51 kDa CYP2A/CYP2B protein but did not induce PROD activity. PB also induced non-constitutive CYP2A/CYP2B protein bands at 49 and 52 kDa in some monkeys. BROD activity was induced less that four-fold by either PB or BNF in monkeys. In conclusion, cynomolgus monkeys expressed a range of constitutive CYP1A, CYP2A or CYP2B, CYP2C and CYP3A proteins similar to man, and a range of AROD monooxygenase reaction rates similar to both man and rat, but the basal MEPB profile of AROD activities in monkeys was more similar to rat than to man. MROD and EROD were good measures of CYP1A induction by polycyclic aromatic hydrocarbons in cynomolgus monkeys, but neither PROD nor BROD were indices of CYP2B induction by PB.

Key words: drug metabolism; species difference; induction; P450

New world monkeys, especially the cynomolgus monkey (*Macaca fascicularis*, also known as the crab-eating macaque monkey) and the marmoset (*Callithrix jacchus*), are regularly used as experimental species during drug development in an attempt to predict drug metabolism and toxicity in man [1–3]. Extrapolation of the data to humans can be improved by a comparative knowledge of the characteristics and properties of drug metabolizing enzymes in each species. An improved ability to extrapolate drug metabolism data from the most commonly used experimental species, rats, to cynomolgus monkeys should help in decisions whether to use this species as a better model for man.

Hepatic cytochrome P450 is a key enzyme in drug metabolism, but there is a dearth of information on its structural and metabolic characteristics in cynomolgus monkeys [3–13] and other monkey species [14–17]. This contrasts with the many reviews on P450 in rats and humans [18–21]. In view of this, we have compared hepatic microsomal P450 in the livers of untreated and inducer-treated cynomolgus monkeys with P450 in rats, mice, hamsters and humans, using immunoblotting to identify the P450 proteins, together with the well-established P450 probe substrates, the alkoxyresorufins, to assess monooxygenase activity characteristics.

MATERIALS AND METHODS

Chemicals. Alkoxyresorufins were synthesized as

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described elsewhere [22], but they can be purchased from Molecular Probes Inc. (4849 Pitchford Ave., Eugene, OR 97402, U.S.A.). BNF* and 3MC were obtained from the Sigma Chemicals Co. (Poole, U.K.), PB sodium and arachis oil were from BDH Ltd (Poole, U.K.), olive oil was BP grade, isosafrole was from ICI (Alderley Park, U.K.), and all other materials were obtained from the usual commercial suppliers.

Animals and pretreatments. Adult male Sprague–Dawley rats (200–250 g), Syrian golden hamsters (120–180 g) and C57/BL10 mice (25–30 g), bred at the University of Aberdeen and fed Oxoid breeding diet and water *ad lib.*, were acclimatized for 1 week on mineral bedding then used either untreated or following treatment with a single inducing agent as follows: PB (0·1% w/v in the drinking water for 6 days, followed by a return to ordinary water for the final 24 hr before death), PCN (50 mg/mL in 1.5% w/v Tween 80, administered at 50 mg/kg by gavage for 4 days), 3MC (1% w/v in olive oil, administered at 80 mg/kg once i.p. 72 hr before death) or ISF (10% w/v in olive oil, administered at 200 mg/kg for 3 days). Untreated, Tween 80-treated and olive oil-treated animals served as vehicle controls for the PB, PCN and 3MC or ISF treatment groups, respectively. Rats, mice and hamsters were killed by cervical dislocation and livers removed immediately into ice-cold 10 mM potassium phosphate buffer, pH 7.6, containing 1.15% KCl (w/v) for microsome preparation.

Adult male and female cynomolgus monkeys (*Macaca fascicularis*, 3–6 kg body weight) were maintained, treated and killed (using an overdose of the barbiturate preparation, Expiral) at Inveresk Research International (Trenent, U.K.). Each animal was individually housed and offered 200 g/day of a complete dry diet of known formulation (SDA Mazuri diet, supplied by Special Diet Services Ltd, Northwich, U.K.) and water *ad lib.* Monkeys were treated with either PB (0.9% w/v in saline at 20 mg/kg i.p. on 5 consecutive days) or BNF (25 mg/mL in arachis oil at 50 mg/kg by gastric gavage on 5 consecutive days). Saline- and arachis oil-treated monkeys served as controls for the PB and BNF treatment groups, respectively. Monkeys were killed 24 hr after the final treatment and livers were excised immediately, cut into 5 g sections, frozen in liquid nitrogen and stored at −80°. The remaining tissues were allocated to other studies.

Human liver samples were obtained with permission from renal transplant donors in the Aberdeen Royal Infirmary within 30 min of circulatory arrest, placed into ice-cold iso-osmotic saline, cut into approximately 1–2 cm cubes and frozen to −80° for storage within 1 hr total elapsed time. Clinical histories for the livers are listed in Table 1.

There were three male rats, mice or hamsters per treatment group. The numbers of male (m) and female (f) monkeys in each treatment group were as follows: untreated, 4 m + 4 f; PB treated, 2 m + 2 f; arachis oil treated, 2 m + 2 f; BNF treated, 2 m + 2 f. Six human livers were studied (2 m and 4 f in the age range 19–63 years, each of whom showed an MEPB profile of comparative MROD, EROD, PROD and BROD activities typical of the mean MEPB profile measured for a total of 47 individuals [23]).

Microsomal preparations. All procedures were performed at 0–4°. Human and cynomolgus liver microsomes were prepared from samples stored at −80°, which were thawed on ice and washed in phosphate-buffered KCl as above. Microsomes were prepared as described previously [24].

Purified P450 and antibodies. P450 forms are named as recently recommended [25]. The P450 forms, CYP1A1 and CYP1A2, were purified from hepatic microsomes of 3MC-treated rats, while CYP2B1, CYP2C6 and CYP3A1/2 were purified from hepatic microsomes of PB-treated rats and CYP2C9 (P450hB20-27) and CYP3A4 (P450hA7) were purified from human liver microsomes, all to a specific content of 16.6–19.3 nmol/mg protein and identified by *N*-terminal amino acid sequencing as described elsewhere [24, 26]. (CYP3A1/2, 52 kDa, is so named to indicate that the *N*-terminal amino acid sequence for 26 residues indicates it to be a mixture of both CYP3A1 and CYP3A2 [27]. Whilst human CYP3A3 and CYP3A4 have 97% similar deduced amino acid sequences and cannot be differentiated immunochemically [21], the expression of CYP3A3 as measured by its mRNA is so low in human liver [19] that the purified human CYP3A P450 used here is much more likely to be CYP3A4 than CYP3A3.) Monoclonal and polyclonal antibodies were prepared as described previously [26, 28].

Analytical procedures. P450 was measured by the method of Omura and Sato [29] using a Cary 219 split-beam spectrophotometer and protein was determined as described by Lowry *et al.* [30]. Microsomal proteins and purified P450 preparations were resolved by discontinuous SDS–PAGE [31], using either a 10% or a 7–10% linear gradient polyacrylamide resolving gel, as described previously [32]. Proteins separated by SDS–PAGE were electroblotted onto nitrocellulose by the method of Towbin *et al.* [33] and immunostained essentially as described by Shaw *et al.* [26]. AROD reactions were measured as described previously [34].

RESULTS

Constitutive catalytic activities

In both control and inducer-treated monkeys hepatic microsomal AROD activities differed by less than 9% between males and females of the same treatment group, with the exception of marginally (20–30%) higher MROD and EROD activities in BNF-treated males compared to females (data not shown). Therefore, the AROD results for the male

* Abbreviations: AROD, alkoxyresorufin O-dealkylation; BNF, β-naphthoflavone; BROD, benzyloxyresorufin O-dealkylation; EROD, ethoxyresorufin O-deethylation; ISF, isosafrole; MEPB profile, the comparative pattern of MROD, EROD, PROD and BROD activities; 3MC, 3-methylcholanthrene; MROD, methoxyresorufin O-demethylation; PAH, polycylic aromatic hydrocarbon(s); PB, phenobarbitone; PCN, pregnenolone 16-α-carbonitrile; PROD, pentoxyresorufin O-dealkylation.

Table 1. Clinical histories for human liver samples

Individual	Sex	Age	Drugs	Diseases/cause of death	Smoking*	Alcohol†
14	F	30	Terminal betamethasone and methylprednisolone	None/accident	Nil/minor	Nil/minor
20	F	62	Papaveratrum	Not specified/accident	Nil/minor	Nil/minor
22	M	54	Diazepam	Gross liver damage/accident	Major	Major
27	F	42	Not known	None/accident	Not known	Not known
28	M	40	Cyclopenthiazide	Hypertension/subarachnoid haemorrhage	Nil/minor	Nil/minor
29	F	63	Nil	None/intracerebral haemorrhage	Not known	Nil/minor

* Smoking is classified as either nil/minor (nil or ≤5 cigarettes/day) or major (≥6 cigarettes/day). None of the individuals were exclusively cigar or pipe smokers.

† Alcohol consumption is classified as either nil/minor (nil, social or occasional drinker) or major (regular or heavy drinker).

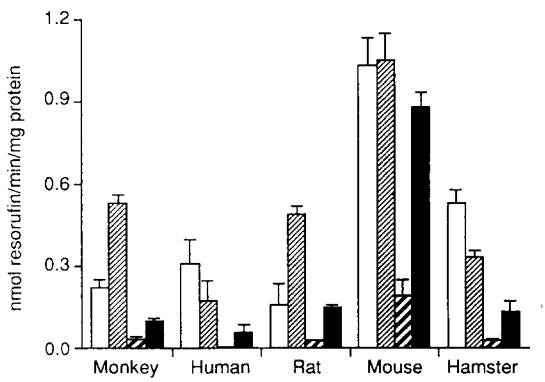

Fig. 1. Basal AROD activities in liver microsomes of cynomolgus monkey, human, rat, mouse and hamster. Data are means ± SEM for eight untreated monkeys (4 m and 4 f), six humans (2 m and 4 f) and three untreated rats, mice and hamsters (all males), for MROD (□), EROD (▨), PROD (▨) and BROD (■).

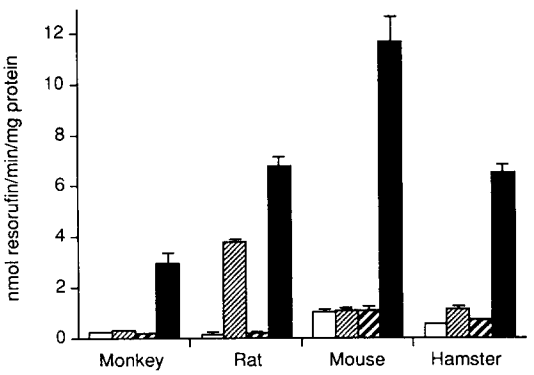

Fig. 2. The effect of BNF and PB treatments on hepatic microsomal MROD activity in cynomolgus monkey, rat, mouse and hamster. Data are means ± SEM for four monkeys (2 m and 2 f) and three rats, mice and hamsters (all males). (□) Untreated or saline-treated animals, (▨) PB-treated animals, (▨) olive oil- or arachis oil-treated animals, (■) 3MC- or BNF-treated animals.

and female monkeys have been combined to provide sufficient replicates for statistical analysis. The pattern of relative MROD, EROD, PROD and BROD activities in a sample of liver microsomes is referred to as the "MEPB profile". Microsomal activities were measured for each sample individually and the results then pooled by treatment group as indicated to provide mean values for each species.

The mean constitutive MROD, EROD, PROD and BROD activities measured in liver microsomes from untreated cynomolgus monkeys were compared with the corresponding activities in liver microsomes prepared from untreated Sprague–Dawley rats, Syrian golden hamsters and C57/BL10 mice and from humans (Fig. 1). The MEPB profile of untreated monkeys resembled the profile of untreated rats, but was different from the MEPB profiles of human and untreated hamsters and mice. The similarities and differences were mainly in EROD and MROD, which were the most active of the reactions in all five species. In both monkey and rat EROD activity was greater than MROD, whereas in human and hamster MROD was greater than EROD, and in mice MROD and EROD were approximately equal. Overall, however, MROD and EROD activities were remarkably similar in all the species, varying less than 7-fold between the least and the most active. BROD activity was much lower than the most active reaction (MROD or EROD, depending on species) in all the species except mouse. Ranking the species in order of activity gave the following: MROD = mouse > hamster > human > monkey > rat; EROD = mouse > monkey ≈ rat > hamster > human; BROD = mouse > rat ≈ hamster > monkey > human.

Induction of catalytic activities in experimental species

Figures 2–5 show the effects of treatment with either of the known rat CYP1A-inducers, 3MC or BNF, or with the rat CYP2B1-inducer, PB, on hepatic microsomal AROD activities in monkey, rat, mouse and hamster. The degrees of induction

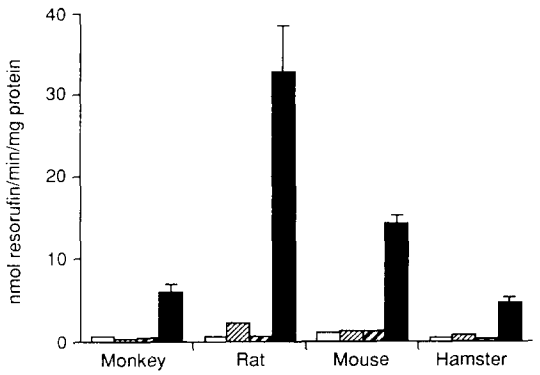

Fig. 3. The effect of BNF and PB treatments on hepatic microsomal EROD activity in cynomolgus monkey, rat, mouse and hamster. Data are means ± SEM for four monkeys (2 m and 2 f) and three rats, mice and hamsters (all males). (□) Untreated or saline-treated animals, (▨) PB-treated animals, (▨) olive oil- or arachis oil-treated animals, (■) 3MC- or BNF-treated animals.

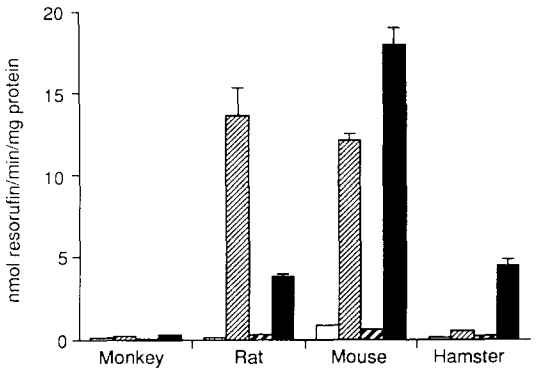

Fig. 5. The effect of BNF and PB treatments on hepatic microsomal BROD activity in cynomolgus monkey, rat, mouse and hamster. Data are means ± SEM for four monkeys (2 m and 2 f) and three rats, mice and hamsters (all males). (□) Untreated or saline-treated animals, (▨) PB-treated animals, (▨) olive oil- or arachis oil-treated animals, (■) 3MC- or BNF-treated animals.

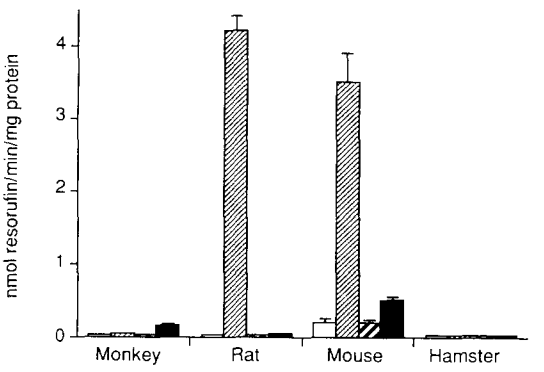

Fig. 4. The effect of BNF and PB treatments on hepatic microsomal PROD activity in cynomolgus monkey, rat, mouse and hamster. Data are means ± SEM for four monkeys (2 m and 2 f) and three rats, mice and hamsters (all males). (□) Untreated or saline-treated animals, (▨) PB-treated animals, (▨) olive oil- or arachis oil-treated animals, (■) 3MC- or BNF-treated animals.

of AROD activities are shown in Table 2. MROD and EROD (Figs 2 and 3) were highly selectively induced by 3MC or BNF compared to PB in monkey, hamster, rat and mouse. The species rank order for the degree of MROD and EROD induction by 3MC or BNF were similar: MROD induction = rat > monkey > mouse ≈ hamster, whereas EROD induction = rat > monkey ≈ hamster > mouse. In rats, but not the other species, there was also a conspicuous (i.e. >3-fold) induction of MROD (24-fold) and EROD (5-fold) by PB. Whereas the effects of inducers on MROD and EROD were broadly similar in all four species, the effects of inducers on PROD and BROD were species-specific. PROD (Fig. 4) was extensively and selectively induced by PB in rat and mouse but not in monkey or hamster. PROD was not induced by 3MC in rat or hamster, but surprisingly, BNF modestly induced PROD in monkey (6-fold) and 3MC induced it slightly in mouse (3-fold). BROD (Fig. 5) was induced extensively by PB in rat (91-fold) and mouse (14-fold) but only minimally in hamster (4-fold), whereas it was markedly induced by 3MC in rat, mouse and hamster (mouse ≈ hamster > rat). PB was a more effective BROD inducer than 3MC in rat, whereas 3MC was more effective as an inducer of BROD than PB in mouse and hamster. In contrast, in the monkey BROD was not notably induced by either PB (2-fold) or BNF (3-fold).

In liver microsomes from BNF-treated monkeys the polyclonal antibody against rat CYP1A1, which also recognises rat and human CYP1A2, inhibited MROD, EROD and PROD activities by 78, 84 and 44%, respectively, at a ratio of 20 mg antibody per mg microsomal protein. For comparison, the same concentration of the antibody inhibited EROD by 94% in liver microsomes from 3MC-treated rats. (Results are means for duplicate determinations, data not shown.)

Immunoblotting characteristics of monoclonal and polyclonal antibodies with rat and human P450

The polyclonal antibody raised against purified rat CYP1A1 specifically recognises CYP1A family proteins (both CYP1A1 and CYP1A2) in both rat and human liver [35]. The polyclonal and monoclonal (RP3) antibodies raised against purified rat CYP2B1 were immunoblotted against purified rat CYP2B1, CYP1A1 and human CYP3A4, against liver microsomes prepared from humans, untreated rats or rats pretreated with either PB or PCN and against lymphoblastoma microsomes containing expressed human CYP2A6 cDNA (generously provided by Dr C. L. Crespi, Gentest Corporation, Woburn, MA, U.S.A.): the results with monoclonal RP3 are shown in Fig. 6, whilst the results with the polyclonal antibody, which were virtually identical, are not

Table 2. Induction of hepatic microsomal AROD activities

Reaction	Inducer	Species			
		Monkey	Rat	Mouse	Hamster
MROD	PB	1.2	23.8	1.0	2.1
	3MC/BNF	18.5	34.7	10.6	9.3
EROD	PB	0.6	4.5	1.1	2.0
	3MC/BNF	15.4	66.0	11.9	15.7
PROD	PB	1.5	168.4	18.0	1.2
	3MC/BNF	5.6	1.3	2.7	0.95
BROD	PB	2.0	90.7	13.7	3.8
	3MC/BNF	3.5	14.4	28.7	28.6

Values are fold-inductions (activity in inducer-treated animal ÷ activity in vehicle control animal), means for three animals per treatment group except for monkey, where four PB-treated and four BNF-treated were used.

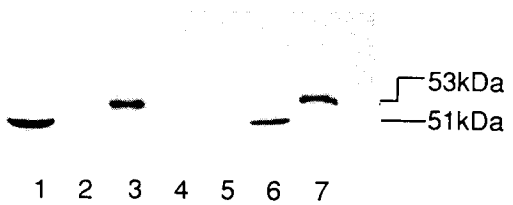

Fig. 6. Immunoblot of human and rat liver microsomes and human CYP2A6 with a monoclonal antibody against rat CYP2B1. Hepatic microsomal proteins were resolved by SDS–PAGE and immunoblotted with the anti-CYP2B1 monoclonal antibody, RM3. Samples (protein loadings in parentheses) are: lane 1, human (10 μg); lane 2, untreated rat (1 μg); lane 3, PB-treated rat (1 μg); lane 4, PCN-treated rat (1 μg); lane 5, non-transfected lymphoblastoma homogenate (10 μg); lane 6, homogenate of lymphoblastoma expressing human CYP2A6 cDNA (10 μg); lane 7, purified rat CYP2B1 (0.5 μg).

shown. Both of the anti-CYP2B1 antibodies recognised purified rat CYP2B1 (53 kDa), expressed human CYP2A6 (51 kDa) and corresponding bands in human (51 kDa) and PB-induced rat (53 kDa) liver microsomes, respectively, but neither recognised purified rat CYP1A1, CYP1A2, CYP2C6 or CYP3A1/2, nor purified human CYP2C9 or CYP3A4, even when loaded at 10-times the amount of CYP2B1 and CYP2A6 (data not shown). A previous batch of polyclonal antibody raised against purified human CYP2C9 recognised on immunoblots both purified and microsomal human CYP2C9 (54 kDa) and an additional weaker band in human liver microsomes at 50 kDa, but not purified rat CYP1A1, CYP1A2, CYP2B1 or CYP3A1/2 or human CYP3A4, while purified rat CYP2C6 was recognised only very weakly [24]. A new batch of this anti-CYP2C9 antibody also recognised on immunoblots a third, weak band (48 kDa) in human liver microsomes (Fig. 7d), but not expressed human CYP2A6 (data not shown). The polyclonal antibody to human CYP3A4 was previously shown to specifically recognise purified and microsomal human CYP3A4 and microsomal rat CYP3A1 and CYP3A2 on immunoblots [26] (this antibody will probably not discriminate between CYP3A3 and CYP3A4 in view of their extensive sequence similarities); it has now also been shown to recognise purified rat CYP3A1/2 but not purified rat CYP1A1, CYP1A2, CYP2B1 or CYP2C6, nor expressed human CYP2A6 or purified human CYP2C9 on immunoblots (data not shown).

Immunoblotting of P450 in monkey, rat and human liver microsomes

A range of hepatic microsomal forms of P450 in the cynomolgus monkey were identified by immunoblotting liver microsomes with antibodies raised against rat or human P450s belonging to the CYP1A, CYP2B, CYP2C and CYP3A families (characterized as described above). Figure 7a–d shows immunoblotting results with polyclonal antibodies for male monkeys, plus rats and humans. Since female monkeys gave virtually identical results with the polyclonal antibodies, the immunoblots for female monkeys are not shown.

Results with a polyclonal anti-rat CYP1A1 antibody are shown in Fig. 7a. Anti-CYP1A1 recognised both CYP1A1 (57 kDa) and CYP1A2 (54 kDa) in liver microsomes from 3MC- and ISF-treated rats (lanes 12 and 13, respectively) and, more weakly, CYP1A2 (54 kDa) at varying intensities in microsomes from three different humans (lanes 9–11). A single band at 54 kDa was also recognised in monkeys: faintly in saline- and arachis oil-treated monkeys (lanes 1 and 2 and 5 and 6, respectively), even more faintly in PB-treated monkeys (lanes 3 and 4) but the 54 kDa band was strongly induced in BNF-treated monkeys (lanes 7 and 8).

Results with a polyclonal anti-rat CYP2B1 antibody are shown in Fig. 7b. Anti-CYP2B1 recognised CYP2B1 (53 kDa) in PB-treated rat liver microsomes, but in monkeys it identified a single band at 51 kDa which was expressed constitutively (lanes 1 and 2 and 5 and 6) and was highly induced by PB (lanes 3 and 4), but was not affected by BNF treatment (lanes 7 and 8). In human liver microsomes the same anti-CYP2B1 antibody recognised a single constitutive band at 51 kDa (lanes 9–11), which was

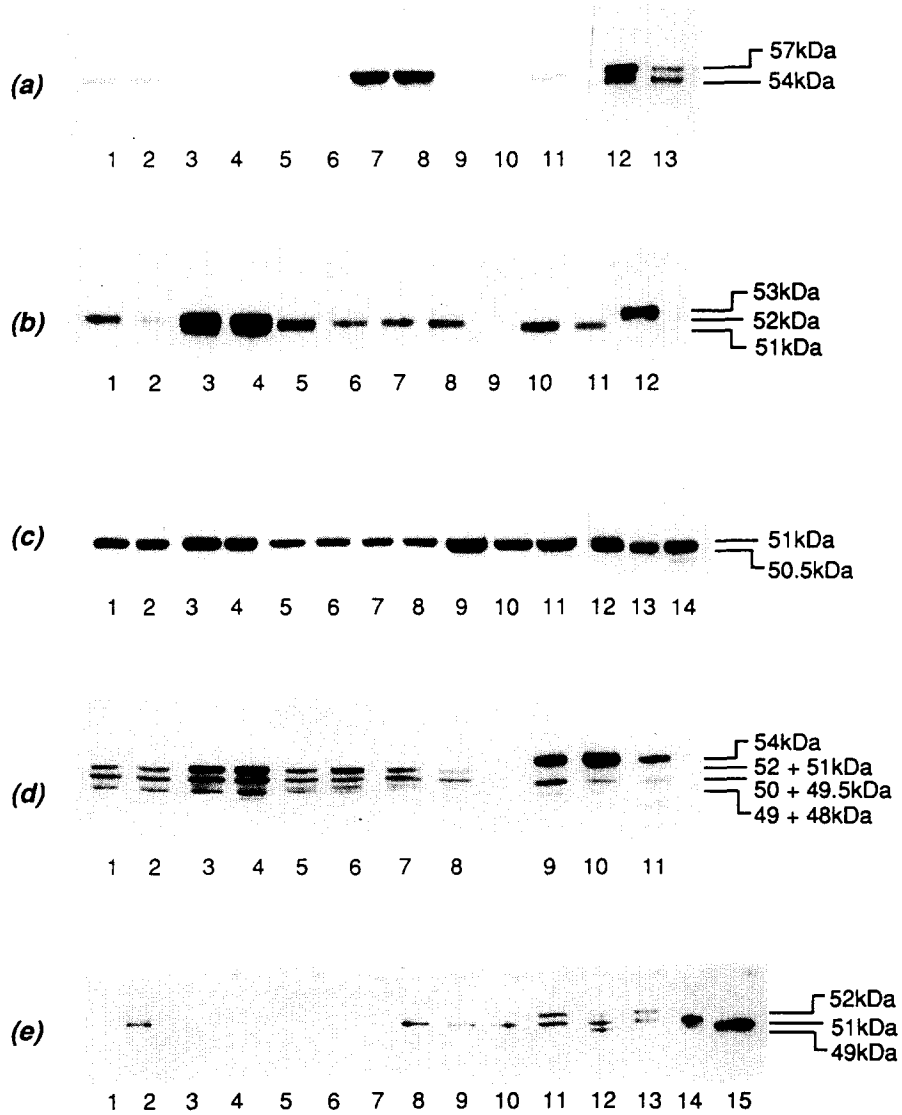

Fig. 7. Immunoblots of cynomolgus monkey, human and rat liver microsomes with antibodies against CYP1A1, CYP2B1, CYP2C9 and CYP3A4. Hepatic microsomal proteins (loaded at 10, 1 and 20 μg protein for monkey, rat and human, respectively) were resolved by SDS–PAGE and immunoblotted with polyclonal antibodies against rat CYP1A1 (part a), rat CYP2B1 (part b), human CYP3A4 (part c) and human CYP2C9 (part d), respectively, and with a monoclonal antibody (RP3) against rat CYP2B1 (part e). Samples in parts a–d are: lanes 1 and 2, saline-treated monkeys; lanes 3 and 4, PB-treated monkeys; lanes 5 and 6, arachis oil-treated monkeys; lanes 7 and 8, BNF-treated monkeys. Additional sample lanes are as follows: (a) lanes 9–11, human (three separate individuals); lane 12, 3MC-treated rat; lane 13, ISF-treated rat; (b) lanes 9–11, human (three separate individuals); lane 12, PB-treated rat; (c) lanes 9–12, human (four separate individuals); lane 13, PCN-treated rat; lane 14, PB-treated rat; (d) lanes 9–11, human (three separate individuals). Samples in part e are: lanes 1 and 2, male arachis oil-treated monkeys; lane 3, female arachis oil-treated monkey; lanes 4 and 5, male BNF-treated monkeys; lanes 6 and 7, female BNF-treated monkeys; lanes 8 and 9, male saline-treated monkeys; lane 10, female saline-treated monkey; lanes 11 and 12, male PB-treated monkeys; lanes 13 and 14, female PB-treated monkeys; lane 15, human.

presumably CYP2A6 by comparison with expressed CYP2A6 cDNA (Fig. 6, lane 6).

Results with a polyclonal anti-human CYP3A4 antibody are shown in Fig. 7c. Anti-CYP3A4 recognised CYP3A4 at 50.5 kDa in human liver microsomes (lanes 9–12). In monkey liver microsomes anti-CYP3A4 recognised a single protein band at 51 kDa which was expressed constitutively (lanes 1 and 2 and 5 and 6), was slightly induced by PB (lanes 3 and 4) but was not affected by BNF (lanes

7 and 8). The same antibody recognised a single band at 50.5 kDa, probably CYP3A1 and/or CYP3A2 [36, 37], in liver microsomes from both PCN- and PB-treated rats (lanes 13 and 14).

Results with a polyclonal anti-human CYP2C9 antibody are shown in Fig. 7d. In human liver anti-CYP2C9 recognised the three CYP2C protein bands (54, 50 and 48 kDa) reported previously [38, 39] (lanes 9–11). In saline- and arachis oil-treated monkeys this antibody recognised four constitutively expressed protein bands: strong bands at 52 and 51 kDa, a weaker band at 49 kDa and an even weaker band at 49.5 kDa (lanes 1 and 2 and 5 and 6). None of these four monkey CYP2C protein bands were affected by BNF treatment (lanes 7 and 8), and the 51 kDa and 49.5 kDa bands were not affected by PB treatment either, but the 52 kDa and 49 kDa bands were induced by PB (lanes 5 and 6). No proteins were recognised by anti-human CYP2C9 antibody in liver microsomes from untreated rats (data not shown).

In monkey liver microsomes there were no marked sex-related differences in the intensities or molecular masses of P450 protein bands recognised by polyclonal antibodies to CYP1A1, CYP2B1, CYP2C9 or CYP3A4 in any of the treatment groups (data not shown). Thus, although the polyclonal antibody immunoblots in Fig. 7a–d show only male monkeys, they are also representative of the females. There were no interindividual variations in the intensities of protein bands recognised by CYP1A1, CYP2C9 or CYP3A4 in either male or female monkeys in any treatment group (Fig. 7a, c and d, plus data not shown). There were, however, interindividual variations in the intensity of the protein band recognised by antibody to CYP2B1 in arachis oil- and saline-treated male monkeys, although not in BNF- or PB-treated male monkeys (Fig. 7b) and not in females of any treatment group (data not shown). Moreover, a monoclonal antibody raised against rat CYP2B1 (antibody RP3) showed an interindividual, non-sex-related, variation in protein band recognition amongst the four PB-treated monkeys (Fig. 7e, which shows both male and female monkeys). RP3 recognised a constitutive 51 kDa protein band in both saline- and PB-treated monkeys (lanes 8–10 and 13 and 14, respectively), which was suppressed in both the arachis oil-treated (lanes 1 and 3) and BNF-treated (lanes 4–7) monkeys, except for one arachis oil-treated monkey (lane 2). RP3 also identified additional bands in certain PB-treated monkeys: a 52 kDa band in one male and one female (lanes 11 and 13) and a 49 kDa band in another male and (more weakly) another female (lanes 12 and 14). In human liver microsomes RP3 recognised a single protein band (presumably CYP2A6) at 51 kDa (lane 15).

DISCUSSION

The cynomolgus monkey is used for drug metabolism and toxicity testing during industrial drug development [1–3]. The decision to use this species, and the extrapolation of the data obtained to humans, will be helped by better prediction of species differences in drug metabolism between the cynomolgus monkey and other experimental species on the one hand, and between this monkey species and humans on the other. Since improved prediction will come from a better knowledge of species differences in the drug metabolizing enzymes, we have compared the incidence and induction of several different P450 forms and their AROD activities between cynomolgus monkeys, rats, mice, hamsters and humans.

Liver microsomes and purified hepatic microsomal P450s from the cynomolgus or crab-eating macaque monkey (*Macaca fascicularis*) have been shown to carry out a number of common hepatic microsomal P450 drug-metabolizing reactions, including hydroxylation (acetanilide, aniline, benzo[*a*]pyrene, phenacetin and testosterone), N-dealkylation (aminopyrine, benzphetamine, ethylmorphine and caffeine), O-dealkylation (ethoxycoumarin, ethoxyresorufin, erythromycin and *p*-nitroanisole) and nifedipine oxidation [7, 8, 11, 13, 15]. In addition, debrisoquine and mephenytoin are hydroxylated *in vivo* [3], while expressed cynomolgus P450 cDNAs metabolically activate aflatoxin [12] and heterocyclic amines [9] to mutagens. AROD reactions using the homologous substrates methoxy-, ethoxy-, pentoxy- and benzyloxyresorufin, respectively, are widely employed for measuring P450 activity. In rats and humans different forms of P450 show differing specificities for these reactions [40–43] and the composite profile of the four reactions in liver microsomes, the so-called MEPB profile, probably reflects the complement of different P450 forms present. In this study the constitutive MEPB profile of cynomolgus monkeys closely resembled that of the rat, but was very different from man, mouse and hamster. Thus in this aspect of P450 reaction specificity the cynomolgus monkey does not appear to be a good experimental model for drug metabolism in humans. Caffeine is another example of a drug which is metabolized very differently by cynomolgus monkeys (mainly N7-demethylation) compared to humans (mainly N3 demethylation) [8]. On the other hand, the hepatic microsomal total P450 concentration and the K_m and V_{max} values for six different demethylation and hydroxylation reactions were similar (<3-fold difference) between cynomolgus monkeys and humans [15]. The different MEPB profiles might be due to species variations in the constitutive levels of expression of individual P450 forms, but they might also arise from species differences in the specificities of orthologous P450 forms expressed at similar levels in various species: for example, in rats CYP1A1 and CYP1A2 selectively catalyse EROD and MROD, respectively, [41], but in rabbits CYP1A2 is more active than CYP1A1 for EROD [44] and in humans CYP1A2 has similar activity for both MROD and EROD [43]. This question cannot be resolved until the AROD specificities of individual cynomolgus monkey P450s have been elucidated.

The constitutive EROD activities measured in the cynomolgus monkey in this study (0.53 ± 0.03 nmol resorufin/min/mg protein, mean ± SEM for four males and four females) were similar to those reported previously (0.60 and 0.56 nmol resorufin/min/mg for male and female, respectively, [11]),

confirming the lack of sex-difference in AROD activities in this species. There are similarly no sex differences in testosterone hydroxylation in cynomolgus monkeys [13]. The EROD results reported here and elsewhere [8, 11] show that the cynomolgus monkey has at least a 10-fold higher basal hepatic EROD activity than the marmoset monkey (0.036–0.038 nmol resorufin/min/mg) [14, 16]. This difference in activities may reflect differences in the levels or specificities of individual P450 forms between cynomolgus and marmoset monkeys, although the total hepatic microsomal P450 concentration is not too dissimilar in the two species (being approximately 2-fold higher in the cynomolgus) [13].

Measurement of the ability of a new drug to induce P450, particularly PAH-like and PB-like induction, is an important aspect of drug metabolism testing during drug development. Since the main use of AROD reactions is to measure in rats the induction of the CYP1A and CYP2B P450 families by PAH and PB, respectively [34], and possibly induction of the CYP3A family by steroids [45], it is probable that these reactions will be routinely used for this purpose in monkeys also. AROD induction is, however, species-dependent, for example, PROD is extensively induced by PB in rats but not in hamsters [46, 47]. Therefore *a priori* assumptions cannot be made about the induction responses of the AROD reactions in cynomolgus monkeys.

MROD and EROD induction in cynomolgus monkeys both showed the typical, highly selective induction by BNF (15–18-fold) compared to PB (0.6–1.2-fold) which is characteristic of EROD in rats [34], mice [22], hamsters [46, 47], rabbits [48] and several other species, including beagle dogs [49]. In fact, in this study the lack of PB induction of MROD and EROD was even more pronounced in the monkey than in the rat. The MROD and EROD reaction rates were lower in BNF-treated monkeys than in 3MC-treated rats, but were more than adequate enough to allow accurate and sensitive measurement. Immunoblotting showed strong BNF-induction of CYP1A immunorelated protein in the monkey, while antibody inhibition (78–84% inhibition by an anti-CYP1A1 antibody) indicated that the BNF-induced MROD and EROD activities in monkey were catalysed virtually entirely by the CYP1A P450 family. MROD and EROD appear, therefore, to be good measures of PAH-type induction of CYP1A in the cynomolgus monkey, combining high sensitivity and high discrimination against PB-induction.

Whereas the induction of MROD and EROD in cynomolgus monkeys was as expected from other species, the effects of inducing agents on PROD and BROD could not be foreseen. PROD was highly selectively induced by PB in rats and mice but not in hamsters, as reported previously [22, 34, 46, 47]. BROD was not induced by PB in monkeys either. Thus, although immunoblotting showed strong PB-induction of CYP2B-immunorelated protein in the monkey, this induced P450 appears to be devoid of PROD activity. A PB-induced P450 was purified from marmoset monkeys and this also had no measurable PROD (or EROD) activity, despite showing high N-demethylation activity and epoxidation activities [4]. A cynomolgus monkey CYP2B protein (P450CMLa) was recently purified, but this was constitutive and its PROD activity was not determined [13]. In cynomolgus monkeys, therefore, as in hamsters and marmoset monkeys, hepatic microsomal PROD apparently cannot be used as an index of PB-type induction. A surprising result was the almost 6-fold induction of PROD by BNF in monkeys. Antibody inhibition (44% inhibition by an anti-CYP1A1 antibody) showed that approximately 50% of this BNF-induced PROD activity was probably catalysed by CYP1A1 or CYP1A2 (which could not be discriminated by the antibody used). Thus, cynomolgus monkey CYP1A, in catalysing significant PROD activity in addition to EROD and MROD, appears to show a very different substrate specificity from either rat or human CYP1A forms, which do not have appreciable PROD activity compared to EROD or MROD [41–43].

The pattern of BROD induction was different in all four experimental species tested, being induced more by PB than 3MC in the rat, more by 3MC than PB in the mouse, by 3MC but not appreciably by PB in the hamster and not appreciably induced by either PB or BNF in the monkey. The BROD results are similar to previous observations in the same strains of male rats and mice [22, 34] and male Wistar rats [50], but differ from a report that in the same strain of hamsters BROD is not appreciably induced by either PB or 3MC [47]. BROD induction in the mouse appears to be very strain and/or sex dependent: in male C57/BL10 mice it was induced more by 3MC than PB (*vide supra*), whereas in female BALB/c [51] or male B6C3F1 [47] mice it is induced more by PB than 3MC or BNF. In rats and humans BROD is metabolized by several forms of P450, albeit at different rates [34, 40, 43, 45], and is the least specific of the alkoxyresorufin substrates. The similarity of basal BROD activities between cynomolgus monkeys, other experimental species and humans shows that constitutive P450 forms in cynomolgus monkeys had significant BROD activity. Since PB and BNF induced P450 in the monkey, as can be seen from the MROD, EROD and immunoblotting results (*vide infra*), the lack of BROD induction in monkeys was presumably due to the induced P450 forms lacking BROD activity. This is a further example of the monkey P450s showing different monooxygenase specificities from other experimental species, and also from humans, where PB and other anticonvulsant treatments induce hepatic microsomal BROD (Ref. 23 and unpublished observations).

The identities of constitutive and induced P450s in cynomolgus monkey liver microsomes were sought by immunoblotting with antibodies known to specifically recognise individual forms of rat and human P450. Assuming that the immunorecognition specificity of the antibodies was maintained across species between rat or man and monkey, cynomolgus monkey liver microsomes expressed members of the CYP1A (54 kDa), CYP2A or CYP2B (51 kDa), CYP2C (four bands, 49, 49.5, 51 and 52 kDa) and CYP3A (51 kDa) families constitutively. We cannot

yet say whether the constitutive monkey P450 protein recognised by the monoclonal and polyclonal anti-rat CYP2B1 antibodies was a CYB2B or a CYP2A form, since although these antibodies are specific for CYP2B (53 kDa) in rats, in human liver they recognise CYP2A6, which has same molecular mass (51 kDa) as the band recognised in monkey. Similarities and differences in electrophoretic molecular masses are, unfortunately, no help in identifying immunorelated P450 forms across different species; for example the relative mobilities of CYP1A1 and CYP1A2 are reversed in humans compared to rats [52]. Since in rats CYP2B1 is PB-inducible and hardly expressed constitutively [53], the possibility of the constitutive expression of a CYP2B in cynomolgus monkey liver form may seem surprising. This was, however, demonstrated by the recent purification from untreated cynomolgus monkeys of a hepatic 49.5 kDa P450 (P450CMLa) with 94 and 65% *N*-terminal amino acid sequence similarity to human CYP2B6 and rat CYP2B1, respectively [13]. Four constitutive CYP2C9-immunoreactive protein bands were identified in cynomolgus monkey in this study, compared to the three which we and others normally see in humans [38, 39]. Thus the CYP2C family appears to be at least as complex in cynomolgus monkeys as in humans [21] and rats [20]. A 50 kDa CYP2C protein (P450-MK1) has been isolated from untreated crab-eating monkey liver, showing 82% *N*-terminal sequence similarity with human CYP2C9 [7]. The single CYP3A protein band identified in untreated cynomolgus liver microsomes had a very similar molecular mass (51 kDa) to the main CYP3A protein in human liver (50.5 kDa) and to a CYP3A protein purified from crab-eating monkeys (P450-MK2, 50.5 kDa), which had high *N*-terminal sequence similarity to human CYP3A4 [7]. The immunoblotting results show that BNF induced CYP1A protein of molecular mass 54 kDa, which is the same size as one of two 3MC-induced CYP1A proteins (P450MC1, 52 kDa and P450MC2, 54 kDa, respectively) purified from crab-eating monkeys [6]. 3MC induces CYP1A1 mRNA in crab-eating monkey liver with almost complete base sequence identity (95%) to human CYP1A1 [9]. PB strongly induced an apparently constitutive CYP2B-immunorelated protein in cynomolgus monkeys and also induced two out of four constitutive CYP2C proteins (49 and 52 kDa) and a CYP3A protein. In addition, it was observed using immunoblotting with a monoclonal antibody that there was an apparent polymorphism in PB-induction of cynomolgus monkey CYP2B-immunorelated proteins of molecular mass 49 and 52 kDa, the former of which had a similar molecular mass to the constitutive CYP2B (P450CMLa, 49.5 kDa) purified from cynomolgus monkey [13]. Our immunoblotting results argue for the presence of at least three constitutive or PB-inducible CYP2A and/or CYP2B immunorelated forms in the cynomolgus monkey.

In summary, the cynomolgus monkey appears to express several different families of hepatic microsomal constitutive, PB-inducible or BNF-inducible P450s which are immunorelated to P450s expressed in humans and rat. However, results for AROD argue for significant differences in the monooxygenase reaction specificities of both constitutive and induced P450s between cynomolgus monkey, human, rat, mouse and hamster. As a result, whilst MROD and EROD are diagnostic for PAH-induced CYP1A in this monkey as in other species, PROD and BROD, which are useful probes for PB-induced P450s in the rat and the mouse, do not serve this function in the monkey. Studies are now underway to compare the specificities of rat, human and cynomolgus monkey P450s for a broader range of P450 substrates.

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