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A Comparison of the Ontogeny of Enterocytic and Hepatic Cytochromes P450 3A in the Rat

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ABSTRACT. Enzymes of the cytochrome P450 3A (CYP3A) sub-family are abundant in adult liver and gut and contribute significantly to the first-pass metabolism of many orally administered drugs. The development of CYP3A enzymes with regard to their expression and activity in enterocytic and hepatic microsomes from 1-day-old through to adult male and female rats has been studied. Microsomes were prepared by calcium precipitation. Enzyme expression was assessed semi-quantitatively by Western blotting using rat polyclonal CYP3A2 and 2C11 antibodies and peptide antibodies specific to rat CYPs 3A1, 3A2, 2C12, and 2C13. The formation of 6β-hydroxytestosterone (6OHT), determined by HPLC, was used as a measure of enzyme activity. Formation of 6OHT by enterocytic microsomes was similar for males and females and showed a sharp increase at weaning. This pattern was mirrored by levels of immunoquantifiable CYP3A2 (CYP3A9), but CYP3A1 followed a more gradual development. CYPs 2C11, 2C12, or 2C13 were not detected in gut microsomes. In contrast, CYPs 3A1, 3A2, 2C11, 2C12, and 2C13 were all expressed in hepatic microsomes. There was no surge in hepatic enzyme expression or hepatic 6OHT formation at weaning, and a marked sex difference in 6OHT formation was apparent from day 25. The surge in gut activity at weaning may be a protective mechanism against ingested toxins. BIOCHEM PHARMACOL **60**;11:1601–1610, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cytochrome P450; rat intestine; rat liver; CYP3A; ontogeny; testosterone 6β-hydroxylase

Enzymes of the cytochrome P450 3A subfamily are abundant in adult liver and gut and contribute to the first-pass metabolism of many orally administered drugs [1, 2]. In humans, CYP3A4§ is the predominant form of cytochrome P450 both in the liver and the small bowel. In the latter, it is expressed in the villous tip and exhibits a gradient from the duodenum to the colon [3, 4]. Rat liver expresses CYPs 3A1 and 3A2, the latter considered by some [5, 6], but not others [7], to be a male-specific form. CYP3A23 is also expressed, but this may be an allelic variant of CYP3A1 [8]. More recently, CYPs 3A9 and 3A18 have been found in livers of both male and female rats [8, 9]. CYP3A1 is present in rat enterocytes [10–12], but evidence for the expression of CYP3A2 is less clear. Some investigators did not detect any mRNA for CYP3A2 [10, 11] and suggest that it is more likely to be CYP3A9, which cross-reacts with CYP3A2 peptide antibodies [7].

Limited information is available on the development of human liver CYP3A4. It increases from birth to adulthood together with a corresponding decrease in CYP3A7, the main fetal form of cytochrome P450 [13]. The urinary 6β-hydroxycortisol:cortisol ratio, an *in vivo* marker of CYP3A4 activity, was shown to be higher in mature compared to premature neonates [14], and the systemic clearance of midazolam, a significant substrate of hepatic CYP3A4, was found to be lower in infants under 2 years of age compared to 3 years and over [15]. Studies of age-related CYP3A activity in rat liver indicate that it rises sharply in the first few days after birth but then falls away rapidly in females by 21 days, while much higher activity is maintained in the males until between 3 and 12 months [7, 16].

To our knowledge, nothing is known about the ontogeny of enterocytic CYP3A enzymes in rat or human. Therefore, we compared their development with regard to expression and activity in enterocytic and hepatic microsomes from 1-day-old through to adult male and female rats. The 6β-hydroxylation of testosterone was used to mark CYP3A activity. The CYP3A subfamily is considered to be mainly responsible for this reaction in rat liver [17], although other forms may contribute including CYPs 1A1/2, 2A2, and 2C13 [18]. Expression was examined using a polyclonal CYP3A2 antibody and specific peptide antibodies raised against CYP3A1 and CYP3A2.

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§ *Abbreviation:* CYP, cytochrome P450.

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MATERIALS AND METHODS

Materials

Acrylamide solution (40% v/v) was purchased from Bio-Rad Micromeasurements, Immobilon-P from Millipore, buffered formaldehyde from Genta Medical, enhanced chemiluminescent reagents (Tropix) from PE Applied Biosystems, trypsin from ICN Biomedicals, BSA from Pierce and Warriner, goat anti-rat polyclonal cytochrome P450 3A2 and 2C11 antibodies and human lymphoblastoma-expressed CYP3A4 microsomes from Cambridge Biosciences, normal rabbit and swine serum, rabbit anti-goat and swine anti-rabbit biotinylated antibodies and streptavidin from Dako, *N*-glycanase from Oxford Glycosciences, and ethyl acetate, methanol, and ethanol from Rathburn Chemicals. All other chemicals were purchased from Sigma or BDH and were of analytical or electrophoresis grade. Specific peptide antibodies to CYPs 3A1, 3A2, 2C12, and 2C13 raised in rabbits were a generous gift from Dr. R. Edwards (Imperial College, University of London).

Animals

Male and female Wistar rats, divided into age groups (days 1, 10, 15, 20, 25, 30, 40, 60, 80, and adults), were obtained from the University of Sheffield Field Laboratories. They were housed in plastic cages and fed a standard cereal-based diet (CRM diet feed); free access was allowed to food and water. The animals were stunned and killed by cervical dislocation according to U.K. Home Office procedure.

Isolation of Enterocytes

Tissue consisting of the first third of the small bowel from the duodenum onwards was dissected from 6 male and 6 female animals in each age group. The bowel sections were immediately placed in ice-cold buffer (PBS containing 5 mM EDTA, 0.5 mM dithiothreitol, and 40 μg/mL of phenylmethylsulphonyl fluoride). The bowel contents were flushed out and the tissue transferred to fresh buffer. A small cross-section of duodenum (1–2 mm) was taken from each bowel length and placed in buffered formaldehyde for histochemical staining. The remaining bowel samples were dissected longitudinally, chopped into 2- to 3-cm lengths and placed in 100 mL ice-cold modified Weiser solution [19]. After stirring gently for 15 min the enterocyte-containing suspension was decanted into a glass beaker on ice. This process was repeated with 100 mL of fresh ice-cold Weiser solution. The two Weiser fractions were then combined and centrifuged in 50-mL aliquots at 800 *g* for 10 min. The cell pellets were then combined, re-suspended, and washed twice with 10 mL of buffer (histidine 5 mM, sucrose 0.25 M, EDTA 0.5 mM, and phenylmethylsulphonyl fluoride 40 μg/mL; pH 7.4), and centrifuged at 800 *g* for 10 min.

Preparation of Microsomes

Enterocytic microsomes were prepared by a calcium precipitation method [20]. All steps were carried out at 0–4°. Washed enterocytes (2–5 g wet weight) were re-suspended in 5 mL of the buffer using 10 up and 10 down strokes of a Potter–Elvehjem homogeniser. The homogenate was then centrifuged at 15,000 *g* for 10 min. The supernatant was carefully removed using a Pasteur pipette and placed in two glass tubes to which was added 1.25 mL of 52 mM calcium chloride solution (the final calcium concentration was approximately 10 mM). After six inversions, the tubes were kept on ice for 15 min and then centrifuged at 2000 *g* for 10 min. The supernatant was discarded and the microsomes were resuspended in 1–2 mL of microsome buffer (20% w/v glycerol, 100 mM Tris–HCl, and 10 mM EDTA; pH 7.4).

Liver (5 G minced wet weight) from at least 4 animals was finely chopped and 15 mL of buffer (0.25 M sucrose, 10 mM Tris–HCl; pH 7.4) was added. The tissue was homogenised using 10 up and 10 down strokes of a Potter–Elvehjem homogeniser, centrifuged at 10,000 *g* for 10 min, and the supernatant carefully removed into glass tubes. Microsomes were sedimented by calcium aggregation as described for intestinal microsomes. The supernatant was discarded and the microsomes were re-suspended in 7 mL 0.15 M potassium chloride solution. After centrifugation at 100,000 *g* for 60 min, the supernatant was again discarded and the microsomes were re-suspended in 5 mL microsome buffer. Microsomal protein content was determined by the method of Lowry *et al.* [21] using BSA as standard.

Measurement of Enzyme Activity

The 6β-hydroxylation of testosterone was used as a measure of microsomal CYP3A activity.

Incubation Procedure

The incubation mixture comprised 200 μL of 1.15% w/v potassium chloride, 50 μL testosterone in 50% methanol/water to give a final concentration of 0–500 μM (100 μM for the ontogeny studies), 200 μL of a NADPH-generating system (0.5 mM NADP, 5 mM glucose 6-phosphate, 5 mM $MgCl_2$, 1 unit glucose 6-phosphate dehydrogenase/mL), and 350 μL of 0.2 M potassium phosphate buffer, pH 7.4. Incubations were carried out in 15-mL uncapped glass tubes at 37° in a shaker water bath (Grant Instruments) for 15 min. Reactions were started by the addition of microsomal suspension (0.2 mL) and stopped by addition of ethyl acetate (2.0 mL) containing 25 μL of a 10 μg/mL solution of 11β-hydroxytestosterone as assay internal standard.

Metabolite Assay

The incubate–ethyl acetate mixture was shaken for 20 min, centrifuged at 2000 *g* for 10 min, and the organic phase removed and evaporated to dryness with a vortex evapora-

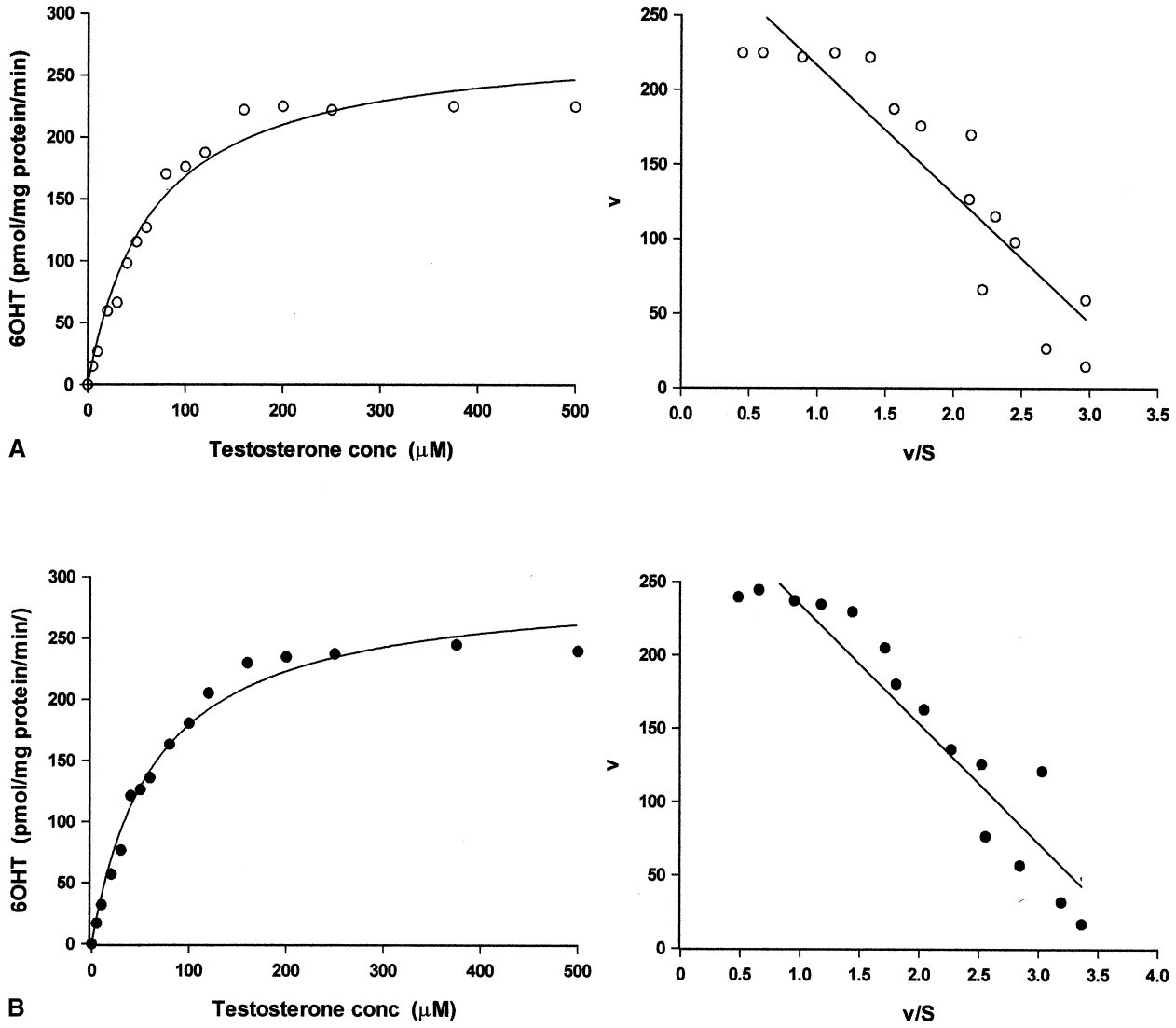

FIG. 1. Velocity (v) vs substrate concentration (S) and Eadie–Hofstee plots for the formation of 6β-hydroxytestosterone (6OHT) by enterocytic microsomes from (a) male adult and (b) female adult rats. Lines are best fits of a single Michaelis–Menten function. Data are means from two experiments performed in triplicate.

tor (Buchler). The residue was then reconstituted in 200 μL of mobile phase, of which 150 μL was injected on column. Chromatography was carried out isocratically using a Spectroflow 400 solvent delivery system (Kratos Analytical Instruments), a Waters manual injector (Waters Corporation), a Waters radial compression unit containing a Novapak C_{18} cartridge (0.8 × 10 cm; 4-μm particle size) fitted with a Guard-Pak column containing a C_{18} insert, a Kratos SF 769 variable wavelength detector (Kratos Analytical Instruments), and a BBC SE120 chart recorder (Goerz Metrawatt). The mobile phase was methanol:water (55:45 v/v) at a flow rate of 3 mL/min. The detection

TABLE 1. Enzyme kinetic data for the 6β-hydroxylation of testosterone by enterocytic and hepatic microsomes from male and female rats

Adult microsomes	V_{max} (nmol/mg protein/min)	K_m (μM)	V_{max}/K_m (μL/min)
Male gut	0.291 ± 0.01	73.5 ± 7.0	4.0 ± 1.4
Male liver	2.50 ± 0.24	12.5 ± 0.6	199 ± 42
Female gut	0.297 ± 0.011	66.5 ± 7.2	4.5 ± 0.61
Female liver	0.535 ± 0.081	18.2 ± 5.5	29.4 ± 15
	1.22 ± 0.95	124.1 ± 110	9.85 ± 8.6

Values N = 6 ± SD.

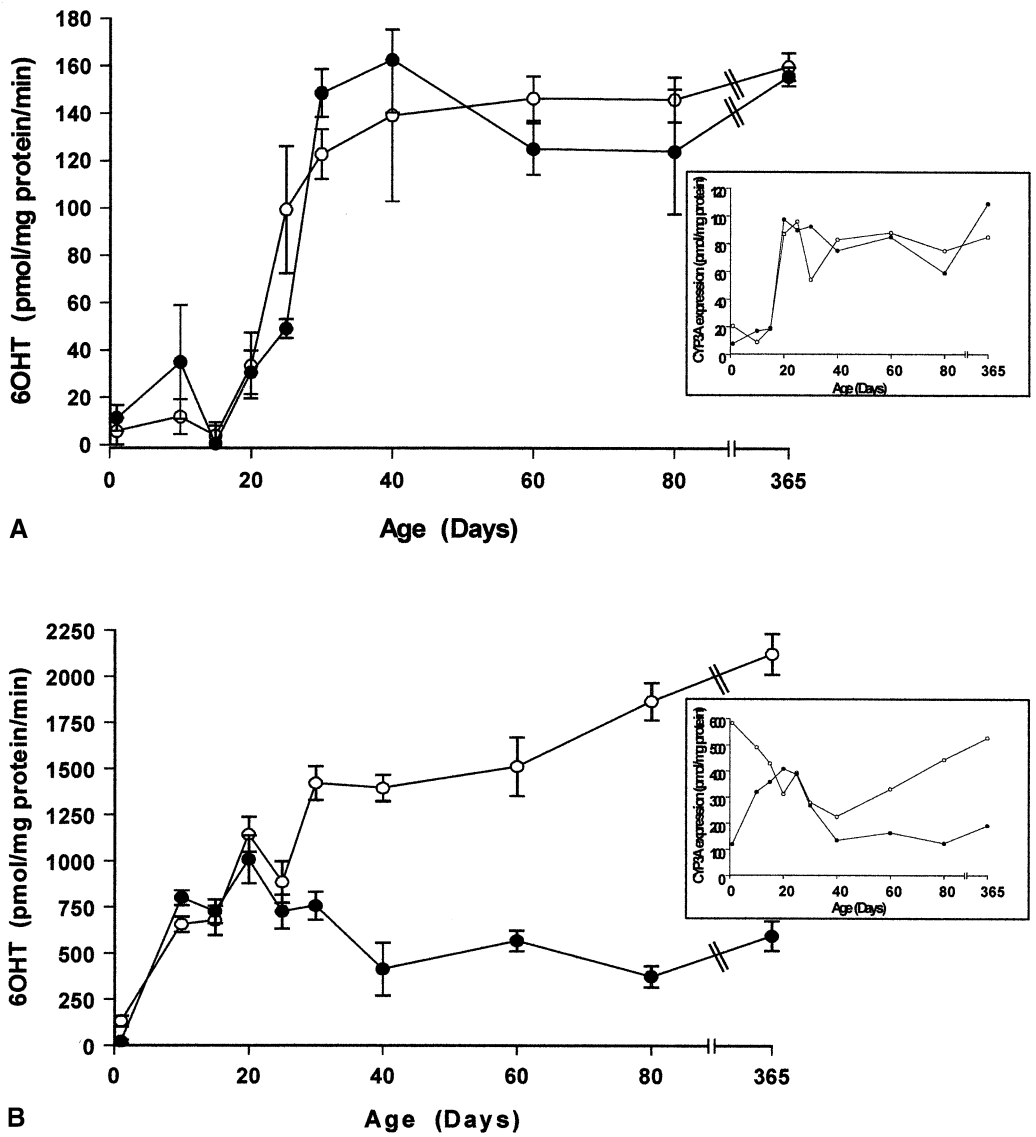

FIG. 2. Development of testosterone 6β-hydroxylase activity in male (○) and female (●) (a) enterocytic and (b) hepatic microsomes. The substrate concentration was 100 μM and 50 μM for enterocytic and hepatic microsomes, respectively. Data points are mean values ± SD for 6 replicate experiments. 6OHT = 6β-hydroxytestosterone. Inset graphs show the corresponding development of CYP3A expression with age quantified by densitometry of immunoblots using a polyclonal CYP3A2 antibody.

wavelength was 240 nm. The limit of detection of the assay was 30 ρmol and the intra-assay coefficient of variation was 8.5% at 150 ρmol (N = 5). For the enzyme kinetic studies, the substrate concentration range was 5–500 μM.

Data Analysis

Enzyme kinetic data were expressed as Eadie–Hofstee plots to obtain initial estimates of K_m and V_{max}. They were then fitted by a Michaelis–Menten function using weighted non-linear least squares regression (Graphit, version 3.0).

Immunohistochemistry

Samples of rat duodenum across the age range were fixed in buffered formaldehyde overnight, dehydrated in a graded series of ethanol solutions, and embedded in paraffin wax. Sections were cut onto glass slides using a Leica Scientific microtome (Leica) and dried at 37°. The paraffin wax was removed with xylene and decreasing concentrations of ethanol solution. The sections were then incubated for 30 min in methanol containing 1.7% v/v (100 vol) hydrogen peroxide to block any endogenous peroxidase activity. All subsequent operations were carried out in a humidified chamber, washing the sections with Tris buffer (pH 7.6) between each incubation. Incubation with 20% normal rabbit serum for 15 min was followed by 30 min with an optimal dilution of primary antibody (1 in 500 goat anti-rat CYP3A2 polyclonal or 1 in 100 rabbit CYP3A1 and CYP3A2 peptide antibodies), 30 min with an optimal dilution of secondary antibody (1 in 400 biotinylated rabbit anti-goat or swine anti-rabbit), and 30 min with a 1 in 600

dilution of streptavidin–biotin–peroxidase complex. Peroxidase activity was visualised using a 0.035% w/v solution of 3,3-diaminobenzidine tetra-hydrochloride containing 0.3% v/v 100 volume hydrogen peroxide.

Immunoblotting

Western blots from microsomal protein were obtained after denaturation and reduction by boiling for 2 min in treatment buffer (62.5 mM Tris–HCl, 2% w/v SDS, 20% w/v glycerol, and 10% v/v mercaptoethanol). A number of blots were also produced under non-reducing, non-denaturing conditions. A Flowgen G31010 electrophoresis unit (Flowgen) was used with 10% SDS–PAGE-resolving and 2.7% stacking gels. Alternate lanes were loaded with 5–10 μg microsomal protein and control buffer along with Novex Mark 12™ and Seeblue™ molecular weight markers. The unit was attached to a Bio-Rad 500/200 power supply (Bio-Rad), adjusted to 150 mV, and left to run for 2 hr. Transfer onto Immobilon-P membrane was performed using a CBS semi-dry blotter (EU-4000) (CBS Scientific Co.) at 80 mA for 50 min. Membranes were probed with goat anti-rat CYP3A2 and CYP2C11 polyclonal antibodies (1:50,000) or the specific peptide antibodies to CYPs 3A1, 3A2, 2C12, and 2C13 (1:5000). Antibody binding was detected using an alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin type G (1:20,000) or alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin type G (1:20,000). The Tropix Western Light™ chemiluminescent detection system was used for visualisation, and the blots were recorded using Kodak X-OMAT film. Western blots were compared both visually and by densitometric analysis. Densitometric analysis was performed using a DC120 digital camera (Kodak) and the KDS 1d image analysis software. CYP3A expression was calculated from the known concentrations in the control microsomes.

RESULTS

Enzyme Kinetics

Preliminary experiments using microsomes from 25-day-old and adult male and female rats established that the 6β-hydroxylation of testosterone proceeded at a constant rate over 20 min with both enterocytic and hepatic microsomes and up to 0.5 mg/mL and 0.25 mg/mL of protein for enterocytic and hepatic microsomes, respectively. Accordingly, enzyme kinetics were evaluated using an incubation time of 15 min and protein concentrations of 0.4 mg/mL and 0.1 mg/mL, respectively, for enterocytic and hepatic microsomes. The minimum detectable rate of metabolism was 14 pmol.mg protein^{-1}.min^{-1}. Figure 1 shows the rate of reaction as a function of substrate concentration and corresponding Eadie–Hofstee plots for incubations with adult male and female enterocytic microsomes. Although there was evidence of systematic deviation from a single-site Michaelis–Menten representation at the higher substrate concentrations, the male and female data were similar and were reasonably described by this model. There was no convincing evidence of sigmoidicity in the plots at lower substrate concentrations. Estimates of K_m, V_{max}, and V_{max}/K_m values are listed in Table 1.

In contrast to the data for enterocytic microsomes, those for adult liver microsomes exhibited a marked sex difference. A single-site model was fitted to the male data, but a two-site model was needed for the female data. Estimates of K_m and V_{max} values are listed in Table 1. The adult male

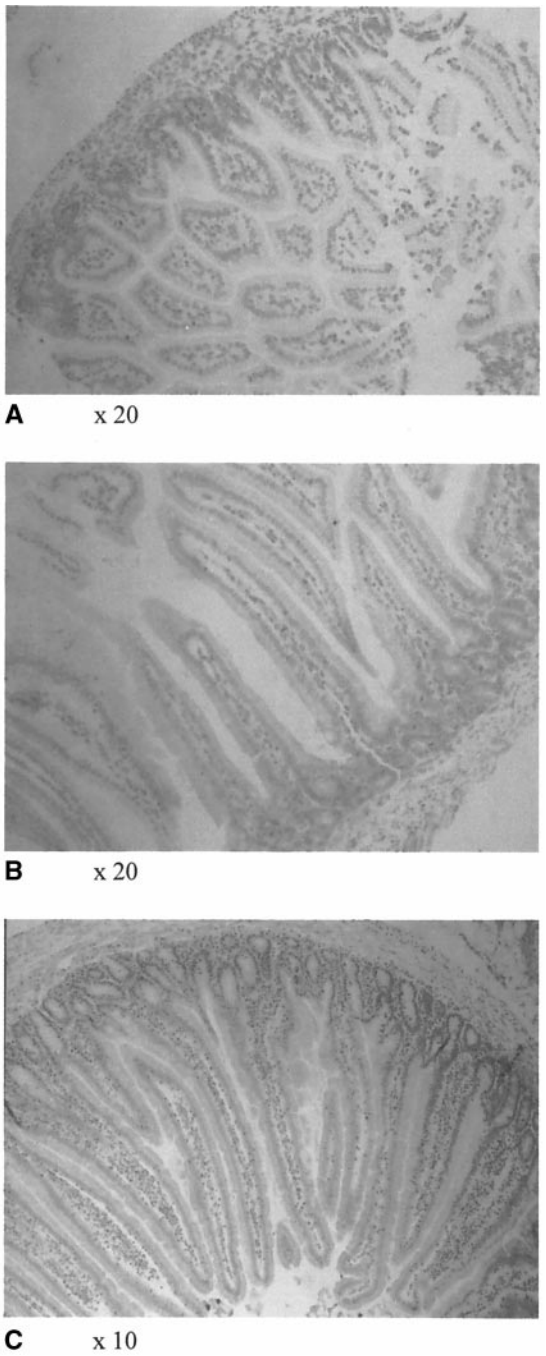

FIG. 3. Cross-sections of male rat duodenum immunostained using a polyclonal goat anti-rat CYP3A2 antibody. (A) 1-day-old animal; (B) 20-day-old animal; (C) 80-day-old animal. The enzyme is indicated by the brown stain.

hepatic microsomes were approximately 50-fold more efficient than enterocytic microsomes, as indicated by V_{max}/K_m values. However, the corresponding difference was on average only 4-fold for adult female microsomes.

Ontogeny of Enzyme Activity

The development of testosterone 6β-hydroxylase activity was evaluated using substrate concentrations of approximately 1.5× the K_m values for adult gut microsomes (100 μM) and 50 μM for the adult rat liver. Changes in activity with age in male and female enterocytic microsomes were similar, with a surge at weaning between 20 and 30 days followed by a plateau up to 80 days (Fig. 2a). No overall statistical difference was found in activity between the sexes ($P = 0.414$, two-way ANOVA), although a difference was apparent at days 25 and 30 ($P = 0.004$, Bonferrioni *t*-test). In contrast, the activity of hepatic microsomes increased gradually in both sexes up to 20 days, and beyond 25 days a marked sex difference was apparent (Fig. 2b). Female activity declined after day 20 while male activity continued to increase up to 1 year.

Immunohistochemistry

Figure 3 shows cross-sections of duodenum from 1-, 20-, and 80-day-old male rats immunostained with the polyclonal CYP3A2 antibody. The presence of enzyme was clearly evident in the brush-border cells of the villi with a relative absence from the crypts. Similar results were obtained using the CYP3A peptide antibodies (data not shown). A subjective analysis of stain intensity suggested an age-related increase with respect to the polyclonal and peptide CYP3A2 antibodies, but not the peptide CYP3A1 antibody (Table 2).

TABLE 2. Intensity of immunostaining of rat duodenal sections from male and female rats as a function of age using three different CYP3A antibodies

Age (Days)	and Sex	Polyclonal 3A2	Peptide 3A2	Peptide 3A1
1	M	+/0	+	
1	F	+	0	+/++
10	M	+	+/++	0
10	F	+/0	+/0	+
15	M	+/0	+	+/++
15	F	+/0	++	+
20	M	++	+++	0
20	F	+/++	+++	+
25	M	+++	+++	+/0
25	F	+/++	++	+/0
30	M	++	+++/++++	+/++
30	F	+++	+++/++++	+
40	M	+++	++++	++
40	F	+++	+	+
60	M	++++	+++/++++	+/++
60	F	+++	++++	++
80	M	+++	++++	+++
80	F	++++	++++	++

Rats were aged 1 to 80 days (N = 3 rats per group). Data were scored subjectively (0–++++) by two observers and the mean score recorded.

Immunoblotting

The chemiluminescence detection system gave a linear response to microsomal standards with respect to CYP3A content over the range 0 to 500 ρmol ($r^2 = 0.94$ to 0.98).

DENATURING AND REDUCING CONDITIONS. Western blots prepared from male and female enterocytic microsomes using the polyclonal CYP3A2 antibody and the peptide CYP3A1 and 3A2 antibodies indicated bands at 50 kDa. The blots for female microsomes, shown in Fig. 4, clearly indicate a marked increase in intensity with respect to the two CYP3A2 antibodies at day 20. A similar effect was apparent for male microsomes. Densitometric analysis of these blots confirmed a similar development for CYP3A expression compared to testosterone 6β-hydroxylase activity (Fig. 2a, inset), although the changes in the latter at weaning lagged slightly behind the increase in protein expression. CYP3A1 protein appeared to show relatively little change in level up to 80 days. Double bands were associated with the polyclonal CYP3A2 antibody from day 20, while the peptide 3A2 antibody detected only a single band. No bands were observed for enterocytic microsomes using either the polyclonal CYP2C11 antibody or the CYP2C12 and 2C13 peptide antibodies. Both CYP2C12 in female liver and CYP2C13 in male liver microsomes showed a gradual development (data not shown). Male and female rat liver microsomes showed a strong single band at 50 kDa using the polyclonal CYP3A2 antibody. The intensity of the blot became noticeably weaker in older female but not in older male liver microsomes (Fig. 5) and apart from in the younger males, densitometric analysis confirmed the developmental pattern found for testosterone 6β-hydroxylation (Fig. 2b, inset).

NON-DENATURING AND NON-REDUCING CONDITIONS. When male and female rat enterocytic microsomes were investigated under these conditions using the polyclonal CYP3A2 and the peptide 3A2 antibodies, a molecular weight shift with age was noted. Thus, microsomes from day 1 to 15 animals exhibited a band at about 100 kDa, with no signal at 50 kDa as observed under reducing conditions. Beyond day 20, the 100-kDa signal became weaker as that at 50 kDa became apparent (Fig. 6). Incubation of the samples with 2.5 units of *N*-glycanase at 37° overnight did not abolish this phenomenon, but denaturation alone (100° for 2 min) did. The molecular weight shift was not observed using the CYP3A1 peptide antibody, nor was it apparent with rat liver microsomes using the CYP3A2 antibodies.

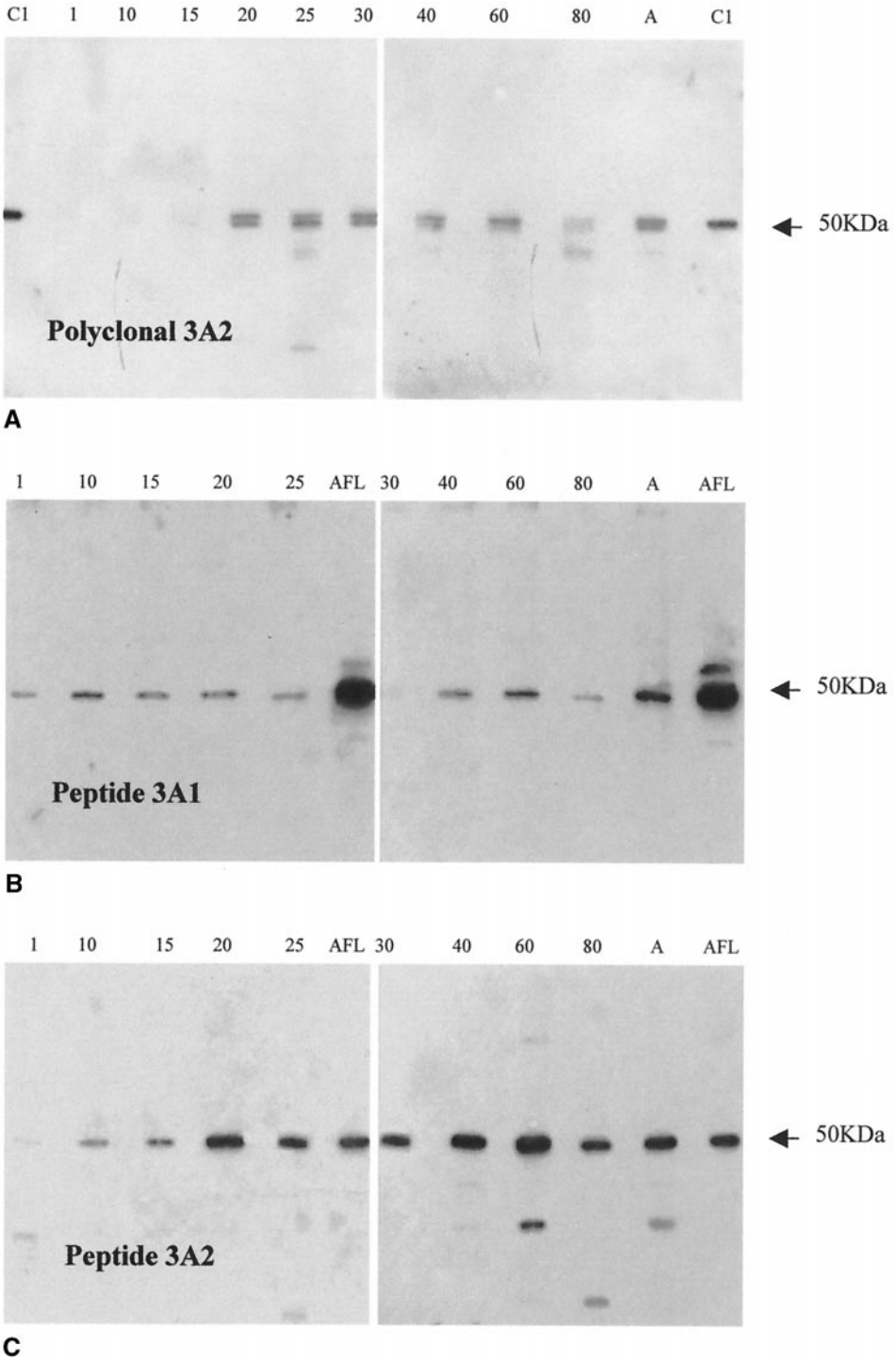

FIG. 4. Western blots of enterocytic microsomes from female rats aged 1 day to 1 year (A, adult) produced using (a) a polyclonal goat anti-rat CYP3A2 antibody, (b) a specific peptide antibody to CYP3A1 raised in rabbit, and (c) a specific antibody to CYP3A2 raised in rabbit. Numbers refer to the age of the animals. AFL refers to adult female liver microsomes and C1 is a human CYP3A4 standard (human lymphoblastoma-expressed CYP3A4 microsomes). The blots were produced under denatured and reduced conditions.

DISCUSSION

The findings of this study indicate significant differences in the 6β-hydroxylation of testosterone by rat enterocytic and hepatic microsomes and in the development of the enzyme(s) involved. The specific activity of the probe reaction was about 10-fold lower in male enterocytic compared to hepatic microsomes; in females this difference was only 4-fold. While a marked sex difference in enzyme kinetics was confirmed for adult hepatic microsomes, there was no such difference with respect to adult enterocytic micro-

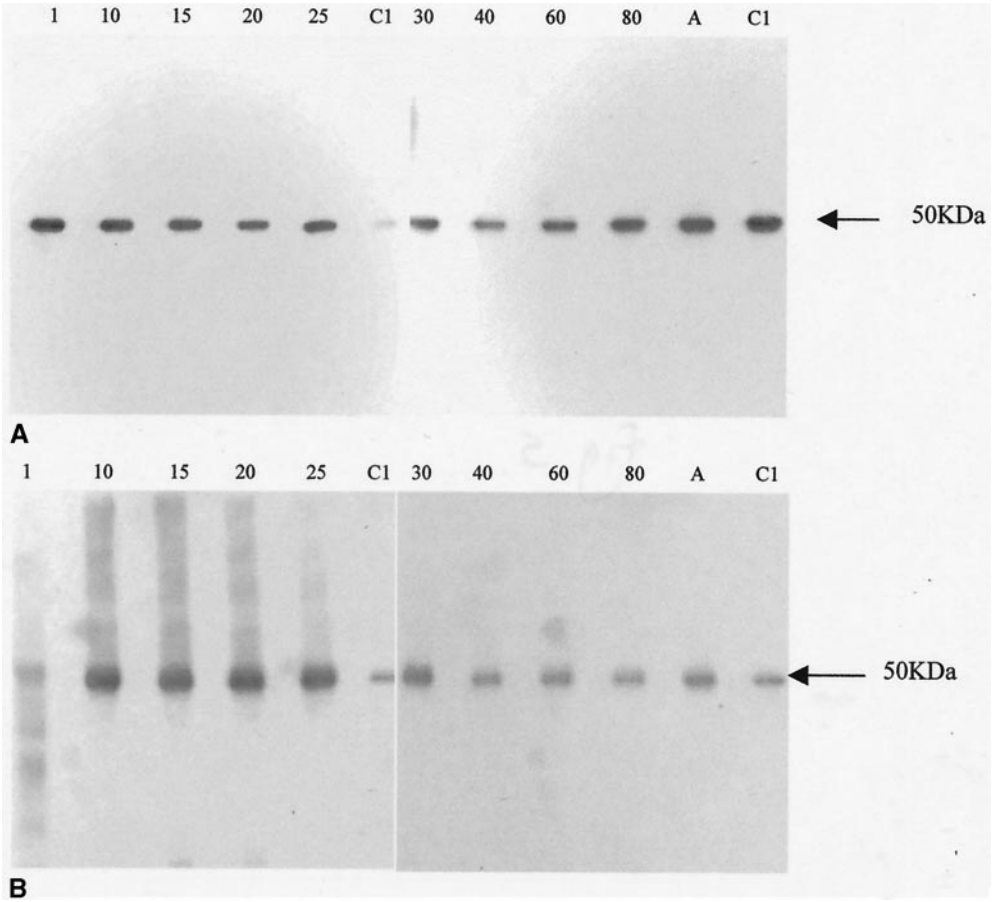

FIG. 5. Western blots of hepatic microsomes from (a) male and (b) female rats aged from 1 day to 1 year (A, adult), produced using a polyclonal goat anti-rat CYP3A2 antibody. Numbers refer to the age of the animals. C1 is a human CYP3A4 standard (human lymphoblastoma-expressed CYP3A4 microsomes). The blots were produced under denatured and reduced conditions.

somes. While enterocytic activity was low before weaning, it surged after 20 days, remaining relatively constant and comparable in both sexes up to 80 days. The development of activity in hepatocytes increased gradually and in parallel in both sexes up to 25 days, beyond which the greater activity seen in adult male microsomes was apparent. Thus, weaning appears to be associated with separate phenomena in enterocytic and hepatic microsomes with respect to the 6β-hydroxylation of testosterone. In the former, it appears to switch the enzyme activity on, presumably as an adaptation to the ingestion of solid food and xenobiotics; in the latter, it precedes a rapid period of sexual differentiation, possibly related to the interplay of steroids and growth hormone [22, 23].

The developmental changes observed in the 6β-hydroxylation of testosterone appear to mirror those in the level of expression of CYP3A2 but not CYP3A1 protein. Thus, the intensity of immunohistochemical staining with CYP3A2 antibody and the immunoblot for CYP3A2 were similar in male and female enterocytic microsomes and showed marked surges at 20 days, while the immunoblot signal was sustained in hepatic microsomes from older male rats but waned in the those from older females. This suggests, but does not prove, that CYP3A2 is the major isoenzyme involved in the 6β-hydroxylation of testosterone. Although the male specificity of this enzyme in rat liver is contentious [6, 7], our data for enterocytic and hepatic microsomes suggest that it may be under different regulation at the two sites. We were unable to detect any CYP2C11, 2C12, or 2C13 protein in male or female enterocytic microsomes, although very low levels of CYP2C were previously detected on immunoblot and confirmed as CYP2C6 and CYP2C11 by RT–PCR (reverse transcriptase–polymerase chain reaction) [10]. However, the CYP2C isoforms are known to be sex-specific [24] and may contribute significantly to the difference in activity seen in hepatic microsomes from male and female animals. Analysis of the enzyme kinetics of testosterone 6β-hydroxylation by rat hepatic microsomes was consistent with the involvement of more than one enzyme, at least in females.

Although a peptide antibody raised specifically against CYP3A2 was used in these studies, there is doubt as to whether this is the protein that is present in rat enterocytes. Others have detected the presence of CYP3A1 in uninduced rat small bowel microsomes, but not CYP3A2 [10, 11]. However, an immunoreactive band at a lower molecular weight than CYP3A2 was observed using a CYP3A2 antibody [12]. Another study of age-related changes in the

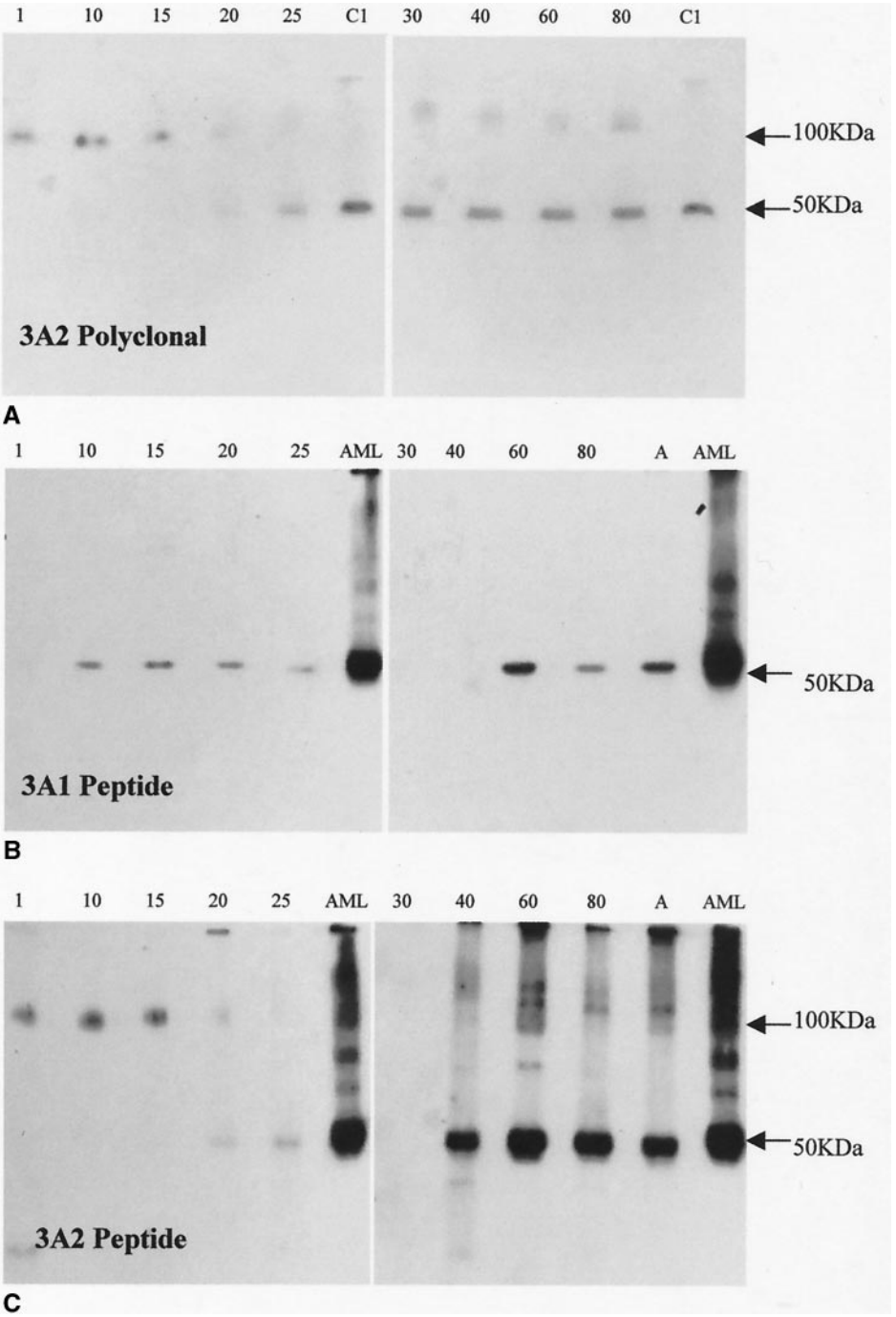

FIG. 6. Western blots of enterocytic microsomes from male rats aged 1 day to 1 year (A, adult), produced using (a) a polyclonal goat anti-rat CYP3A2 antibody, (b) a specific peptide antibody to CYP3A1 raised in rabbit, and (c) a specific peptide antibody to CYP3A2 raised in rabbit. Numbers refer to the age of the animals. AML refers to adult male liver microsomes and C1 is a human CYP3A4 standard (human lymphoblastoma-expressed CYP3A4 microsomes). The blots were produced under non-denatured and non-reduced conditions.

metabolic capacity of the rat intestine did not detect any CYP3A using a polyclonal antibody [25]. More recently, Northern blotting indicated an absence of the message for CYP3A2 in rat enterocytes [10, 11]. These discrepancies may be resolved by the fact that CYP3A2 shares a 97% homology with CYP3A9, the message for which has been detected in rat enterocytes [11] and by the observation that the two forms cross-react with the peptide antibody [7]. CYP3A18 is unlikely to cross-react with the peptide 3A2 antibody, as the C5-terminal amino acid sequence is totally different [11]

CYP3A9 mRNA has been shown to be present in male and especially female rat livers from 5 weeks gestation [8]. The relative contribution to testosterone 6β-hydroxylation is as yet unknown, necessitating caution in the interpretation of the liver ontogenic data.

Another difference between enterocytic and hepatic microsomes with respect to CYP3A2/3A9 was the observation of a molecular weight shift in the immunoblot produced from the former when the microsomes were not denatured or reduced. Although precise molecular weights cannot be deduced, the apparent halving of the value at day 20 might reflect breaking of a dimer or dissociation from another protein of similar molecular weight to CYP3A2/3A9. The higher molecular weight form did not disappear on incubation with *N*-glycanase, suggesting that glycosylation was not involved. Its presence was independent of whether reducing conditions were used or not, which is consistent with an absence of disulphide bonds in cytochromes P450 [26]. The functional significance of the observed molecular weight shift is unknown. However, the fact that it disappeared at weaning correlates with the surge in enzyme activity and may be related to the activation of the enzyme. This observation merits further investigation at the mRNA level.

In conclusion, our findings on the differential ontogeny of testosterone 6β-hydroxylase activity and CYP3A isoforms in rat enterocytic and hepatic microsomes have implications for the use of the rat as an animal species for the toxicity testing of CYP3A substrates and point to the need for parallel studies in children.

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