Regulation of Cytochrome P-450j, a High-Affinity *N*-Nitrosodimethylamine Demethylase, in Rat Hepatic Microsomes

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ABSTRACT: Polyclonal antibodies were produced in rabbits against purified cytochrome P-450j isolated from isoniazid-treated adult male rats. The monospecificity of immunoadsorbed antibody to cytochrome P-450j was demonstrated by Ouchterlony double diffusion analyses, enzyme-linked immunosorbent assays, and immunoblots. Immunoquantitation results indicated that rat liver microsomal cytochrome P-450j content decreases between 3 and 6 weeks of age in both the male and female animal. Several xenobiotics, such as Aroclor 1254, mirex, and 3-methylcholanthrene, repressed cytochrome P-450j levels when administered to male rats. Isoniazid, dimethyl sulfoxide, pyrazole, 4-methylpyrazole, and ethanol were inducers of cytochrome P-450j in rat liver although these compounds showed different inducing potencies. Microsomes from adult male rats with chemically induced diabetes also contained elevated levels of cytochrome P-450j compared to untreated animals. Cytochrome P-450j levels were measurable in kidney, whereas this isozyme was barely detectable in lung, ovaries, and testes; however, extrahepatic cytochrome P-450j was inducible by isoniazid. Approximately 80–90% of microsomal *N*-nitrosodimethylamine demethylation was inhibited by antibody to cytochrome P-450j whether the microsomes were isolated from untreated rats or animals administered inducers or repressors of cytochrome P-450j. The residual catalytic activity resistant to antibody inhibition may be a reflection of the inaccessibility of a certain amount of cytochrome P-450j due to interference by NADPH–cytochrome P-450 reductase based on results obtained with the reconstituted system. There was a good correlation ($r^2 = 0.87$) between cytochrome P-450j content and *N*-nitrosodimethylamine demethylase activity in microsomes from rats of different ages and treated with various xenobiotics. The evidence presented indicates that cytochrome P-450j is the primary, and perhaps sole, microsomal catalyst of *N*-nitrosodimethylamine demethylation at substrate concentrations relevant to hepatocarcinogenesis induced by *N*-nitrosodimethylamine.

Cytochrome P-450j has recently been purified from rats treated with either isoniazid or ethanol and shown to be orthologous to cytochrome P-450 LM3a purified from ethanol- or imidazole-treated rabbits (Ryan et al., 1985, 1986; Koop et al., 1982). Purified cytochrome P-450j, like isozyme 3a (Morgan et al., 1982), is an efficient catalyst of aniline and butanol oxidation. Furthermore, metabolism of these substrates by hepatic microsomes from ethanol- or isoniazid-treated rats is primarily cytochrome P-450j mediated on the basis of inhibition studies with antibody against rabbit isozyme 3a (Ryan et al., 1986).

The potent hepatocarcinogen *N*-nitrosodimethylamine (NDMA)[1] requires metabolic activation to exert its toxic and carcinogenic effects. The decisive bioactivation step, α-hydroxylation to yield formaldehyde and an unstable intermediate, has been reported to be cytochrome P-450 mediated and is the rate-limiting step in activation (Preussmann & Stewart, 1984). Demethylation of NDMA by hepatic microsomes has been proposed on the basis of kinetic analyses (Lake et al., 1974; Arcos et al., 1977; Sipes et al., 1978; Tu et al., 1981) to proceed via a high-affinity, low-K_m enzyme and a low-affinity, high-K_m enzyme. These two enzymes are subject to distinct regulatory controls since they respond differently to induction. The low-K_m enzyme is induced by compounds such as ethanol, pyrazole, acetone, and 2-propanol but is repressed by inducers of the high-K_m enzyme such as phenobarbital, pregnenolone 16α-carbonitrile, 3-methylcholanthrene, and Aroclor 1254 (Sipes et al., 1973, 1978; Tu et al., 1981, 1983; Arcos et al., 1975; Frantz & Malling, 1975; Lake et al., 1976; Mostafa et al., 1981; Garro et al., 1981; Peng et al., 1982; Evarts et al., 1982). On the basis of concentrations of NDMA found in liver, only the high-affinity, low-K_m enzyme plays a significant role in demethylation in vivo (Argus & Arcos, 1978; Kuntz et al., 1978; Magee, 1980). Among 11 rat liver isozymes purified in this laboratory, only cytochrome P-450j has high activity toward demethylation of NDMA at low substrate concentrations (0.5–5.0 mM) (Levin et al., 1986). The $K_{m_{app}}$ of cytochrome P-450j in the reconstituted system with cytochrome b_5 (0.56 mM) approaches that obtained with microsomes from ethanol-treated rats (0.35 mM) (Levin et al., 1986). Tu and Yang (1985) have reported that a partially purified preparation of cytochrome P-450 from ethanol-treated rats, which may correspond to cytochrome P-450j, has high *N*-demethylase activity toward NDMA. Ethanol-inducible cytochrome P-450 LM3a has been shown to have the highest NDMA demethylase activity of six purified rabbit liver cytochromes P-450 (Yang et al., 1985).

This report describes experiments designed to clarify the regulation of cytochrome P-450j and its role in microsomal NDMA demethylation. Polyclonal antibody to cytochrome P-450j has been produced and made monospecific by appropriate absorption chromatography. Cytochrome P-450j has been quantified in hepatic microsomes from male and female rats of different ages as well as animals treated with several compounds. The cytochrome P-450j content and catalytic activity of extrahepatic tissues have also been examined.

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[1] Abbreviations: NDMA, *N*-nitrosodimethylamine; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 0.2 mM EDTA, and 10 mM sodium phosphate buffer, pH 7.4); IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid.

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Finally, the demonstration of cytochrome P-450j as a high-affinity, low-K_m NDMA demethylase in microsomes has been evaluated by antibody inhibition studies.

Experimental Procedures

Chemicals. Imidazole was purchased from Sigma Chemical Co., and 4-methylpyrazole hydrochloride was obtained from Aldrich Chemical Co. Dimethyl sulfoxide was purchased from Burdick and Jackson, Inc., Muskegon, MI. The sources for the remaining chemicals have been reported previously (Thomas et al., 1981, 1983; Bandiera et al., 1986).

Xenobiotic Treatment of Rats and Preparation of Microsomes. Long Evans rats (Blue Spruce Farms, Altamont, NY) were maintained on a 12-h light cycle in wire-bottom cages or on granulated corn cob bedding. Rats were allowed free access to water and Ralston Purina Rodent Chow 5001.

Immature (50–60 g, approximately 4 weeks old) or adult (170–300 g, 7–10 weeks old) male rats were treated with the following xenobiotics at the doses listed: imidazole (200 mg kg^{-1} day^{-1}), phenobarbital (75 mg kg^{-1} day^{-1}), pyrazole (200 mg kg^{-1} day^{-1}), 4-methylpyrazole (200 mg kg^{-1} day^{-1}), and SKF-525A (75 mg kg^{-1} day^{-1}) were each dissolved in water; 3-methylcholanthrene (25 mg kg^{-1} day^{-1}), pregnenolone 16α-carbonitrile (25 mg kg^{-1} day^{-1}), *trans*-stilbene oxide (300 mg kg^{-1} day^{-1}), γ-chlordane (50 mg kg^{-1} day^{-1}), clofibrate (320 mg kg^{-1} day^{-1}), Kepone (20 mg kg^{-1} day^{-1}), phenothiazine (100 mg kg^{-1} day^{-1}), mirex (50 mg kg^{-1} day^{-1}), and triacetyloleandomycin (500 mg kg^{-1} day^{-1}) were dissolved in corn oil. Compounds were administered by intraperitoneal injection on 3 or 4 consecutive days with all rats killed 24 h after the last treatment. Dimethyl sulfoxide was administered intraperitoneally at 2 mL (neat) kg^{-1} twice per day for 4 days. Aroclor 1254 (300 mg kg^{-1}), dissolved in corn oil, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (10 μg kg^{-1}), dissolved in dioxane (33.3 μg mL^{-1}), were each given as a single intraperitoneal injection 4 days before death. Cholestyramine was added to pulverized Ralston Purina Rodent Chow 5001 (3% w/w) for 1 week prior to death while controls received the same diet without the drug. Streptozotocin (90 mg kg^{-1}), dissolved in 0.1 M citrate, pH 4.5, was given as a single intraperitoneal injection to 5 week old rats, and animals with a positive urinary glucose >250 mg/dL were killed 3 weeks later. Isoniazid was administered in the drinking water (0.1% w/v) for 10 days. Ethanol was administered in liquid diet (6.4% v/v) as per the Lieber and De Carli (1970) method for 3 weeks before death. Control animals were fed an equivalent volume of isocaloric liquid diet minus ethanol.

Hepatic and extrahepatic microsomes from untreated and treated rats were prepared as described previously (Thomas et al., 1983). Total microsomal cytochrome P-450 was determined from the CO-reduced difference spectrum as described (Omura & Sato, 1964). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Purification of Rat Hepatic Microsomal Enzymes. Cytochromes P-450a–P-450k were purified to apparent electrophoretic homogeneity from Long Evans rats (Blue Spruce Farms, Altamont, NY) as previously reported (Ryan et al., 1979, 1980, 1982, 1984, 1985; Bandiera et al., 1986). NADPH–cytochrome P-450 reductase was purified from phenobarbital-treated rats to a specific activity of 40 000 nmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$ at 22 °C by the method of Dignam and Strobel (1975) through their Sephadex A-25 column step followed by affinity chromatography as described by Yasukochi and Masters (1976). The concentration of the reductase was determined from the A_{456} (E = 21.4 mM^{-1} cm^{-1}) of the oxidized enzyme (French & Coon, 1979).

Preparation of Monospecific Anti-P-450j. Antibodies to electrophoretically homogeneous cytochrome P-450j were raised in rabbits by previously described methods (Thomas et al., 1981; Ryan et al., 1986). IgG was purified from a pool of heat-inactivated high-titer antisera derived from multiple bleedings of several rabbits as described (Thomas et al., 1976). IgG concentration was determined spectrophotometrically at 280 nm, $E^{1\%}_{1cm}$ = 13, in PBS. As originally purified (i.e., before absorption), this antibody cross-reacted weakly with several cytochrome P-450 isozymes (cf. Table I and Figures 1 and 2). In a manner analogous to that used to make other antibodies monospecific (Thomas et al., 1981; Reik et al., 1982; Bandiera et al., 1986), we looked for a suitable absorbent which would remove the antibodies to cross-reacting proteins with a minimal loss of antibodies against cytochrome P-450j.

Liver microsomes from rats treated with Aroclor 1254 were used as an absorbent since they contained very little cytochrome P-450j but did contain cross-reacting cytochrome P-450 isozymes. These liver microsomes were detergent solubilized and immobilized by absorption to a column of *n*-octyl-Separose 4B as previously described (Kamataki et al., 1983). Anti-P-450j which passed through this column (column equilibration buffer: 0.1 M potassium phosphate, pH 7.4, with 1 mM EDTA) did not recognize any of the cross-reacting cytochrome P-450 isozymes except cytochrome P-450i and to a lesser extent cytochrome P-450f. To further absorb anti-P-450j, a partially purified fraction of cytochrome P-450i was isolated from detergent-solubilized mature female rat liver microsomes (Bandiera et al., 1986). Partially purified cytochrome P-450i was bound to a column packed with *n*-octyl-Sepharose 4B which was used to further absorb the anti-P-450j. This final absorption chromatography step was most effective when carried out in low ionic strength buffer (5 mM potassium phosphate buffer, pH 7.4, 65 mM NaCl, and 0.1 mM EDTA). While anti-P-450j was not absorbed against cytochrome P-450f, the absorption against cytocrome P-450i also removed the weak cross-reaction with cytochrome P-450f because the cross-reacting antibody recognized immunochemically identical epitopes on these two isozymes. This absorbed anti-P-450j is monospecific by the criteria to be described.

Other Assays. Agarose Ouchterlony double diffusion plates were prepared without detergent as described (Thomas et al., 1981). SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) in a 7.5% acrylamide separating gel which was 0.75 mm thick. The SDS gels were stained for protein, or proteins were transferred to nitrocellulose and probed with antibody by using procedures described previously (Thomas et al., 1984). Quantitative determinations of cytochrome P-450j in microsomal samples were performed by competitive ELISA using monospecific anti-P-450j in an analogous manner to that developed for the determinations of cytochromes P-450f and P-450g (Bandiera et al., 1986) except that the concentration of anti-P-450j was 10 μg/mL. The metabolism of [^{14}C]NDMA to H^{14}CHO was determined by the method of Hutton et al. (1979) as modified by Hawke and Welch (1985). Additional details of the assay including concentrations of components used in the microsomal and cytochrome P-450j reconstituted systems have been described (Levin et al., 1986).

Results and Discussion

Characterization of Anti-P-450j. Antibodies to highly purified cytochrome P-450j were produced in New Zealand

Table I: ELISA Reactivity of Anti-P-450j with 11 Cytochrome P-450 Isozymes before and after Absorption To Remove Cross-Reacting Antibody[a]

anti-P-450j		purified rat liver cytochrome P-450 (% of cytochrome P-450j)							
treatment	concn (μg/mL)	P-450j	P-450a	P-450b–P-450e	P-450f	P-450g	P-450h	P-450i	P-450k
before absorption	2	100	18	<3	8	10	10	16	8
after absorption (monospecific)	4	100	<3	<3	<3	<3	<3	<3	<3

[a] Antibodies were prepared and noncompetitive ELISA was performed as described under Experimental Procedures. The level of binding of antibody to P-450j was set at 100%. The absorbance values at 490 nm for 100% reaction with antibody before and after absorption were 1.5 and 1.1, respectively. A reaction of less than 3% of that obtained with cytochrome P-450j is considered negative.

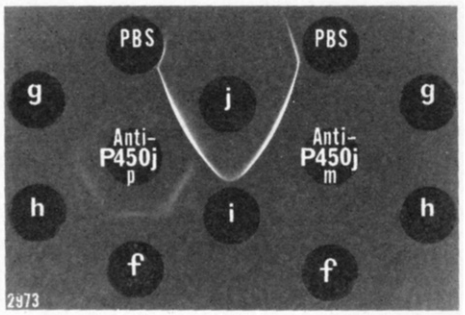

FIGURE 1: Ouchterlony double diffusion analysis of the specificity of antibodies against cytochrome P-450j. The central well on the left was filled with anti-P-450j (16 mg of IgG/mL) before absorption (p, polyspecific) whereas the central well on the right contained anti-P-450j (36 mg of IgG/mL) after absorption (m, monospecific). Peripheral wells were filled with purified cytochrome P-450 isozymes corresponding to the letters in the wells. Purified cytochromes P-450 were present at 5 μM except cytochrome P-450j which was present at 9 μM. Wells marked "PBS" were filled with phosphate-buffered saline. The agarose gel (0.9%) was formulated without detergent, and the wells were filled with 11 μL of the indicated solution.

white rabbits, and IgG was isolated from high-titer antisera. The specificity of anti-P-450j was tested in Ouchterlony double diffusion plates prepared in the absence of detergent to maximize cross-reactions with heterologous proteins (Figure 1). Prior to immunoabsorption, anti-P-450j formed a single, strong precipitin band with the antigen of immunization and also reacted with the immunochemically related family of cytochromes P-450f–P-450i, but not P-450k. Cytochromes P-450b–P-450e were not recognized by anti-P-450j (data not shown). The recognition of cytochrome P-450a by anti-P-450j was observed in Ouchterlony plates when stained for protein but not when examined under dark-field illumination (results not shown). If detergent (0.2% Emulgen 911) was included in the immunodiffusion medium, no cross-reactions were detected with any heterologous isozymes (data not shown). After immunoabsorption as detailed under Experimental Procedures, anti-P-450j reacted only with cytochrome P-450j (Figure 1). These results indicate that the cross-reactions of anti-P-450j with cytochromes P-450f–P-450i were eliminated by immunoabsorption, and the final antibody preparation appeared to be specific for cytochrome P-450j.

The monospecificity of anti-P-450j after immunoabsorption was also evaluated in a noncompetitive ELISA, as illustrated in Table I. Before absorption, the levels of binding of cross-reactive isozymes (cytochromes P-450a, P-450f–P-450i, and P-450k) to anti-P-450j were 8–18% of the level observed with cytochrome P-450j. No significant binding (<3%) was detected between anti-P-450j and cytochromes P-450b–P-450e. Although no cross-reaction was detected between anti-P-450j and cytochrome P-450k in Ouchterlony immunodiffusion plates, the antibody did recognize this isozyme in an ELISA. The basis of this difference is unknown, but such differences in the immunoreactivity of cytochrome P-450 isozymes in various assays have been reported previously (Reik et al., 1985). More importantly, however, after immunoabsorption, monospecific anti-P-450j showed significant binding only to cytochrome P-450j. The extent of binding of the absorbed anti-P-450j to the 10 heterologous isozymes was <3% of that observed with the antigen. Therefore, by the ELISA data listed in Table I, anti-P-450j is monospecific after immunoabsorption. To achieve monospecificity, there was a significant loss of antibody as shown in Table I. Before absorption, an A_{490} of 1.5 was attained with 2 μg of IgG/mL whereas after absorption 4 μg of IgG/mL was necessary to obtain an A_{490} of 1.1 in an ELISA.

Although after immunoabsorption anti-P-450j did not react with any of the heterologous purified cytochromes P-450 in Ouchterlony plates or ELISA, additional experiments were conducted to determine if other microsomal proteins were recognized by the antibody. Hepatic microsomes from control or treated rats were electrophoresed in SDS gels, transferred to nitrocellulose, and probed with anti-P-450j, yielding the results shown in Figure 2. The left portion of Figure 2 shows the mobilities of purified cytochromes P-450a, P-450f–P-450i, and P-450j, as well as the microsomal preparations stained for protein. The microsomes had been isolated from control adult female and adult male rats, and adult males treated with either Aroclor 1254 or ethanol. Before absorption, anti-P-450j recognized the heterologous cytochromes P-450 as well as the corresponding isozymes in microsomes (center portion, Figure 2). An additional microsomal protein of lower molecular weight was detected in the sample from ethanol-treated rats. However, as shown in the right portion of Figure 2, immunoabsorbed anti-P-450j reacted only with purified cytochrome P-450j and a single band in microsomes whose mobility corresponds to the antigen. These results provide additional evidence for the monospecificity of anti-P-450j after immunoabsorption. Since the staining intensity of the band is a reflection of the amount of cytochrome P-450j, microsomes from adult male and female control rats contain approximately equivalent amounts of this isozyme. Interestingly, the level of cytochrome P-450j in microsomes from Aroclor 1254 treated male rats appears to be significantly repressed relative to the other microsomal preparations. Ethanol treatment of adult male rats results in an induction of cytochrome P-450j so that less microsomal protein (2 μg) was applied to the gels for comparison to the level of cytochrome P-450j seen in microsomes (5 μg of protein) from control rats.

Regulation of Cytochrome P-450j. Monospecific anti-P-450j was used in a competitive ELISA to determine the levels of cytochrome P-450j in hepatic microsomes from untreated male and female rats with the results illustrated in Figure 3. Levels of cytochrome P-450j are expressed both as a percent of total spectral cytochrome P-450 and as the picomoles of cytochrome P-450j per milligram of microsomal protein. The cytocrome P-450j content in male rats (Figure 3, part A) was highest in the 3-week-old rat (158 pmol/mg, approximately 24% of total cytochrome P-450) and declined dramatically to slightly less than 8% of total cytochrome P-450 (43 pmol/mg) by 8 weeks of age. Thereafter, levels of this isozyme remained relatively constant. In Figure 3, part B, the results of im-

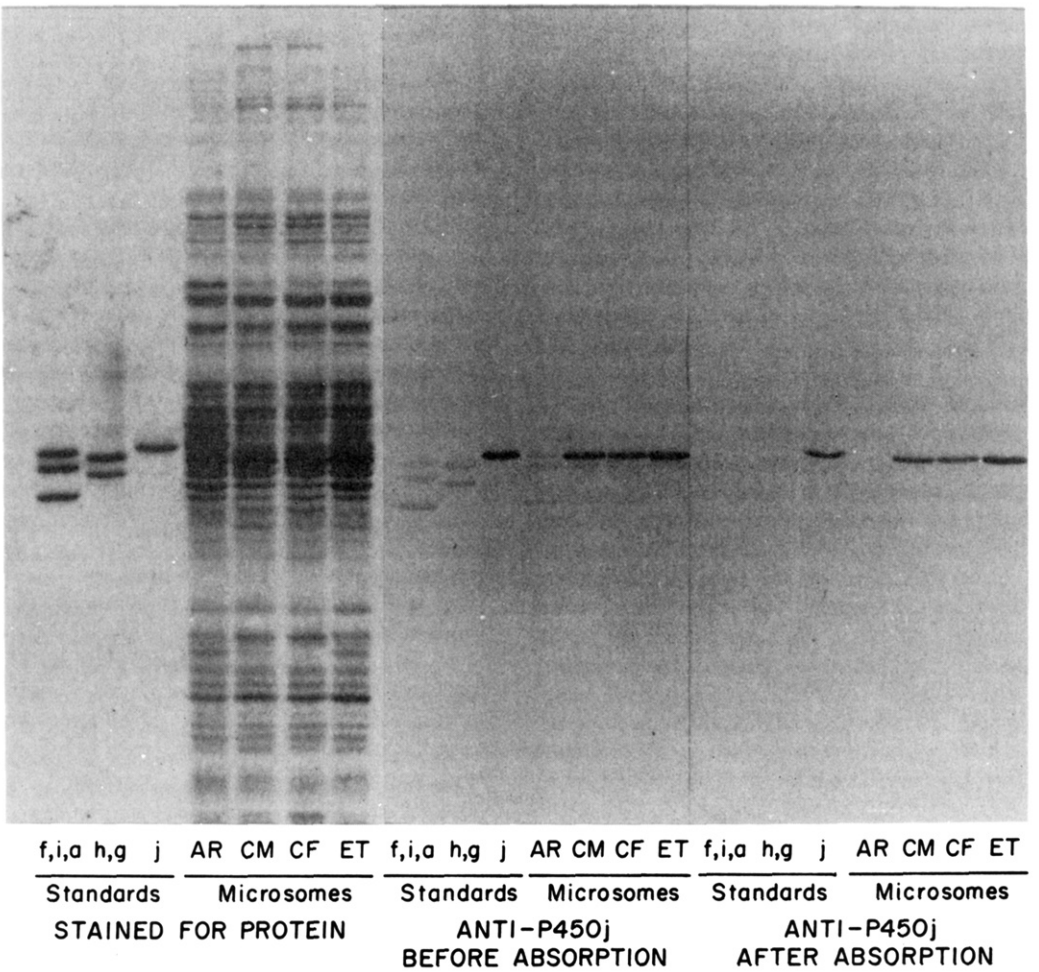

FIGURE 2: Protein-stained SDS gel and blots probed with anti-P-450j. The SDS–polyacrylamide gel on the left was stained with Coomassie Blue R250, whereas the blots in the center and right were probed with anti-P-450j before absorption (5 μg of IgG/mL) and after absorption (10 μg of IgG/mL), respectively. The lower case letters are arranged left to right to correspond to the purified cytochrome P-450 standards in that track from top to bottom, respectively. All cytochrome P-450 standards were present at 0.2 μg of protein/track except cytochrome P-450j which was present at 0.05 μg of protein/track on the immunoblots. The abbreviations for liver microsomes from xenobiotic-treated rats are as follows: AR, Aroclor 1254 treated; CM, control male (untreated); CF, control female (untreated); and ET, ethanol treated. The liver microsomes were all present at 5 μg of protein/track except for liver microsomes from ethanol-treated rats where 2 μg of protein/track was used for the blots only.

munoquantitation of cytochrome P-450j in hepatic microsomes from female rats also indicated an age-dependent decrease in the level of this isozyme. Microsomes from 3-week-old females contained approximately 174 pmol of cytochrome P-450j/mg which was about 21% of total cytochrome P-450. Subsequently, the level of cytochrome P-450j declined with age to 84 pmol/mg (approximately 10–11% of total cytochrome P-450) at 12 weeks of age. Although there were slight differences in the rates of decrease in the content of cytochrome P-450j with age between males and females, expression of cytochrome P-450j is not markedly sex dependent. The amount of cytochrome P-450j in hepatic microsomes is, however, age dependent in both sexes.

The effects of treatment of immature male rats with several xenobiotics on the expression of microsomal cytochrome P-450j are listed in Table II. Included in Table II are the total picomoles of cytochrome P-450 per milligram of microsomal protein and the cytochrome P-450j content expressed as the percent of total spectral cytochrome P-450 as well as the picomoles of cytochrome P-450j per milligram of microsomal protein. When immature male rats were given Aroclor 1254, pregnenolone 16α-carbonitrile, chlordane, 3-methylcholanthrene, SKF-525A, triacetyloleandomycin, or phenothiazine, a marked induction in total microsomal cytochrome P-450 was observed accompanied by a repression of cytochrome P-450j levels. In animals treated with any of these compounds, less than 3% of total microsomal cytochrome P-450 was cytochrome P-450j (22–57 pmol of cytochrome P-450j/mg of protein), whereas in untreated male rats at 4–5 weeks of age, cytochrome P-450j constituted 11% of total cytochrome P-450 (91 pmol of cytochrome P-450j/mg of protein). The repression of cytochrome P-450j was especially apparent in rats treated with the polychlorinated biphenyl mixture Aroclor 1254, in which the greatest induction of total cytochrome P-450 and the lowest level of cytochrome P-450j were observed among the compounds tested. The repressive effect of Aroclor 1254 on cytochrome P-450j content was also visually apparent in the immunoblots shown in Figure 2.

As listed in Table II, total microsomal cytochrome P-450 was slightly decreased in rats fed cholestyramine. Whereas cholestyramine exerted a repressive effect on the amount of cytochrome P-450j (49 pmol/mg of protein), the value obtained with the control diet (73 pmol/mg of protein) approached the levels of this isozyme found in untreated animals. A range of 61–82 pmol of cytochrome P-450j/mg of protein (3.6–8.8% of total microsomal cytochrome P-450) was observed in rats administered chlorpromazine, phenobarbital, *trans*-stilbene oxide, Kepone, or clofibrate. This range of values was also somewhat lower than that determined for the untreated animal.

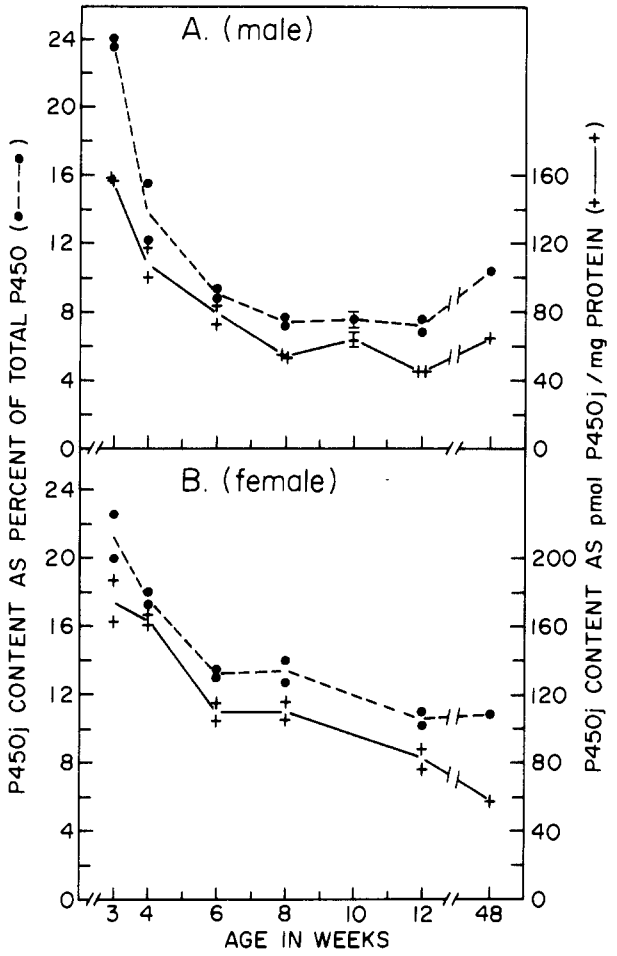

FIGURE 3: Immunoquantitation of hepatic cytochrome P-450j in male and female rats of different ages. A competitive ELISA was used to determine the levels of cytochrome P-450j, and these results are expressed in two ways. The scale on the left ordinate is given in cytochrome P-450j as a percent of the total spectrally determined microsomal cytochrome P-450. The scale on the right ordinate is in units of picomoles of cytochrome P-450j per milligram of microsomal protein. For each point, microsomes from a pool of two to four livers were immunoquantitated with one exception. In part A, the calculation for the amount of cytochrome P-450j in 10-week-old males was derived from immunoquantitation of microsomes from nine individual rats.

Interestingly, imidazole was not an inducer of cytochrome P-450j in the immature male rat. This compound has been demonstrated to be an effective inducer of isozyme 3a in the rabbit (Koop & Coon, 1984; Koop et al., 1985), an isozyme shown previously to be orthologous to cytochrome P-450j (Ryan et al., 1986). Therefore, the inducing capability of imidazole is apparently species dependent as had been suggested in an earlier study by Reinke et al. (1985).

As shown in Table II, total microsomal cytochrome P-450 and cytochrome P-450j levels are essentially the same in corn oil treated as in untreated immature male rats. Isoniazid and pyrazole induce cytochrome P-450j levels approximately 3.6–4.5-fold relative to the control level when expressed in picomoles per milligram of protein. No increase in total microsomal cytochrome P-450 was observed when immature male rats were administered isoniazid. Therefore, the induction of cytochrome P-450j by isoniazid to 40% of the total microsomal cytochrome P-450 (330 pmol of cytochrome P-450j/mg of protein) is probably accompanied by repression of other cytochrome P-450 isozymes. The highest level of cytochrome P-450j (410 pmol/mg of protein) was observed in microsomes from immature male animals treated with pyrazole. Cytochrome P-450j constituted 28% of the total microsomal cytochrome P-450 in immature male rats administered pyrazole.

During maturation, the amount of cytochrome P-450j in hepatic microsomes decreases in the untreated rat (Figure 3).

Table II: Immunochemical Quantitation of Cytochrome P-450j in Hepatic Microsomes from Immature Male Rats Treated with Various Xenobiotics[a]

rat treatment	pmol of P-450/mg of protein: total	pmol of P-450/mg of protein: P-450j	P-450j % of total P-450
untreated, n = 7	840 ± 35	91 ± 9	11 ± 0.7
Aroclor 1254, n = 4	2500 ± 310	22 ± 8	1.1 ± 0.5
pregnenolone 16α-carbonitrile	1400	33	2.3
γ-chlordane	1900	41	2.2
3-methylcholanthrene, n = 2	2100	44	2.1
SKF-525A	1800	46	2.5
cholestyramine in diet	640	49	7.6
control diet for cholestyramine	690	73	11
triacetyloleandomycin	2300	53	2.3
phenothiazine	2300	57	2.5
chlorpromazine	970	61	6.3
phenobarbital, n = 2	1800	65	3.6
trans-stilbene oxide	1600	66	4.2
kepone	1400	70	5.0
clofibrate	930	82	8.8
imidazole	1100	100	9.4
corn oil, n = 2	920	120	13
isoniazid	820	330	40
pyrazole	1500	410	28

[a] Levels of cytochrome P-450j were determined by competitive ELISA as described under Experimental Procedures. Immature male rats were 4–5 weeks old when killed. Each determination was made on microsomes prepared from a pool of two or more rat livers. When more than one pool was used, "n" indicates the number of pools averaged ± the standard error of the mean. The table was arranged in the order of increasing levels of cytochrome P-450j (pmol/mg of protein) following administration of the various compounds.

Table III: Immunochemical Quantitation of Cytochrome P-450j in Hepatic Microsomes from Adult Male Rats Treated with Various Xenobiotics[a]

rat treatment	pmol of P-450/mg of protein: total	pmol of P-450/mg of protein: P-450j	P-450j % of total P-450
untreated, n = 11	880 ± 35	66 ± 3.7	7.6 ± 0.4
mirex	1000	13	1.2
3-methylcholanthrene	1400	26	1.9
2,3,7,8-tetrachlorodibenzo-p-dioxin	1300	26	2.0
phenobarbital, n = 2	2000	37	1.9
triacetyloleandomycin	1500	42	2.8
pregnenolone 16α-carbonitrile	1400	47	3.3
cholestyramine	840	48	5.7
clofibrate	1000	50	5.0
kepone	1900	52	2.7
corn oil	830	54	6.5
streptozotocin	760	130	17
pyrazole	970	200	20
dimethyl sulfoxide	890	210	23
4-methylpyrazole	1600	230	14
isoniazid (in drinking water), n = 3	960 ± 100	310 ± 36	33 ± 5
ethanol, n = 4	1700 ± 180	520 ± 48	30 ± 0.6
control diet for ethanol, n = 4	940 ± 40	48 ± 4.5	5.2 ± 0.4

[a] Levels of cytochrome P-450j were determined by competitive ELISA as described under Experimental Procedures. Male Long Evans rats between the ages of 7 and 10 weeks were used, and each determination was made on microsomes prepared from a pool of two or more rat livers. When more than one pool of livers were used, "n" indicates the number of pools averaged ± the standard error of the mean. The table was arranged in the order of increasing levels of cytochrome P-450j (pmol/mg of protein) following administration of the various compounds.

The ability of several xenobiotic treatments to alter the expression of cytochrome P-450j was also studied in adult male rats with the results listed in Table III. As was observed in the study with immature animals (Table II), administration of several of the xenobiotics to adult male rats resulted in lower amounts of cytochrome P-450j relative to the control level. Treatment of adult male rats with mirex, a chlorinated insecticide, resulted in the most dramatic repression of cytochrome P-450j of the compounds studied. Hepatic microsomes isolated from mirex-treated animals contained 13 pmol of cytochrome P-450j/mg of protein compared to a level of 66 pmol of cytochrome P-450j/mg of protein in the untreated rat. Cytochrome P-450j content was decreased to varying degrees by the administration of 3-methylcholanthrene, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, phenobarbital, triacetyloleandomycin, pregnenolone 16α-carbonitrile, cholestyramine, clofibrate, or Kepone to adult male rats.

Although total microsomal cytochrome P-450 was affected differently by treatment of rats with streptozotocin, pyrazole, dimethyl sulfoxide, 4-methylpyrazole, isoniazid, and ethanol, each of these compounds was an inducer of cytochrome P-450j. The microsomal sample from streptozotocin-treated rats was prepared from animals that had developed chemically induced diabetes. Cytochrome P-450j constituted 17% of the total microsomal cytochrome P-450 in diabetic animals. Treatment of adult male rats with dimethyl sulfoxide resulted in an induction of cytochrome P-450j but not total microsomal cytochrome P-450 content.[2] Whereas isoniazid induced the level of cytochrome P-450j approximately 3.5-fold in the immature rat (Table II), a 4.5-fold induction of this isozyme (expressed as picomoles of cytochrome P-450j per milligram of protein) was observed in the adult animal (Table III). Although total spectral cytochrome P-450 content was higher in microsomes from 4-methylpyrazole-treated rats (1600 pmol/mg of protein) compared to microsomes from pyrazole-treated rats (970 pmol/mg of protein) as previously described (Krikun et al., 1986), both compounds resulted in similar elevated levels of cytochrome P-450j (200–230 pmol/mg of protein). The administration of ethanol to adult animals resulted in the highest microsomal cytochrome P-450j content (approximately 520 pmol/mg of protein) of the compounds tested. The cytochrome P-450j content, expressed in picomoles per milligram of protein, was induced approximately 10-fold in ethanol-treated rats compared to the control diet. Cytochrome P-450j has been purified to homogeneity from adult male rats treated with either isoniazid or ethanol (Ryan et al., 1986). Pyrazole, isoniazid, and ethanol are effective inducers of cytochrome P-450j and isozyme 3a in the rat and rabbit, respectively, in contrast to the results with imidazole (Ingelman-Sundberg & Jornvall, 1984; Koop et al., 1985).

Role of Cytochrome P-450j in Microsomal NDMA Demethylation. Purified cytochrome P-450j has been shown previously to be an efficient catalyst of NDMA demethylation at substrate concentrations ranging from 0.5 to 5.0 mM, whereas 10 other purified isozymes (cytochromes P-450a–P-450i and P450-k) have little or no activity toward this substrate (Levin et al., 1986). Anti-P-450j was, therefore, used to evaluate the contribution of cytochrome P-450j to NDMA metabolism in microsomes from untreated or ethanol-treated rats with the results illustrated in Figure 4. NDMA demethylation in either microsomal preparation was markedly inhibited up to a maximum of 90% by anti-P-450j, indicating that cytochrome P-450j is the primary catalyst of this reaction. Lower amounts of anti-P-450j were necessary to inhibit the reaction in microsomes from untreated rats than required for inhibition of metabolism in microsomes from ethanol-treated rats. This difference is a reflection of the different amounts of cytochrome P-450j in the two microsomal preparations, since ethanol is an effective inducer of this isozyme (Table III). Approximately 10–15% of NDMA demethylation was not inhibited by anti-P-450j in either microsomal sample even at relatively high antibody concentrations. This residual activity could be due to the presence of other cytochrome(s) P-450 that metabolize NDMA or to the inaccessibility of a certain amount of microsomal cytochrome P-450j to the antibody.

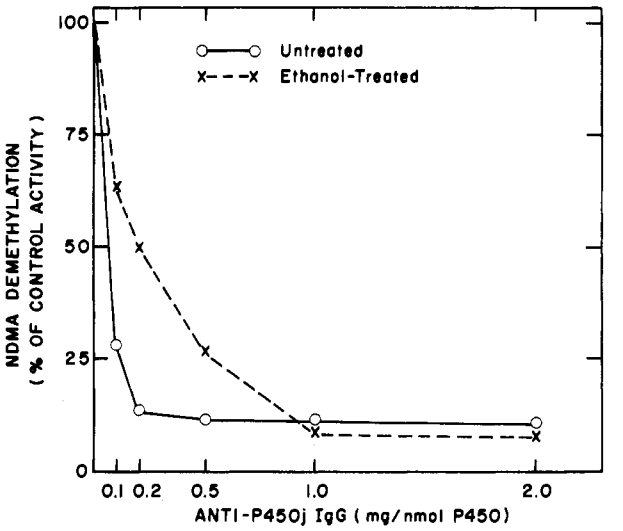

FIGURE 4: Effect of anti-P-450j on NDMA demethylation by rat liver microsomes. Liver microsomes from untreated or ethanol-treated rats (0.25 nmol of total cytochrome P-450) were preincubated 10 min at 22 °C with increasing amounts of anti-P-450j, and then metabolism was assayed at 1 mM [^{14}C]NDMA for 10 min at 37 °C as described under Experimental Procedures. The turnover numbers (in the presence of control IgG) for liver microsomes from untreated and ethanol-treated rats were 0.36 and 3.44 nmol of formaldehyde min^{-1} (nmol of P450)$^{-1}$, respectively.

Table IV: Anti-P-450j Inhibition of NDMA Metabolism Catalyzed by Rat Liver Microsomes

treatment, age (weeks), and sex	NDMA (1 mM) metabolism [nmol of HCHO min^{-1} (nmol of P-450)$^{-1}$] control IgG	anti-P-450j	% of control
untreated, 3, male	1.86 (1.25)[a]	0.29	16
untreated, 3, female	2.01 (1.63)	0.23	11
untreated, 4, male	0.97 (0.90)	0.12	12
Aroclor 1254, 4, male	0.13 (0.28)	0.02	15
untreated, 8, male	0.66 (0.47)	0.10	15
untreated, 8, female	0.88 (0.73)	0.11	13
phenobarbital, 8, male	0.19 (0.36)	0.05	24
3-methylcholanthrene, 8, male	0.27 (0.37)	0.04	14
pregnenolone 16α-carbonitrile, 8, male	0.28 (0.40)	0.05	17
pyrazole, 8, male	3.72 (3.61)	0.41	11
4-methylpyrazole, 8, male	2.01 (3.21)	0.23	12
ethanol, 8, male	3.44 (5.09)	0.36	10
isoniazid, 8, male	3.51 (3.54)	0.40	11
dimethyl sulfoxide, 8, male	1.84 (1.63)	0.20	11

[a] The numbers in parentheses give the nanomoles of HCHO formed per minute per milligram of microsomal protein. Both control IgG (nonimmune) and anti-P-450j were added at 1.5 mg of IgG/nmol of cytochrome P-450. NDMA metabolism was effected for 10 min at 37 °C as described under Experimental Procedures.

[2] The observation that dimethyl sulfoxide is an inducer of cytochrome P-450j probably explains early observations that treatment of rats with this compound resulted in increased hepatic metabolism of aniline (Stock et al., 1970) and NDMA (Argus et al., 1980), both of which are substrates for this isozyme.

Table V: Effect of Order of Combining Anti-P-450j with Cytochrome P-450j, NADPH–Cytochrome P-450 Reductase, and Lipid on NDMA Metabolism

condition	order of addition[a]	IgG	HCHO formed [nmol min^{-1} (nmol of P-450)$^{-1}$]	% of control
1	IgG, P-450, incubate 5 min, reductase, lipid, incubate 5 min	control	7.52	100
		anti-P-450j	0	0
2	P-450, lipid, IgG, incubate 5 min, reductase, incubate 5 min	control	6.42	100
		anti-P-450j	0.02	0.3
3	P-450, reductase, IgG, incubate 5 min, lipid, incubate 5 min	control	8.99	100
		anti-P-450j	0.89	10
4	P-450, reductase, lipid, IgG, incubate 10 min	control	10.25	100
		anti-P-450j	1.57	15
5	same as condition 3 except 8 mg of IgG/nmol of P-450j	control	10.81	100
		anti-P-450j	2.48	23
6	P-450, reductase, lipid, cholate, IgG, incubate 10 min	control	13.34	100
		anti-P-450j	2.27	17

[a] Components were combined at room temperature (22 °C) in the order listed from left to right in a final volume of 65 μL. The following amounts of proteins were added when indicated: 0.1 nmol of cytochrome P-450j, 0.4 mg of IgG (4 mg of IgG/nmol of P-450), 0.4 nmol of NADPH–cytochrome P-450 reductase, and 15 nmol of dilauroylphosphatidylcholine. Sodium cholate was added at a final concentration of 0.5%. After the indicated incubations, the samples were placed in ice where remaining buffers, substrate (5 mM final concentration), and NADPH were added (final volume 0.5 mL), and the assay was completed as described under Experimental Procedures.

There were two reasons for conducting the experiments shown in Table IV on anti-P-450j inhibition of NDMA metabolism catalyzed by liver microsomes from rats of different ages and treated with various xenobiotics. The first goal was to establish the potency of anti-P-450j, at a ratio of 1.5 mg of IgG/nmol of cytochrome P-450, as an inhibitor of this metabolic pathway in untreated rats and animals administered repressors or inducers of cytochrome P-450j. The results in Figure 4 indicated that this antibody to cytochrome P-450 ratio yielded maximum inhibition of microsomal NDMA metabolism. As shown in Table IV, regardless of the source of the microsomes, demethylation of NDMA was primarily cytochrome P-450j mediated since 76–90% of the catalytic activity was inhibited by the antibody. The relatively constant extents of antibody inhibition and residual activity suggested that cytochrome P-450j might be responsible for all the microsomal metabolism of this substrate. If additional cytochromes P-450 participated in the metabolism of NDMA, then microsomes from rats treated with xenobiotics having very different inducing and repressing selectivities would have shown large differences in susceptibility to antibody inhibition of catalytic activity. Furthermore, if the residual NDMA demethylase activity that is resistant to antibody were due to other cytochromes P-450, those putative enzyme contents would have to vary in concert with cytochrome P-450j levels in the microsomal samples.

The second goal of the experiments shown in Table IV was to determine if NDMA metabolism correlated with cytochrome P-450j content in the various microsomal samples. Untreated male and female rats showed an age-dependent decline in the ability to N-demethylate NDMA from 1.9–2.0 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$ at 3 weeks of age to 0.7–0.9 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$ at 8 weeks of age which corresponds to the observed decrease in cytochrome P-450j content during maturation (Figure 3). Davies et al. (1976) first noted an age-dependent decrease in NDMA metabolism by microsomes from male rats between 4 and 8 weeks of age, but the reason for this decline was unknown.

Aroclor 1254 treatment of immature rats results in repression of microsomal cytochrome P-450j content (Table II) and, from the data in Table IV, a dramatic reduction in NDMA metabolism relative to untreated animals. When expressed per milligram of protein, microsomes from Aroclor 1254 treated rats had 31% of the catalytic activity and 24% of the cytochrome P-450j content (Table II) observed in untreated animals. As was shown in Table III, the expression of cytochrome P-450j is repressed to varying extents by treatment of adult male rats with phenobarbital, 3-methylcholanthrene, or pregnenolone 16α-carbonitrile. Metabolism of NDMA (Table IV) was also depressed in microsomes from animals treated with any of these xenobiotics.

NDMA demethylation in rat liver microsomes was inducible by the same compounds, pyrazole, 4-methylpyrazole, ethanol, isoniazid, and dimethyl sulfoxide, that increased the levels of cytochrome P-450j as determined immunochemically. For example, ethanol treatment of animals resulted in the greatest induction of cytochrome P-450j content (520 pmol/mg of protein; Table III) and the highest catalytic activity [5.09 nmol of HCHO min^{-1} (mg of protein)$^{-1}$; Table IV] of any of the xenobiotics tested. Overall, the metabolism data presented in Table IV and the immunoquantitation data in Tables II and III revealed a strong correlation between hepatic levels of cytochrome P-450j and NDMA metabolism.

A plot of NDMA demethylation vs. the amount of cytochrome P-450j in microsomes (Figure 5) yielded a coefficient of determination (r^2) of 0.87. This regression line was derived from data on microsomal samples from rats of different ages and treated with various xenobiotics. Therefore, it appears that NDMA (1 mM) metabolism in liver microsomes provides a good estimate of cytochrome P-450j content. However, other factors such as NADPH–cytochrome P-450 reductase, cytochrome b_5, and/or lipid content of a microsomal sample may also influence the rate of microsomal demethylation of NDMA. Furthermore, the presence of catalytically inactive cytochrome P-450j will be detected in the immunochemical but not the catalytic assay.

As evidenced from the data in Figure 4 and Table IV, anti-P-450j did not completely inhibit microsomal NDMA metabolism, and the residual catalytic activity could be a reflection of microsomal cytochrome P-450j that was not accessible to the antibody. To investigate the basis of this possible inaccessibility, the potency of anti-P-450j as an inhibitor of NDMA demethylation in the reconstituted system containing purified cytochrome P-450j, NADPH–cytochrome P-450 reductase, and lipid was evaluated (Table V). If cytochrome P-450j was incubated with the antibody prior to the addition of other components (condition 1), complete inhibition of NDMA metabolism was observed at 4 mg of IgG/nmol of cytochrome P-450j. As shown in condition 2, all of the catalytic activity was also inhibited by anti-P-450j if cytochrome

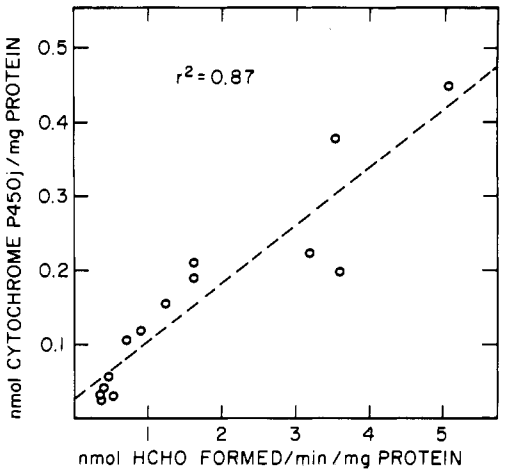

FIGURE 5: Correlation of cytochrome P-450j protein with NDMA metabolism by rat liver microsomes. The level of cytochrome P-450j was determined by competitive ELISA, and the metabolism of [^{14}C]NDMA was assayed by measuring formation of H^{14}CHO as described under Experimental Procedures.

P-450j and dilauroylphosphatidylcholine were combined prior to the addition of the antibody. Apparently, the presence of this lipid did not prevent the access of anti-P-450j to cytochrome P-450j. However, when cytochrome P-450j and NADPH–cytochrome P-450 reductase were mixed together and anti-P-450j subsequently added (condition 3), 10% of the catalytic activity was resistant to inhibition. Doubling the antibody concentration (condition 5) had no further effect on catalytic activity. Approximately 15% of the cytochrome P-450j mediated demethylation of NDMA was resistant to antibody inhibition if the three components of the reconstituted system were combined prior to the addition of anti-P-450j (condition 4). Finally, inclusion of the ionic detergent sodium cholate with the reconstituted system prior to the addition of anti-P-450j did not facilitate complete inhibition of metabolism (condition 6). Although the addition of cytochrome b_5 to the reconstituted system containing cytochrome P-450j causes a 6-fold decrease in $K_{m_{app}}$ of NDMA demethylation as previously reported (Levin et al., 1986), the presence of cytochrome b_5 did not affect the results shown in Table V (data not shown). The presence of the three components (cytochrome P-450, reductase, and lipid) prior to the addition of the antibody is analogous to the microsomal membrane, although the relative ratios of cytochrome P-450j (0.1 nmol), NADPH–cytochrome P-450 reductase (0.4 nmol), and lipid (15 nmol) are different in the two systems. Since complete inhibition could not be attained when cytochrome P-450j was mixed with the reductase alone, or with the reductase and lipid, prior to incubation with anti-P-450j, it would appear that the interaction of the cytochrome with the reductase plays an imporant role in preventing complete antibody inhibition of catalytic activity. On the basis of these results presented in Table V, complete inhibition of microsomal NDMA by antibody may not be achieved due to interference by NADPH–cytochrome P-450 reductase.

Extrahepatic Cytochrome P-450j and NDMA Metabolism. NDMA has been demonstrated to exhibit organ specificity in carcinogenesis in the rat (Magee & Barnes, 1956, 1962). Whereas long-term treatment with NDMA was associated with a high incidence of hepatic carcinomas, short-term intensive feeding with NDMA resulted in the appearance of a significant number of kidney tumors. Tumors of the lung were less commonly observed and consisted of primary pulmonary adenomas and secondary tumors from the kidney and liver

Table VI: Comparison of Cytochrome P-450j Levels and NDMA Metabolism in Extrahepatic Tissues[a]

	P-450j levels (μg of P-450j/mg of protein)	NDMA (1 mM) metabolism [pmol of HCHO min^{-1} (mg of protein)$^{-1}$]	
		control IgG	anti-P-450j
untreated female			
liver	7.1	nd[b]	
kidney	0.4	nd	
lung	≤0.05	nd	
ovaries	0.1	nd	
untreated male			
liver	7.0	nd	
kidney	0.39	29.6	5.6 (19)
lung	0.06	<5	<5
testes	0.07	<5	<5
isoniazid-treated male			
liver	32.4	nd	
kidney	1.2	158	16.4 (10)
lung	0.14	7.9	<5
testes	0.25	7.8	<5

[a] Levels of cytochrome P-450j were determined by competitive ELISA as described under Experimental Procedures on tissues from five or more rats. Data are expressed as micrograms of cytochrome P-450j per milligram microsomal protein due to difficulties in accurately determining cytochrome P-450 spectrally in extrahepatic tissues. NDMA metabolism in extrahepatic tissues was effected for 30 min at 37 °C as described under Experimental Procedures. As appropriate, control IgG or anti-P-450j was added at 0.5 mg of IgG/mg of microsomal protein. Values in parentheses are percent of control activity. [b] nd, not determined.

(Magee & Barnes, 1962). Both cytochrome P-450j content and NDMA metabolism were determined in extrahepatic tissues of the rat, and the results are shown in Table VI. The levels of cytochrome P-450j are expressed as micrograms of P450j per milligram microsomal protein because of difficulties in accurately determining cytochrome P-450 spectrally in extrahepatic tissues.

Kidney microsomes from untreated female or male rats contained measurable levels of cytochrome P-450j (0.4 μg/mg of protein), but kidney cytochrome P-450j content was much less than observed in liver (7.0 μg/mg of protein). Microsomes from lung, ovaries, or testes contained barely detectable amounts of this isozyme (≤0.05–0.1 μg of cytochrome P-450j/mg of protein). When male rats were treated with isoniazid, increased cytochrome P-450j expression was observed in all the tissues examined. Approximately a 3-fold induction in cytochrome P-450j levels were detected in kidney, lung, and testes microsomes. NDMA metabolism correlated with the isozyme content as evidenced by the data in Table VI. Kidney microsomes from untreated rats metabolized NDMA at a rate of 29.6 pmol of HCHO min^{-1} (mg of protein)$^{-1}$ whereas demethylation proceeded at 158 pmol of HCHO min^{-1} (mg of protein)$^{-1}$ in kidney microsomes from isoniazid-induced rats. Catalytic activity toward NDMA was barely measurable in lung and testes of untreated animals but easily determined in lung and testes microsomes from isoniazid-treated rats. That extrahepatic microsomal NDMA demethylation was catalyzed by cytochrome P-450j was indicated by the ability of anti-P-450j to inhibit 80–90% of NDMA metabolism.

A second approach was also used to verify the identity of extrahepatic cytochrome P-450j as illustrated in Figure 6. Hepatic microsomes (5 μg of protein) and extrahepatic microsomes from kidney, lung, and testes (60 μg of protein each) were electrophoresed in SDS gels, transferred to nitrocellulose, and probed with anti-P-450j. On the basis of relative staining intensities, hepatic microsomes contain significantly more cytochrome P-450j than kidney microsomes especially when

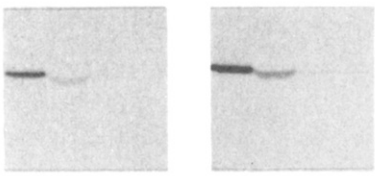

FIGURE 6: Immunoblots of hepatic and extrahepatic microsomes from isoniazid-treated and control (untreated) rats probed with anti-P-450j. The abbreviations used and the amount of microsomal protein loaded were as follows: H, hepatic (5 μg/track); K, kidney (60 μg/track); L, lung (60 μg/track); and T, testes (60 μg/track).

the difference in the amount of protein applied to the gel is taken into consideration. The results shown in Figure 6 also indicate that hepatic, kidney, lung, and testes microsomal cytochrome P-450j content is inducible by isoniazid in the rat. Whereas cytochrome P-450j could not be detected in immunoblots of control long and testes microsomes, a stained band corresponding to this isozyme was observed in these tissues from isoniazid-treated animals. It is tempting to speculate that the relative amounts of cytochrome P-450j in hepatic and extrahepatic tissues could be an important determinant in the organ specificity of NDMA as a carcinogen in the rat.

In conclusion, NDMA is a carcinogen of potential critical concern to humans who are exposed to the compound via a multitude of exogenous sources including air, water, tobacco smoke, soil, cheese, vegetables, meat, fish, alcoholic beverages, pesticides, and drugs as well as endogenous formation of the carcinogen in the digestive tract (Preussmann & Eisenbrand, 1984). Recently, a human liver cytochrome P-450, HLj, has been identified and shown to have significant structural homology to rat liver cytochrome P-450j and rabbit isozyme 3a (Wrighton et al., 1986). HLj was reported to function as the major NDMA demethylase in human liver microsomes, and levels of HLj were shown to be elevated in patients with high blood levels of ethanol and in a patient treated with isoniazid. Human liver HLj content may be relevant to several clinical consequences such as susceptibility to NDMA carcinogenesis and toxic responses to acetaminophen and carbon tetrachloride, both of which are known substrates for orthologous rabbit isozyme 3a (Morgan et al., 1983; Johansson & Ingelman-Sundberg, 1985).

Added in Proof

After submission of the manuscript, a paper appeared (Ding et al., 1986) which described the detection of isozyme 3a, the rabbit ortholog of cytochrome P-450j (Ryan et al., 1986), in kidney and nasal mucosa microsomes of untreated rabbits. They also found that kidney isozyme 3a was inducible by ethanol treatment of rabbits.

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Molecular Cloning of Human Cathepsin G: Structural Similarity to Mast Cell and Cytotoxic T Lymphocyte Proteinases†

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ABSTRACT: Human cathepsin G is a serine proteinase with chymotrypsin-like specificity found in both polymorphonuclear leukocytes (neutrophils) and the U937 leukemic cell line. Utilizing RNA from the latter, we have constructed a cDNA library in λgt11 and isolated a clone which apparently codes for the complete amino acid sequence of this enzyme. Analysis of the sequence reveals homology with rat mast cell proteinase II (47%) but a greater degree of identity (56%) with a product of activated mouse cytotoxic T lymphocytes. The close relationship between the three proteins indicates similarities in substrate specificity and in biosynthesis which we predict involves removal of a two amino acid activation peptide during or just before packaging into their respective storage granules.

In humans, the polymorphonuclear leukocyte (neutrophil) is the most abundant circulating white blood cell. Among the several properties of these cells, perhaps their most important functions are the ability to control the growth of microbial pathogens and to partake in the generation and regulation of inflammation (Lisiewicz, 1980). To accomplish these tasks, neutrophils contain specialized granules whose contents, including the serine proteinases cathepsin G (cat G)[1] and elastase, may participate in the killing and digestion of engulfed pathogens, and in connective tissue remodeling at sites of inflammation (Starkey, 1977). In the absence of controlling inhibitors, elastase appears to cause the abnormal degradation of elastin and proteoglycan, resulting in the development of pulmonary emphysema (Janoff, 1985). Cathepsin G, on the

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[1] Abbreviations: cat G, cathepsin G; RMCP II, rat mast cell proteinase II; CCP I, putative protein from mouse cytotoxic T lymphocytes; SDS, sodium dodecyl sulfate; pfu, plaque-forming unit; bp, nucleotide base pairs; SSC, 0.15 M NaCl and 0.015 M sodium citrate, pH 7; ds, double stranded; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography.

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