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IDENTIFICATION OF A HUMAN LIVER CYTOCHROME P-450 EXHIBITING CATALYTIC AND IMMUNOCHEMICAL SIMILARITIES TO CYTOCHROME P-450 3a OF RABBIT LIVER*

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Abstract—Immunoblot analysis of liver microsomes from nine patients demonstrated that each contained a cytochrome P-450 that reacted with an antibody directed against the ethanol-inducible rabbit liver cytochrome, P-450 3a. Two of the liver specimens exhibited high concentrations of the immunoreactive protein, high rates of aniline hydroxylation and *N*-nitrosodimethylamine demethylation, and extensive inhibition of activity in the presence of antibody to P-450 3a. One other liver specimen exhibited a very low rate of aniline hydroxylation with significantly less antibody inhibition. The variability witnessed was independent of the alcohol history of the individual patients, suggesting that the human cytochrome may be under some other environmental, dietary or genetic regulation. Its inducibility by ethanol was not directly studied in this investigation. However, we conclude that there is a cytochrome P-450 present in human liver which is immunochemically and catalytically similar to the ethanol-inducible P-450 of rabbit liver.

The microsomal hemoproteins, known collectively as cytochrome P-450, consist of a family of multifunctional forms encoded by distinct genes. The multiplicity of these cytochromes has been clearly established in animals by isolation and characterization [1]; however, the human liver P-450 system has not been investigated extensively. Recently, evidence was presented which confirms that human liver contains multiple forms of cytochrome P-450 which are similar to the animal cytochromes—catalytically, immunochemically, and in their responses to inducing agents [2, 3]. A recent investigation [4] determined that at least one form of human liver P-450 is induced by macrolide antibiotics and dexamethasone. It resembles the pregnenolone 16α-carbonitrile (PCN) inducible form of rat liver, P-450p, in its ability to metabolize erythromycin and form a metabolic-intermediate complex from troleandomycin (TAO). In addition, Quatrrochi *et al.* [5] demonstrated, by isolation and characterization of two different human cDNAs, that there exist at least two forms of human P-450 that are homologous to the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible forms in other species. Other investigators [3, 6, 7] have purified and characterized human liver P-450s but have not determined whether compounds known to be inducers in animals also produce induction of similar cytochromes in human liver.

Recently, several studies focused on the ethanol-inducible P-450 in various animal species. The induction of this isozyme is associated with an increase in the metabolism of aniline [8] and ethanol [9], and an increase in the toxicity of certain compounds including *N*-nitrosodimethylamine (NDMA) [10, 11] and acetaminophen [12]. The ethanol-inducible form of rabbit liver, cytochrome P-450 3a, exhibits high activity towards the conversion of acetaminophen to a reactive intermediate [13]. Clinical observations demonstrated that hepatotoxicity from large doses of acetaminophen occurs more frequently in the alcoholic than the non-alcoholic [14, 15]. This led us to hypothesize that human liver also contains an ethanol-inducible P-450 similar to rabbit P-450 3a. In this investigation, we attempted to establish whether a similar cytochrome exists in humans. We also determined whether it was immunochemically and catalytically similar to rabbit P-450 3a. Immunoquantitation of the cytochrome in a small number of human liver samples led us to the conclusion that there is variability among various individuals in the concentration of the P-450, as well as in its catalytic activity.

EXPERIMENTAL PROCEDURES

Preparation of microsomes. Human liver specimens were obtained at surgery from nine patients (six females, three males) during major hepatic resection for hepatic or colon cancer. Portions were submitted for routine histopathologic examination, while the rest (tumor-free) were transported from the operating room on ice. The samples were minced and homogenized, and microsomes were prepared immediately by the method of van der Hoeven and

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Coon [16]. Only one patient (patient #4) had a history of drug therapy, this consisting of a daily injection of NPH insulin. None of the patients consumed alcohol except on social occasions, and their ages ranged from 47 to 59.

Preparation of antibody. Antibody to cytochrome P-450 3a was raised in female sheep as previously described [17]. Purified IgG was utilized in all the experimental procedures.

Determination of catalytic activity. Aniline *p*-hydroxylation was determined in reaction mixtures containing 0.5 nmol microsomal P-450, 0.5 mM aniline and 1 mM NADPH in a total volume of 1 ml of 50 mM potassium phosphate buffer (pH 7.4). After incubation for 15 min at 37°, the reaction was stopped by addition of 0.3 ml of 20% trichloroacetic acid (TCA), and *p*-aminophenol was assayed by the method of Schenkman *et al.* [18]. Results are expressed as nmol *p*-aminophenol/min/nmol microsomal P-450.

The N-demethylation of *N*-nitrosodimethylamine (NDMA) was assayed by the radioactive formaldemethone method previously described [19, 20]. The incubation medium consisted of 100 mM potassium phosphate buffer (pH 6.5), 2 mM NADPH, 10 mM semicarbazide, 2 mM NDMA containing 0.2 μCi [^{14}C]NDMA and 0.5 nmol microsomal P-450. After a 30-min incubation at 37°, the reaction was terminated with TCA. Demethylation of NDMA was determined by measuring the H[^{14}C]HO which was precipitated by formaldemethone using 8.0 mg of the carrier formaldehyde. Radioactivity was measured by scintillation spectrometry. Results are expressed as nmol HCHO/min/nmol microsomal P-450.

Erythromycin N-demethylation was determined in a 1 ml reaction mixture containing 0.5 nmol microsomal P-450, 50 mM potassium phosphate buffer (pH 7.4), 0.5 mM erythromycin and 1 mM NADPH. The incubation proceeded for 15 min at 37° and the reaction was concluded by addition of 0.3 ml of 20% TCA. The formaldehyde formed was assayed by the method of Nash [21], and results are expressed as nmol HCHO/min/nmol microsomal P-450.

To determine the effect of the antibody to P-450 3a on aniline hydroxylation, 3 mg of either sheep anti-3a IgG or preimmune IgG per nmol microsomal P-450 was preincubated for 3 min at 37°. At the end of the preincubation, the assay components were added, and activity was determined in the presence of sheep anti-3a IgG or preimmune IgG as described.

Immunoblot analysis. Appropriate quantities of microsomes from each patient were subjected to electrophoresis on polyacrylamide gels (7.5%). At completion of electrophoresis, the separated proteins were electrophoretically transferred to nitrocellulose paper. Coomassie staining of gels was used to determine if there had been complete transfer of protein.

The nitrocellulose sheets containing the protein were incubated overnight at 4° in 10 mM sodium phosphate buffer (PBS), pH 7.4, containing 0.15 M NaCl with 3% bovine serum albumin. The sheets were washed five times with PBS and then incubated with 1 μg/ml of the anti-3a IgG in PBS for 2 hr at room temperature. After five consecutive washes with PBS, the sheets were then incubated in a 1:300 dilution of rabbit anti-sheep IgG in PBS. After 30 min at room temperature, washing was carried out. Sheep peroxidase anti-peroxidase at a 1:5000 dilution was incubated with the nitrocellulose paper for 30 min at room temperature, followed by washing. The peroxidase activity was detected with 4-chloro-1-naphthol and H_2O_2 as described by Nielsen *et al.* [22]. The sheets were washed in water and dried, and the relative staining intensity was quantitated with a spectrodensitometer model SD 3000 densitometer (Schoeffel Instrument Corp.).

Immunoquantitation. Quantitation of the immunoblots was determined using densitometric values of staining intensity for each of the human liver microsomal samples. Linearity was assessed by applying 5–20 pmol of microsomal P-450 per sample, and both correlation coefficients and slopes of the relative staining intensity versus microsomal P-450 concentration were determined. Linear regression analysis of microsomal P-450 concentration versus aniline hydroxylase activity was also performed.

Other methods. Protein was determined by the method of Lowry *et al.* [23] with bovine serum albumin as the standard. Cytochrome P-450 content was determined and calculated by the method of Omura and Sato [24].

Materials. Aniline, erythromycin and 4-chloro-1-napthol were purchased from the Sigma Chemical Co. NADPH was obtained from Boehringer Mannheim and *p*-aminophenol was from Kodak Laboratory Chemicals. Rabbit anti-sheep immunoglobulin was purchased from Miles Laboratory, and sheep peroxidase anti-peroxidase was obtained from Cooper Biomedical. Reagents for polyacrylamide gel electrophoresis and nitrocellulose paper were obtained from Bio-Rad Laboratories. All other materials were of reagent grade or better.

RESULTS

Aniline hydroxylase activity in human liver microsomes. Human liver microsomes exhibited marked variability in their rates of aniline hydroxylation. The cytochrome P-450 content among the individuals was also variable and demonstrated no correlation with aniline hydroxylase activity (Table 1).

N-Demethylation of NDMA by human liver microsomes. Since previous investigations [10, 11] have demonstrated that ethanol treatment of both rabbits and rats results in an increase in the rate of microsomal N-demethylation of NDMA, this activity was also determined in microsomes from human liver. When microsomes from eight individuals (an insufficient quantity of microsomes was available from one liver) were analyzed for NDMA demethylase activity, the variability in the rates was similar to the pattern observed for aniline hydroxylation (Table 1). Microsomes exhibiting high rates of aniline metabolism also exhibited high rates of NDMA demethylation. Linear regression analysis (Fig. 1) revealed a strong correlation ($r = 0.87$) between the two activities.

Erythromycin N-demethylation by human liver microsomes. Erythromycin N-demethylation is thought to be catalyzed by HLp [4], the human P-

Table 1. Properties of human liver microsomes

Patient No.	Cytochrome P-450*	Aniline hydroxylase†	NDMA *N*-demethylase‡	Erythromycin *N*-demethylase‡
1	0.70	0.34	0.24	3.72
2	0.75	0.81	0.44	7.27
3	0.40	0.79	0.55	2.46
4	0.57	0.94	0.71	3.40
5	0.34	1.10	1.14	4.40
6	0.56	0.84	0.80	4.19
7	1.13	0.33		
8	0.57	0.67	0.38	6.17
9	0.57	0.86	0.53	4.22

* Values are expressed as nmol/mg protein.

† Each value represents the mean of three determinations and is expressed as nmol *p*-aminophenol/min/nmol microsomal P-450.

‡ Each value represents the mean of two determinations and is expressed as nmol HCHO/min/nmol microsomal P-450.

450 homologous to the PCN form of rat liver. Thus, it was expected that no correlation would occur between aniline hydroxylation and erythromycin *N*-demethylation. When erythromycin was utilized as the substrate and linear regression analysis was performed between the N-demethylase and the hydroxylase, no correlation was observed ($r = < 0.1$) (Table 1). This suggests that aniline hydroxylase is a different P-450 from that of erythromycin *N*-demethylase.

Immunochemical reactivity. The antibody to cytochrome P-450 3a was used to probe immunoblots of human liver microsomal preparations. As shown in Fig. 2, the antibody cross-reacts with two proteins of human liver, one with a higher minimum M_r than purified P-450 3a. This staining pattern was observed for all of the human liver specimens examined. The staining intensity of the high M_r protein varied among the individual liver specimens, while that of the lower band exhibited a different pattern of variability. When blots of human microsomes were stained with anti-P-450 3a IgG that had been back-absorbed against P-450 3b, the lower M_r band decreased in intensity while the higher M_r band retained the same staining intensity observed prior to back-absorption of the antibody. Thus, the immunoreactive protein migrating in the higher M_r region is believed to be a human P-450 immunochemically related to P-450 3a. Densitometric scans were also performed on immunoblots containing variable concentrations of microsomal P-450 for each of the individual human liver preparations. The plot of relative staining intensity versus concentration of microsomal P-450 revealed linearity in a concentration range of 5–20 pmol. Correlation coefficients for each of the samples ranged from 0.85 to 0.996.

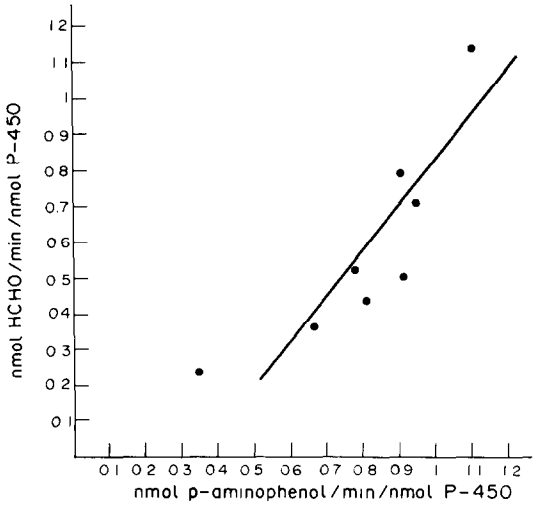

Fig. 1. Correlation of human liver microsomal aniline hydroxylase and NDMA demethylase activities. Linear regression analysis displayed a correlation between aniline hydroxylase and NDMA demethylase activity (nmol/min/nmol P-450) in preparations of microsomes from eight individual human liver specimens. A correlation coefficient of 0.87 and a slope of 0.70 nmol/min/nmol P-450 were obtained. Each value represents the mean of two determinations.

Aniline hydroxylase activity was determined in the presence of either preimmune IgG or anti-P-450 3a IgG for the remaining eight human liver specimens. The amount of antibody used was that concentration which produced the greatest amount of inhibition as determined by titration (3 mg IgG/nmol microsomal P-450). Examination of human liver aniline hydroxylase activity in the presence of the anti-P-450 3a IgG demonstrated variability in the amount of activity that was inhibited among the eight individual liver specimens (Fig. 3). Those microsomes exhibiting the highest rates of hydroxylation also exhibited the greatest decrease in activity in the presence of the anti-P-450 3a antibody (0.45 and 0.68 nmol/min/nmol P-450 for patients #4 and #5, respectively), whereas microsomes exhibiting a low rate of hydroxylation (patient #1) displayed the least amount of change (0.19 nmol/min/nmol P-450).

Quantitative analysis of immunoblots indicated that patients #4 and #5 had the highest concentrations of the immunoreactive protein and patients #1 and #7 had the lowest concentrations with the remaining samples exhibiting concentrations intermediate of the high and low expression microsomes. When staining intensities of the high M_r protein for the nine patients were obtained from the

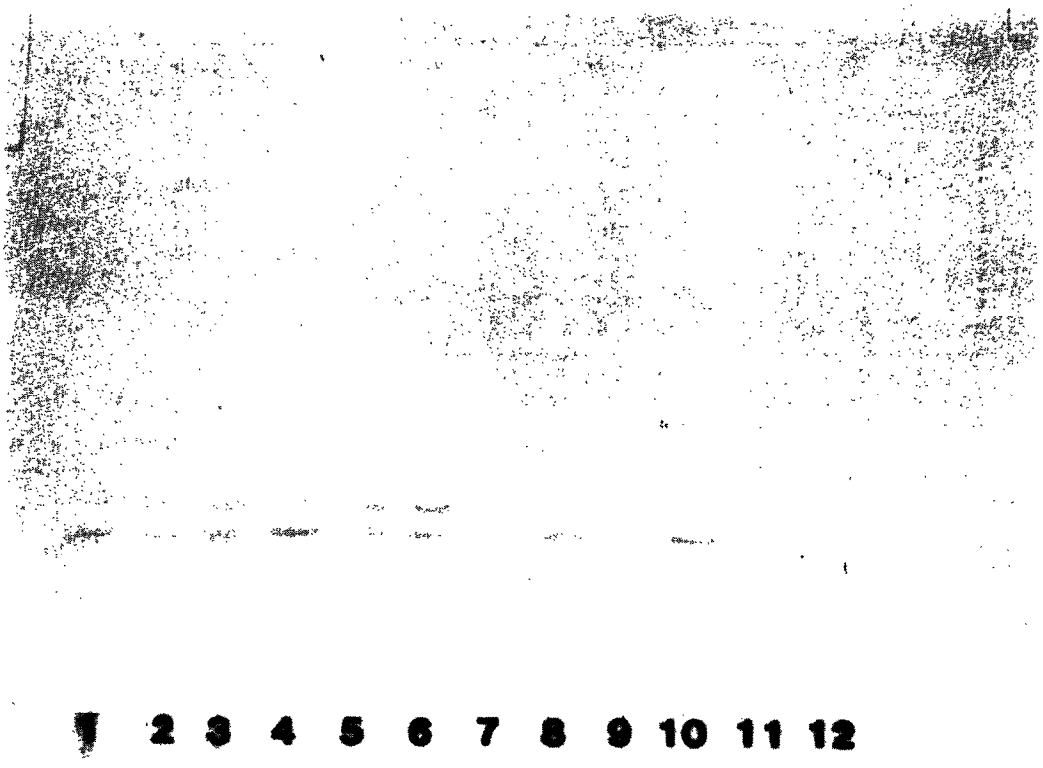

Fig. 2. Immunoblot analysis of human liver microsomes: Immunochemical reaction of human liver microsomal proteins to an antibody prepared against rabbit P-450 3a. Each lane was loaded with 10 pmol of microsomal P-450 from nine individual human liver specimens (lanes 2–6 corresponding to patients 1–5, and lanes 8–11 corresponding to patients 6–9). Purified rabbit P-450 3a was applied to lanes 1, 7 and 12 at a concentration of 50 ng. Microsomal protein was immunoblotted as described in Experimental Procedures.

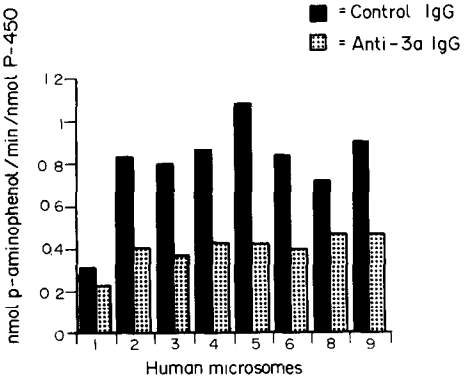

Fig. 3. Effect of anti-P-450 3a IgG on aniline hydroxylation catalyzed by human liver microsomes. Aniline hydroxylase activity was determined in microsomes from eight individual human liver specimens with 0.5 mM aniline and 0.5 nmol of microsomal P-450 in the presence of either anti-3a IgG or pre-immune IgG. The differences in the activities in the presence of the anti-3a IgG and pre-immune IgG are 0.19, 0.42, 0.43, 0.45, 0.68, 0.42, 0.25 and 0.44 for patients 1–6 and 8 and 9 respectively. Each value represents the mean of three determinations.

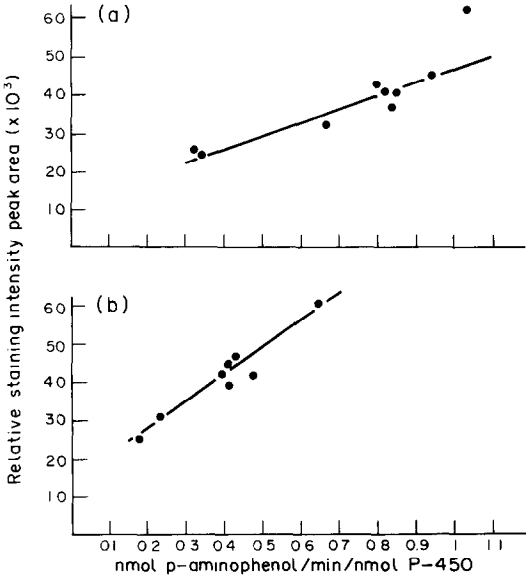

Fig. 4. Linear regression analysis correlating (a) the relative staining intensity of the high M_r protein band with aniline hydroxylase activity ($r = 0.91$) and (b) the antibody-inhibited rate of aniline hydroxylation versus the relative staining intensity of the high M_r protein ($r = 0.93$). The values obtained for the relative staining intensity were obtained by scanning the immunoblot on a Schoeffel 5300 spectrodensitometer. Intensities of staining were integrated as peak areas.

immunoblots and plotted against aniline hydroxylase activity, linear regression analysis revealed a high correlation ($r = 0.91$) (Fig. 4a). In contrast, linear regression analysis of aniline hydroxylase activity and the staining intensity of the low M_r protein demonstrated a very low correlation ($r = 0.60$). Thus, the high M_r protein appears to be the aniline hydroxylase and NDMA demethylase. The degree of inhibition (the antibody-inhibited rate of aniline hydroxylation) was also correlated ($r = 0.93$) with the relative staining intensity of the high M_r protein (Fig. 4b), confirming its identification as the P-450 with characteristics similar to rabbit P-450 3a. No correlation was observed between the inhibited rate and the low M_r band ($r = < 0.1$).

DISCUSSION

A sampling of the human liver population revealed differences in the rates of aniline hydroxylation and NDMA demethylation among individuals. Of the nine patients examined, liver microsomes from two individuals exhibited high rates of both aniline hydroxylation and NDMA demethylation, whereas two other liver specimens displayed low catalytic activities. These differences could not be attributed to any particular drug therapy nor to chronic ethanol ingestion.

Immunoblots with anti-P-450 3a IgG and the individual human liver microsomes produced blots exhibiting two protein bands with different molecular weights. The high M_r band was most likely a P-450 analogous to rabbit P-450 3a. This was confirmed by a close correlation between aniline hydroxylase activity and the relative staining intensity of the high M_r protein. The correlation decreased when the same activity was compared to the staining intensity of the low M_r protein. The rate of aniline hydroxylation in the presence of antibody also correlated highly with the staining intensity of the upper band but did not correlate when compared to the low M_r protein. In addition, when cross-reaction with P-450 3b was removed by back-absorption against purified P-450 3b, the low M_r protein band was absent. Similar results were reported in the cross-reaction of anti-P-450 3a IgG with rat liver microsomes [25, 26].

Antibody inhibition of aniline hydroxylase activity demonstrated that the greatest inhibition occurred in microsomes exhibiting high rates of hydroxylation. There was a basal level of activity (about 0.4 nmol/min/nmol P-450) which was achieved in the presence of the antibody irrespective of the rate of hydroxylation (Fig. 3). Previous investigations have shown that several forms of P-450, other than P-450 3a, catalyze aniline hydroxylation [27]. Thus, the basal aniline hydroxylase activity produced in the presence of the anti-P-450 3a IgG in human liver may reflect the contributions of these forms. The differences in activities in the presence and absence of anti-P-450 3a IgG among the human liver specimens appear to reflect the differences in concentration of a cytochrome similar to P-450 3a.

The inducible forms of liver P-450 in untreated animals represent a small fraction of the total. Cytochrome P-450 3a represents about 5% of the total cytochrome P-450 in untreated rabbits, and microsomes from these animals exhibit low rates of aniline hydroxylation [28]. Higher rates of aniline hydroxylation are observed in microsomes from animals treated with either ethanol, acetone, isoniazid or pyrazole [28]. However, the high rates of hydroxylation observed among the human individuals examined in this study were probably typical of the variability of a normal population and, thus, not related to chronic exposure to ethanol or other xenobiotics.

The physiological significance of a human hepatic cytochrome similar to P-450 3a with high catalytic activity towards aniline and NDMA is unknown. It is unlikely that it is present for the sole purpose of metabolizing ethanol since most ethanol oxidation is performed by alcohol dehydrogenase. Therefore, the physiological role of this P-450 in human liver may be in the metabolism of an endogenous substrate. Rats treated with acetone, made diabetic, or fasted exhibit an increase in the rate of microsomal aniline hydroxylation [29, 30]. Plasma acetone levels have also been shown to be elevated in humans during fasting [31]. Koop and Casazza [26] demonstrated that P-450 3a is the acetone and acetol monooxygenase, suggesting a role for the enzyme in the initial steps leading to gluconeogenesis. In humans with elevated plasma acetone levels, incorporation of [^{14}C]acetone into glucose has been identified [31]. This suggests a possible role for the human P-450 in facilitating the conversion of high levels of acetone to glucose in either fasted or diabetic individuals.

In our investigation, only one patient was diabetic (patient #4) and receiving insulin. Microsomes obtained from this individual exhibited one of the highest rates of aniline hydroxylation, in addition to displaying a high relative staining intensity on the immunoblot. However, since this was only one individual, and a liver specimen from a non-diabetic displayed similar properties, it cannot be confirmed that insulin-controlled diabetics have a higher concentration of the P-450 resembling P-450 3a. It may be possible that diabetic individuals not receiving anti-hyperglycemic therapy exhibit a greater expression of the enzyme.

In this investigation, we demonstrated the presence of a human liver P-450 that is catalytically and immunochemically related to the ethanol-inducible P-450 3a of the rabbit. The enzyme resembles P-450 3a in its ability to catalyze the demethylation of NDMA and the hydroxylation of aniline. The activity and concentration of the P-450 appear to differ among individuals; whether these variations reflect a genetic polymorphism or the susceptibility of the cytochrome to environmental or dietary factors has yet to be determined.

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