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Evidence for involvement of multiple forms of cytochrome P-450 in aflatoxin B_1 metabolism in human liver

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ABSTRACT **Liver cancer is a major cause of premature death in many areas of Africa and Asia and its incidence is strongly correlated with exposure to aflatoxin B_1 (AFB_1). Because AFB_1 requires metabolic activation to achieve a biological response, there is a need for detailed knowledge of the mechanism of activation to assess individual risk. We have carried out an extensive study using a total of 19 human liver samples to determine the individual variability in the metabolism of the toxin to mutagenic or detoxification products and to identify the specific cytochrome P-450 forms involved in these processes. Metabolism to the toxic 8,9-epoxide or to products mutagenic in the Ames test was found to exhibit very large individual variation. The rates of metabolic activation were highly correlated with both the level of proteins of the P450IIIA gene family and with the total cytochrome P-450 content of the microsomes. In agreement with this, antibodies reacting with P450IIIA proteins were strong inhibitors of both the metabolism and mutagenicity in the majority of the samples. However, the inhibition varied between 50% and 100%. The expression of a protein in the P450IIC gene family also correlated with AFB_1 metabolism and mutagenicity. This result therefore indicated the involvement of cytochromes other than P450IIIA in the activation of AFB_1 by human liver microsomes. This hypothesis was strongly supported by the finding that antibodies to P450IA2 and P450IIA1 were also effective inhibitors of metabolism in many of the samples. These data demonstrate that, although P450IIIA probably plays an important role in AFB_1 activation, several other cytochrome P-450 forms have the capacity to activate the toxin. Similar considerations apply to detoxifying metabolism to aflatoxin Q_1 and aflatoxin M_1. The levels of expression of many of the forms of cytochrome P-450 involved in AFB_1 metabolism are known to be highly sensitive to environmental factors. This indicates that such factors will be an important determinant in individual susceptibility to the tumorigenic action of AFB_1.**

Liver cancer is one of the major causes of premature death in certain regions of Africa and Asia. Two major environmental factors have been implicated in the etiology of this disease: infection with hepatitis B virus and exposure to the chemical carcinogen aflatoxin B_1 (AFB_1) (1, 2). AFB_1 is a mycotoxin produced by species of *Aspergillus*, and human exposure results principally from the ingestion of stored foodstuffs contaminated with the mold. The carcinogenicity of this compound is associated with its conversion to aflatoxin 8,9-oxide by the hepatic cytochrome P-450 (P450)-dependent monooxygenase system (3, 4). It is therefore of considerable importance to establish which human P450 isozymes are involved in the metabolism of this compound and to understand the genetic and environmental factors which determine their level of expression.

In animal model systems, evidence for the involvement of a wide variety of P450 isozymes, from several gene families, in the metabolism of AFB_1 has been forthcoming. These include proteins from the 3-methylcholanthrene-inducible family P450IA (5–7), the phenobarbital-inducible P450IIB subfamily (8, 9), and P450IIC subfamily (10), the dexamethaxone-inducible P450IIIA family (11), and possibly P450s from the family P450IVB (8).

The complexity of the rodent system does not permit easy identification of candidate human P450s involved in the activation and metabolism of this compound. Induction of specific AFB_1 metabolic pathways in rodents, however, has indicated the catalytic roles played by the induced P450 species. The administration of polycyclic hydrocarbons induces predominantly 4-hydroxylation, forming aflatoxin M_1 (AFM_1), and to a much lesser extent epoxidation, whereas phenobarbital induces epoxidation and aflatoxin Q_1 (AFQ_1) formation to approximately equal extents (12). In the case of human microsomes, Shimada and Okuda (11) concluded that constitutively expressed forms were probably involved in AFB_1 activation. They excluded cytochromes from the P450IA and P450IIB gene families, as antibodies raised against the rat homologues of these proteins did not inhibit AFB_1 metabolism in human liver. Studies using immunochemical inhibition indicated that the P450 associated with the polymorphic metabolism of debrisoquine, subfamily P450IID, was also not involved in the metabolism of AFB_1 either to a DNA-binding species or to AFQ_1 (13). Shimada and Guengerich (14) have recently reported that the major cytochrome P450 associated with AFB_1 activation is a member of the P450IIIA family, a protein also associated with the oxidation of nifedipine. Their evidence was based principally on correlations between level of the P450IIIA cytochrome and the rate of AFB_1 metabolism to mutagenic products or products that bound to DNA. However, their experiments using reconstituted cytochrome systems indicated that the activation of AFB_1 to mutagenic species was not confined to P450IIIA, although this enzyme had the highest activity of the three tested. These workers selected a single liver sample for further study, and found a surprisingly high activity towards sterigmatocystin (>2 × the activity towards AFB_1) and a very low activity towards aflatoxin G_1 (AFG_1). This is unexpected in view of the potent carcinogenic and toxic properties of AFG_1 and the low carcinogenicity of sterigmatocystin noted in animal experiments. Since the liver sample used also had an unusually high P450 level, the question is raised as to how typical this sample is of the range of human microsomes. In view of the broad range of P450s with the

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Abbreviations: AFB_1, aflatoxin B_1; AFQ_1, aflatoxin Q_1; AFM_1, aflatoxin M_1; P450, cytochrome P-450. The nomenclatures for the P450 isozymes and gene families are described by Nebert *et al.* (19).

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capacity to metabolize AFB_1 in the rat, and the finding that the relative concentrations of different P450 forms can have profound effects on carcinogenicity (10), we have determined the individual variability in pathways which lead to the activation or detoxification of AFB_1 in 19 human liver samples and the relative roles of a broad range of P450 forms in these reactions.

MATERIALS AND METHODS

Materials. All chemicals were obtained from the usual commercial sources and were of the highest purity.

Preparation of Microsomes. A total of 19 human livers were obtained from kidney-transplant donors. The donors were 8 to 68 years old and consisted of 12 males, 6 females, and one of unknown sex. Samples were frozen in liquid nitrogen within 1 hr of removal. Microsomal fractions were prepared as previously described and were stored in 10 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose at −70°C. Protein concentrations were measured by using the method of Lowry *et al.* (15). P450 levels in these samples were 0.10–0.47 nmol/mg of microsomal protein. There was no evidence of any P450 degradation to cytochrome P-420, and the variability in cytochrome content could be due to drugs given to the transplant donors. Unfortunately very little information on drug history was available. Human liver microsomes were sterilized for mutation assays by irradiation [40,000 rads (1 rad = 0.01 Gy)] using a cesium-137 source followed by filtration. This treatment did not affect the metabolic capacity of the microsomal samples.

Preparation and Use of Antisera. Antibodies were raised against purified rat P450 isozymes as previously described (16–18). When used as inhibitors, antibodies (IgG fraction) at various concentrations were incubated with the microsomal samples at room temperature for 15–20 min prior to the assay.

A representative Western blot showing the proteins identified by the antibodies used is shown in Fig. 1. In certain cases—for example, the P450IIC gene subfamily—several protein bands were identified. This is consistent with the reports of the expression of multiple members of this gene family in human liver (20) and the isolation of cDNA clones encoding several distinct proteins (21). Where possible, antibody specificity was demonstrated by showing specific reactivity with the expressed recombinant P450 proteins (a generous gift of Dr. F. Gonzalez). The recombinant proteins also had the same electrophoretic mobilities as the proteins identified in the microsomes by Western blot analysis (see legend to Fig. 1).

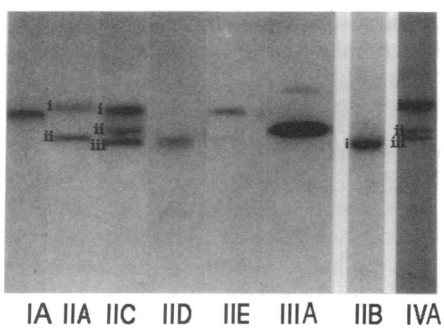

FIG. 1. Proteins identified in human liver by using antibodies raised to rat liver P450 proteins. Microsomal proteins from a single human liver (7.5 μg per track) were loaded across a 7% polyacrylamide slab gel and separated by electrophoresis. The proteins were transferred to nitrocellulose and the filter was inserted into an apparatus that allows simultaneous exposure to several P450 antibodies without cutting the nitrocellulose sheet (Decca probe; Hoefer). The antibodies used were raised to the following rat liver P450s: P450IA2, P450IIA1, P450IIB1, P450IIC6, P450IID6 (human monoclonal antibody), P450IIE1, P450IIIA1, and P450IVA1 (see ref. 19 for the nomenclatures used). The estimated molecular masses are given in Table 2. Samples P450IIB and P450IVA were run on a separate gel. The antibodies used reacted specifically with recombinant human P450s from the gene families to which the antibodies were raised and, on the basis of relative mobility in SDS gels, the P450s were categorized according to the nomenclature of Nebert *et al.* (19) as: P450IIAii = P450IIA3, P450IIB = P450IIB6, P450IICi = P450IIC9, P450IICii = P450IIC8, and P450IIIA = P450IIIA3 or P450IIIA4. These latter two recombinant proteins reacted with the antibody to P450IIIA1 and have the same mobility on SDS gels.

Immunoblotting. Microsomal samples (7.5 μg) were separated by SDS 9% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and exposed to the P450 antibodies (1:500 dilution) as previously described (22, 23). Following exposure to ^{125}I-labeled staphylococcal protein A (0.19 MBq) bands were visualized by autoradiography and relative concentrations of P450 isozymes in the different samples were determined by laser scanning of the autoradiographs. Relative P450 content was established by preparing standard curves from the microsomal samples loaded in a series of concentrations.

HPLC Analysis of AFB_1 Metabolites. Human microsomal protein (0.05–0.4 mg) was incubated with AFB_1 (5 μM) and a NADPH-generating system in 0.1 M Tris·HCl, pH 7.4, for 15 min at 37°C (24). In this incubation system AFB_1 8,9-dihydrodiol reacts with Tris buffer to form a highly fluorescent, stable, Schiff base. Metabolites were extracted and analyzed by HPLC as previously described (25).

Mutagenicity Testing. The metabolic activation of AFB_1 to mutagenic proteins was assessed by using the standard bilayer plate incorporation test developed by Ames *et al.* (26). Microsomal protein (0.1 mg) was added to a cofactor mixture containing 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose 6-phosphate, and 4 mM $NADP^+$ in 50 mM sodium phosphate buffer, pH 7.4. The mixture was poured onto prepoured agar plates (Difco) immediately after the addition of 5 μM AFB_1, 0.1 ml of an overnight culture of *Salmonella typhimurium* strain TA98, and 2 ml of 0.6% agar containing 0.6% NaCl, 50 μM L-histidine hydrochloride, 50 μM biotin, and ampicillin at 250 μg/ml. Plates, in triplicate, were incubated for 48 hr at 37°C. The bacterial strain was routinely tested for ampicillin resistance, histidine requirement, and deep rough character (tested by using crystal violet) and with reference mutagens in discs impregnated with 10 μl of sodium azide at 100 μg/ml, 4-nitro-*o*-phenylenediamine at 600 μg/ml, or nitrofurantoin at 10 μg/ml as described by Zeiger *et al.* (27). Mutation frequency was determined from the number of histidine-independent revertant colonies.

RESULTS

P450 isozyme levels in a range of human liver samples, determined by Western blotting, were compared with the ability of the samples to metabolize AFB_1 to products mutagenic to *S. typhimurium*, to the carcinogenic AFB_1 8,9-epoxide (assayed as AFB_1 8,9-dihydrodiol), and to the detoxification products AFQ_1 and AFM_1. Both AFQ_1 and AFB_1 8,9-dihydrodiol were produced in significant amounts by the human liver samples and represented up to 80% of the total soluble metabolites formed with lower amounts of AFM_1 (Table 1). Of the P450 forms detected by Western blot analysis (Fig. 1) there was a high correlation between AFB_1 metabolism to the carcinogenic AFB_1 8,9-epoxide (assayed as AFB_1 8,9-dihydrodiol) and the microsomal content of P450IIIA3/P450IIIA4 (Table 2). Interestingly, the formation of AFQ_1, an AFB_1 detoxification product, also correlated strongly with the expression of these proteins (Table 2). It is perhaps significant that the formation of these products was also highly correlated with the total P450 content. The expression of P450IIIA3/P450IIIA4, as well as total P450

Table 1. Metabolism of AFB_1 by human liver microsomes

Microsomal sample	Rate of AFB_1 metabolism, μg product per incubation			Total metabolism, ng/min	Unchanged AFB_1, μg
	Diol	AFQ_1	AFM_1		
L4	0.45	2.80	0.02	296.0	0.64
L5	0.12	1.37	0.05	137.2	3.02
L7	0.22	2.48	0.04	287.7	0.76
L8	0.28	2.78	0.04	312.8	0.39
L9	0.17	1.93	0.02	192.6	2.19
L10	0.13	1.68	0.02	165.4	2.60
L11	0.12	1.40	0.02	123.9	3.22
L12	0.24	2.86	0.09	279.1	0.89
LW1	0.09	0.98	0.02	95.5	3.65
LB1	0.22	2.56	0.06	280.1	0.88

Incubations at 37°C were carried out for 15 min in mixtures containing 400 μg of microsomal protein. Other details are described in refs. 25 and 26. Total AFB_1 recovery was 68–99%.

content, was also highly correlated with AFB_1-induced mutation rate (Fig. 2, Table 2). In the 19 liver samples studied, the level of P450IIIA protein was subject to a 26-fold variation, compared with a 13-fold variation in mutation frequency and a 5-fold variation in the rate of AFB_1 8,9-dihydrodiol formation.

For the liver panel shown in Table 2 AFB_1-induced mutagenicity and metabolism to the AFB_1 8,9-dihydrodiol and AFQ_1 also correlated with the level of a protein in the P450IIC gene subfamily (band P450IICii, P450IIC8). In further studies, using a second panel of human liver microsomes, the high correlation with P450IIIA3/P450IIIA4, as well as P450 content, was confirmed. A correlation with a member of the P450IVA family (band P450IVAiii, $P < 0.05$) was also observed (results not shown). In this panel the association of metabolic activation with the level of P450IIC8 protein did not reach statistical significance. In these latter studies the metabolism of AFB_1 to AFM_1 also correlated with the expression of what appears to be P450IA2 ($P < 0.05$) (data not shown).

The above data indicated that several human P450 forms have the capacity to metabolize and activate AFB_1. The

Table 2. Correlations between P450 isozyme expression and AFB_1 metabolism and mutagenicity

P450 band	kDa	Spearman rank coefficient		
		AFB_1 mutagenicity	AFB_1 8,9-diol	AFQ_1
P450IA (IA2)	53.0	0.00	0.00	0.14
P450IIAi	54.5	0.10	−0.16	−0.08
P450IIAii (IIA3)	51.5	0.48	0.30	0.29
P450IIBi (IIB6)	51.0	0.19	0.47	0.47
P450IICi (IIC9)	54.5	0.57	0.24	0.32
P450IICii (IIC8)	52.5	0.74*	0.62*	0.65*
P450IICiii	51.0	0.49	0.42	0.39
P450IID (IID6)	51.5	−0.36	−0.18	−0.06
P450IIE (IIE1)	54.5	−0.36	−0.28	−0.41
P450IIIA (IIIA3/A4)	52.5	0.96**	0.86**	0.88**
P450IVAii	ND	0.036	−0.09	0.21
P450IVAiii	ND	0.36	0.41	0.32
Total P450	—	0.87**	0.86**	0.83**
P450 reductase	—	0.50	0.38	0.45

AFB_1 metabolism was assayed either by an *S. typhimurium* mutagenicity test or by HPLC for the AFB_1 8,9-diol and AFQ_1. Probable nomenclature according to Nebert *et al.* (19) is given in parentheses. ND, not determined.
*$P < 0.05$.
**$P < 0.005$.

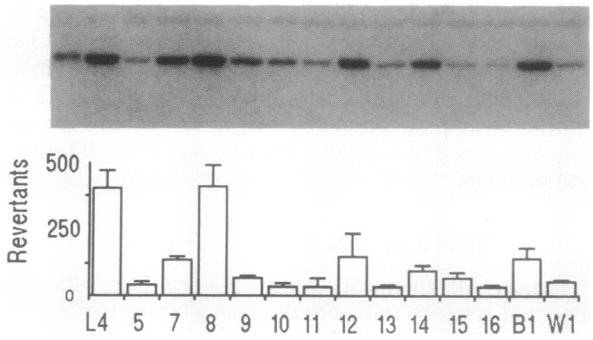

FIG. 2. Comparison of the metabolic activation of AFB_1 with the expression of P450IIIA-related proteins in human liver. (*Upper*) Western blot analysis on a panel of human liver microsomal samples. The leftmost band is a rat liver P450IIIA1 standard. (*Lower*) Ability of these microsomal samples to convert AFB_1 to mutagenic products, measured by the number of *S. typhimurium* histidine-independent revertants in the Ames test. Revertants are expressed per 0.1 mg of microsomal protein per assay after subtraction of background values. The values shown are mean ± SD for triplicate determinations.

strong correlation with P450IIIA proteins is because they represent the major P450 form(s) in human liver. To assess the relative role of different P450s in AFB_1 metabolism the ability of antibodies to inhibit AFB_1 activation was determined. The antibodies used have been shown to be effective inhibitors of other P450-mediated monooxygenase reactions in human liver (refs. 28 and 29; unpublished data).

The antibody to P450IIIA1 was found to be a potent inhibitor of AFB_1-induced mutagenesis in all of the liver samples tested, irrespective of the capacity of the individual microsome samples to metabolize the compound to mutagenic products (Figs. 2 and 3). In all four of the liver samples that were used to give the results detailed in Fig. 3, more than 80% inhibition of mutagenicity was observed at the highest concentration of IgG used. In two separate experiments using human liver microsomal preparations, anti-P450IIIA1 was found to be an inhibitor of AFB_1 metabolism to AFB_1 8,9-dihydrodiol (Figs. 4 and 5). The level of inhibition, however, varied significantly between samples and ranged

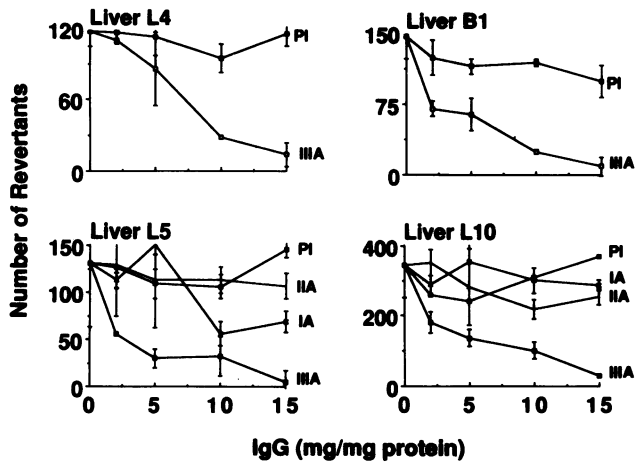

FIG. 3. Inhibition of AFB_1-induced mutagenicity by P450 antibodies. The amount of microsomal protein for each assay plate was 0.25 mg for livers L5 and L10 and 0.1 mg for L4 and B1. Different amounts were used to optimize the number of revertants obtained. Control mutation frequencies were obtained in the presence of an equivalent concentration of preimmune serum (PI). The antibodies used were to the rat P450s P450IA2 (IA), P450IIA1 (IIA), and P450IIIA1 (IIIA). Assays were carried out in triplicate and results are expressed ± SD.

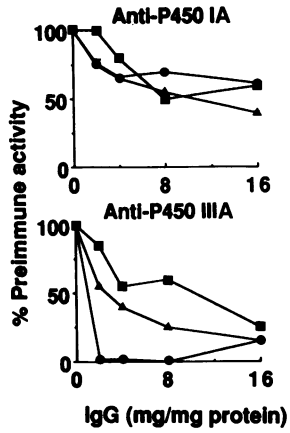

FIG. 4. Effect of P450 antibodies on AFB_1 metabolism to AFB_1 8,9-dihydrodiol. Antibodies used were to P450IA2 (*Upper*) and P450IIIA1 (*Lower*). Values are expressed as percentage of control values obtained in the presence of preimmune serum. Experimental points are the means of duplicate determinations. Liver samples used were liver L4 (■), liver L5 (▲), and liver L12 (●). AFB_1 dihydrodiol production of control uninhibited liver samples was 6.02 ng by L4, 1.13 ng by L5, and 3.62 ng by L12. Incubation mixtures contained 50 μg of microsomal protein.

between 50% and 100%. In addition, inhibition was also observed in many of these samples with an antibody to P450IA2 and ranged between 0% and 62%. This antibody also inhibited AFB_1-induced mutagenicity in liver L5 by 54% (Fig. 3). The slight differences in the ability of P450IA2 or P450IIIA1 antibodies to inhibit 7,8-diol formation vs. AFB_1 mutagenicity (Fig. 4 vs. Fig. 5) may be related to the different metabolic endpoints measured. Of the other antibodies tested, to P450IIA1, P450IIB1, P450IIC6, and P450IVA1, only the antibody to P450IIA1 inhibited AFB_1 8,9-dihydrodiol formation in some samples (Fig. 5). Significant variability in the effectiveness of this antibody was observed. The inhibition ranged between 13% and 47%. These data support the involvement of multiple forms of P450 in AFB_1 metabolism in human liver. There did not appear to be a direct relationship between the level of P450IA2 or P450IIA3 proteins and the ability of antibodies to these cytochromes to inhibit AFB_1 metabolism. Also, it might have been expected that inhibition by P450IA2 or P450IIA1 antibodies would be observed in samples containing low levels of P450IIIA protein. However, this was not the case. It is worthy of note that the summed antibody inhibition of metabolism or mutagenesis, resulting from the use of all the antibodies in a single liver microsomal preparation, in some cases exceeded 100%. The reason for this is unclear. However, similar observations have been made in other P450 antibody inhibition and immunoquantitation studies.

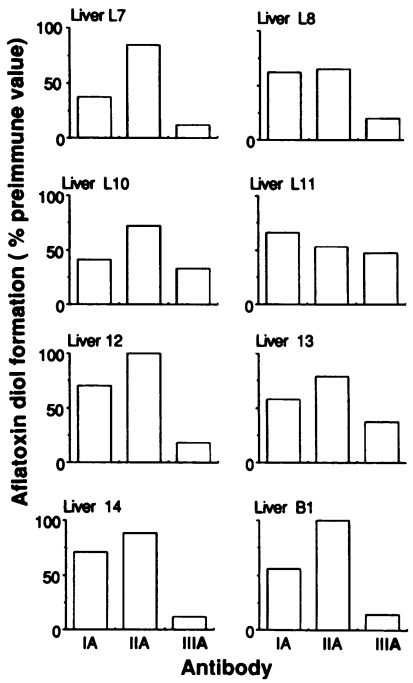

FIG. 5. Inhibition of AFB_1 metabolism to AFB_1 8,9-dihydrodiol in human livers. The antibodies to rat P450IA2 (IA), P450IIA1 (IIA), and P450IIIA1 (IIIA) were used at 10 mg of IgG per mg of microsomal protein. The results shown are means of duplicate determinations and are expressed as percentages of the rates obtained in the presence of preimmune serum. Antibodies to P450IIB1, P450IIC6, and P450IVA1 were also tested but did not inhibit more than 20% in any of the samples.

DISCUSSION

We provide evidence that proteins from several distinct human P450 gene families have the capacity to metabolize AFB_1 to both mutagenic products and the carcinogenic 8,9-epoxide. The most consistent and potent inhibitor of AFB_1 metabolism and mutagenicity was an antibody to a member of the P450IIIA gene family. This antibody recognizes three distinct recombinant human P450IIIA proteins. Two, which are highly homologous at the amino acid level (P450IIIA3 and P450IIIA4), have the same mobility as the protein identified in human liver by Western blot analysis (L.M.F., F. J. Gonzalez, and C.R.W., unpublished data). The involvement of proteins encoded by the P450IIIA gene family in AFB_1 activation is in agreement with the findings of Shimada and Guengerich (14). The P450 identified by these workers (P450IIIA4) catalyzes the oxidation of the calcium channel-blocking drug nifedipine. The finding that the metabolic activation of AFB_1 is also highly correlated with the total P450 content indicates that P450IIIA proteins are often the major P450 forms in human liver. Indeed, analysis of our data shows that in the two liver panels used in this study the expression of P450IIIA was significantly correlated with total P450 content, with correlation coefficients (R_s) of 0.92 ($P < 0.005$) and 0.56 ($P < 0.05$). This indicates that the correlation between P450IIIA3/P450IIIA4 expression and AFB_1 metabolism is because it is present in high concentration and not because it is the only P450 with the capacity to metabolize this substrate. The potential of other P450s to metabolize AFB_1 is substantiated by the inability of P450IIIA1 antibody to completely inhibit metabolism in many of the liver samples and by the finding that antibodies to P450IIA1 and particularly P450IA2 were effective inhibitors of 8,9-diol formation.

Current evidence indicates that the toxic or carcinogenic effects of AFB_1 are intimately linked to both the rate of activation and the rate of detoxification at both the primary and secondary levels of metabolism (30). Primary detoxification by metabolism conversion to AFQ_1 appears also to be catalyzed predominantly by proteins of the P450IIIA gene family. Whether P450IIIA3, P450IIIA4, or both proteins are involved in both the metabolic activation and the formation of the AFQ_1 remains to be determined. It is worthy of note that these two pathways of AFB_1 metabolism are coinduced in the rat by treatment with phenobarbital (12). It is also interesting that the formation of AFB_1, a further detoxification product, was correlated with the expression of P450IA2.

Our studies strongly support the conclusion that although P450s in the P450IIIA gene family play an important role in the activation of AFB_1 other species of cytochrome can play a major role. The variability in the relative role of different enzymes is probably due to the large variation in their level of expression. Studies in animals and humans indicate that

the level of proteins encoded by the P450IA gene family is regulated by cigarette smoke components such as polycyclic aromatic hydrocarbons and plant alkaloids such as safrole (31). Hepatic levels of members of the P450IIIA gene family are regulated by glucocorticoids and a wide variety of foreign compounds (32). Recent studies have also indicated that in the mouse P450IIA proteins are highly inducible by pyrazole and to a lesser degree by phenobarbital (33). It is interesting that P450IIA proteins catalyze coumarin hydroxylation (34, 35) and that AFB_1 also contains the coumarin structure. The apparent inducibility of many of the P450s involved in AFB_1 metabolism suggests that the relative importance of specific forms in its activation will be determined by environmental and/or hormonal factors. Whether genetic factors are also involved remains to be established.

A genetic polymorphism in the P450IID6 in humans, which results in a loss of the function of this protein, has been associated with reduced susceptibility to liver cancer (36). This indicates that this P450 is involved in the activation of liver carcinogens. However, no correlation between AFB_1 activation or AFQ_1 metabolism and the level of P450IID6 protein was found (Table 2). Indeed, in individual livers not expressing this enzyme metabolism was often high—e.g., liver L7 (Table 1). Wolff *et al.* (13) have also shown that in rats antibodies to P450IID proteins do not inhibit AFB_1 activation or detoxification. The lack of correlation between the genetic and biochemical data is difficult to reconcile unless other factors are involved in the disease association observed.

Evidence for the involvement of AFB_1 in the pathogenesis of liver cancer in humans is strong but circumstantial. Identification of the cytochrome P450s involved in the pathways of AFB_1 metabolism may help elucidate factors that contribute to susceptibility to this disease.

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