Involvement of Cytochrome P450, Glutathione *S*-Transferase, and Epoxide Hydrolase in the Metabolism of Aflatoxin B_1 and Relevance to Risk of Human Liver Cancer

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In recent years there has been considerable interest in the effect of variations in activities of xenobiotic-metabolizing enzymes on cancer incidence. This interest has accelerated with the characterization of human enzymes, both those involved in activation and detoxication, and the development of methods for analyzing genetic polymorphisms. However, progress in epidemiology has been slow and the contributions of polymorphisms to risks from individual chemicals and mixtures are often controversial. A series of studies is presented to show the complexities encountered with a single chemical, aflatoxin B_1 (AFB_1). AFB_1 is oxidized by human cytochrome P450 enzymes to several products. Only one of these, the 8,9-*exo*-epoxide, appears to be mutagenic and the others are detoxication products. P450 3A4, which can both activate and detoxicate AFB_1, is found in the liver and the small intestine. In the small intestine, the first contact after oral exposure, epoxidation would not lead to liver cancer. The (nonenzymatic) half-life of the epoxide has been determined to be approximately 1 sec at 23°C and neutral pH. Although the half-life is short, AFB_1-8,9-*exo*-epoxide does react with DNA and glutathione *S*-transferase. Levels of these conjugates have been measured and combined with the rate of hydrolysis in a kinetic model to predict constants for binding of the epoxide with DNA and glutathione *S*-transferase. A role for epoxide hydrolase in alteration of AFB_1 hepatocarcinogenesis has been proposed, although experimental evidence is lacking. Some inhibition of microsome-generated genotoxicity was observed with rat epoxide hydrolase; further information on the extent of contribution of this enzyme to AFB_1 metabolism is not yet available. — Environ Health Perspect 104(Suppl 3):557–562 (1996)

Key words: aflatoxin B_1, cytochrome P450, glutathione, glutathione *S*-transferase, epoxide hydrolase, metabolism of mycotoxins, metabolism of chemical carcinogens

Introduction

The role of enzymatic transformation in the activation and detoxication of chemical carcinogens has been recognized for half a century (*1*). Studies on these enzymes have shown their multiplicity in many cases, as well as the large extent of variation of levels of individual enzymes that can occur (*2*). With experimental animal systems, there is considerable evidence that alterations in levels of some of these enzymes can have dramatic effects in influencing the incidence of cancer from chemical carcinogens, both in the classical initiation and promotion phases (*3–7*). This background in experimental models has led to hypotheses that variations in the activities of individual enzymes involved in xenobiotic transformation influence cancer incidence in humans (*7,8*). In the late 1980s it became possible to assign roles in the activation of many chemical carcinogens to individual P450 enzymes on the basis of *in vitro* results (*8,9*) (Table 1). The same approaches have been used with other enzymes such as GST and *N*-acetyltransferase. In some cases, the dominance of a particular enzyme (especially cytochrome P450) in the metabolism of a drug has made it possible to make *in vivo* evaluations of the contributions to the human metabolism of a carcinogen, especially if low levels of the carcinogen can be administered to humans (*7,8,11*).

The above information has led to studies involving what is often referred to as molecular epidemiology, particularly in the effort to associate cancer risks with levels of enzymes or with genetic polymorphisms in the enzymes. The difficulty in the approach may be exemplified by the results of efforts to relate levels of P450 2D6 with tobacco-induced lung cancer, where equivocal results have been obtained in different laboratories over the course of a decade (*12–15*). Part of the difficulty in this situation may be a result of the myriad of potential carcinogens found in tobacco smoke and the lack of P450 2D6 to dominate in the activation of any of these (*16–18*).

Aflatoxin B_1 (AFB_1) is generally considered to play a major role in human liver cancer in some parts of the world (*19,20*), and much is now known about its mechanism of genotoxicity, which appears to be the result of the formation of a single initial DNA adduct (at the guanyl N7 atom) (*21,22*). We have considered some of the complexities of the metabolism of AFB_1 and the relevance to efforts to implicate individual enzyme levels as factors in risk.

Methods

Chemicals

AFB_1 was purchased from Sigma Chemical Co. (St. Louis, MO) and was further purified by thin-layer chromatography under dim light (silica gel G) before use in

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Abbreviations used: AFB_1, aflatoxin B_1; AFQ_1, aflatoxin Q_1; AFM_1, aflatoxin M_1; GSH, glutathione; GST, glutathione *S*-transferase; EH, epoxide hydrolase; GS-AFB_1, 8-(*S*-glutathionyl),9-hydroxy AFB_1.

Table 1. Major carcinogenic substrates for human P450 enzymes.

P450	Substrate
1A1	Polycyclic hydrocarbons
1A2	Aryl amines Heterocyclic amines
2A6	Nitrosamines
2E1	Nitrosamines Benzene Vinyl monomers Halogenated hydrocarbons
3A4	Aflatoxins Polycyclic hydrocarbon dihydrodiols Pyrrolizidine alkaloids Some aryl amines Mercapturic acids

Data from Guengerich and Shimada (*8,10*).

the genotoxicity experiments. AFB_1-8,9-epoxide was synthesized with the use of dimethyldioxirane (*23*) by T. M. Harris, and the *exo* isomer was crystallized from a $(CH_3)_2CO$:$CHCl_3$ mixture (*24*). 8-(*S*-glutathionyl), 9-hydroxy AFB_1 (GS-AFB_1) (*25*) was also a gift from Harris' laboratory. AFB_1-8,9-dihydrodiol was prepared by the addition of aqueous HCl to AFB_1-8,9-epoxide.

Enzymes

Recombinant human P450s 1A2 (*26*) and 3A4 (*27*) were produced in *Escherichia coli* and purified as described. Rabbit liver NADPH-P450 reductase (*28*) and cytochrome b_5 (*29*) were isolated as described. Recombinant (rat) glutathione *S*-transferase (GST) 3-3 was a gift from R. N. Armstrong. Rat and human liver epoxide hydrolases (EHs) were purified using modifications of procedures published elsewhere (*30*).

Assays

GS-AFB_1 (products of *exo* and *endo* epoxides) and AFB_1-8,9-dihydrodiol were separated by high-performance liquid chromatography (HPLC) and quantified (A_{360}) as described elsewhere (*25,31*). Genotoxicity assays involved measurement of the *umu* (SOS) response in *Salmonella typhimurium* TA1535 harboring the plasmid pSK1001 using general procedures described elsewhere (*32*). Experiments involving kinetics of AFB_1-8,9-*exo*-epoxide hydrolysis were done using an Applied Photophysics SX-17-MV system (Leatherbarrow, U.K.) equipped with fluorescence and UV-diode array detectors and software for analysis of results. Kinetic simulations were done with the KINSIM (*33*) software run on a Macintosh PowerMac computer (Apple Computer, Inc., Cupertino, CA) equipped with a Software FPU coprocessing simulator.

Results

Oxidation of AFB_1 by P450 Enzymes

P450 3A4 has been shown to play a major role in the activation of AFB_1 due to its intrinsic activity towards this substrate and the high level of this enzyme present in human liver (*9,31,34–37*) (Table 2). P450 1A2 and some other human P450s can also contribute, but they play a lesser role, even at relatively low AFB_1 concentrations (*31,35,36,38,39*). P450 3A4 forms only the genotoxic AFB_1-8,9-*exo*-epoxide; P450 1A2 forms both the *exo* and the nongenotoxic *endo* isomers (Figure 1)(*31*).

P450s can also detoxicate AFB_1 (Figure 1). P450 3A4 forms AFQ_1, the 3α-hydroxylation product, which does not appear to be a good substrate for epoxidation (*37*). P450 1A2 forms AFM_1 (by 9a hydroxylation), which also seems to be a detoxication product (*31,37*). In animal studies, the induction of P450 1A2 and production of AFM_1 have been reported to account for the lower levels of AFB_1-induced hepatocellular cancer after administration of polycyclic hydrocarbons (*40,41*). Which of the human P450s form aflatoxin P_1 is not known.

Table 2. Rates of oxidation of AFB_1 by recombinant human P450s.

	Product formed, nmol/min/nmol P450			
			AFB_1-8,9-epoxide	
P450	AFQ_1	AFM_1	*exo*	*endo*
1A1	<0.05	0.14	<0.05	<0.05
1A2	0.2	1.5	0.15	0.15
3A4	6.8	<0.05	1.35	<0.05

Data from Ueng et al. (*31*).

Flavonoids can modulate the metabolism of AFB_1, as first reported by Conney's laboratory (*42,43*). α-Naphthoflavone inhibits all activities of P450 1A2 (*31*); it also inhibits AFB_1 3α-hydroxylation (to form AFQ_1) by P450 3A4 but stimulates 8,9-epoxidation (*31,37*). The influence on the kinetic profiles is postulated to reflect an allosteric mechanism (*44*).

Conjugation of AFB_1-8,9-epoxides with Glutathione

Several lines of evidence suggest that the extent of glutathione (GSH) conjugation of AFB_1-8,9-epoxide is a major factor in influencing the risk of different experimental animal species to AFB_1-induced hepatocellular carcinoma (Figure 2)(*6,45*). At neutral pH, there is essentially no nonenzymatic reaction of GSH with AFB_1-8,9-epoxide (*25*). With crude liver cytosolic

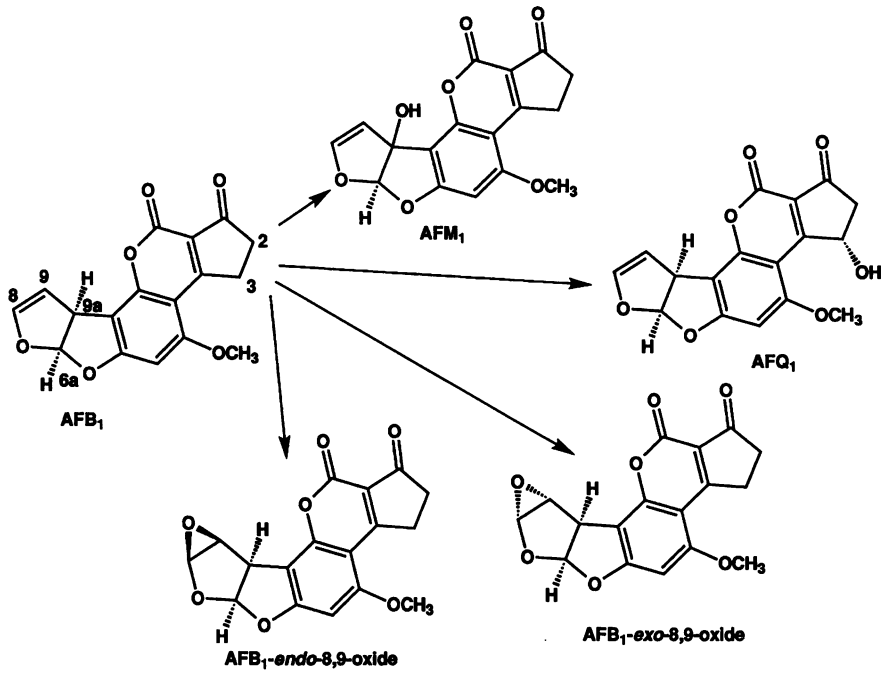

Figure 1. Oxidation of AFB_1 by P450s.

fractions, the order of (enzymatic) GST activity is mouse > rat > human (*25*). The relative extent of GSH-AFB_1 conjugate formation by some human GSTs is shown in Table 3.

AFB_1-8,9-epoxide Hydrolysis

Previous work has shown that the *exo* epoxide, the major isomer formed (*24,31*), is also less stable in CH_3OH/H_2O mixtures (*46*). Other work has suggested that the half-life ($t_{1/2}$) of the *exo* epoxide was < 10 sec (*47*).

Preliminary studies have indicated that the UV spectrum of AFB_1-8,9-dihydrodiol is distinct from that of the epoxide (Figure 3A). The fluorescence spectra are even more distinct, with the dihydrodiol having more than two orders of magnitude more fluorescence than the epoxide. Kinetics of hydrolysis were measured in a stopped-flow apparatus in a pH 7.0 buffer, with 9% $(CH_3)_2CO$ present (final concentration). The $t_{1/2}$ was approximately 1 sec when either UV or fluorescence traces were measured (Figure 3B).

Further studies indicated that the observed hydrolysis rate constant was rather constant between pH 4 and pH 9 but was increased at <pH 4, with a slope of the $\log_{10}$ observed versus pH having a slope of unity.

Interaction of AFB_1-8,9-*exo*-epoxide with Glutathione *S*-Transferase 3-3 and DNA

In earlier studies the relative rates of reaction of GSTs with AFB_1-8,9-epoxides had been estimated by quantitation of GSH-AFB_1 with fixed concentrations of GSTs and time points (Table 3) (*25*). To examine the aspects of these interactions, we measured the extent of GS-AFB_1 formation after mixing varying concentrations of AFB_1-8,9-*exo*-epoxide and GST 3-3 in the presence of GSH (Figure 4).

To estimate constants for the reactions, we set up the equations

$$A + B \underset{}{\overset{K_{eq}}{\rightleftharpoons}} AB \xrightarrow{k_1} C$$

and

$$A \xrightarrow{k_o} D$$

where A = [AFB_1-8,9-*exo*-epoxide]
B = [GST 3-3]
C = [GS-AFB_1]
and D = [AFB_1-8,9-dihydrodiol]

and utilized the KINSIM program (*33*) to evaluate various values of K_{eq} and k_1 that would give match values of C and D experimentally measured at various concentrations of A and B (Figure 4). The same approach was used with DNA and previously measured values (*48*) and with B = DNA and C = DNA N^7-guanyl adduct. The estimates are presented in Table 4, along with the measured k_o.

Table 3. Conjugation of AFB_1-8,9-epoxides by purified human GSTs.

GST	GSH-AFB_1 conjugate formed, nmol/mg GST protein[a]	
	exo-8,9-Epoxide	*endo*-8,9-Epoxide
A1-1	0.04	<0.01
A2-2	0.02	<0.01
M1-1	0.55	1.35
M3-3	<0.14	<0.14
P1-1	<0.06	<0.06

Data from Raney et al. (*25*). [a] AFB_1 (10:1 *exo*:*endo* mixture) was added to a 50 µl solution of each enzyme to give a final concentration of 55 µM AFB_1-8,9-epoxide. CH_3CO_2H was added to precipitate protein, and the GSH-AFB_1 conjugates were analyzed by HPLC. The results presented are means of duplicate experiments (*25*).

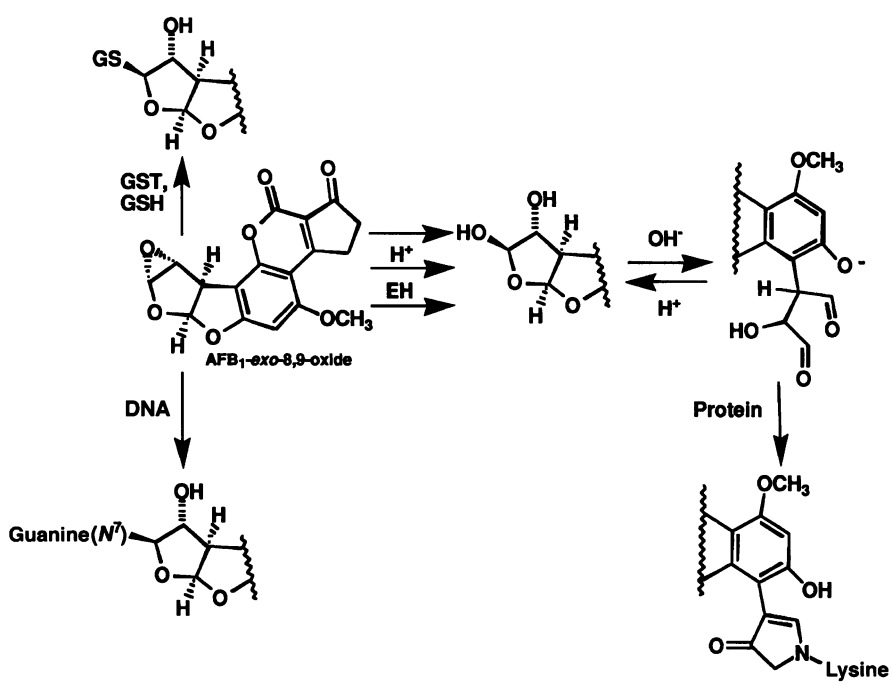

Figure 2. Reactions of AFB_1 8,9-oxides.

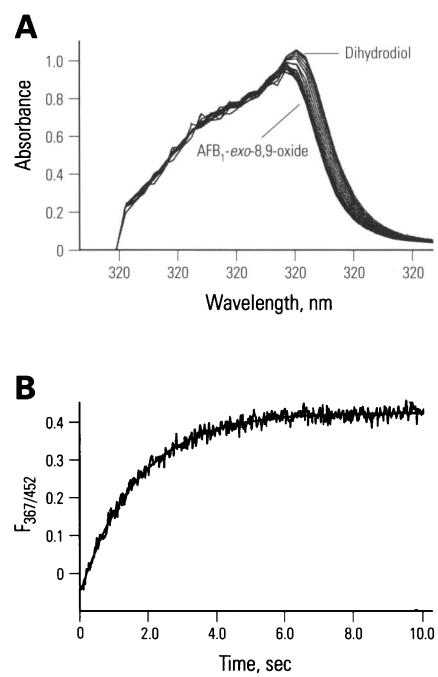

Figure 3. Nonenzymatic hydrolysis of AFB_1-8,9-*exo*-epoxide. *A*, A solution of AFB_1-8,9-*exo*-epoxide [in dry $(CH_3)_2CO$] was mixed with 10 volumes of 50 mM potassium phosphate buffer (pH 7.0) at 23 °C in the stopped-flow spectrophotometer and UV spectra were recorded approximately every 20 msec with the use of a diode array detector. *B*, In a similar experiment, the increase in fluorescence (excitation 357 nm, emission 452 nm) was recorded as a function of time.

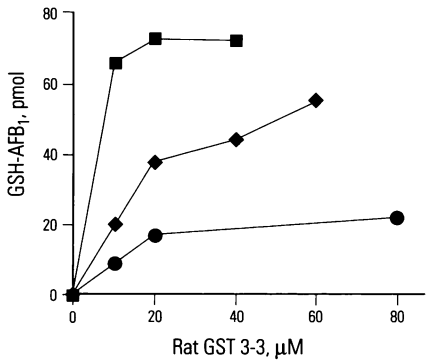

Figure 4. Formation of GS-AFB_1 by GST 3-3. AFB_1-8,9-*exo*-epoxide, in dry acetone, was mixed with varying amounts of GST 3-3 and 10 mM GSH in 50 µl of 50 mM potassium phosphate buffer (pH 7.4) at 23°C. The final concentration of AFB_1-8,9-*exo*-epoxide was 4 (●), 12 (◆), or 24 (■) mM. After 15 sec, 20 ml of 2.0 M aqueous CH_3CO_2H was added and the protein was precipitated by centrifugation at $3 \times 10^3 \times g$ for 10 min. Aliquots of the supernatant were analyzed for AFB_1-8,9-dihydrodiol and GS-AFB_1 by HPLC as described elsewhere (*25,31*).

Table 4. Reactions of AFB_1 8,9-*exo* oxide with GST 3-3 or DNA.

$k_o = k_{H^+} a_{H^+} + k_{o'}$

$k_{o'} = 0.58$/sec

$k_{H^+} = 2.0 \times 10^3$/M/sec

B	K_{eq}	k_1
GST 3-3	0.03 µM	0.08/sec
DNA	0.05 µM	0.20/sec

(See text for kinetic model.)

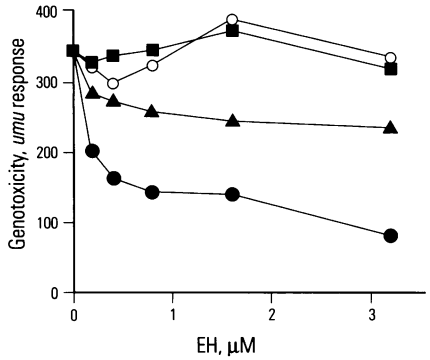

Figure 5. Effect of purified EH on genotoxicity of AFB_1 activated by P450 3A4. AFB_1 (20 µM) was activated in the presence of a recombinant P450 3A4 (10 nM)-based oxidation system (*31*) in the presence of an NADPH-generating system, *S. typhimurium* TA1535 containing plasmid pSK1001, and the indicated concentrations of purified rat (●) or human (▲,■) EH (the latter two samples were prepared from human liver samples, HL96 and HL105, of two different individuals). The response to heat-inactivated rat EH is also shown (○). The *umu* response was monitored by β-galactosidase response and is expressed as described by Shimada et al. (*32*).

Interactions of AFB_1-8,9-*exo*-epoxide and Epoxide Hydrolase

The action of EH on AFB_1-8,9-*exo*-epoxide has been postulated before (*49,50*), but definite results regarding this possibility are not available. The rapid rate of nonenzymatic hydrolysis (Figure 3B, Table 4) renders direct measurement of the reaction difficult. We could not see a definite effect of purified rat human EH on the recovery of N^7-guanyl DNA adducts in experiments where AFB_1-8,9-epoxide was mixed with DNA or where EHs were added to a system containing P450 3A4 (plus all accessory components needed for oxidation) and a suboptimal amount of GST. However, in other experiments we have been able to increase the observed rate of AFB_1-8,9-*exo*-epoxide hydrolysis (from 0.64 to 0.78/sec) in the presence of 19 µM rat EH. This result needs to be further evaluated.

We also used another system in which a very low concentration of P450 3A4 (and accessory components) was used to activate AFB_1 in an *S. typhimurium* system in which an *umu* end point was measured (Figure 5). Under such conditions a very high ratio of EH:P450 can be achieved. Both rat and human EH could partially inhibit the genotoxicity response under these conditions.

Summary

Further studies are needed to evaluate the role of EH in the metabolism of AFB_1-8,9-epoxide. The report of McGlynn et al. (*49*) is surprising in that the EH allelic variant was linked with higher levels of AFB_1–albumin adduct, even though the major AFB_1 protein adduct is thought to be derived from AFB_1-8,9-dihydrodiol (*51*). The dihydrodiol results from the enzymatic or nonenzymatic hydrolysis of the epoxide. The possibility of direct reaction of protein with the epoxide cannot be ruled out at this time; however, the effect of the allelic variation on the catalytic activity of EH is not well established. The report of lower activity is the result of lower levels of expression in a transient system, not intrinsic catalytic activity (*52*).

The complexity of the enzyme systems involved in the metabolism of AFB_1 points out the difficulties in doing molecular epidemiology studies, even when a single chemical carcinogen has been identified. The roles of at least two P450s in the activation process have been considered. There is suggestive evidence that human GSTs in the alpha, mu, and theta families may all have roles in the detoxication of the epoxide (*25,53–55*). Further work is needed to establish whether there is a role for EH. With all of the enzymes, there is a need

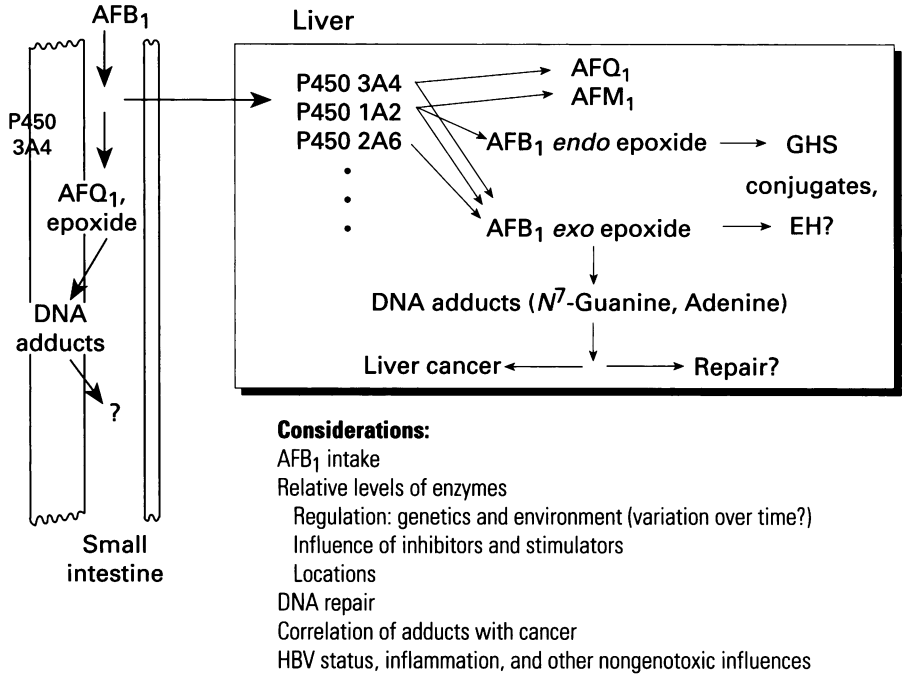

Figure 6. Complications involved in the metabolism of AFB_1 and relevance to hepatocellular cancer.

to consider the stereochemistry of the epoxide, which has been shown to be critical in genotoxicity.

We anticipate that more careful analysis of the kinetics of the reactions under consideration here will provide insight into competition of detoxication enzymes with DNA (Figure 2). However, even with this information there are considerable problems in the knowledge base underlying efforts in molecular epidemiology (Figure 6). The P450s both activate and detoxicate AFB_1, and the effect of inducing individual P450s is not easy to predict. Moreover, P450 3A4 is expressed in small intestine, the site of absorption of orally ingested AFB_1. The extent of detoxication there is unknown. Further, even activation of AFB_1 and DNA alkylation in the small intestine may be considered to be a detoxication process since the cells are sloughed rapidly and cancers of the small intestine are very rare. Other aspects regarding enzymes of activation and detoxication mentioned in Figure 6 have been discussed above. Other aspects not discussed here, but which may be involved, include whether the N^7-guanyl adduct or its ring-opened form is more mutagenic, the role of DNA repair, aflatoxin intake, the intraindividual variability of levels of the enzymes under consideration during the course of tumor initiation and development, and hepatitis B virus status.

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