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AN INVESTIGATION OF THE FORMATION OF CYTOTOXIC, PROTEIN-REACTIVE AND STABLE METABOLITES FROM CARBAMAZEPINE *IN VITRO*

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Abstract—The formation of chemically reactive metabolites from carbamazepine (CBZ) in the presence of mouse and human liver microsomes has been investigated using cytotoxicity and irreversible binding of radiolabelled compound as quantitative end-points. For comparison, the formation of the stable CBZ-10,11-epoxide (CBZ-10,11-E) has been measured. The formation of the cytotoxic, protein-reactive and stable metabolites of CBZ was increased by induction of the cytochrome P450 enzymes by phenobarbitone and reduced by co-incubation *in vitro* with ketoconazole (10–250 μM), suggesting that the formation of these metabolites is cytochrome P450 dependent. All human livers tested (N = 6) bioactivated CBZ to a protein-reactive metabolite, the mean covalent binding increasing from 0.08 ± 0.01% (without NADPH) to 0.27 ± 0.09% (with NADPH; $P \leq 0.05$). The formation of the chemically reactive metabolites was reduced by a subphysiological concentration of reduced glutathione (GSH) (500 μM), while ascorbic acid (100 μM) had no effect. Neither compound affected the formation of CBZ-10,11-E. Microsomal epoxide hydrolase (mEH), but not cytosolic epoxide hydrolase, caused a concentration-dependent inhibition of cytotoxicity reaching a maximum of 60% at 100 U of mEH. Covalent binding was also reduced by 60% by 100 U mEH. The separated T- and B-lymphocytes showed no difference in sensitivity when incubated with CBZ and mouse microsomes. The study demonstrates that the balance between activation of CBZ by the cytochrome P450 enzymes to a chemically reactive arene oxide metabolite and its detoxification by mEH and GSH may contribute to individual susceptibility to CBZ idiosyncratic toxicity.

The primary function of oxidative (phase I) metabolic pathways is the conversion of lipophilic, non-polar compounds to hydrophilic, polar compounds to facilitate excretion from the body. However, phase I metabolism can also lead to the formation of chemically reactive metabolites [1] which, if not adequately detoxified, can react by covalent linkage with cellular or autologous macromolecules resulting in either necrosis or secondary immune reactions [1–3]. Therefore, the balance between activation to a chemically reactive metabolite and its detoxification may be a critical determinant of the likelihood of developing idiosyncratic toxicity with a compound.

Carbamazepine (CBZ‡), a widely used anticonvulsant, is extensively metabolized with over 30 metabolites having been identified *in vivo* [4]. The most important pathway involves the formation of the stable CBZ-10,11-epoxide (CBZ-10,11-E) and subsequent hydrolysis by epoxide hydrolase to the *trans*-10,11-dihydroxy-10,11-dihydro CBZ [4, 5]. CBZ can also be converted to CBZ-10,11-E *in vitro*, although the rate of conversion has been shown to be variable between different human livers [6].

CBZ is associated with a wide range of idiosyncratic adverse effects including cutaneous reactions [7], aplastic anaemia [8] and hepatitis [9]. These are thought to result from the formation (by phase I metabolic pathways) of toxic, chemically reactive metabolites [10, 11]. CBZ can be bioactivated by human liver microsomes to a cytotoxic metabolite [12] and it has been shown that cells from CBZ-hypersensitive patients are more sensitive to oxidative drug metabolites generated by a murine hepatic microsomal system than appropriate controls [11, 12], suggesting that these patients have a deficiency of cellular detoxification. The chemically reactive metabolite, thought to be responsible for the idiosyncratic toxicity of CBZ, has been postulated to be an arene oxide [10–12] on the basis that the metabolism-dependent cytotoxicity of CBZ *in vitro* can be enhanced by trichloropropene oxide (TCPO) [10], an inhibitor of epoxide hydrolase. However, deduction of the possible nature of the toxic metabolite from this evidence is complicated by the fact that TCPO is also known to deplete glutathione [13] and inhibit cytochrome P450 [14].

In view of the above considerations, in this *in*

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‡ Abbreviations: ASC, ascorbic acid; CBZ, carbamazepine; CBZ-10,11-E, CBZ-10,11-epoxide; CRM, chemically reactive metabolite; cEH, cytosolic epoxide hydrolase; GSH, reduced glutathione; HSA, human serum albumin; mEH, microsomal epoxide hydrolase; MNL, mononuclear leukocytes; NAc, *N*-acetyl cysteine; TCPO, 1,1,1-trichloropropene-2,3-oxide; PB, phenobarbitone; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

vitro study, we have investigated the characteristics of the stable, cytotoxic and protein-reactive metabolites formed from CBZ in the presence of human and mouse liver microsomes, and factors which affect the formation of these metabolites.

MATERIALS AND METHODS

Chemicals. CBZ, 1,1,1-trichloropropene-2,3-oxide (TCPO), reduced glutathione (GSH), *N*-acetylcysteine (NAc), ascorbic acid (ASC), ketoconazole and human serum albumin (HSA; fraction V) were purchased from the Sigma Chemical Co. (Poole, U.K.). Reduced NADPH (tetrasodium salt) was obtained from BDH Chemicals (Poole, U.K.). [10,11-^{14}C]CBZ (radiochemical purity 99%) and CBZ-10,11-E were gifts from Ciba-Geigy Pharmaceuticals (Basle, Switzerland). All solvents were of HPLC grade and were products of Fisons plc (Loughborough, U.K.).

Purification of microsomal and cytosolic epoxide hydrolases (mEH and cEH). mEH was purified to homogeneity from guinea pig liver by the method of Lu *et al.* [15] and was dialysed in 10 mM sodium phosphate buffer (pH 7.4). cEH was prepared from guinea pig liver [16] and the semi-purified product was dialysed in 10 mM Tris buffer and 0.1 mM EDTA (pH 8.0). The activities of these enzymes were determined using styrene oxide [15] and *trans*-stilbene oxide [16] as substrates for mEH and cEH, respectively, and are expressed as units (U), where one unit converts one nanomole of substrate to the diol product per minute.

Preparation of human and murine hepatic microsomes. Human liver microsomes from histologically normal livers (N = 6; age range 27–66 years) obtained from kidney transplant donors were prepared as reported previously [17]. Ethical consent was obtained from the local ethical committee. Microsomes were also prepared from groups of six phenobarbitone (PB)-pre-treated mice (60 mg/kg body weight per day, i.p. in 0.9% w/v saline for 3 days) and six control mice (which received 0.15 mL of 0.9% saline once a day for 3 days) by the same centrifugation procedure used to obtain washed human liver microsomes. The microsomal pellets were stored at −80° until used. The cytochrome P450 content was measured by the method of Omura and Sato [18] and microsomal protein content estimated by the method of Lowry *et al.* [19].

Isolation of human cells from peripheral blood. Peripheral blood mononuclear leukocytes (MNL) were isolated from fresh heparinized venous blood from healthy male volunteers (age range 22–37 years) as described previously [20]. Their viability upon isolation was >95%.

T- and B-lymphocytes were isolated using magnetic monosized polymer microspheres (Dynabeads M-450, Dynal (UK) Ltd, Wirral, U.K.) using a previously published procedure [21] with minor modifications [22]. The purity of the isolated T- and B-lymphocyte subpopulations was 95 and 90%, respectively, and the viability upon isolation was >95%.

Determination of the metabolism-dependent cytotoxicity of CBZ. Isolated MNL (1×10^6) in HEPES-buffered balanced salt medium (1 mL) [23] were incubated with CBZ (50 μM) and murine or human liver microsomes (0.5–2.0 mg microsomal protein/incubation) in the presence or absence of NADPH (1 mM) for 2 hr at 37°. The concentration of CBZ used was not directly cytotoxic [12] and the drug was added in 10 μL HPLC grade methanol, which as a 1% solution (v/v) was non-toxic. All incubations were carried out in quadruplicate.

In some incubations, with PB mouse microsomes, varying concentrations of mEH and cEH were added, with control incubations receiving an equivalent volume of the dialysing buffer. In addition, in other experiments, the effect of pre-incubation of cells with GSH (500 μM), NAc (50 μM) and ASC (100 μM) was assessed.

After 2 hr, the cells were sedimented and resuspended in a drug-free medium (HEPES-buffered medium containing 5 mg/mL HSA). Incubations were continued for 16 hr at 37° and aliquots removed in order to determine cell viability by Trypan blue dye exclusion (0.2% Trypan blue) as reported previously [20].

Experiments with the separated T- and B-lymphocyte subpopulations were carried out with PB mouse microsomes (0.5 mg) in the same manner as above, except that the final incubation volume was 0.5 rather than 1 mL.

Metabolism of CBZ by human and murine hepatic microsomes. [^{14}C]CBZ (50 μM, 0.15 μCi) was incubated with murine (1 mg protein) or human (2 mg protein) hepatic microsomes and 1×10^6 MNL in HEPES-buffered medium (pH 7.4; final volume 1 mL). The reactions were initiated by the addition of NADPH (1 mM; omitted in control incubations) followed by incubation in a shaking water bath for 2 hr at 37°. Some incubations also contained TCPO (30 μM), mEH (100 U), GSH (500 μM), NAc (50 μM), ASC (100 μM) or ketoconazole (10–250 μM). The reactions were terminated by the addition of 3 mL acetonitrile and left overnight at −20° to precipitate the protein. After centrifugation (2000 *g* for 10 min) the supernatants were removed for analysis of stable metabolites.

Irreversible binding of radiolabelled material. Radiolabelled material irreversibly bound to precipitated protein was determined by exhaustive solvent extraction. The protein was extracted successively with methanol (5 mL × 2) and 70% (v/v) methanol (5 mL × 2) by mixing on a rotary mixer for 20 min. The protein was dissolved in 1 M NaOH (1 and 2 mL for murine and hepatic microsomes, respectively) at 60°. Aliquots were taken for quantification of radioactivity (in 12 mL scintillant) and for protein estimation. Irreversible binding of radiolabelled CBZ is expressed as a percentage of the initial radioactivity bound to the incubated microsomal protein.

Analysis of the stable metabolite by radiometric HPLC. The supernatants from the mouse or human microsomal incubations with [^{14}C]CBZ were analysed for unchanged CBZ and its 10,11-epoxide essentially as described by Regnaud *et al.* [24] using an HPLC (LKB, Bromma, Sweden) linked to a radiometric detector (Flo-one Beta, Canberra, Packard). The volume of the supernatant was

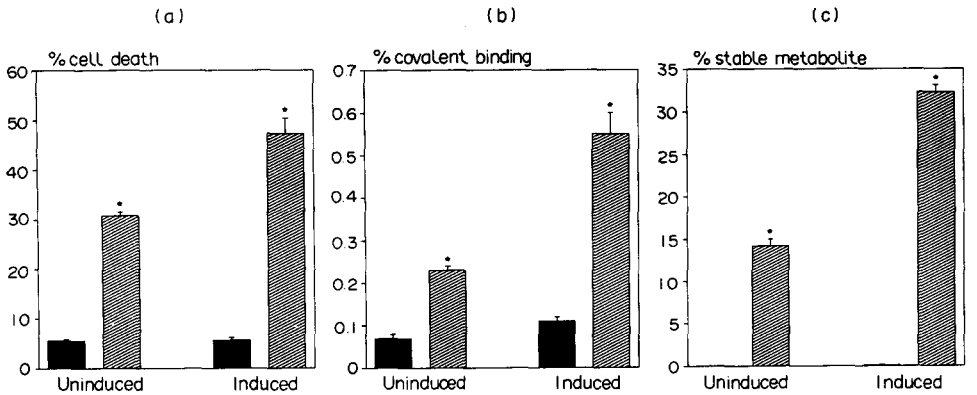

Fig. 1. A comparison of the effect of uninduced (1 mg) and PB-induced (1 mg) mouse liver microsomes on the formation of cytotoxic (a), protein-reactive (b) and stable (CBZ-10,11-E; c) metabolites from CBZ (50 μM) either in the absence (■) or presence (▨) of NADPH (1 mM). The results represent the means ± SEM of triplicate or quadruplicate incubations. The control cell death values in the absence of drug were 4.8 ± 1.0 and 6.6 ± 0.7% for uninduced and induced liver microsomes, respectively. Statistical analysis performed by comparing incubations with and without NADPH: *$P \leq 0.002$.

reduced to *ca.* 1 mL by vacuum centrifugation at 40° for 40 min. The resulting solution was filtered through a 0.45 μm filter (Millipore, U.K.) and 50 μL was injected onto a C_{18} Ultratech 50DS column (20 cm; HPLC Technology, Macclesfield, U.K.). The solvent system (30% acetonitrile: 10% methanol: 60% 0.005 M tetrabutyl ammonium chloride in water) was delivered at a flow rate of 1.2 mL/min and the absorbance of the eluant was monitored at 230 nm. CBZ-10,11-E and CBZ were identified by comparison of their retention times (6.3 and 11.9 min for CBZ-10,11-E and CBZ, respectively) with those of co-injected authentic compounds. Radioactivity was monitored throughout the run and peaks were integrated and expressed as percentage radioactivity eluting from the column. Integration of radioactivity over the full 15-min run time showed that >95% of injected radioactivity was recovered.

Statistical analysis. Statistical analyses were performed by one-way analysis of variance accepting $P \leq 0.05$ as significant. Data are presented as means ± SEM.

RESULTS

Effect of enzyme induction on metabolite formation from CBZ

The cytochrome P450 content of control (saline-treated) mice and PB-treated mice was 1.3 and 1.9 nmol P450/mg protein, respectively. The addition of NADPH (1 mM) significantly increased metabolite formation from CBZ with both uninduced (1 mg) and induced (1 mg) mouse microsomes (Fig. 1). The formation of the cytotoxic ($P \leq 0.01$), protein-reactive ($P = 0.005$) and stable (CBZ-10,11-E; $P \leq 0.001$) metabolites was significantly higher with PB-treated mouse microsomes than with the untreated mouse microsomes.

Table 1. The NADPH-dependent conversion of CBZ (50 μM) to protein-reactive and stable (CBZ-10,11-E) metabolites by human liver microsomes (2 mg)

Liver	Irreversible binding (%)	CBZ-10,11-E formation (%)
L1	0.15	7.7
L2	0.10	4.3
L3	0.10	8.8
L4	0.10	5.9
L5	0.11	3.7
L6	0.58	8.5

Values represent the means of triplicate determinations and have been determined by subtracting the result obtained in incubations without NADPH from incubations with NADPH.

Bioactivation of CBZ to a protein-reactive metabolite by human liver microsomes

In a previous study, it has been shown that human livers are capable of bioactivating CBZ (50 μM) to a cytotoxic metabolite [12]. In the present study, experiments with six human livers showed that all of them were capable of forming protein-reactive (and stable) metabolites with the addition of NADPH (Table 1), the mean covalent binding (N = 6 livers) increasing from 0.08 ± 0.01% (without NADPH) to 0.27 ± 0.09% (with NADPH; $P \leq 0.05$). The addition of TCPO (30 μM; N = 3 livers) resulted in a non-significant increase in covalent binding (0.18 ± 0.01 vs 0.22 ± 0.02%; NS).

Effect of ketoconazole on the formation of metabolites from CBZ

Ketoconazole, a cytochrome P450 inhibitor [25], reduced irreversible binding and stable metabolite

Table 2. The effect of ketoconazole on the metabolism-dependent irreversible binding of radiolabelled CBZ (50 μM) and stable (CBZ-10,11-E) metabolite formation in the presence of either PB-induced mouse (1 mg) or human (2 mg) liver microsomes

Ketoconazole concentration (μM)	PB mouse liver microsomes		Human liver microsomes	
	Covalent binding (%)	CBZ-10,11-E formation (%)	Covalent binding (%)	CBZ-10,11-E formation (%)
0	0.37 ± 0.01	23.1 ± 1.3	0.7 ± 0.02	8.6 ± 0.1
10	0.16 ± 0.03*	8.9 ± 1.1*	0.32 ± 0.06*	2.4 ± 0.3*
100	0.05 ± 0.00*	2.8 ± 0.06*	0.25 ± 0.01*	0.5 ± 0.1*
250	ND	ND	0.20 ± 0.01*	0.2 ± 0.1*

The results represent the means ± SEM of triplicate incubations performed with pooled mouse liver microsomes or microsomes prepared from one human liver (L6). Statistical analysis performed by comparing incubations with and without added ketoconazole, *P ≤ 0.001. ND, not determined.

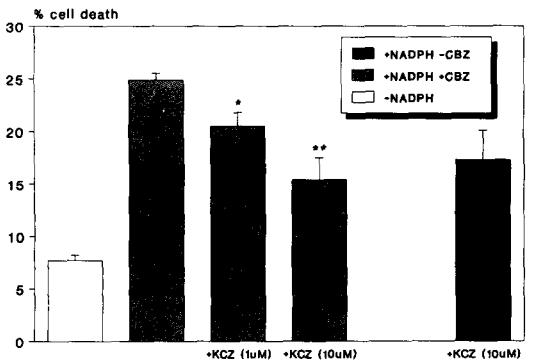

Fig. 2. The effect of ketoconazole (KCZ; 1 and 10 μM) on the bioactivation of CBZ (50 μM) to a cytotoxic metabolite by PB-induced mouse liver microsomes (1 mg) either in the presence or absence of NADPH (1 mM). The cytotoxicity of ketoconazole (10 μM) (in the absence of CBZ) is also shown. The results represent the means ± SEM of quadruplicate incubations. The control cell death value in the absence of drug was 6.6 ± 0.9%. The inhibition of CBZ cytotoxicity has been analysed by comparing incubations with and without ketoconazole (in the presence of NADPH), *P ≤ 0.05, **P = 0.005.

formation with both PB mouse microsomes (1 mg) and human liver microsomes (2 mg; Table 2).

With PB mouse microsomes (1 mg), ketoconazole also inhibited the metabolite-dependent cytotoxicity of CBZ (Fig. 2), although the maximum inhibition of CBZ cytotoxicity was limited by the cytotoxicity of ketoconazole (10 μM) itself (Fig. 2), thus precluding the use of higher ketoconazole concentrations.

Effect of thiols and ASC on metabolite formation from CBZ

Inclusion of GSH (500 μM) significantly reduced metabolism-dependent cytotoxicity and protein-reactivity of CBZ, while ASC (100 μM) had no effect (Table 3). *N*-acetylcysteine (50 μM) reduced metabolism-dependent cytotoxicity but not irreversible binding of radiolabelled compound (Table 3). Experiments with one human liver (L1; 2 mg; incubations in quadruplicate) showed that although overall bioactivation of CBZ to a cytotoxic metabolite was less than with PB mouse microsomes, the effect of co-incubation with thiols and ASC was similar to that seen with PB mouse microsomes, with GSH and NAc reducing metabolism-dependent cytotoxicity (91 and 71% inhibition for GSH and NAc, respectively), while ASC (4% inhibition) had no significant effect. The formation of CBZ-10,11-E was not affected by either GSH, NAc or ASC with either human liver microsomes or PB mouse microsomes.

Effect of epoxide hydrolase on the bioactivation of CBZ

With PB mouse microsomes (0.5 mg), co-incubation of MNL with purified mEH resulted in a concentration-dependent decrease in the metabolism-dependent cytotoxicity of CBZ, reaching a maximum at 100 U (Fig. 3). Control incubations containing HSA at an equivalent protein concentration to that of mEH did not reduce cytotoxicity (data not shown). Co-incubation of TCPO (30 μM) with 100 U of mEH resulted in a statistically significant (P ≤ 0.02) increase in cytotoxicity (Fig. 3).

In contrast, semipurified cEH caused no decrease in metabolism-dependent cell death up to 100 U (29.9 ± 1.5, 32.9 ± 2.1 and 31.8 ± 2.2% at 0, 50, and 100 U of cEH, respectively). An increase in the concentration of cEH to 200 U significantly increased cell death to 39.9 ± 1.6% (P = 0.003). However, use of heat-inactivated cEH (60° for 10 min) at a similar concentration also increased cytotoxicity (relative to the incubations without cEH), suggesting that a contaminant in the enzyme preparation was responsible for the increase in cell death.

Co-incubation of mEH (100 U) with radiolabelled CBZ and PB mouse microsomes resulted in a significant decrease in *in vitro* covalent binding (0.37 ± 0.01 vs 0.15 ± 0.00%; P ≤ 0.001) and CBZ-

Table 3. The effect of GSH (500 μM), NAc (50 μM) and ASC (100 μM) on the metabolism-dependent cytotoxicity and irreversible binding of CBZ (50 μM) with either PB-induced (PB) mouse (1 mg) or human (2 mg) liver microsomes

	PB mouse liver microsomes		Human liver microsomes
Compound	Cytotoxicity inhibition (%)	Covalent binding inhibition (%)	Covalent binding inhibition (%)
GSH	59 ± 3*	63 ± 2*	51 ± 8*
NAc	33 ± 2*	28 ± 13	4 ± 9
ASC	0.7 ± 21	12 ± 17	−11 ± 13

Results for PB-induced mouse liver microsomes represent the means ± SEM for three experiments with pooled microsomes (incubations in triplicate or quadruplicate) while for human liver microsomes, the results are the means ± SEM for four human livers (incubations in triplicate). Statistical analysis performed by comparing degree of inhibition obtained by co-incubation with the above compounds with incubations without any added compounds, *$P \leq 0.001$.

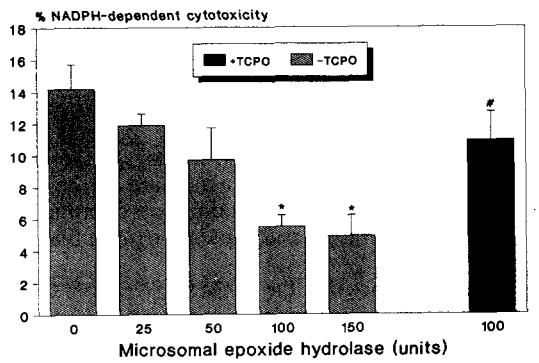

Fig. 3. The effect of addition of mEH on the cytotoxicity of CBZ (50 μM) in the presence of PB-induced mouse liver microsomes (0.5 mg) and NADPH (1 mM), and in the presence or absence of TCPO (30 μM), expressed as NADPH-dependent cytotoxicity (results represent means ± SEM of quadruplicate incubations). The control cell death values either in the absence of drug or absence of NADPH were 7.2 ± 0.5 and 7.3 ± 1.5%, respectively. Statistical analysis performed by comparing incubations with and without mEH, *$P \leq 0.005$, and for incubations containing 100 U mEH with and without TCPO, # $P \leq 0.02$.

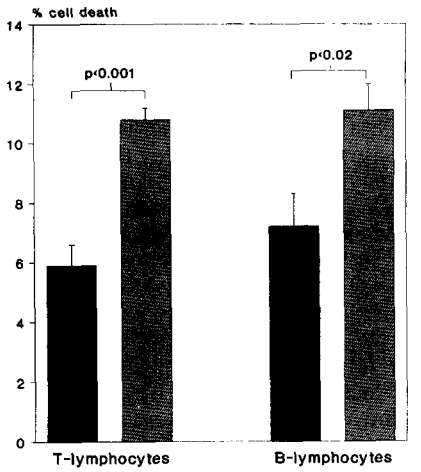

Fig. 4. A comparison of the sensitivity of separated T- and B-lymphocyte subpopulations to the cytotoxic metabolite of CBZ generated *in vitro* by a murine microsomal system (0.5 mg). Results represent the means ± SEM of three separate experiments (incubations in quadruplicate). Statistical analysis within the same group performed by comparing the incubations with (▨) and without NADPH (■), with significance values as marked on the graph. Analysis of any difference in the sensitivity of the two cell types performed by comparing the NADPH-dependent cytotoxicity of the two types (no significant difference).

10,11-E formation (23.1 ± 1.3 vs 8.2 ± 0.3%; $P \leq 0.001$).

Metabolism-dependent cytotoxicity of CBZ with T- and B-lymphocyte subpopulations

Although there was an increase in cell death of both the T- and B-lymphocytes in the presence of a full metabolising system (Fig. 4), there was no significant difference in the sensitivity of the T- and B-cell subpopulations to the CBZ metabolite.

DISCUSSION

Idiosyncratic adverse drug reactions can result from the formation of chemically reactive metabolites [1]. For some drugs such as sulphonamides [22] and dapsone [26], these toxic metabolites have been characterized. However, for a wide variety of drugs, including CBZ, the nature of the chemically reactive metabolite is unknown, largely due to the fact that such metabolites (by definition) are often too unstable to be characterized by routine analytical methods such as HPLC and mass spectrometry. Therefore, in this *in vitro* study, we have used two techniques, irreversible protein binding of radiolabelled material and MNL cytotoxicity, as

indirect markers for the formation of chemically reactive metabolites (CRM) from CBZ and, for comparison, the formation of CBZ-10,11-E, a stable metabolite of CBZ [4, 5], has been measured using radiometric HPLC.

In vivo, the ability of a drug to cause an adverse reaction depends on the balance between the formation of a toxic metabolite and its cellular detoxication. Therefore, although the use of covalent binding and metabolism-dependent cytotoxicity as markers for the formation of CRM may not accurately reflect the *in vivo* toxicology of a drug [27], these techniques are useful in that the conditions used may be varied, as in this study, to determine which factors lead to the critical imbalance between activation and detoxication *in vivo* and thus predispose to idiosyncratic toxicity. An added advantage of the *in vitro* cytotoxicity assay devised by Spielberg [23] is that inter-individual variability in the detoxication of drug metabolites can be assessed by using peripheral blood MNL from different individuals. Indeed, cells from patients known to be hypersensitive to CBZ show greater sensitivity to metabolites generated *in situ* by a murine microsomal system than those from appropriate controls [11, 12]. This evidence, taken together with the ability of human livers to bioactivate CBZ to a cytotoxic metabolite [12] and, from the present study, to a protein-reactive metabolite (Table 1), suggests that CBZ-hypersensitive patients have a deficiency in cellular detoxication.

Based largely on the observation that TCPO enhances the *in vitro* cytotoxicity of CBZ [10, 11], it has been suggested that the hypersensitive patients have a deficiency of epoxide hydrolase [11]. However, further evidence is needed to substantiate this hypothesis since TCPO has multiple actions [13, 14] and at least two forms of epoxide hydrolase, with different substrate specificities [28], exist. Of the two major forms of EH, the microsomal enzyme (mEH) is located mainly in the endoplasmic reticulum and catalyses the hydrolysis of xenobiotic epoxides (including arene oxides) [28] while the cytosolic form (cEH), located both in cytosol and peroxisomes, catalyses the hydrolysis of *trans*-substituted styrene oxides [28]. mEH is in fact known to hydrolyse CBZ-10,11-E to its major urinary metabolite, CBZ-dihydrodiol [29]. Indeed, in accordance with this, we found that the formation of CBZ-10,11-E *in vitro* was reduced by the addition of exogenous mEH. Furthermore, mEH but not cEH also reduced the metabolism-dependent cytotoxicity of CBZ (Fig. 3), providing further evidence that the toxic metabolite is an arene oxide. The reduction in cytotoxicity is unlikely to be due to the addition of exogenous protein since equivalent amounts of HSA did not affect cytotoxicity and co-incubation of mEH with TCPO reduced the inhibition of cytotoxicity. A similar reduction in irreversible binding of radiolabelled CBZ with mEH suggests that the protein-reactive metabolite may be the same metabolite as that responsible for cytotoxicity. Therefore, these results indicate that CBZ-hypersensitive patients may have a deficiency of the microsomal, rather than the cytosolic form of the enzyme.

The glutathione redox cycle is an important protective mechanism for the cell [30, 31]. GSH acts as a nucleophilic "scavenger" conjugating, either non-enzymatically or enzymatically, with different types of electrophile, including arene oxides, leading to their detoxication [31, 32]. Thus, a subphysiological concentration of GSH inhibited both metabolism-dependent cytotoxicity and protein-reactivity (Table 3), but had no effect on the formation of the stable 10,11-epoxide. The inhibition of irreversible binding *in vitro* by GSH supports the concept that the irreversible binding observed with radiolabelled CBZ in this study represents covalently bound drug metabolites and not non-extractable metabolites. The role of GSH in protecting against idiosyncratic toxicity with CBZ *in vivo* is unclear; however, as has been suggested for detoxication of *trans*-β-ethylstyrene 7,8-oxide by cEH [33], the concentration of the CRM metabolite may be important with mEH being more important than the glutathione transferases at low concentrations. Another possibility raised by the detoxication of the CRM of CBZ by both mEH (Fig. 3) and GSH (Table 3) is that some CBZ-hypersensitive patients may have more than one pharmacogenetic disorder, i.e. a double genetic polymorphism. However, a preliminary study in hypersensitive patients has shown that the polymorphic enzyme [34], glutathione transferase μ, which is known to detoxify certain epoxides [35], does not show a different distribution from that seen in the general population (unpublished data).

Further evidence that the CRM formed from CBZ is an arene oxide is provided by the lack of effect of ASC on the *in vitro* toxicity of CBZ (Table 3). This concentration of ASC has been shown previously to reduce the cytotoxicity associated with dapsone [26] and sulphapyridine [22], drugs whose CRM are known to be other than epoxide metabolites. Interestingly, NAc reduced metabolism-dependent cytotoxicity but had a minimal effect on covalent binding to microsomal protein (Table 3) suggesting that protection for cells may have been afforded by virtue of its ability to enter cells and subsequently serve as a precursor for GSH formation [31].

The *in vitro* cytotoxicity assay devised by Spielberg [23] uses MNL as target cells. This is a mixed cell population consisting of mainly lymphocytes (T- and B-cells) and monocytes [36]. In this study, we separated the T- and B-lymphocytes, obtaining high purity sub-populations, and found that there is no difference in the sensitivity of the isolated cells, presumably reflecting an equivalent capacity for the detoxification of the CRM of CBZ.

The epoxidation of CBZ to the stable 10,11-epoxide is cytochrome P450 dependent [5]. Consistent with this, the formation of CBZ-10,11-E was enhanced by enzyme induction with PB (Fig. 1) and inhibited by ketoconazole (Table 2), a non-specific cytochrome P450 inhibitor [25]. Our results also indicate that the formation of the CRM of CBZ is also cytochrome P450 dependent, since cytotoxicity (Fig. 5) and protein reactivity (Fig. 1 and Table 2) were affected in a similar manner to that seen with the stable epoxide. CBZ is well known to cause both auto- and hetero-induction of the cytochrome P450

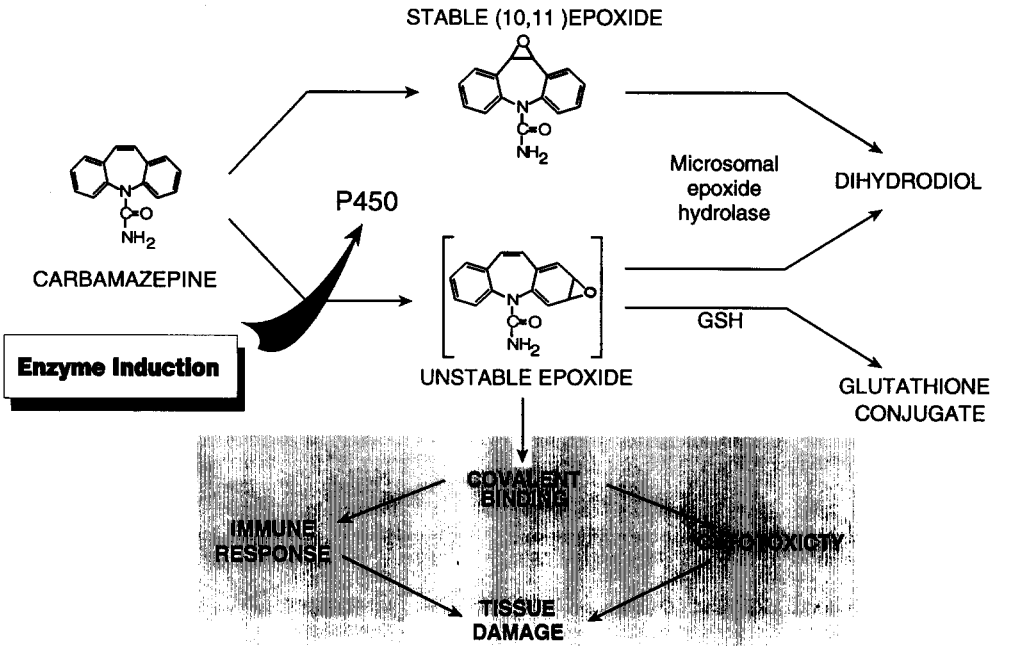

Fig. 5. Schematic representation of the importance of the balance between activation of CBZ to a chemically reactive epoxide metabolite and its detoxication in predisposing to idiosyncratic toxicity.

enzymes [5, 37], and thus will to some extent induce the formation of its own reactive epoxide metabolite. However, CBZ is also known to induce the epoxide hydrolase system [6, 24], but several studies have found that the inducibility of the CBZ-epoxidase pathway is higher compared with the hydration (i.e. epoxide to dihydrodiol) pathway [5, 6, 38], suggesting that enzyme induction (either autoinduction or induction by other concurrently administered anti-epileptics such as phenytoin and phenobarbitone) may serve as a contributory risk factor by further altering the balance between activation and detoxication in certain patients.

In summary, our results indicate that CBZ is bioactivated by the cytochrome P450 enzymes to a chemically reactive metabolite, thought to be an arene oxide, which is detoxified by mEH and GSH. The unique predisposition of patients to develop idiosyncratic toxicity with CBZ may be due to a deficiency in cellular detoxication, predominantly mEH, with enzyme induction serving as a contributory risk factor (Fig. 5). The inadequate detoxication of the CRM may lead to covalent binding with subsequent toxicity either directly or indirectly by initiating an immune response. The latter is thought to be more likely for CBZ in view of the accompanying symptomatology [11, 12], the demonstration of reactive T-lymphocytes [39, 40] and the presence of autoantibodies in hypersensitive patients [41]. Further studies are required in patients to demonstrate any enzyme deficiency and the possible long-term consequences of such a deficiency.

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