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HUMAN ANTI-ENDOPLASMIC RETICULUM AUTOANTIBODIES PRODUCED IN AROMATIC ANTICONVULSANT HYPERSENSITIVITY REACTIONS RECOGNISE RODENT CYP3A PROTEINS AND A SIMILARLY REGULATED HUMAN P450 ENZYME(S)

Robert J. Riley[1#], Gillian Smith[2], C. Roland Wolf[2], Vicki A. Cook[3] and J. Steven Leeder[3]

[1]Fisons Pharmaceuticals, Department of Biochemistry, Drug Metabolism Section, Bakewell Road, Loughborough, Leics., UK

[2]Imperial Cancer Research Fund, Molecular Pharmacology Laboratory, Hugh Robson Building, George Square, Edinburgh, UK

[3]Division of Clinical Pharmacology and Toxicology, Department of Paediatrics, The Hospital for Sick Children, Toronto, Ontario, Canada

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SUMMARY: Hypersensitivity reactions to aromatic anticonvulsants are associated with anti-liver microsomal antibodies which recognise rodent proteins. The reactivity of these antibodies, the regulation of the rodent antigens and the identity of the human autoantigen have been investigated. Dexamethasone elevated markedly the levels of an immunoreactive mouse protein(s) which exhibited a *Mr* (53 kDa) and inducibility consistent with the major *Cyp3a* product. Immunoblots conducted with hepatic microsomes from control and induced rats and purified rat P450s confirmed that these antibodies also recognised constitutive (3A2) and inducible (3A1) rat *CYP3A* products. Negligible reactivity was observed with microsomes from human B-lymphoblastoid cell lines expressing CYP1A1, 1A2, 2A6, 2D6, 2E1, 3A4 or epoxide hydrolase. Analysis of a phenotyped human liver bank revealed that the antibodies recognised a 52.5 kDa microsomal protein which exhibited marked heterogeneity in its expression and appeared to be regulated co-ordinately with human CYP2C8 and 3A3/4. The inter-individual variation in the expression of this protein(s) and its potential induction by anticonvulsant therapy together with an inherited deficiency in drug detoxification capacity may explain predisposition to these immunoallergic reactions. © 1993 Academic Press, Inc.

#To whom correspondence should be addressed.

ABBREVIATIONS: AC(s), anticonvulsant(s); PB, phenobarbitone; BNF, β-naphthoflavone; Dex, dexamethasone; EH, epoxide hydrolase; HRP, horseradish peroxidase; DMSO, dimethyl sulphoxide; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; *Mr*, apparent relative molecular weight.

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Patients receiving structurally-related anticonvulsants (ACs), including phenobarbitone (PB), phenytoin and carbamazepine, may develop rare, severe and potentially life-threatening "hypersensitivity reactions" between a week and several months into therapy which are characterised by fever, skin rash, lymphadenopathy and toxicity to other organ systems (1-3). Such idiosyncratic reactions are not predictable from a knowledge of the pharmacological properties of the drugs, are dose-independent and are therefore most feared by physicians. While drug-specific antibodies or sensitized T-cells have yet to be identified in patients receiving AC therapy, the clinical manifestations of these reactions are consistent with an immunological origin (4).

Although the metabolic basis of AC-induced hypersensitivity reactions remains largely obscure, several studies have suggested they may arise from an imbalance in activation and detoxification pathways as a result of an inherited deficiency in epoxide hydrolase (EH; 5,6) and an increase in the form(s) of P450 responsible for metabolising ACs to reactive intermediates (2,5). Either of these metabolic idiosyncrasies could conceivably lead to increased levels of covalent adducts which could then serve as potential immunogens and trigger the formation of antibodies directed against drug-modified or native proteins.

In support of this hypothesis, we have identified recently the presence of specific anti-endoplasmic reticulum antibodies in 14 patients presenting with potential hypersensitivity reactions to AC therapy which recognise a PB-inducible 53 kDa rodent microsomal protein (7-9). These antibodies have been shown to react with members of the P450 supergene family as described for sera from patients suffering from immunoallergic reactions to tienilic acid (CYP2C9; 10), dihydralazine (CYP1A2; 11) and, more recently, halothane (possibly CYP2E1; 12). Despite the diversity of organs involved in the toxic reactions, the reactivity of the sera was similar for all the AC patients and preliminary data suggest that the isoforms of P450 detected are quite distinct from those produced during these other adverse drug reactions (9, 10). No such reactivity was observed with serum from appropriate control individuals. Here, we have extended our characterisation of these antibodies from one affected individual. This report examines the specific reactivity of the antibodies and the regulation of the immunoreactive protein(s) by employing a more extensive panel of purified rat P450 proteins; examining the inducibility of the rat antigen and contrasting its expression to other candidate proteins; and comparing the pattern of expression of the human autoantigen with the major human P450 isoforms.

MATERIALS AND METHODS

Chemicals

Electrophoresis reagents were obtained from Bio-Rad Laboratories (Watford, Herts., UK). Horseradish peroxidase (HRP)-conjugated protein A, HRP-conjugated sheep anti-human immunoglobulin antibodies, chemiluminescence detection reagents, X-ray film and nitrocellulose membranes were purchased from Amersham (Aylesbury, Bucks., UK). Rabbit polyclonal antisera against various rat P450 isoforms were purchased from OXYgene (Dallas, TX, USA) or prepared as described previously (13). Highly purified rat P450 proteins were prepared and characterised as detailed elsewhere (13). Microsomes from lymphoblastoid cell

lines transfected with cDNAs for the major human P450s and EH were purchased from the GENTEST Corp. (Woburn, MA, USA). Rabbit polyclonal anti-human EH antiserum was a generous gift from Dr. Urs Meyer (Basel, Switzerland). All other reagents were purchased from Sigma (Poole, Dorset, UK).

Patient Information and Clinical Course of Toxic Reaction

The patient was a 24 year old male who had been treated with phenytoin for grand mal and partial seizure activity. After 1 month of therapy, he developed fever to 39.8 °C and a generalised pruritic erythoderma with mucous membrane involvement. There was also marked, generalised lymphadenopathy which was present for approximately 2 weeks. *In vitro* challenge to examine the individual's cellular capacity to detoxify reactive AC metabolites (14) confirmed phenytoin as a potential causative agent. The percentage dead cells above baseline (ie. in incubations conducted in the absence of the drug) for the patient were 21.3%, 14.5% and 36.5% for phenytoin, PB and carbamazepine, respectively, compared to 0 - 0.9% for the control (means, n = 3). PB and carbamazepine were included in the *in vitro* rechallenge because of the apparent cross-reactivity of the aromatic ACs (2) and the clinical importance of determining which alternative AC might be safe for this patient. Based on population data, the upper limits of normal (expressed as s.d. above the mean) for phenytoin, PB and carbamazepine are 3.6%, 4.5% and 9.9% (2). These data indicated that the patient's mononuclear cells were more susceptible to a standardized challenge with reactive phenytoin metabolites than control cells, and that the patient was also potentially at risk for toxicity from carbamazepine and PB.

Hepatic Microsomal Samples

Microsomes were prepared from the livers of male Balb/c or National Institutes of Health General Purpose Swiss mice [N:GP(SW)] which had been injected intraperitoneally with PB (60 mg/kg in 0.9% saline) or dexamethasone (Dex; 100 mg/kg in corn oil) once daily for 3 days (5). Control groups of animals received the equivalent dose volume of vehicle (saline or corn oil; 10 ml/kg). Hepatic microsomes were also prepared from male Sprague-Dawley rats pre-treated (once daily i.p. for 3 days) with saline, corn oil, PB (75 mg/kg) or β-naphthoflavone (BNF; 50 mg/kg). The case histories of the human liver donors and the preparation of human microsomes have been detailed elsewhere (15). Protein concentrations were determined according to the method of Lowry *et al* (16). The erythromycin and benzphetamine *N*-demethylase activities of the murine microsomes were determined at a substrate concentration of 1 mM by measuring the rate of formaldehyde formation according to Nash (17).

SDS-PAGE and Immunoblotting

SDS-PAGE (18) and immunoblotting (19) were conducted according to standard methods with minor modifications (9). Microsomal samples were loaded at 5 - 20 μg per 5 mm lane. Purified P450s were loaded at 0.8 - 1.6 pmol/lane. The nitrocellulose sheets were incubated with appropriate dilutions of sera containing antibodies directed against specific microsomal proteins (1/100 for OXYgene antibodies as suggested by the manufacturer; 1/500 of a 10 mg/ml stock for the other anti-P450 antisera employed; and 1/500 for the polyclonal rabbit anti-human EH antibody) or patient serum (diluted 1/250). After a 1 - 3 hr incubation at room temperature, the antibody solutions were removed and the blots washed extensively. The nitrocellulose membranes were then incubated with appropriate dilutions of secondary antibody (HRP- or alkaline phosphatase-conjugated) for 1 hr at room temperature, washed and bound antibody visualised using enhanced chemiluminscence detection (9) or colorimetrically using 4-chloro-1-naphthol/H_2O_2 or 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium, as appropriate.

RESULTS AND DISCUSSION

Aromatic ACs are metabolised by PB-inducible P450 enzymes to reactive epoxide intermediates (2,5,20) which could produce toxicity either directly (2,5) or through the formation of immunogenic drug-macromolecule conjugates (4). Previous *in vitro* investigations of the metabolic basis for predisposition to adverse reactions to these drugs have suggested that they may be a consequence of a rare combination of enhanced metabolic activation by P450 (5,6) and decreased detoxication, possibly by EH (2,5). Immunoblotting analyses have shown that sera from patients experiencing AC hypersensitivity reactions, but not from appropriate control individuals, contain circulating antibodies which recognise PB-inducible rodent hepatic microsomal proteins with a *Mr* of ~ 53 kDa (7-9). These findings are consistent with the clinical features of the reactions which suggest that they have an immunological component (1-3). The present study demonstrates that these antibodies, as with similar toxicities caused by tienilic acid, dihydralazine and halothane (10-12), appear to be directed against P450 proteins.

Preliminary work showed that the 53 kDa protein(s) recognised in rodent liver microsomes is induced by PB more markedly in the rat than in the mouse (7,8). Given the disparate effects of PB in these two species, initial experiments focused on the regulation of the mouse 53 kDa protein by Dex, another prototype P450 inducer. Figure 1 shows that, although weak reactivity was observed with microsomes from vehicle-treated animals, treatment of mice with Dex elevated markedly the levels of the 53 kDa protein recognised by the patient serum. This effect was similar to that observed previously with phenytoin and more pronounced than with PB (7,8). An additional minor 50 kDa 3a-related protein, which may be the recently characterised $P450_{UT}$ (21), was also detected by the patient serum in microsomes from Dex-induced mice. Immunoblots

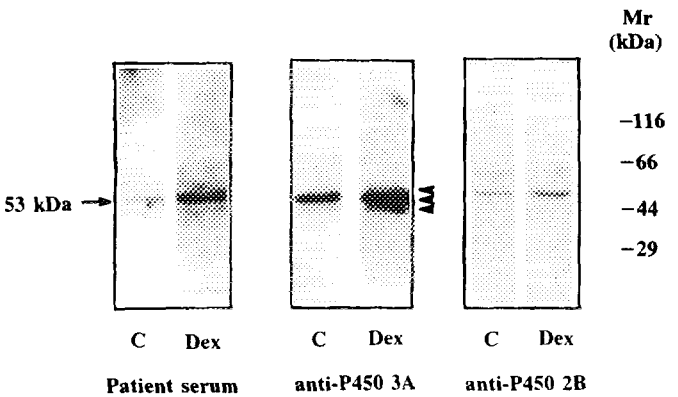

FIGURE 1. Immunoblot of hepatic microsomes (5 μg per track) from corn oil (C)- and dexamethasone (Dex)-treated Balb/c mice probed with the positive patient serum (diluted 1/250) and antisera to rat CYP2B1/2 and 3A1 (from OXYgene; diluted 1/100). Electrophoresis and immunoblotting were conducted as described in 'Materials and Methods'. The polyacrylamide gel concentration was 10%. Values on the right denote the migration positions of the molecular weight standards. The arrow indicates the position of migration of the 53 kDa polypeptide recognised by the patient serum. Arrowheads adjacent the anti-P4503A blot signify the immunoreactive *Cyp3a* proteins.

conducted with antisera to rat CYP3A1 and 2B1 suggested that the apparent *Mr* and relative expression of the 53 kDa protein was similar to that observed for the major, steroid-inducible mouse 3a protein. This corresponded with one of three proteins (two of which were constitutive) recognised by the anti-CYP3A antiserum in Dex-induced mouse liver microsomes. Thus, as many as three products of the *Cyp3a* subfamily may be expressed in mouse liver as demonstrated in the rat (22). The increase in immunoquantifiable 3a protein(s) produced by Dex was parallelled by a similar rise in the activity of erythromycin *N*-demethylase, a specific probe the 3A isoforms (11.63 ± 1.22 nmol/min/mg *versus* 1.91 ± 0.08 nmol/min/mg; mean ± s.d. n = 4, $p < 0.01$).

As demonstrated previously (23), Dex also induced weakly a 2b protein(s) in Balb/c mice (possibly 2b-9 or 2b-10) which was reflected in an increased benzphetamine *N*-demethylase activity, a reaction diagnostic for inducible 2B proteins (12.85 ± 0.46 nmol/min/mg *versus* 6.12 ± 0.92 nmol/min/mg; mean ± s.d. n = 4, $p < 0.01$). However, the apparent *Mr* (55 kDa) and relative increase (≤ 2-fold) in this protein supported earlier data which showed that antibodies in the patient serum are not directed against the major PB-inducible rat P450.

Because of a relative paucity of background information regarding mouse P450 isoforms, further experiments were aimed at examining the regulation of expression of the rat antigen and characterisation of the human orthologue. Figure 2A compares the patterns of recognition of rat hepatic microsomal proteins from vehicle-treated and PB- and BNF-induced animals by the AC patient serum and antisera to the major rat drug metabolising P450 subfamilies. BNF treatment increased the levels of a constitutive 1A protein (1A2) and resulted in detectable levels of a second larger protein (57 kDa *versus* 54 kDa, although not well-resolved on this 10% gel; 1A1); PB induced the levels of 2B1/2, 2C (probably a composite of elevated 2C6, 2C7 and repressed 2C11) and 3A1/2 proteins. The apparent *Mr* of the major protein detected by the patient serum and its differential regulation by BNF (which appeared to suppress the levels of immunoreactive protein) and PB were consistent with it being a *CYP3A* product. Minor bands of 43 and 45 kDa were also detected in PB-induced microsomes.

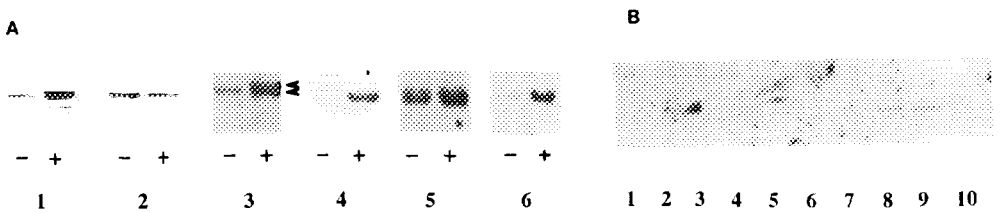

FIGURE 2. A) Comparison of effects of hepatic enzyme induction with phenobarbitone (blots 1, 4, 5 and 6) and β-naphthoflavone (blots 2 and 3) on the expression of the rat microsomal antigen recognised by the patient serum (diluted 1/250) and rat P450 proteins of the *CYP1A*, *2B*, *2C* and *3A* subfamilies. '-' and '+' refer to microsomes (10 μg per track) from vehicle-treated (controls) or induced animals, respectively. Immunoblots were reacted with the patient serum (blots 1 and 2) or antisera (diluted to 20 μg/ml) to rat CYP1A2 (blot 3), 2B1 (blot 4), 2C6 (blot 5) and 3A1 (blot 6). Arrowheads denote the positions of migration of CYP1A1 and 1A2.
B) Immunoblot of purified rat P450s probed with the anticonvulsant patient serum (diluted 1/250). Lanes 1-10 contained 0.8 pmol of CYP1A1, 1A2, 2A1, 2B1, 2B2, 2C6, 2C11, 3A2, 3A1 and 4A1, respectively. Only migration regions of interest are shown to facilitate comparison.

Studies with an extensive panel of purified rat P450 enzymes confirmed that the major proteins recognised in microsomes from vehicle-treated and PB-induced animals were CYP3A2 and 3A1, respectively (Figure 2B). No reactivity was noted towards purified CYP1A1, 1A2, 2A1, 2B1, 2B2, 2C6, 2C11 or 4A1. Interestingly, the major constitutive rat antigens identified to date are male-specific (2C11 and 3A2) and earlier work speculated that the bioactivation of phenytoin to reactive epoxides in the rat may be significantly higher in males (24). Following microsomal bioactivation, [^{14}C]-phenytoin became irreversibly bound to proteins with apparent *Mr* between 52 - 55 kDa (data not shown) which could be the P450 enzymes that catalyse this toxifying reaction. A mechanism of autoantibody generation similar to that proposed for dihydralazine (11,25) may thus be in operation whereby these drugs induce the human P450 enzymes involved in their bioactivation which may be haptenated and rendered immunogenic.

Attempts to identify the human orthologue of the rodent antigens were performed with microsomes from lymphoblastoid cell lines which stably express recombinant human P450 isoforms (26). Figure 3A demonstrates that, with the exception of several feint bands in the 45 - 60 kDa range in microsomes from the cell line expressing 3A4, no significant immunoreactivity was noted towards any of the recombinant human proteins. The high *Mr* material ($\geq$ 64 kDa) detected in several of the microsomal preparations appears to be an artefact of the expression system employed. Similar results were obtained with 2B6 and 2C9 (data not shown) and support negative findings with lysates from vaccinia virus-infected HepG2 cells which express human CYP2C8, CYP2C9, CYP3A4 and CYP3A5 (8 and unpublished data). Although the expression of some of

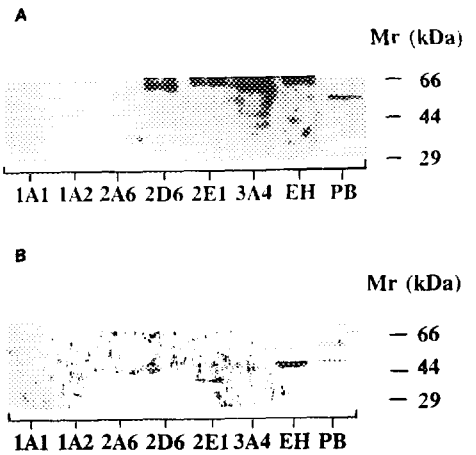

FIGURE 3. Comparison of reactivity of patient serum (A) and polyclonal rabbit anti-human epoxide hydrolase antiserum (B) towards microsomes from human B-lymphoblastoid cell lines expressing human CYP1A1, 1A2, 2A6, 2D6, 2E1, 3A4 and epoxide hydrolase. Hepatic microsomes from phenobarbitone-treated mice (PB track) were included as a positive control. Microsomes (20 μg per track) were separated by SDS-PAGE, blotted on nitrocellulose and probed with the positive human serum sample (diluted 1/250) or the anti-epoxide hydrolase antiserum (diluted 1/500). Immunoblot A was developed using enhanced chemiluminescence detection whereas an alkaline phosphatase-conjugated goat anti-rabbit IgG and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was used to visualise the bound antibody in immunoblot B. The positions of migration of the molecular weight standards are shown on the right.

these P450 isoforms may be lower in these cells than in some human livers (31), immunoblotting experiments with anti-rat P450 antisera confirmed the expression of these proteins at immunodetectable levels.

Theoretically, autoantibodies produced in AC hypersensitivity reactions could be directed against an aberrant form of EH. However, Figure 3B shows that this enzyme was neither the human autoantigen nor the protein recognised in PB-induced mouse hepatic microsomes: the antiserum raised towards human EH detected a single 49 kDa protein in the microsomes from the transfected cells as anticipated (27); no proteins were recognised by the patient serum in these same microsomes; and the protein recognised by the anti-EH antiserum in PB-induced mouse microsomes had an apparent *Mr* (50 kDa) distinct from the 53 kDa protein recognised by the positive serum sample.

Further attempts to define the human neoantigen were conducted with a previously phenotyped human liver panel (15). Figure 4 compares the expression of proteins recognised in human liver microsomes by antisera to rat CYP2C6 (2C8, 9 and an unidentified *CYP2C* product), CYP3A1 (3A3/4 which exhibited identical electrophoretic mobility) and the positive patient serum

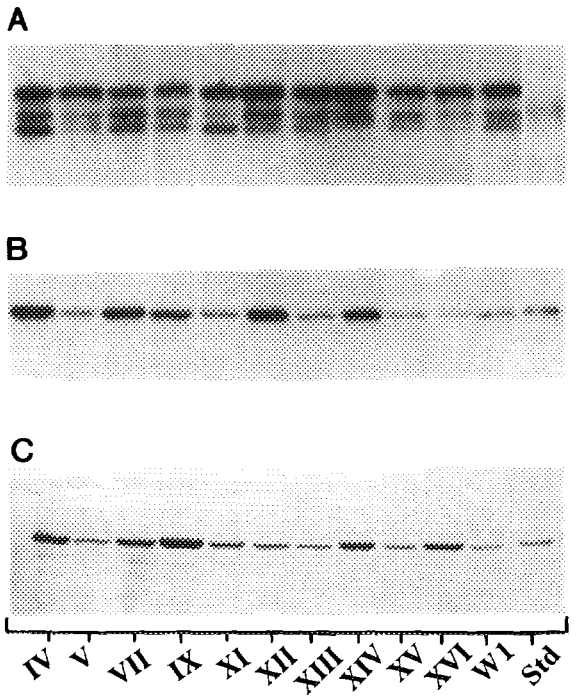

FIGURE 4. Immunoblots of a human liver panel reacted with antisera to rat CYP2C6 (A), CYP3A1 (B) and the human serum examined (C). Microsomes (15 μg protein per track) from 11 renal transplant donors and the respective purified rat standards (1.6 pmol per track; CYP2C6 for A and CYP3A1 for B and C) were electrophoresed on 9% polyacrylamide and immunoblotted with anti-CYP2C6, anti-CYP3A1 (both diluted to 20 μg/ml) or the anticonvulsant patient serum (diluted 1/250). The three immunoreactive bands in B represent CYP2C8 (upper band), 2C9 (middle band) and an as yet unidentified *CYP2C* product (lower band) (15). Two minor, smaller proteins were also detected in some livers by the patient serum as described previously (9).

used in the present study. Both the anti-CYP3A antiserum and the patient serum recognised purified rat 3A1 and a protein with an apparent *Mr* of 52.5 kDa in human liver which exhibited marked inter-individual variation in its expression. Considering that AC patient sera react primarily with rat CYP2C11, 3A1 and 3A2 (9 and this study), possible candidates for the human orthologue (based on cDNA and amino acid homology) would be CYP3A3/4/5/7 and CYP2C9 (28). It is tempting to speculate that the human autoantigen is CYP3A3. Although the high degree of homology between this protein and CYP3A4 ($\geq$ 98%) perhaps makes this suggestion unlikely, recent studies have indicated that sera from individuals suffering from dihydralazine hepatitis caused by this compound produce anti-human 1A2 antibodies which are oligoclonal or, possibly, monoclonal (25). Alternatively, the reactivity in Figure 4C may be to an as yet unidentified human *CYP3A* product which is expressed largely extra-hepatically, the existence of which has been proposed in experiments with the non-invasive CYP3A probes cortisol and erythromycin (29). This could explain why not all affected patients demonstrate overt hepatic involvement (9).

It is worthy of note that proteins of the human *CYP3A* and *CYP2C* subfamilies appear to be regulated co-ordinately and may be increased by both AC (15,30,31) and steroid therapy (31). Interestingly, only human livers from donors who had received AC therapy prior to surgery activated phenytoin to a reactive metabolite (5) or expressed significant amounts of immunoreactive protein (9). Studies with tienilic acid have shown that anti-microsomal antibodies associated with this compound recognise rat CYP2C11, 2B1/2 and human CYP2C9 (the human orthologue of 2C11) (32), which catalyses tienilic acid bioactivation. Although recent studies have shown that CYP2C9 can also hydroxylate phenytoin (33), several laboratories have concluded that there is no simple direct relationship between phenytoin 4-hydroxylation and reactive metabolite formation (5,6,34). Moreover, no significant immunoreactivity was evident with purified rat 2B1/2 or recombinant human 2C9 with the serum used in the present study.

Extrapolation of the rodent data generated to the identification of the human autoantigen may be obfuscated by inter-species variation in both metabolic and immunological properties of P450 proteins. For example, although mephenytoin 4-hydroxylase is a *CYP3A* protein in the rat, this enzyme is a *CYP2C* protein in man (possibly 2C18; 35) and antibodies to purified rat P450 proteins are known to recognise epitopes on human proteins assigned to different subfamilies (15,36). Nevertheless, it seems likely that the human autoantigen is a *CYP3A* protein or a similarly regulated member of the *CYP2C* subfamily (perhaps 2C18 or 19). Ultimately, identification of the human autoantigen should increase our understanding of the enzymes involved in AC bioactivation and may culminate in the development of a simple assay for both the early diagnosis of immunoreactions to ACs and the detection of predisposed individuals.

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