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Epitope Mapping Studies with Human Anti-Cytochrome P450 3A Antibodies

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SUMMARY

A subset of patients with hypersensitivity reactions to the aromatic anticonvulsants phenytoin, carbamazepine, and phenobarbital have circulating antibodies that recognize members of the rat cytochrome P450 (CYP) 3A subfamily. These antibodies do not recognize related human CYP3A proteins despite the high degree of structural similarity. To investigate the relationship between P450-mediated drug metabolism and the development of anti-P450 antibodies, we initiated epitope mapping studies by screening a library of fusion proteins constructed from rat CYP3A1 with an anti-CYP3A1-positive patient serum sample. Positive signals from colony lifts were confirmed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblotting, and a 26-amino acid sequence corresponding to amino acids 342–367 of the CYP3A1 protein (NKAPPTYDTVMEMEYLDMVLNETLRL) was identified as containing the epitope recognized by IgG_3 antibodies in this serum sample. By subjecting inserts from two clones into a second round of library construction and screening by immunoblot analysis, we further defined the epitope to EYLDMVLNETLRL. Single amino acid deletions identified DMVLNETLRL as the minimum amino acid sequence required for antibody binding. The corresponding sequence in the four human CYP3A proteins differs by only one amino acid (DMVVNETLRL) This amino acid is critical to antibody recognition as immunoreactivity of the L361V mutant is markedly reduced. Anti-CYP3A antibodies in nine of nine additional sera also recognized the 13-amino acid epitope; for five of these sera, the minimum antibody binding sequence was DMVLNETLRL. The proximity of this epitope to a region determining substrate specificity may provide the link among reactive metabolite production, hapten formation, and the production of anti-P450 antibodies in anticonvulsant-induced idiosyncratic reactions.

The CYPs constitute a gene superfamily that plays a major role in the biotransformation of drugs and environmental toxins to, for the most part, more easily excreted and less toxic products [CYP nomenclature according to Nelson *et al.* (1)]. In this manner, they provide a crucial function in protecting the host from small molecule (chemical) insults, analogous perhaps to the protective role against large molecules played by the immune system and its immunoglobulin, T cell receptor, and MHC gene superfamilies. There appear to be, however, situations where various CYP enzymes become the target of an immune response; these include CYP2D6 (2, 3) and, less commonly, CYP1A2 (4) in type II chronic autoimmune hepatitis, CYP11A1 in autoimmune polyendocrine syndrome type I (5), and CYP17 and CYP21 in Addison's disease (5–7). Anti-P450 antibodies have also been observed in idiosyncratic drug reactions such as tienilic acid hepatitis (anti-CYP2C9) (8), dihydralazine hepatitis (anti-CYP1A2) (9), and hypersensitivity reactions to the aromatic anticonvulsants phenytoin, phenobarbital, and carbamazepine (anti-CYP3A) (10, 11). In the latter case, patient antibodies recognize rat CYPs 3A1 and 3A2 and, to a lesser extent, CYP2C11 (10). Although an antigen expressed in human liver microsomes appears to be similarly regulated (11), recombinant CYPs 2C9/10 and 3A4 are not recognized by these antibodies despite a relatively high degree of similarity with the rat antigens.

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ABBREVIATIONS: CYP or P450, cytochrome P450; DDEP, 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine; HCV, hepatitis C virus; MHC, major histocompatability; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TNT, Tris/sodium chloride/Tween-20 buffer; M-TNT, Tris/sodium chloride/Tween-20 buffer with 4% skim milk powder; PCR, polymerase chain reaction; IgG, immunoglobulin G.

The mechanisms by which CYPs become antigenic are not known. Three groups have mapped independently the epitope recognized by anti-LKM_1 (anti-CYP2D6) antibodies to a linear segment centering around the consensus sequence DPAQPPRD (Asp-Pro-Ala-Gln-Pro-Pro-Arg-Asp). Homologies have been found between the mapped amino acid segments and herpes simplex virus type 1 and HCV proteins, suggesting that mimicry may occur between CYP2D6 and viral antigens (12–14).

In the case of drug-induced disorders with immunological etiologies, it has been proposed that drug bioactivation to reactive metabolites capable of covalently modifying cellular macromolecules (proteins) leads to the formation of drug/protein conjugates (haptens) that may be perceived as "nonself" by the host immune system. The clinical manifestations of drug hypersensitivity reactions are thus believed to represent an immune response directed against new antigenic determinants derived from the drug and/or the carrier protein (15). The CYP2C9 target of antibodies in tienilic acid hepatitis is the P450 responsible for tienilic acid bioactivation to a reactive metabolite, possibly a thiophene *S*-oxide derivative, that covalently binds to human liver microsomal proteins (16, 17). It appears that the reactive metabolite binds in or near the CYP2C9 active site (18) and ultimately results in inactivation of the enzyme (17). The specific epitope has not yet been identified, but current data do not indicate a strict requirement for covalently bound drug.

In anticonvulsant hypersensitivity reactions, several findings suggest that the sequence of events leading to the production of anti-CYP antibodies is more complicated than would be predicted by the hapten hypothesis. For example, the primary antibody response to new immunogenic determinants derived from a drug-modified protein would be expected to be of the IgM class, followed later by an IgG response. In fact, the absence of an IgM anti-CYP response has been a consistent finding in all sera tested, including those obtained 10–17 days after the onset of clinical symptoms of anticonvulsant hypersensitivity; immunoglobulin class- and subclass-specific reagents indicated that the antibodies were predominantly IgG_1 and IgG_3, although some IgA antibodies were also observed (19). Also, anti-CYP3A may persist in patients for several years after the acute hypersensitivity event has resolved, even in the absence of continued administration of aromatic anticonvulsants. Furthermore, antibodies have been detected in the sera of two individuals with no history of exposure to anticonvulsant (or other) medication.[1] One interpretation of these findings is that the anti-CYP3A antibodies represent a response to an antigen independent of drug administration, possibly of infectious origin, and become pathogenic only in the presence of drug.

The purpose of the present study was to map the epitope on CYP3A1 recognized by patient sera to better understand the differential recognition of rat and human CYP3A proteins and to further investigate the relationship between drug metabolism and the immune response in the pathogenesis of anticonvulsant hypersensitivity reactions.

[1] A. Gaedigk, X. Lu, and J. S. Leeder. Manuscript in preparation.

Methods

Chemicals and reagents. Electrophoresis reagents were obtained from ICN Biomedical (Cleveland, OH). Molecular weight standards, thimerosal, Tween 20, Tris base, glycine, NaCl, and Ponceau S concentrate were obtained from Sigma Chemical Co. (St. Louis, MO). HRP-conjugated mouse anti-human IgG_1 (World Health Organization clone HP6069) and IgG_3 (World Health Organization clone HP6047) were obtained from Zymed (South San Francisco, CA). Nitrocellulose membranes, chemiluminescence detection reagents, and film were obtained from Amersham Canada Ltd. (Oakville, Ontario, Canada). All other reagents were of the highest grade commercially available.

Construction of CYP3A1 library. CYP3A1 cDNA was generously provided by Dr. Frank Gonzalez (Laboratory of Molecular Carcinogenesis, National Institutes of Health, National Cancer Institute, Bethesda, MD). A library of fusion proteins containing CYP3A1-derived peptide fragments was generated using the NovaTope System (Novagen, Madison, WI). CYP3A1 cDNA (1.6 kb) was digested with DNase I in the presence of Mn^{2+} and size-fractionated on a 2% agarose gel. The band corresponding to 100–150-bp fragments was excised, and the DNA was recovered with use of the QIAEX gel extraction kit (Qiagen, Chatsworth, CA). Ends of the digested fragments were made blunt, and single 3′ dA residues were added by sequential reactions with T4 DNA polymerase and *Tth* DNA polymerase. The modified fragments were ligated directly into pTOPE T-vector prepared by *Eco*RV digestion and the addition of single 3′ dT residues (Novagen). Ligated plasmids were transformed into *Escherichia coli* strain NovaBlue(DE3), and target sequences were expressed as fusions with the T7 gene *10* protein. The identity of inserts was confirmed by sequencing with T7 gene *10* or T7 terminator primers using the T7-Sequencing Kit protocol (Pharmacia Biotechnology, Montreal, Quebec, Canada).

Immunoscreening of expressed CYP3A1 fragments. Transformants were plated at ~400–500 colonies/100-mm plate. Colonies were transferred to nitrocellulose filters (Amersham Canada Ltd.), lysed by chloroform vapor, and denatured with 20 mM Tris·HCl, pH 7.9, 6 M urea, and 0.5 M NaCl. Filters were blocked overnight at room temperature with TNT (10 mM Tris, 154 mM NaCl, 0.2% w/v Tween 20, 0.1% w/v thimerosal) containing 4% w/v skim milk powder (M-TNT). Immunoscreening was performed with a patient serum sample containing IgG_3 anti-CYP3A1 antibodies. Both the patient serum and the HRP-conjugated mouse anti-human IgG_3 secondary antibody were preadsorbed overnight at room temperature with 1 mg/ml lysate protein from NovaBlue(DE3) containing vector without insert. Incubation with human serum was carried out at room temperature for 3 hr, followed by washing in TNT for 30 min at 5-min intervals. The washed blots were incubated with HRP-conjugated mouse anti-human IgG_3 diluted 1:2000 in M-TNT for 1 hr at room temperature. After washing (5 min × 6), bound antibody was visualized by enhanced chemiluminescence detection (Amersham).

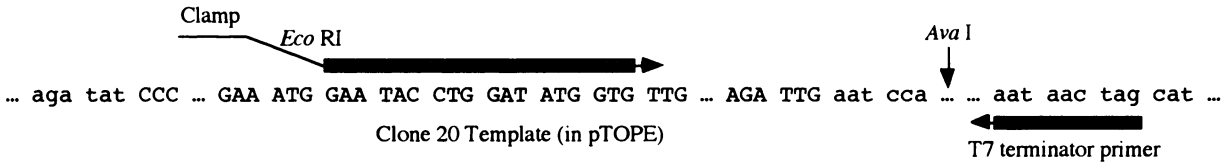
Immunoscreening by Western blotting. SDS-PAGE was conducted according to Laemmli (20) on 10% or 12% resolving minigels (5.0 cm long, 1.5 mm thick) using an LKB model 2050 Midget Electrophoresis Unit (LKB Produkter AB, Bromma, Sweden). Colony lysate protein was diluted in sample buffer (1% SDS, 5% glycerol, 50 mM Tris, pH 6.8, 0.4 M mercaptoethanol, and 0.002% Bromophenol blue final) and boiled for 2 min. The samples were electrophoresed at 28 mA/gel for 90 min and transferred electrophoretically (0.8 mA/cm^2 for 60 min) to nitrocellulose (Hybond-ECL, Amersham) using an LKB model 2117 Novablot Electrophoresis Unit (LKB Produkter AB) and a buffer consisting of 48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20% methanol.

Location of the molecular weight markers on the nitrocellulose was verified using Ponceau S staining according to the manufacturer's directions (Sigma). The nitrocellulose membranes were then blocked with M-TNT overnight at room temperature with gentle rocking. Immunoblotting with preadsorbed patient serum and en-

1. Primer Construction:

Clamp	*Eco*RI Site	E	Y	L	D	M	V
CGCGC	GAATTC	GAA	TAC	CTG	GAT	ATG	GTG

2. Amplify fragment of desired length using primers incorporating either *Eco*RI or *Ava*I sites



3. Digest PCR product with *Eco*RI and *Ava*I, and ligate into prepared pTOPE vector

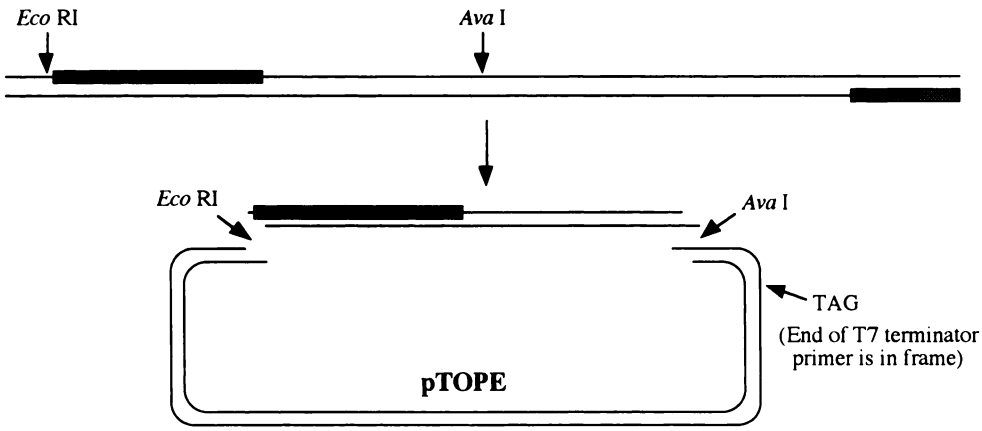


Fig. 1. Strategy for epitope fine mapping.

TABLE 1
PCR primer pairs for fine mapping of epitope

Desired construct	Forward primer	Reverse primer
EYLDMVLNETLRL	5'-CGCGCGAATTCGAATACCTGGATATGGTG-3'	T7 termination primer[a]
EYLDMVLNETLR	T7 gene *10* primer[b]	5'-CGCGCTCGAGATTTCTGAGGGTTTCAT-3'
EYLDMVLNETL	T7 gene *10* primer	5'-CGCGCTCGAGGGTTTCATTCAACAC-3'
DMVLNETLRL	5'-GCGCGAATTCGATATGGTGTTGAATG-3'	T7 termination primer
MVLNETLRL	5'-GCGCGAATTCATGGTGTTGAATGAA-3'	T7 termination primer
VLNETLRL	5'-GCGCGAATTCGTGTTGAATGAAACC-3'	T7 termination primer
LNETLRL	5'-GCGCGAATTCTTGAATGAAACCCTCAG-3'	T7 termination primer
NETLRL	5'-GCGCGAATTCAATGAAACCCTCAGA-3'	T7 termination primer

[a] T7 termination primer: 5'-GCTAGTTATTGCTCAGCGG-3' (Novagen #69337-1).
[b] T7 gene *10* primer: 5'-TGAGGTTGTAGAAGTTCCG-3' (Novagen #69341-1).

hanced chemiluminescence detection was conducted as described above.

Fine mapping of epitope. The general strategy for fine mapping the epitope by using PCR is illustrated in Fig. 1. The template was a fragment (designated clone 20) derived from the DNase digestion of CYP3A1 cDNA cloned onto pTOPE. This fragment coded for CYP3A1 amino acids Pro^{310} to Leu^{367} and produced a fusion protein immunoreactive with anti-CYP3A1 patient antibodies. A series of oligonucleotide primers was synthesized (General Synthesis and Diagnostics, Toronto, Ontario, Canada) to generate gene *10* fusion proteins containing the CYP3A1-derived amino acid sequence EYLDMVLNETLRL and sequential amino acid deletions (Table 1).

PCR amplification was conducted with a Cetus Perkin-Elmer thermal cycler under the following conditions: initial denaturation at 94° for 3 min followed by 35 cycles of denaturation, 30 sec at 94°; annealing, 30 sec at 50°; extension, 45 sec at 72°; and a final 7-min extension at 72°. The reaction mixture consisted of 3 ng template DNA, 1 mM Tris·HCl, pH 8.3, 50 mM KCl, 1 mM $MgCl_2$, 0.1% gelatin, 0.1 mM dNTPs, 20 pmol each forward and reverse primers, and 1.25 units AmpliTaq polymerase (Perkin-Elmer Applied Biosystems Canada, Mississauga, Ontario, Canada) in a total volume of 50 μl. To clone the PCR product into pTOPE for expression, it was precipitated with ethanol, digested with *Eco*RI and *Ava*I, and ligated directly into pTOPE vector prepared by *Eco*RI/*Ava*I digestion. The presence and integrity of each insert were confirmed by PCR and dideoxy sequencing as described above.

To generate a gene *10*-EYLDMVLNETLRL fusion protein, PCR was performed on clone 20 DNA with 5'-CGCGCGAATTCGAATAC-CTGGATATGGTG-3') and T7 terminator (Novagen) primers. Table 1 lists the PCR primers used to generate fusion proteins in which single amino acids were deleted from the EYLDMVLNETLRL template. PCR conditions were as described above except that the annealing temperatures were adjusted to 42–52° depending on the melting temperature of the respective primers, and the construct coding for EYLDMVLNETLRL was used as a template. Restriction digests, ligations, immunoscreening, and confirmation of insert identity by sequencing were conducted as described above.

Site-directed mutagenesis. Because human CYP3As differ from CYP3A1 by only one amino acid in this region (DMV-VNETLRL), an L361V mutation was introduced in the 10-amino acid construct DMVLNETLRL by PCR. Conditions were as described above using the forward primer 5'-GCGCGAATTCGATATGGTGGT-GAATG-3', T7 terminator primer, and an annealing temperature of 43°. *Eco*RI/*Ava*I digestion and ligation of the PCR product were also conducted as described. Incorporation of the nucleotide mutation was confirmed by dideoxy sequencing.

Patient and control sera. Sera were obtained from 10 patients who had experienced hypersensitivity reactions to phenytoin and/or carbamazepine and who had circulating anti-P450 antibodies in their sera (10, 19). The negative control serum used in these studies was obtained from an individual with phenytoin hypersensitivity, including hepatic involvement, who tested positive to *in vitro* rechallenge (21) but did not have antibodies against denatured CYPs 1A2, 2B1, 2B2, 2C6, 2C7, 2C9/10, 2C11, 2C13, 2E1, 3A1, 3A2, or 3A4. Also included for comparison was a serum sample obtained from an individual with no history of exposure to anticonvulsants that contained anti-microsomal antibodies directed against CYPs 2C11, 3A1, and 3A2 (19).

Results

Mapping of epitope by immunoblotting. Initial immunoscreening was conducted with a patient serum sample containing IgG_3 anti-CYP3A1 antibodies and colony lifts that had been denatured with 6 M urea. Under these conditions, it was difficult to differentiate positive signals from background signal. Sequencing of ~40 "positive" colonies revealed three clusters of clones. One cluster was located near the amino terminus of the CYP3A1 protein, corresponding roughly to amino acids Thr^{11} to Gly^{48}. Of these clones, 5 of 14 were in frame. A second group of clones corresponded to the carboxyl terminus (3 of 12 clones in frame). Ten randomly picked colonies were uniformly distributed throughout the CYP3A1 sequence. The third cluster of sequenced clones was localized to a region between amino acids Pro^{310} and Gly^{392}, and of these, 11 of 14 were in frame.

These latter results were confirmed by SDS-PAGE and immunoscreening. For these studies, nonspecific background antibody binding was minimized by preadsorbing the patient serum with 0.5 mg each of native and boiled NovaBlue(DE3) lysate protein. The results presented in Fig. 2 indicate that the epitope is contained within a 26-amino acid sequence corresponding to amino acids 342–367 of the CYP3A1 protein, NKAPPTYDTVMEMEYLDMVLNETLRL.

To further define the epitope, inserts from clones 21 (235 bp) and 29 (218 bp) were subjected to a second round of library construction yielding fragments ~30–60 bp long. After immunoscreening by immunoblot analysis, several colonies, both immunoblot positive and negative, were sequenced. Comparison of the signals obtained with clones 20 (presence of signal) and 29–18 (absence of signal) indicated

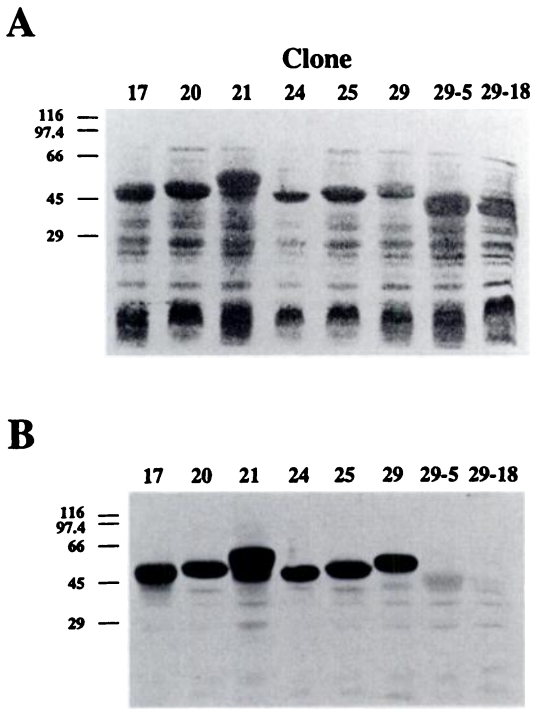


Fig. 2. Mapping of CYP3A1 epitope to the 13-amino acid sequence E^{355}YLDMVLNETLRL^{367}. Several colonies expressing CYP3A1 peptides immunoreactive and nonreactive with IgG_3 antibodies in a patient serum were selected and cultured overnight. Cell lysate protein was electrophoresed on 12% SDS-PAGE minigels and transferred to nitrocellulose membranes. A, Ponceau S-stained nitrocellulose membrane. B, Immunoblot of membrane in A. Both the patient serum (1:500 in TNT) and the HRP-conjugated mouse monoclonal antibody to human IgG_3 (1:2000 in TNT) were preadsorbed with 1.0 mg/ml NovaBlue(DE3) lysate protein overnight at room temperature. Bound antibody was visualized by chemiluminescence:

clone 17, N^{342}KAPPTYDTVMEM**EYLDMVLNETLRL**Y···G^{392};
clone 20, P^{310}···N^{342}KAPPTYDTVMEM**EYLDMVLNETLRL***;
clone 21, P^{326}···N^{342}KAPPTYDTVMEM**EYLDMVLNETLRL**Y···H^{403};
clone 24, P^{319}···N^{342}KAPPTYDTVMEM**EYLDMVLNETLRL**YP*;
clone 25, P^{310}···N^{342}KAPPTYDTVMEM**EYLDMVLNETLRL***;
clone 29, T^{289}···N^{342}KAPPTYDTVMEM**EYLDMVLNETLRL**YP···;
clone 29–5, N^{342}KAPPTYDTVMEM**EYLDMVLN***;
clone 29–18, Q^{329}···N^{342}KAPPTYDTVMEM*.

Overlapping amino acid sequences derived from clones 17 and 20 localize the epitope to the sequence N^{342}KAPPTYDTVMEMEYLDMVLNETLRL^{367}. The lack of reactivity with clone 29–18 (Q^{329} to M^{354}) further maps the epitope to the sequence EYLDMVLNETLRL. *, Termination of translation.

that the epitope recognized by IgG_3 antibodies in this patient serum was located within the 13-amino acid sequence ^{355}EY-LDMVLNETLRL^{367} (Glu-Tyr-Leu-Asp-Met-Val-Leu-Asn-Glu-Thr-Leu-Arg-Leu; Fig. 2). Furthermore, the weaker signal obtained with clone 29–5 (lacking the ETLRL sequence of the 13-amino acid epitope) suggested that these amino acids were important for antibody binding.

To determine whether recognition of this amino acid sequence was unique to the patient serum used for the initial epitope mapping, a screening experiment was conducted with sera from six additional anti-CYP3A1-positive anticonvulsant hypersensitivity patients. Comparison of the reactivities with clone 20 and clone 29–18 indicated that all patients had IgG_1 or IgG_3 antibodies, which recognized the clone 20 fusion protein but not the clone 29–18 fusion protein. Serum from a patient with anticonvulsant hypersensitivity but no anti-

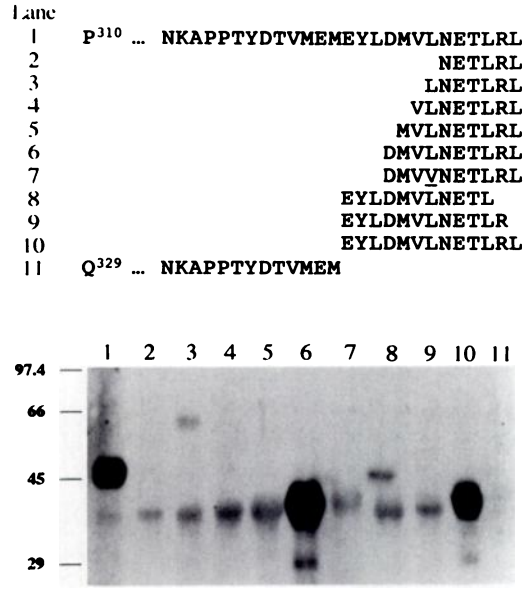


Fig. 3. Identification by immunoblot analysis of the minimum amino acid sequence required for anti-CYP3A1 antibody binding. Electrophoresis and immunoblotting were conducted as described in the legend to Fig. 2. Aliquots of clones 20 (*lane 1*) and 29–18 (*lane 11*) were included as positive and negative controls, respectively. The blot was incubated with the patient serum used for the original epitope mapping studies and HRP-conjugated mouse anti-human IgG_3 secondary antibody. The 10-amino acid construct DMVLNETLRL (*lane 6*) was the shortest amino acid sequence associated with antibody recognition; D^{358} and L^{367} are apparently essential for antibody recognition. An $L^{361}V$ mutation corresponding to the sequence of the human CYP3As (*lane 7*) was associated with considerable loss of antigenicity.

CYP3A antibodies did not recognize either fusion protein (not shown). These data provided indirect evidence that antibodies directed against the CYP3A1-derived amino acid sequence EYLDMVLNETLRL were a consistent finding among anticonvulsant hypersensitivity patients with anti-CYP3A antibodies.

Fine mapping of epitope. Direct evidence for patient antibody recognition of the 13-amino acid sequence was obtained by constructing a gene *10*-EYLDMVLNETLRL fusion protein (Fig. 3). Initially, the strategy for fine mapping the epitope involved successive three-amino acid deletions from the amino terminus of the EYLDMVLNETLRL construct. With the same serum sample as used in Fig. 2, it was apparent that full antigenicity was retained in the sequence DMVLNETLRL. Although further fine mapping was carried out with single amino acid deletions from both termini, this 10-amino acid sequence, DMVLNETLRL, was the minimum epitope recognized by the IgG_3 antibodies in this sample. Deletion of the leucine residue at the carboxyl terminus of the peptide was sufficient to abolish immunoreactivity, consistent with the data for clone 29–5 in Fig. 2. Likewise, deletion of the aspartate residue (the methionine of the epitope was preceded by a phenylalanine derived from the *Eco*RI site in the gene *10* protein) also resulted in loss of antigenicity (Fig. 3).

Similar studies with additional patient sera indicated that although clone 20 was recognized by antibodies in all samples, the patients could be divided into two groups based on the patterns of reactivity with the EYLDMVLNETLRL sequence and deletions (Fig. 4). In five patients, there was a progressive increase in immunoreactivity as the CYP3A1-derived expressed fragment increased in size from the six-amino acid sequence NETLRL to the 10-amino acid epitope DMVLNETLRL; this fusion protein produced a considerably more intense signal than those containing the nine-amino acid sequence MVLNETLRL. In four of these five patients (patients 4, 9, 17, and 18), the 10-amino acid sequence resulted in a more intense signal than the 13-amino acid construct despite similar amounts of bacterial lysate protein in each lane (Coomassie-stained gel; Fig. 4A).

Three of the remaining patient sera (patients 2, 3, and 14) recognized the longer amino acid sequences rather uniformly, whereas sera from patient 15 and the anti-CYP3A1-positive sample from the individual not exposed to aromatic anticonvulsants (C) demonstrated only minimal reactivity with the longest constructs. Visualization of antibody binding with the anti-CYP3A1-positive control serum required a much longer exposure time (6 min) compared with the patient sera (3 min).

Site-directed mutagenesis. Because rat and human CYP3A proteins differ by only one amino acid within this epitope, DMVLNETLRL versus DMVVNETLRL, and human CYP3A isoforms are not well recognized by these patient antibodies, we examined the effect of a leucine-to-valine mutation (Fig. 3). This single, conservative amino acid change resulted in a dramatic decrease in immunoreactivity, explaining to some extent the discrepancy between the recognition of rat and human CYP3As. All sera recognizing DMVLNETLRL produced the same result (not shown).

Discussion

Epitope mapping studies with human autoantibodies have been conducted for several reasons. One purpose is to investigate possible similarities between the autoantigen and antigenically related environmental pathogens that may provide clues to the pathogenesis of the disease. In this context, the relationship between HCV infection and anti-CYP2D6 antibodies in type II autoimmune hepatitis has been of particular interest to several groups. There now appear to be two distinct populations of patients with anti-CYP2D6 antibodies: (i) LKM_1-positive patients without HCV markers, who tend to be young females with other associated autoimmune disorders or a family history of autoimmune hepatitis and response to immunosuppressive therapy; and (ii) LKM_1-positive patients with HCV markers, who are more likely to be older males without other autoimmune manifestations and may respond better to antiviral therapy. Recent data suggest that anti-LKM_1 antibodies from patients with autoimmune hepatitis type II recognize a linear epitope, whereas those from HCV-positive patients recognize a conformational epitope or epitopes (22). Thus, LKM_1 autoantibodies arising from infectious challenge appear to be different from those associated with autoimmune disease.

Autoantibodies to CYPs involved in steroid biosynthesis have been identified in the sera of patients with Addison's disease and autoimmune polyendocrine syndrome type I. The major autoantigen in Addison's disease is CYP21A2 (steroid 21-hydroxylase), which is expressed only in the adrenal cortex (6, 23). CYP17 (steroid 17α-hydroxylase) has also been reported as an autoantigen in one study (7), but this finding

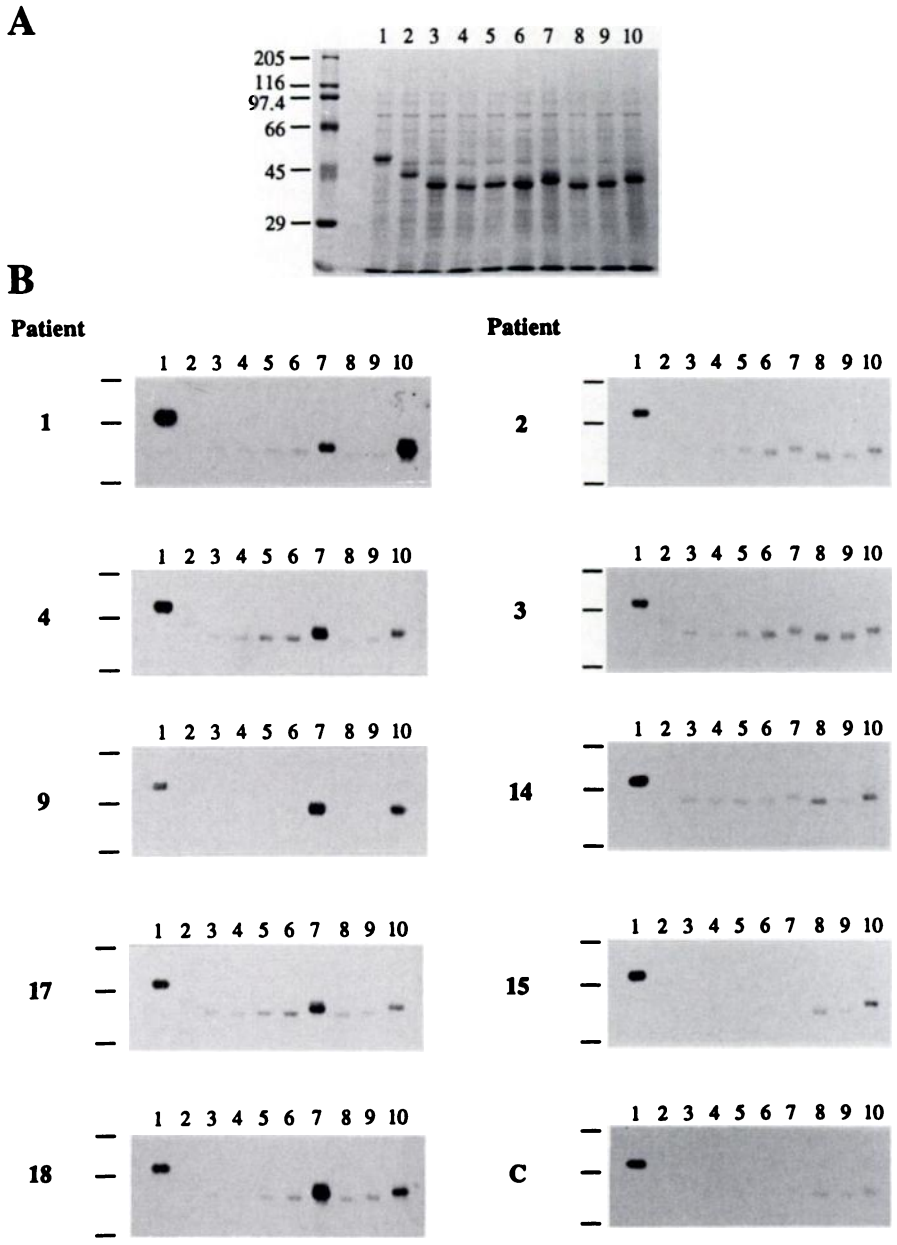


Fig. 4. Minimum amino acid sequence required for anti-CYP3A1 antibodies from patients with anticonvulsant hypersensitivity. Gene *10* fusion proteins were constructed with the following CYP3A1-derived amino acid sequences, *lane 1*, clone 20; *lane 2*, clone 29–18; *lane 3*, NETLRL; *lane 4*, LNETLRL; *lane 5*, VLNETLRL; *lane 6*, MVLNETLRL; *lane 7*, DMVLNETLRL; *lane 8*, EYLDMVLNETL; *lane 9*, EYLDMVLNETLR; and *lane 10*, EYLDMVLNETLRL. Electrophoresis and immunoblotting were conducted as described in the Legend to Fig. 2. A, Coomassie-stained gel. B, Immunoblots with patient sera and HRP-conjugated anti-human IgG_1 (patients 1, 2, 3, 14, 15, and 18 and control) or IgG_3 (patients 4, 9, and 17) secondary antibodies. Patient sera (1:500 in TNT) and the secondary antibodies (1:1500 in TNT) were preadsorbed with 1.0 mg bacterial lysate protein. Patient identification is from Ref. 18.

could not be confirmed in another (6). In contrast, CYP11A1 (cholesterol side-chain cleavage enzyme) is an autoantigen in autoimmune polyendocrine syndrome type I (5). A major CYP21A2 epitope has been mapped to amino acids 298–356 and contains most of the putative steroid hormone-binding site (24). Mapped epitopes on CYP17 are equally large (25), making identification difficult of antigenically related proteins of infectious origin.

Similar epitope mapping studies have not been reported for anti-P450 antibodies involved in tienilic acid and dihydralazine hepatitis. Our interest in mapping the CYP3A1 epitope recognized by antibodies from patients with anticonvulsant hypersensitivity reactions was 2-fold. First, we were interested in possible molecular mimicry with antigens of infectious origin that might help define factors determining susceptibility to these reactions. Second, we hoped that a well-defined epitope would aid in identifying the human (auto)antigen recognized by these antibodies because recognition of human CYPs 3A has been uniformly negative. The data from the present investigation indicate that the 13-amino acid sequence EYLDMVLNETLRL and, more specifically, the 10-amino acid sequence DMVLNETLRL were a common feature among anti-CYP3A-positive anticonvulsant hypersensitivity patients. This region is highly conserved among the CYP3A subfamily, with L361V being the only difference among rat, mouse, rabbit, and human enzymes; rat CYP3A1 and CYP 3A2 and mouse 3A11 share the DMV*L*··· sequence, whereas mouse CYP3A13, rabbit 3A6, and all four human CYP3As (3A3, 3A4, 3A5, and 3A7) have the DMV*V*··· sequence. There also is a high degree of homology (at least 7 of 10 amino acid identity) with thromboxane synthetase (CYP5) and the yeast alkane-inducible P450s (i.e., CYPs 52A1, 52A2, and 52A3).

The DMVLNETLRL epitope has several interesting features. First, it contains a potential *N*-glycosylation site, N-X-

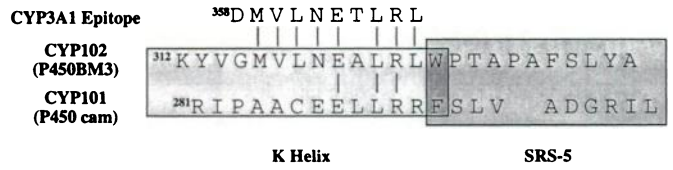


Fig. 5. Alignment of minimum antibody-binding site in CYP3A1 with the K-helices of CYP102 ($P450_{BM3}$) and CYP101 ($P450_{cam}$). The alignment of $P450_{BM3}$ and $P450_{cam}$ is according to Ravichandran *et al.* (26).

S/T, where X is any amino acid except proline, and a potential protein kinase C phosphorylation site, S/T-X-R/K (26). Second, it contains the E-X-X-R motif, which is well conserved among P450s and is a hallmark of the K-helix. Although no crystallographic data are available for eukaryotic P450s, alignment of the E-X-X-R region with the corresponding regions in CYP101 ($P450_{cam}$) and CYP102 ($P450_{BM-3}$) suggests that this epitope is located in the K-helix of CYP3A1 (Fig. 5). This epitope (DMVLNETLRL) is very similar (8 of 10 amino acid identity) to the *G*MVLNE*A*LRL sequence of CYP102, providing strong evidence for this. A further implication is that the X-X residues of E-X-X-R must be more important for antibody binding than the E and R residues because not all P450s are recognized by the patient antibodies.

Localization of the epitope to the K-helix has two major implications. First, structural models based on crystallographic data indicate that the K-helix is buried within the P450 molecule (27). The means by which drug-modified P450s become exposed to the immune system represent an area of some interest in drug-induced toxicities associated with anti-P450 antibodies. It has been proposed that small amounts of CYPs 1A2, 2C, 2D6, 2E1, and 3A4 are present on hepatocyte plasma membranes and thus could be targets of immune effectors (28). However, although plasma membrane localization of P450s is generally accepted, there is some controversy as to whether the orientation is cytosolic, corresponding to their orientation in the endoplasmic reticulum, or extracellular (29). For an epitope located in the K-helix, these considerations are largely moot because even with an extracellular orientation, it is unlikely that the epitope within the K-helix would be accessible to either antibodies or cytotoxic T cells. This implies that there must be alternative means by which P450s or P450-derived peptide fragments are presented to the immune system.

Studies of P450 degradation in cells may provide some insight. Administration of the nifedipine analogue DDEP to untreated, phenobarbital-pretreated, or dexamethasone-pretreated rats results in time-dependent losses of hepatic P450 content, specifically, CYPs 2C6, 2C11, 3A1, and 3A2. DDEP-mediated destruction is more prominent in microsomes from animals pretreated with CYP3A inducers and is prevented by troleandomycin, implicating members of the CYP3A subfamily in the process. The mechanism of CYP3A inactivation involves heme fragmentation to reactive metabolites that irreversibly bind to the protein (30). Heme alkylation of DDEP-inactivated CYP3A appears to structurally damage the apo-P450s and expose targetable residues (i.e., lysine) for ubiquitin conjugation, marking the P450s for rapid degradation by hepatic ubiquitin-dependent proteases (31, 32). A similar, ubiquitin-dependent pathway has been proposed for the processing of intracellular proteins to peptides for MHC class I-restricted antigen presentation (33). This represents a potential link between P450 damage by reactive metabolites and presentation of P450 fragments to the immune system in the appropriate context for an immune attack on hepatocytes expressing these MHC-bound peptides on their surface. Until the intracellular disposition of CYP3A-derived peptides is characterized in more detail, such a link is purely speculative, but it merits further study.

The second implication of epitope localization to the K-helix relates to its possible role in substrate binding and orientation within the active site of the enzyme. As shown in Fig. 5, a hypervariable region or substrate recognition sequence believed to be important in substrate binding (specifically, SRS-5) is located immediately adjacent to the carboxyl terminus of the K-helix (34). Based on a search for homologous sequences in various steroidogenic enzymes and steroid receptors, Picado-Leonard and Miller (35) have proposed the following steroid-binding consensus sequence:

$$L\begin{matrix}L\\P\end{matrix}LL\pm OOO\begin{matrix}RE\\KD\end{matrix}OL\begin{matrix}R\\K\end{matrix}L\pm PV$$

where $\pm$ is a charged amino acid, and 0 is an uncharged amino acid. An alignment of this consensus sequence with the 13-amino acid epitope and flanking amino acids derived from CYP3A1 is presented in Fig. 6; also included are the corresponding regions of the human CYP3As. The number of amino acid matches between the consensus sequence and CYP3As ranges from 11 with 3A1 to 13 matches with 3A7.

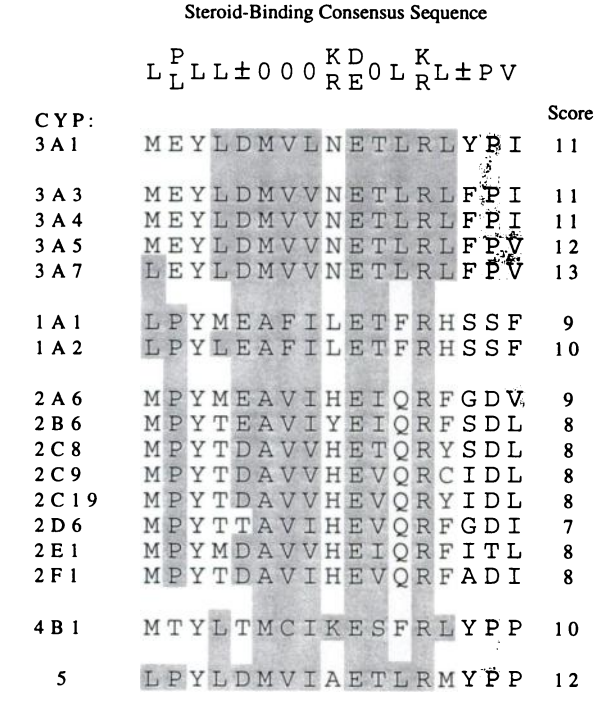


Fig. 6. Comparison of CYP3A amino acid sequences around the 13-amino acid epitope with a postulated steroid-binding consensus sequence. The amino acid sequences of CYP3A1 containing the 13-amino acid epitope recognized by human anti-CYP3A antibodies and human CYPs 3A3, 3A4, 3A5, and 3A7 are compared with the steroid-binding consensus sequence proposed by Picado-Leonard and Miller (34). Also included are the corresponding K-helix regions of human CYPs in families 1, 2, 4, and 5 that have been aligned to the CYP3As by the E-X-X-R motif. *Shaded area*, amino acid matches with the consensus sequence; *Score*, number of matches for each CYP.

This compares favorably with scores of 11–13 with the steroid receptors and sex steroid-binding protein and 10 with CYP19 but is less than those for CYP11A1 (score of 15), CYPs 11B1 and 21 (16 each), and CYP17 (score of 17).

It appears, however, that not all of the consensus sequence is specific for steroid binding as human P450s in the CYP1 and CYP2 families have a reasonable amount of homology with this region as well (Fig. 6). Much of this is due to the stretch of three uncharged residues, which impart a general helical structure. Of these human P450s, only CYP1A2 [estradiol 2- and 4-hydroxylation (36)] and CYP2C8 [hydroxylation of testosterone, androstenedione, and progesterone to unknown metabolites (37)] have any steroid-metabolizing capacity. On the other hand, the human (and rodent) CYP3As have well-documented steroid-hydroxylating activities, particularly 2β-, 6β-, and 15β-hydroxylation of testosterone, androstenedione, and progesterone (37). The 10-amino acid CYP3A1-derived epitope identified in this study and the three amino acids that follow it have particularly high homology with the carboxyl terminus, E0LRL ± PV (0 and ± refer to any uncharged or charged amino acids, respectively), of the consensus sequence. In addition, the LRL sequence appears to be particularly important for antigenicity (at least for half the patients) as deletion of the terminal leucine residue results in loss of antigenicity. It is tempting to speculate that this region might be important for anticonvulsant binding to human CYP3As and that perhaps any reactive intermediate formed interacts covalently in, or near, this substrate binding region. Accelerated degradation of inactivated enzyme could result in the production of drug-modified peptide fragments, possibly processed by the ubiquitin-protosomal pathway as demonstrated for rat CYP3As (31, 32).

The precise role of human CYP3As in phenytoin or carbamazepine bioactivation is unknown. Biotransformation of phenytoin to 4'-hydroxy phenytoin (presumably via an arene oxide) correlates well with markers of CYP2C9 activity such as tolbutamide methyl hydroxylation and (*S*)-warfarin 7-hydroxylation but not with immunoquantified CYP2C9 or CYP2C8. This has been attributed to allelic variants of CYP2C9, which may differ in their catalytic properties or, less likely, alternative P450s (38). On the other hand, CYP3As are responsible for carbamazepine bioactivation in murine liver (39), and although a role of CYP3A4 in the biotransformation of carbamazepine to its 10,11-epoxide formation has been documented (40), this stable epoxide is probably not the "reactive" metabolite implicated in the pathogenesis of hypersensitivity reactions. Because anticonvulsants are frequently prescribed in combinations, the presence of multiple, competing bioactivation pathways would not be surprising.

We have identified an amino acid sequence that forms a common epitope recognized by antibodies in the sera of patients with hypersensitivity reactions to phenytoin, carbamazepine, and phenobarbital. However, as has also been demonstrated for anti-CYP2D6 antibodies (12), probably not all patient antibodies recognize precisely the same epitope, and it must be remembered that this epitope is just one target of what is likely an oligoclonal antibody response. However, our patient antibodies can be divided into two main groups according to the reactivity to the EYLDMVLNETLRL epitope (Fig. 4 and Table 2). One group is characterized by pronounced recognition of the 10-amino acid sequence DMVLNETLRL. These patients (with one exception) predominantly recognized CYP3A1 and had no CYP2C11 reactivity. Drug therapy in three of the five patients included carbamazepine, and in four patients therapy consisted of phenytoin in combination with carbamazepine (two patients) or phenobarbital (two patients). Two of these patients had hepatic involvement, whereas the liver was not affected in any of the other patients.

The second group includes four patients who experienced hypersensitivity reactions after phenytoin monotherapy and the anti-CYP-positive control serum. These patient sera showed preferential reactivity against the EYLDMVLNETLRL/gene *10* fusion protein and shorter EYL-containing constructs. This suggests that some amino acids preceding this amino acid sequence are also important in defining the epitope, a possibility that is being pursued experimentally. These sera are further distinguished from the other group by their recognition of CYP2C11 in addition to CYP3A1 when tested against purified rat P450s. Differentiation on the basis of CYP3A1 epitope pattern represents the first parameter by

TABLE 2

Characteristics of anticonvulsant hypersensitivity patients with antibodies recognizing the amino acid sequence "DMVLNETLRL" compared with those with less defined epitopes

Patient identification and intensity of immunoreactivity as presented in reference (18). The intensity of antibody recognition was arbitrarily assigned a value of + to +++; n.r. = no reactivity. In vitro rechallenge: Peripheral blood mononuclear cells are challenged with drug metabolites generated by a murine microsomal metabolizing system as described by Shear et al. (20). A "positive" result is defined as cytotoxicity exceeding 3.6, 4.5, and 9.9% for phenytoin, phenobarbital and carbamazepine, respectively, based on population data.

Patient	Anticonvulsant therapy	*In vitro* rechallenge	Immunoreactivity[a]		
			2C11	3A1	3A2
Defined Epitope "DMVLNETLRL"					
1	phenytoin, phenobarbitol	Positive	n.r.	+++	+
4	carbamazepine	Positive	+	+	+
9	carbamazepine, phenytoin	Positive	n.r.	+++	+
17	phenobarbitol, phenytoin	Negative	n.r.	+++	++
18	carbamazepine, phenytoin	Positive	n.r.	+++	+++
Less Defined Epitope					
2	phenytoin	Positive	++	+++	+
3	phenytoin	Positive	+	+++	n.r.
14	phenytoin	Positive	+	+++	++
15	phenytoin	Positive	+++	+++	+
Control	None	Negative	+++	+	+

which patients with anticonvulsant hypersensitivity reactions can be subcategorized.

The exact identity of the human (auto)antigen remains unclear. Although the human and rodent CYP3A sequences differ by only one amino acid with the identified epitope, the valine residue in the human CYP3As dramatically reduces antigenicity compared with the leucine of the rat enzymes. It is possible that susceptible individuals have a single nucleotide mutation in exon 11 of one or more of the CYP3A genes that results in a valine-to-leucine change and predisposes them to a hypersensitivity reaction to phenytoin or carbamazepine on exposure. Given the location of the epitope, it is possible that such a valine-to-leucine mutation may have dramatic effects on the production of reactive metabolites. Alternatively, the human antigen may be a non-CYP3A protein (e.g., thromboxane synthetase) that has high homology to CYP3As within the epitope region. A final possibility is that covalently bound drug is critical for epitope formation in human CYP3A. These hypotheses are undergoing experimental evaluation, and the results may provide clearer understanding of the relationships between drug bioactivation and immune function that underlie the development of drug hypersensitivity reactions in susceptible individuals.

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