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Tienilic Acid-Induced Autoimmune Hepatitis: Anti-Liver and -Kidney Microsomal Type 2 Autoantibodies Recognize a Three-Site Conformational Epitope on Cytochrome P4502C9

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SUMMARY

Tienilic acid-induced hepatitis is characterized by the presence of anti-liver and -kidney microsomal (anti-LKM2) autoantibodies in patient sera. Cytochrome P4502C9 (CYP2C9), involved in the metabolism of tienilic acid, was shown to be a target for tienilic acid-reactive metabolites and for autoantibodies. To further investigate the relationship between drug metabolism and the pathogenesis of this drug-induced autoimmune disease, the specificity of anti-LKM2 autoantibodies toward CYP2C9 was first determined, and the antigenic sites on CYP2C9 were localized. By constructing several deletion mutants derived from CYP2C9 cDNA and by probing the corresponding proteins with different anti-LKM2 sera, we defined three regions (amino acids 314–322, 345–356, and 439–455); they interacted to form a major conformational autoantibody binding site. This binding site was immunoreactive with 100% of sera and allowed removal of the entire reactivity of the sera tested by immunoblotting. Epitope mapping studies have been performed for CYP2D6, CYP17, CYP21A2, and, recently, CYP3A. Those data were compared with the results obtained in the current study with CYP2C9 in an attempt to elucidate one of the mechanisms by which CYP becomes immunogenic.

Xenobiotic metabolizing enzymes, and particularly CYPs, may be involved in the pathogenesis of autoimmune diseases because some of them have been identified as specific targets for autoantibodies. Indeed, anti-CYP2C9[1] autoantibodies (anti-LKM2) and anti-CYP1A2 autoantibodies (anti-LM) have been found in tienilic acid- and dihydralazine-induced hepatitis, respectively (1, 2). Furthermore, Leeder *et al.* (3) showed that antibodies recognizing CYP3A were found in anticonvulsant-induced hepatitis. CYPs are also recognized by other autoantibodies in autoimmune diseases but apparently not as the result of the use of a drug: in chronic active hepatitis, anti-LKM1 autoantibodies specifically recognized CYP2D6 (4), and for some of the patients, CYP1A2 (5); autoantibodies appearing with adult-onset Addison's disease were directed against CYP21A2 (steroid 21a-hydroxylase) (6, 7); also, antibodies recognizing CYP17 (17a-hydroxylase) (8) and CYP11A1 (cholesterol side-chain cleavage enzyme) (9, 10) were found in autoimmune polyendocrine type I syndrome.

In the case of tienilic acid-induced hepatitis, anti-LKM2 autoantibodies have been shown to recognize CYP2C9 (1, 11), which was involved in the metabolism of tienilic acid (12) and was a specific target for tienilic acid-reactive metabolites (11). These different characteristics are summarized in Fig. 1.

To further investigate the relationship between drug metabolism and the pathogenesis of this drug-induced autoimmune disease, we attempted to elucidate antigenic sites on CYP2C9 recognized by anti-LKM2. Indeed, identification of the CYP2C9 epitope or epitopes could provide insight into the triggering of the immune response (Fig. 1, *step 1*). We wanted to address the following specific points: Is the CYP2C9 epitope the same peptide as that covalently bound by tienilic acid-reactive metabolites? Is this epitope unique? Is this epitope localized at the same sites as those of other CYPs identified as targets of autoantibodies?

For this purpose, 13 sera from patients were used to identify the antigenic sites on CYP2C9. Various fragments of CYP2C9 cDNA were cloned into a bacterial expression vector, and the corresponding truncated proteins were tested by immunoblotting. Anti-LKM2 antibodies recognized a unique conformational epitope constituted in three peptides. The results obtained in this study with CYP2C9 were compared

[1] CYP450 international nomenclature according to Nelson *et al.* (25).

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ABBREVIATIONS: CYP, cytochrome P450; anti-LKM2, anti-liver and -kidney microsomal antibodies type 2; anti-LM, anti-liver microsomal antibodies; anti-LKM1, anti-liver and -kidney microsomal antibodies type 1; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

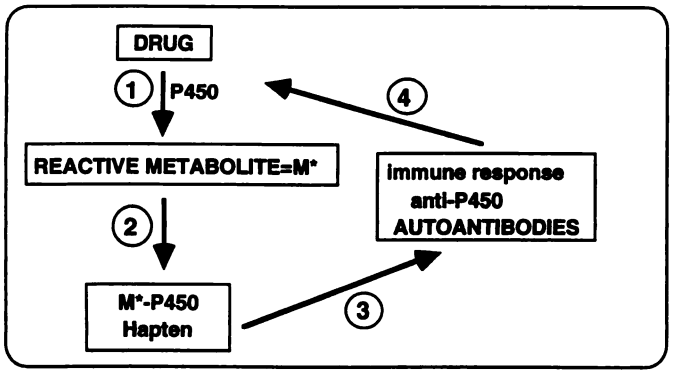

Fig. 1. Initial events postulated in tienilic acid-induced autoimmune hepatitis (1, 11). *1*, Step 1, formation of reactive metabolites (*M**) by CYP2C9. *2*, Step 2, covalent binding of M* on CYP2C9: formation of a neoantigen. *3*, Step 3, immunological response, including production of autoantibodies. *4*, Step 4, specific recognition of CYP2C9 by autoantibodies.

with data from the literature from other CYP epitope mapping studies (CYP2D6, CYP17, CYP21A2, and CYP3A; Refs. 13–19).

Materials and Methods

Chemicals and reagents. Electrophoresis reagents were purchased from Serva Fine Biochemicals (Heidelberg, Germany); nitrocellulose sheet was from Bio-Rad (Richmond, CA); peroxidase-conjugated anti-human and anti-rabbit immunoglobulins were from Dako (Copenhagen, Denmark); Luminol (enhanced chemiluminescence; ECL Western kit) was from Amersham (Buckinghamshire, UK); molecular biology products, including restriction enzymes (*Bam*HI, *Eco*RI, *Sac*I, *Hin*cII, and *Hin*dIII) and T4 DNA ligase were from New England Biolabs (Beverly, MA); NuSieve agarose were from FMC BioProducts (Rockland, ME); isopropyl-β-D-thiogalactoside and Triton X-100 were from Boehringer (Mannheim, Germany); oligonucleotides were from Genosys (Cambridge, UK); T7 sequencing kit was from Pharmacia LKB (St. Quentin, France); bacterial expression vector pET was from AMS Biotechnology (Lugano, Switzerland); and bacterial expression vector pGEX was from Pharmacia LKB. All other reagents were of the highest quality available (Prolabo, Paris, France, or Sigma Chemical Co., St. Louis, MO).

Patient sera. Thirteen serum samples containing anti-LKM2 antibodies were obtained from patients with tienilic acid-induced hepatitis. (20). They were identified by a three-letter code. All patient sera specifically recognized CYP2C9 by immunoblotting. As negative controls, sera from patients with other markers of autoimmune hepatitis (e.g., anti-LM, anti-LKM1, halothane-induced hepatitis) were used. None of them recognized CYP2C9 protein or, in human liver microsomes, a protein comigrating with CYP2C9.

Rabbit antibodies. Anti-CYP2C9 antibodies were rabbit polyclonal antibodies prepared in the laboratory against human CYP2C9. The human CYP2C9 cDNA-coding sequence (21) was amplified by PCR and inserted into bacterial expression vector pET (22) as described previously (23). Rabbits were immunized with 100 μg of the corresponding purified protein homogenized in 1 ml of complete Freund's adjuvant. Three subcutaneous injections at 10-day intervals were administered before the rabbits' death. These antibodies were tested on human liver and yeast microsomes by immunoblotting, and they specifically recognized CYP2C.

Plasmid constructions. For cloning into the pET bacterial vector, various deletions of CYP2C9 cDNA were carried out by cutting at different restriction enzyme sites (*Sac*I, *Hin*dIII, *Hin*cII, and *Bam*HI) (Fig. 2). The restriction enzymes used enabled maintenance of the reading frame. The modified genes cloned into the vector were then expressed in *Escherichia coli* as described by Belloc *et al.* (23). The pGEX bacterial vector (the structure is described in Ref. 24) was used in most cases with 2C9 cDNA constructs amplified by PCR. The oligonucleotides were designed according to the published sequence of 2C10 cDNA, which is an allelic variant of 2C9 (21, 25); they included *Bam*HI (5′ end) and *Eco*RI (3′ end) restriction sites for cloning. Constructions with internal deletion were prepared using two couples of oligonucleotides, including *Bam*HI (5′ end) and *Hin*dIII (3′ end) restriction sites for the first fragment and *Hin*dIII (5′ end) and *Eco*RI (3′ end) for the second fragment. The two fragments were amplified by PCR, purified on agarose low melting point gel, cut, and ligated for cloning into the pGEX 2T vector. Expression of the heterologous proteins as fusion proteins with the glutathione-*S*-transferase of *Schistosoma japonicum* was performed in *E. coli*, as described previously (23). The fusion proteins were harvested in sonicated lysates of *E. coli* and detected with rabbit anti-2C9 antibodies by immunoblotting.

Sequence analysis. All cDNA constructions were purified by using the Qiagen plasmid purification kit (Studio City, CA). The double-stranded cDNA inserts were sequenced by the dideoxynucleotide chain-termination procedure (26) using T7 DNA polymerase. All constructions were sequenced in both directions with the primers used to amplify the corresponding cDNA inserts, and all of the sequences were identical to the original one (21).

Electrophoresis and immunoblotting. SDS-PAGE was performed according to the method of Laemmli (27) using 4% stacking and 9% separating gels. *E. coli* extracts containing the recombinant protein were solubilized in SDS gel loading buffer [30% glycerol (v/v), 1% SDS (w/v), 0.2 M Tris·HCl, pH 6.8, 0.01% pyronine] and heated for 2 min at 100°. After electrophoresis, the proteins were electrotransferred (1 hr, 400 mA) to a nitrocellulose sheet (28) in a 25 mM Tris/192 mM glycine/20% methanol (v/v) buffer. Blots were incubated in 1% polyvinyl pyrrolidone diluted in phosphate-buffered saline and Tween-20 [135 mM NaCl, 15 mM KH_2PO_4, 81 mM Na_2HPO_4, 27 mM KCl, 0.05% Tween 20 (v/v)] for 30 min to block the free binding sites. They were then probed with a rabbit anti-human CYP2C9 (diluted 1:50,000 in polyvinyl pyrrolidone) or the different patient sera (BOU, BOV, DIC, MAR, and PLA diluted 1:5000; PIJ, JOR, KUT, THI, TAI, and CAI diluted 1:10,000; and MIG and SAM diluted 1:20,000). Immunoblots were performed using anti-human IgG labeled with peroxidase (diluted 1:20,000) as second antibody, with Luminol as substrate (according to the manufacturer's recommendations).

Absorption of antibody reactivity. The specificity of autoantibody reactivity was studied by absorption of anti-LKM2 antibodies with crude lysates of *E. coli* containing either proteins produced by unmodified plasmid, full-length 2C9 (construct A1), or fragments A3, A4, and C6. Sera MIG and SAM (final dilution of 1:50,000) were incubated with 0.5 mg of sonicated lysates of the expression clones in 2 ml of phosphate-buffered saline. Absorption was carried out at 4° for 18 hr on a rocker. The sera absorbed were spun for 3 min at 10,000 × *g* and probed against the full-length CYP2C9 by immunoblotting. As controls, sera were absorbed with proteins produced from unmodified plasmid and with a clone expressing a fragment that was not recognized by the anti-LKM2 antibodies (fragment A3).

Results

Specificity of Anti-LKM2 Antibody Recognition against CYP

Serum autoantibodies were all specifically directed against CYP2C9 and did not recognize CYP2C8, CYP2C18, or CYP2C19 under our conditions of immunoblotting, despite a high degree of similarity between these CYPs (Fig. 3). Recognition of CYP2C19 by anti-CYP2C9 rabbit antibodies was weaker than was recognition of the other CYP2C; the cross-reactivity of the rabbit antibodies showed little difference from the other CYP2C isozymes. For determination of the absence of recognition of CYP2C8, CYP2C18, and CYP2C19, the quantity of these loaded CYPs was twice that of CYP2C9.

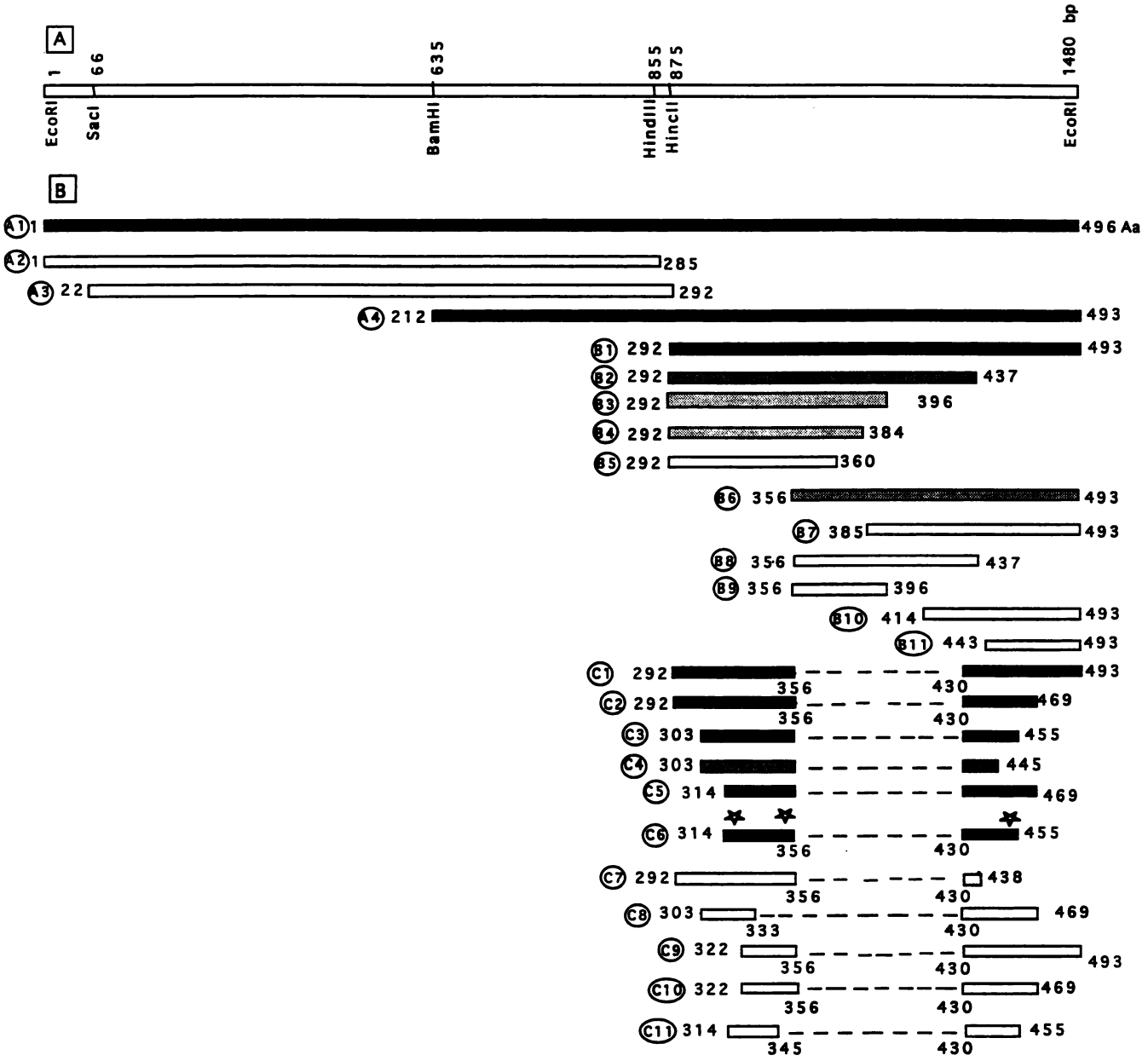

Fig. 2. Strategy for epitope mapping studies. A, CYP2C9 cDNA restriction enzyme in schematic form. B, Schematic representation of CYP2C9 fragments tested with patient sera. Localization of the fragments is indicated by amino acid numbers. The first four constructs were cloned into the pET vector using indicated restriction sites on A. The other constructs were amplified by PCR before cloning into pGEX vector at *Bam*HI/*Eco*RI restriction sites. The fragments become darker as the percentage of positive sera increases. ⋆, Putative sites of anti-LKM2 binding. Code for fragments given within *circles*.

Strategy for Expression of CYP2C9 Fragments Cloned into a Bacterial Vector

Twenty-six cDNA fragments covering the complete human CYP2C9 were inserted into the pGEX or pET bacterial expression vector. The strategy for expression is schematically represented in Fig. 2. The corresponding peptides were produced as fusion proteins to glutathione-*S*-transferase when the pGEX vector was used (fragments B1–11 and C1–11) or to 11 amino acids of the protein 10 from bacteriophage T7 when the pET vector was used (fragments A1–4). The electrophoretic migrations of fusion proteins were directly related to the size of cDNA inserts. Because mutation might be generated by PCR and could modify the epitope, all of the cDNA inserts were sequenced; in comparison with the 2C9 sequence (21), no change was found in any of the clones used in this work.

Recognition of the CYP2C9 Fragments by a Rabbit Polyclonal Antibody

All fragments except one were recognized in immunoblotting by polyclonal rabbit antibodies raised against human CYP2C9 (Fig. 4, A–C). The fragment B9 remained unrecognized. This fragment was located in an extremely hydrophobic region of the protein and then was probably found inside the folded protein. An antigenic index of CYP2C9 showed that the fragment was very weakly antigenic. This little fragment was expressed as a fusion protein with glutathione-

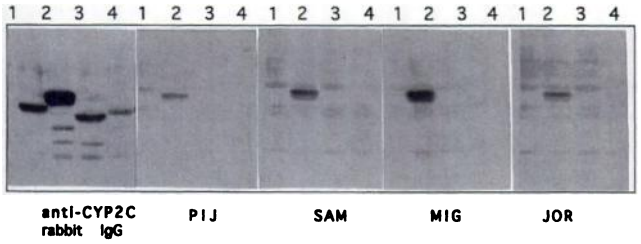

Fig. 3. Recognition of CYP2C8, CYP2C9, CYP2C18, and CYP2C19 expressed in yeast by anti-LKM2 and by rabbit IgG human ant-CYP2C. Yeast microsomes corresponding to 5 pmol of CYP2C8 (*lane 1*), 2.5 pmol of CYP2C9 (*lane 2*), 5 pmol of CYP2C18 (*lane 3*), and 5 pmol of CYP2C19 (*lane 4*) were separated by SDS-PAGE and transferred to a nitrocellulose sheet. Immunoblots were performed with anti-LKM2 sera (*three-letter code*) diluted 1:10,000 or rabbit IgG diluted 1:50,000. The second antibody, labeled with peroxidase, was diluted 1:20,000, and blots were stained with Luminol.

S-transferase; after renaturation, the fusion protein probably folded with this hydrophobic fragment inside the protein structure, and therefore this fragment was probably inaccessible to the antibodies. The cDNA corresponding to this fragment was checked, and the fusion protein expressed was of expected size. Some of the proteins gave proteolysis products, also recognized by the rabbit antiserum.

Reactivity of Human Sera against CYP2C9 Fragments

Table 1 and Fig. 5 provide summaries of the analysis of autoantibodies binding to full-length and modified recombinant CYP2C9. Human liver microsomes and full-length CYP2C9 were used as positive controls, whereas proteins produced by unmodified pET and pGEX plasmids were used as negative controls.

Fragments cloned into pET. None of the 13 sera from patients tested recognized the amino-terminal portion of the protein (fragment A3: amino acids 22–292) (Fig. 5A). All patients tested recognized the carboxyl-terminal fragment (fragment A4: amino acids 212–493), and deletion of the amino-terminal region (fragment B1) had no effect on antibody binding.

Fragments cloned into pGEX. Peptides from the carboxyl-terminal region defined above were not recognized by all sera (Fig. 5B). Recognition decreased with the size of the fragment; the shorter carboxyl-terminal fragments were not recognized. All sera recognized fragment B1; 4 of 13 sera recognized fragment B2; 2 of 11 sera weakly recognized fragment B6; and 1 of 11 sera recognized fragment B4. This recognition was lost when either the amino- or carboxyl-terminal extremities of the carboxyl-terminal fragments were eliminated.

These initial results were in good agreement with a conformational epitope. To shorten this epitope without losing the recognition, we tried to remove internal amino acids from fragment B1.

Constructions with internal deletions cloned into pGEX. Eleven constructions, which included internal deletions, were inserted into the pGEX vector and tested with all sera (an example is shown with one serum in Fig. 5C). The deletion of amino acids 357–429 on fragment B1 led to the strong recognition of the construction (fragment C1) by all sera. Finally, the shortest construction recognized by all sera corresponded to amino acids 314–356 ligated to amino acids 430–455 (fragment C6). The comparison of the constructs C2, C5, and C10 led to identification of amino acids 314–321 as

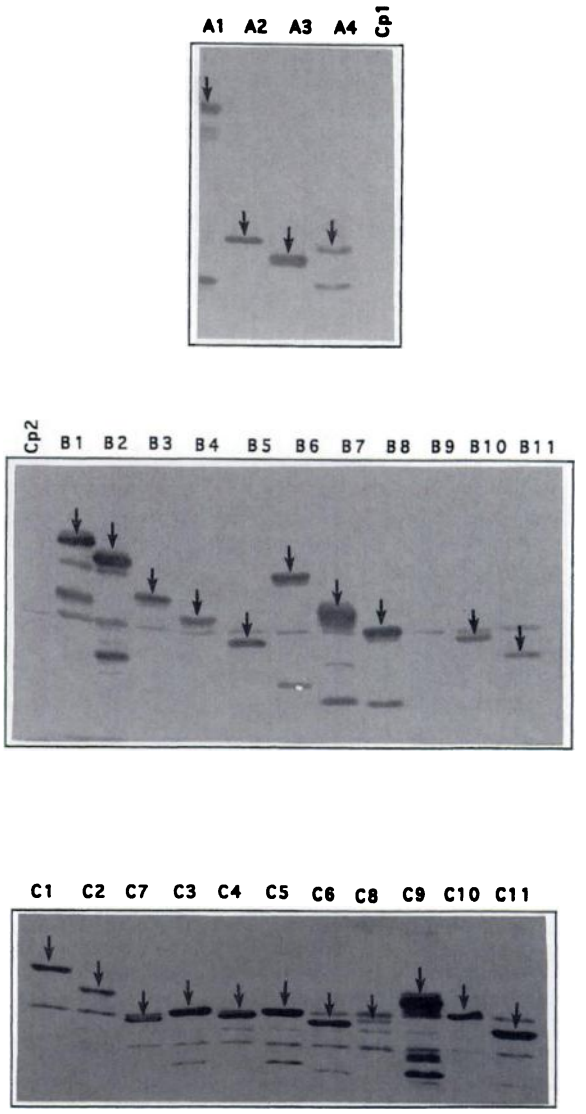

Fig. 4. Recognition of CYP2C9 fragments by anti-human CYP2C rabbit IgG. Lysates of *E. coli* containing each fragment were separated by SDS-PAGE and transferred to a nitrocellulose sheet. Immunoblots were tested with rabbit IgG diluted 1:50,000 and anti-rabbit IgG labeled with peroxidase diluted 1:20,000. Blots were stained with Luminol. For the codes of each fragment, see Fig. 3B. *Top*, constructs cloned into the pET vector. *Cp1*, control proteins produced by the unmodified pET plasmid. *Middle*, constructs cloned into the pGEX vector. *Cp2*, control proteins produced by the unmodified pGEX plasmid. *Bottom*, constructs with internal deletions cloned into the pGEX vector.

being important in the recognition. The comparison of C6, C8, and C11 identified amino acids 345–356, and the comparison of C3, C4, and C7 showed that a portion of amino acids 438–455 was necessary for the recognition. For some of the sera, constructs with fragment 292–356 (C1–2) were slightly better recognized than were constructs with fragment 314–356 (C5–6), indicating that amino acids 292–314 may also be useful for the recognition, participating in the conformational structure of the epitope. A comparison of constructs C3–4 and C5–6 (Table 1) indicated that amino acids 456–469 may be somewhat inhibitory in antibody binding, probably by modifying the conformational structure of the epitope. Most of the sera had the same recognition pattern.

TABLE 1
Reactivity of the different anti-LKM2 sera of patients

	Construct[a]	BOU[b]	BOV	CAI	PIJ	DIC	MIG	JOR	KUT	PLA	SAM	THI	TAI	MAR	Number of positive sera
A1	1–493	■	■	■	■	■	■	■	■	■	■	■	■	■	13/13
A2	1–285														0/13
A3	22–292														0/13
A4	212–493	■	■	■	■	■	■	■	■	■	■	■	■	■	13/13
B1	292–493	■	■	■	■	■	■	■	■	■	■	■	■	■	13/13
B2	292–437	▩	▩	▩	▩										4/13
B3	292–396				▩										1/13
B4	292–384				▩										1/13
B5	292–360														0/13
B6	356–493	□	□												2/13
B7	385–493														0/13
B8	356–437														0/13
B9	356–396														0/13
B10	414–493														0/13
B11	443–493														0/13
C1	(292–356)–(430–493)	■	■	ND	■	■	■	■	■	■	■	■	ND	ND	10/10
C2	(292–356)–(430–469)	■	■	ND	■	■	■	■	■	■	■	ND	ND	ND	9/9
C3	(303–356)–(430–455)	▩	▩	ND	■	▩	■	▩	▩	▩	■	ND	ND	ND	9/9
C4	(303–356)–(430–445)	□	□	ND	□		□		□		□	ND	ND	ND	6/9
C5	(314–356)–(430–469)	▩	▩	ND	□	▩	▩	□	□	▩	■	ND	ND	ND	9/9
C6	(314–356)–(430–455)	■	■	ND	■	■	■	■	■	■	■	ND	ND	ND	9/9
C7	(292–356)–(430–438)			ND								ND	ND	ND	0/9
C8	(303–333)–(430–469)			ND								ND	ND	ND	0/9
C9	(322–356)–(430–493)			ND								ND	ND	ND	0/9
C10	(322–356)–(430–469)			ND								ND	ND	ND	0/9
C11	(314–345)–(430–455)			ND								ND	ND	ND	0/9

[a] A1, A2, . . . are the codes for the different constructs. They are characterized by their amino-acid location.
[b] BOU, BOV, CAI, . . . are the codes for the different patient sera.
■, Strong recognition by the serum; ▩, Medium recognition; □, Weak recognition; ND, not determined.

Checking the Amounts of Expressed Proteins

A Coomassie blue-stained SDS-polyacrylamide slab gel was performed with the different constructs using the same amounts of *E. coli* lysates as used for the immunoblots. This was carried out to verify that approximately equal amounts of each fragment were loaded on electrophoresis. As an example, a Coomassie blue stain representing exactly the same pattern as those in Figs. 4C and 5C is shown in Fig. 6.

Absorption of Sera with the Shorter Deleted Fragment

To verify that the shorter deleted fragment recognized by the anti-LKM2 antibodies included a unique epitope, we tried to remove all reactivity of the sera by absorption with the shorter fragment C6. The ability to remove the reactivity of the sera was studied by immunoblotting. Fig. 7 shows the result obtained with serum MIG: absorptions were performed with full-length CYP2C9 (as positive control), with proteins produced by unmodified plasmids, with fragment A3 (as negative controls), and with fragments A4 and C6. Serum MIG diluted 1:50,000 was totally absorbed by the two positive fragments A4 and C6 and completely lost its reactivity against the full-length protein. Absorption with proteins from unmodified plasmids and with the negative fragment A3 had no effect on the recognition of CYP2C9. Results were identical when the serum was diluted 1:50,000 or 1:100,000. These experiments were also performed with serum SAM (same dilutions), and similar results were obtained.

The lower bands in Fig. 7 probably corresponded to a proteolysis product of CYP2C9.

Discussion

The main objective of this study was to map the epitope or epitopes of CYP2C9 recognized by anti-LKM2 autoantibodies. Because sera specifically recognized CYP2C9 on immunoblotting (Fig. 2), we thought it should be possible to more precisely map antigenic determinants on 2C9 by immunoblotting. By construction of several deletion mutants derived from CYP2C9 cDNA and by probing the corresponding proteins with different anti-LKM2 sera, we were able to define three regions (amino acids 314–322, 345–356, and 439–455) that interacted to form a major autoantibody binding site. This binding site was immunoreactive with 100% of the sera tested and was able to remove the reactivity of two sera by immunoblotting.

The involvement of such an extensive region of the molecule suggested that the binding site was generated by three-dimensional folding; the antibody binding site could be formed by three distinct regions that would interact. The fact that the epitope was probably conformational was not in contradiction with the positive results from immunoblotting experiments (in SDS-PAGE, the proteins migrate under reducing and denaturing conditions). This indicated that peptide renaturation was allowed to occur because SDS was probably stripped off the proteins during electrotransfer (29). Furthermore, the blotted proteins were placed under renaturing conditions before interaction with autoantibodies (30). Indeed, some immunoblotting experiments performed in other epitope mapping studies led to the same conclusion, that of a conformational epitope (16).

The correct alignment of the three peptides corresponding to the antigenic sites was maintained, although a section of the protein had been deleted. This was possible because we had deleted a peptide corresponding to a loop, relatively separate from the remainder of the protein, in the tridimensional structure of the entire protein. We thought that the deletion of this loop would not drastically modify the deleted

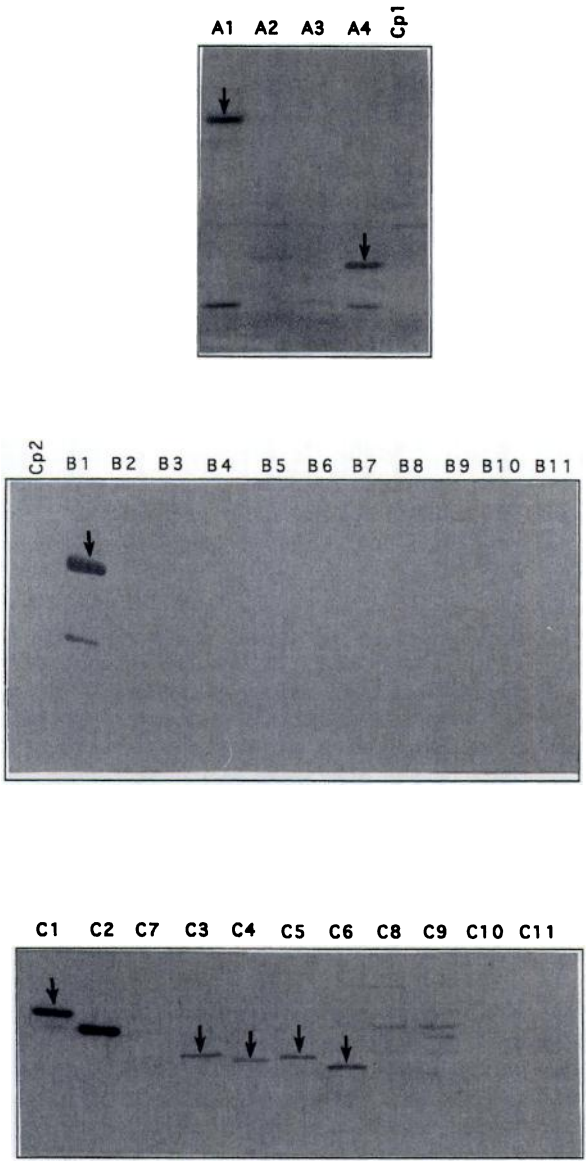

Fig. 5. Example of recognition of CYP2C9 fragments by a patient serum containing anti-LKM2. Lysates of *E. coli* containing each fragment were separated by SDS-PAGE and transferred to a nitrocellulose sheet. Immunoblots were tested with serum MIG diluted 1:20,000 and anti-human iummunoglobulins labeled with peroxidase diluted 1:20,000. Blots were stained with Luminol. For the codes of each fragment, see Fig. 3B. *Top,* constructs cloned into the pET vector. *Cp1*, control proteins produced by the unmodified pGEX plasmid. *Middle*, constructs cloned into the pGEX vector. *Cp2*, control proteins produced by the unmodified pGEX plasmid. *Bottom,* constructs with internal deletions cloned into the pGEX vector.

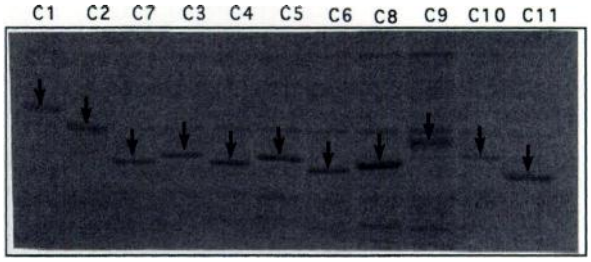

Fig. 6. Coomassie blue-stained SDS-polyacrylamide slab gel showing the constructs with internal deletions cloned into the pGEX vector. The quantities of *E. coli* lysates loaded were the same as those loaded for Figs. 4C and 5C.

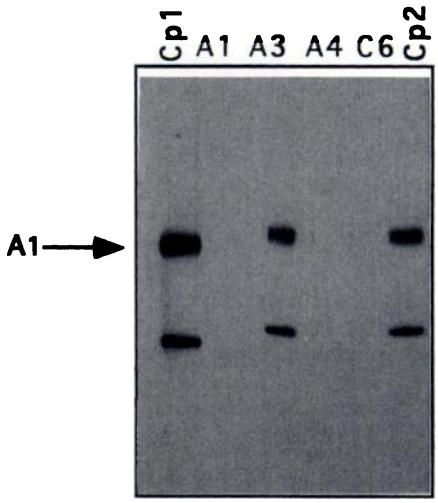

Fig. 7. Absorption of anti-LKM2 with different expression clones. Anti-LKM2 (an example is shown with serum MIG), diluted 1:50,000, was incubated with 0.5 mg of lysates of *E. coli* containing CYP2C9; fragments A3, A4, and C7; and control proteins produced by either an unmodified pET plasmid or unmodified pGEX plasmid. The serum absorbed was spun for 3 min at 10,000 × *g* and probed against CYP2C9 (construct A1) by immunoblotting. Blot was stained with Luminol. *Cp1* and *Cp2*, control proteins produced by the unmodified pET and pGEX plasmids, respectively. For the codes of the different fragments, see Fig. 3B.

peptide folding. Furthermore, the constructs with internal deletions were clearly recognized by the autoantibodies, with a very high titer; it could be very unlikely to recover, by deletion, a peptide completely different from the native one and so well recognized by the autoantibodies because these antibodies were shown to be very specific.

On the basis of molecular modeling of antibodies reacting with antigens, ≥90% of B cell epitopes are thought to be conformational (31). In contrast to T cell epitopes, which are short, linear peptides, B cell epitopes may be more complex, as demonstrated by the recent mapping of a number of B cell epitopes: CYP2D6 in chronic active hepatitis (32), CYP21A2 (21a-hydroxylase) in adult-onset Addison's disease (16), thyroid peroxidase in Hashimoto's thyroiditis (33), glutamate decarboxylase in insulin-dependent diabetes mellitus (34), and the E2 component of the branched-chain 2-oxo-acid dehydrogenase complex in primary biliary cirrhosis (35). All of the authors of the above studies reported a major conformational epitope on these CYPs or on other enzymes that are targets for autoantibodies.

In the current study, we report that the major epitope actually involved amino acids 314–356 and 430–455. The three sites corresponding to amino acids 314–322, 345–356, and 439–455 were no doubt essential in the binding of the anti-LKM2 antibodies and the folding of the peptide because removal of each of these three sites led to a partial or total loss of recognition by autoantibodies. Because anti-LKM2 antibodies were highly specific and did not recognize the other CYP2C on immunoblotting, it was interesting to compare the four sequences of CYP2C8, CYP2C9, CYP2C18, and CYP2C19 for the three relevant regions. CYP2C9 sequence was shown to differ from the other CYP2Cs by two amino acids: alanine instead of glycine at position 439 and glycine instead of arginine at position 442. These two amino acids

may have a crucial role in the binding of the autoantibodies or for the structure of the epitope, but it will be necessary to confirm this hypothesis through additional experiments, such as site-directed mutagenesis.

None of the patient sera tested recognized the NH_2-terminal region (amino acids 1–294), indicating that this region was not involved in antibody recognition and binding. This result was in good agreement with epitope mapping work on CYP21A2 (16, 17), CYP2D6 (13, 14), and CYP3A1 (18, 19), in which epitope sites have been identified in the carboxyl-terminal half of the protein. More precisely, Wedlock *et al.* (16) reported a central and a terminal region (amino acids 281–379 and 380–494) of the CYP21A2, interacting to form at least one major conformational autoantibody binding site. Song *et al.* (17) indicated a major epitope located at amino acids 298–356 for the same protein. In other cases of endocrinal autoimmune disease, such as diabetes, Richter *et al.* (34) reported that a central region and a carboxyl-terminal region of the glutamate decarboxylase were involved, forming two major conformational epitopes. In a case of chronic active hepatitis, Manns *et al.* (13) found a small epitope recognized by anti-LKM1 sera. This epitope was localized at amino acids 263–270. Yamamoto *et al.* (14) reported other immunogenic sites in CYP2D6: amino acids 321–351, 373–389, and 410–429. Studies on anticonvulsant-induced hepatitis reported a CYP3A1 epitope located at amino acids 342–367 (18, 19). According to the alignment data of Korzekwa *et al.* (36) and Hasemann *et al.* (37), the epitope sites on CYP2D6 and CYP3A1 were localized in the same region as the immunogenic sites for CYP2C9.

Although no crystallographic data are available for eukaryotic CYPs, alignment of the regions concerned with CYP_{terp}, CYP_{cam}, and CYP_{BM-3} (36, 37) suggested that the three sites corresponded to J helix (amino acids 314–322), K helix (amino acids 345–356), and the half-end of the L helix (amino acids 439–455). They corresponded to highly conserved structures in the neighborhood of the heme. The structures involved in substrate recruitment and binding were not involved. A structural model based on crystallographic data indicated that the three helices were next to each other (38, 37); this is in good agreement with a conformational epitope. These results should be compared with those for anticonvulsant-induced autoimmune hepatitis, in which patient sera recognized the K helix of CYP3A1 (18). It was thus striking that for these two cases of drug-induced autoimmune hepatitis, the recognized epitopes had a similar structure and location in the protein. This could indicate a common mechanism of triggering of the immune response for the two types of hepatitis.

Two hypotheses of triggering of the autoimmune response can be advanced: first, a certain CYP fragment near the active site of the enzyme could be the preferential target for drug-reactive metabolites and thus would be modified. After cell death (consecutive to a slight cytolytic effect of the drug), the same CYP fragment could be presented to the immune response cells and be recognized as nonself by T cells. The activated T cells would stimulate the antibody response. By cross-reaction, the activated B lymphocytes would recognize modified and native CYPs. The epitope found in this work is localized near the active site of the protein; the reactive metabolites generated during CYP metabolization of tienilic acid would thus be able to bind the enzyme *in situ*, immediately after their formation, constituting a neoantigen and leading to naive T lymphocyte activation. These results are in agreement with the first data presented by Lecoeur *et al.* (11). We were aware that several CYP2C subfamily members were able to metabolize tienilic acid, but it was striking that CYP2C9 was the only CYP to be covalently bound by tienilic acid-reactive metabolites, with this covalent binding leading to suicidal inactivation of the enzyme (12).

An alternative hypothesis is the triggering of the immune response due to autoreactive B cells that recognize a native epitope of the CYP (39). After cell death, these B cells could capture the CYP and present either a native epitope or an alkylated epitope, in association with class II major histocompatibility molecules, to T cells. The presentation of an alkylated peptide could stimulate the T cell response to this alkylated peptide. The T cell response would then enhance autoantibody production. This latter hypothesis is acceptable if the native epitope recognized by the autoreactive B cells is located superficially on the protein. In fact, the location of the CYP2C9 epitope on helix J, helix K, and the end of the helix L, compared with the tridimensional structure of CYP_{cam} (38), seemed to belong to the external portion of the protein.

Our work showed that all sera from the patients, whose serum probably was not sampled at the same time in the evolution of the disease, had the same pattern of recognition. All sera were specifically directed against a small part of the protein. However, 4 of 13 sera recognized a part of the protein that was slightly different from the three sites discussed above (fragment B2 was recognized by four sera, fragment B4 by one serum, and fragment B6 by two sera); the epitope mentioned above is probably the major one, but some autoantibodies may be directed against a CYP region slightly displaced in comparison to the major epitope.

The experiments on absorption with two sera showed that this epitope was in large part dominant and, for some of the sera, probably the only epitope. Because most of the sera seemed to present the same pattern of recognition, absorption experiments were performed with two sera that had a particularly high titer and a high specificity of recognition. The absorption of these sera on the epitope (C6) removed 100% of the immunoreactivity. However, we could not be absolutely certain that this epitope was unique because we performed all of the experiments by immunoblotting; it is possible that some recognition of other conformational epitopes was lost. This could be confirmed by the use of an enzyme-linked immunosorbent assay, for example, or experiments on immunoinhibition of CYP2C9 activities with preabsorbed sera.

In conclusion, our work showed that (a) anti-LKM2 autoantibodies recognized à conformational epitope on CYP2C9; (b) this epitope was localized near the active site of the enzyme, on the J, K, and L helices; (c) this epitope was unique; and (d) all sera tested had the same pattern of recognition.

This epitope included a structure similar to that identified in anticonvulsant-induced autoimmune hepatitis, suggesting a common mechanism in the triggering of the disease.

These findings led us to formulate certain hypotheses concerning events occurring between covalent binding of reactive metabolites on CYP and the outbreak of autoantibodies. Up to now, the peptide alkylated by tienilic acid metabolites had not been precisely determined. This will be necessary before definitive conclusions can be made. Epitope mapping studies on

CYP1A2, the target of anti-LM autoantibodies in dihydralazine-induced hepatitis, would also be very useful because the mechanism of triggering of the disease seemed similar to that induced by tienilic acid (40). Finally, an animal model of drug-induced hepatitis would be necessary for elucidation of the exact role of the autoantibodies in the development of active disease. This is now under investigation in our laboratory.

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