

ANTI-LIVER KIDNEY MICROSOME ANTIBODY RECOGNIZES  
A CYTOCHROME P450 FROM THE IID SUBFAMILY

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Children with autoimmune chronic active hepatitis can be separated into two groups depending on the presence in their serum of either anti-smooth muscle antibody (SMA) or anti-liver-kidney microsomal antibody (LKMA) (1). LKMA-positive autoimmune chronic active hepatitis may be detected early in life, present different clinical patterns, and is frequently associated with extrahepatic autoimmune manifestations (2). The antigen recognized by LKMA has been identified as a protein with an  $M_r$  of 50 kD, present in a higher concentration in smooth than in rough microsomes. This 50-kD protein is an integral membrane protein exposed on the cytoplasmic side of the microsomal membrane (3). These properties are typical of the cytochromes, a family of heme protein monooxygenase isozymes present in large amounts in hepatocytes. It was recently shown that LKMA crossreacts with two methylcholantrene-inducible isozymes of cytochrome P450 on dot-blot analysis, suggesting that the antigen, present in noninduced microsomes, has some common epitopes with these P450 isozymes and could be a constitutive form of cytochrome P450 (4). Here, we show that LKMA recognizes cytochrome P450 forms from the IID subfamily.

Materials and Methods

**Affinity Purification of Human Anti-50-kD Antibodies.** Sera were obtained from three children with autoimmune hepatitis with high titers of LKMA in immunofluorescence ( $>1:100,000$ ) and anti-50 kD on immunoblot analysis ( $>1:1,000$ ). Rat liver microsomes were prepared as described previously (5), and resuspended in PBS at a final protein concentration of 3 mg/ml. Microsomes were solubilized as described by Bar-Nun et al. (6) with sodium deoxycholate 0.4%, during 30 min, at 0°C. After centrifugation at 100,000 g (Ti70 rotor; Beckman Instruments, Inc., Palo Alto, CA) during 2 h at 4°C, proteins from the supernatant were coupled to Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) following the protocol from the manufacturer. Solubilized microsomal proteins bound to Affi-Gel 10 were poured into a column and used for purification of anti-50-kD antibodies from LKMA-positive human sera, as described (7). Fractions eluted from the column were lyophilized and dialyzed. Their specificity for the 50-kD protein was checked by immunoblot analysis against total rat liver microsomal proteins. The same technique was used for titration of the antibody solution before use for the screening of the rat liver cDNA library.

**Screening of Rat Liver cDNA Library.** A rat liver cDNA library (Normal female adult Sprague-

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Dawley) in phage  $\lambda$ GT-11 (Clontech, Palo Alto, CA) was screened as described by Young and Davis (8). Approximately  $4 \times 10^5$  recombinant phages were plated on lawns of *Escherichia coli* Y 1090. Production and recovery of fusion proteins was obtained overlying nitrocellulose filters impregnated with 10 mM isopropylthiogalactoside (Sigma Chemical Co., St. Louis, MO). Immunopositive clones were detected by immunoblot technique using affinity-purified human anti-50-kD antibodies at a dilution of 1:25. All these clones were purified and used to lysogenize Y1089 cells (9). To show that the fusion protein was responsible for the signal, total extract proteins from *E. coli* Y1089, and from lysogenes obtained with immunopositive clones, or nonrecombinant clones, with or without  $\beta$ -galactosidase induction were performed, and analyzed by immunoblotting. Four clones called LKMC1, LKMC2, LKMC3, and LKMC4 were further analyzed.

*Analysis of Crossreactivity Between Fusion Proteins and the 50-kD Protein.* The following procedures were used: (a) Isolated immunopositive LKMC1 clone was plated and fusion protein was recovered following the technique used for the cDNA library. Filters were blocked and incubated with affinity-purified human anti-50-kD antibodies at a dilution of 1:25, at 4°C overnight. After washing, bound antibodies were eluted by treatment of the filters with 50 mM glycine, pH 3, 3 min at room temperature, and the pH of the solution was immediately neutralized with 1 M Tris, pH 8. Eluted antibodies were analyzed by immunoblotting against total rat liver microsomal proteins. (b) Purified fusion protein prepared from clone LKMC4 as described (10), was injected to a rabbit according to standard procedures (11). Pre-immune rabbit serum and serum after three injections were tested by immunoblot analysis on total rat liver microsomal protein. (c) Purified fusion protein from clone LKMC4 was tested in immunoblot against LKMA positive total human sera.

*Immunoblot Analysis.* SDS-polyacrylamide gels of 10% were used for the electrophoresis of total rat liver microsomal proteins and of 7.5% in the case of total extracts or purified fusion protein. 5  $\mu$ g of  $\beta$ -galactosidase were loaded as an  $M_r$  marker. Electroblothing onto nitrocellulose and different steps of the immunoblot procedure were essentially done as described by Towbin et al. (12). Normal human serum (control), affinity-purified human anti-50-kD antibodies and LKMA-positive human sera were used for immunoblot analysis at a dilution between 1:25 and 1:1,000. Second antibodies, goat anti-human and anti-rabbit IgG, labeled with horseradish peroxidase (Byosis, Compiègne, France), were used at 1:1,000 dilution. Immunoblots were developed for 1 min with diaminobenzidine in 50 mM Tris-HCl, pH 7.5 (0.5 mg/ml), and H<sub>2</sub>O<sub>2</sub> (final concentration of 0.01%).

*Restriction Mapping and Sequencing of cDNA of Immunopositive Clones.* cDNA from clones LKMC1, LKMC2, LKMC3, and LKMC4 were prepared and subcloned in the Eco R1 site of the vector pGEM2 (Promega Biotec, Madison, WI). A restriction map was obtained from these clones with the following enzymes: ApaI, AvaI, BamHI, BglI, BglII, HindII, HindIII, HpaI, NcoI, and PstI (Boehringer Mannheim Biochemicals, Penzberg, Federal Republic of Germany). The Hind III-Bam HI 608-bp fragment of LKMC1 was subcloned in phages M13 mp10 and mp11 and sequenced using the method of Sanger et al. (13).

## Results and Discussion

Immunoscreening of the rat liver cDNA library in phage  $\lambda$ GT-11 with purified human anti-50-kD antibodies allowed us to detect twelve independent immunopositive clones from a total of  $4 \times 10^5$  recombinant phages tested. Because of the known crossreactivity of the LKMA with the native forms of various inducible P450s in rat liver (4), fusion proteins were tested under denaturing conditions. Eight clones gave a positive signal, and four of them (called LKMC1 to LKMC4) were used for further testing. Fig. 1 A, lane c shows the positive signal for LKMC4.

The crossreactivity between fusion proteins of two subclones (LKMC1 and LKMC4) and the 50-kD endoplasmic reticulum protein was demonstrated by three methods (Fig. 1): (a) Human antibodies eluted from nitrocellulose filters containing LKMC1 fusion protein also recognized a 50-kD protein on immunoblot of to-

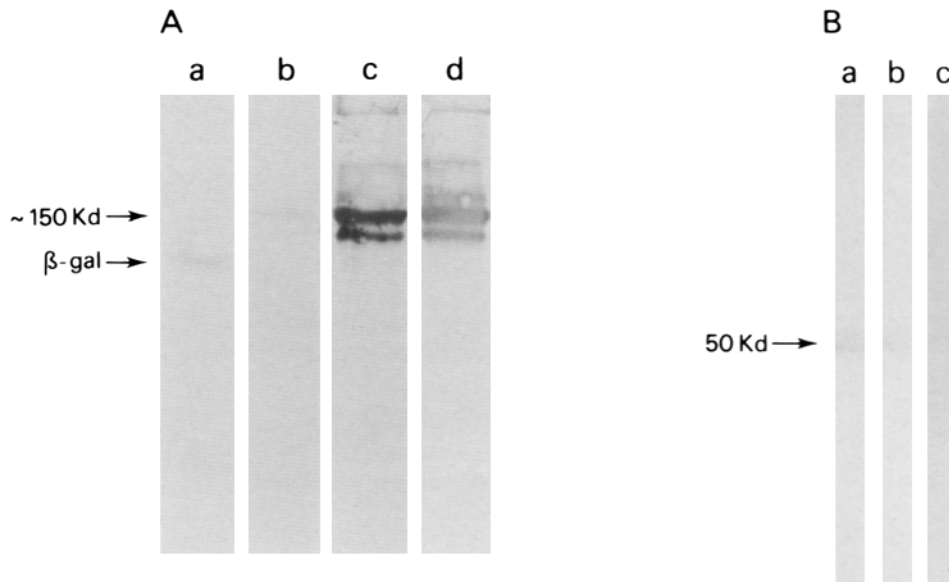


FIGURE 1. Analysis of crossreactivity between fusion proteins from clones LKMC1 and LKMC4 and the 50-kD rat microsomal protein. (A) LKMC4-purified fusion protein was analyzed by immunoblot technique. (a) 5  $\mu$ g of  $\beta$ -galactosidase. (b-d) 5  $\mu$ g of purified LKMC4 protein ( $\sim$ 150 kD). Lanes a and b were stained with Ponceau red. Lanes c and d were revealed with affinity-purified human anti-50-kD antibodies (1:25 dilution) and LKMA-positive human serum (1:1,000 dilution), respectively. (B) Rat liver total microsomal proteins were electroblotted on a nitrocellulose paper and revealed using: (a) LKMA-positive serum (dilution 1:1,000); (b) antibodies eluted from nitrocellulose filters, where LKMC1 fusion protein was previously bound (dilution 1:25); and (c) serum from a rabbit immunized against LKMC4-purified fusion protein (dilution 1:1,000).

tal rat liver microsomal proteins (Fig. 1 B, lane b); (b) serum from the rabbit immunized against LKMC4 fusion protein also recognized a 50-kD protein under the same conditions (Fig. 1 B, lane c); (c) LKMA-positive human sera recognized LKMC4-purified fusion protein on immunoblot (Fig. 1 A, lane d).

The identification of cDNA inserts was done by restriction mapping. We compared the restriction maps of LKMC1, LKMC2, LKMC3, and LKMC4 (Fig. 2) with those from already sequenced rat P450s, because previous work had shown that human LKMA recognizes methylcholantrene-inducible forms of rat liver cytochrome P450. With the enzymes used in this study, we found that the maps of LKMC1 and LKMC2 were identical to that of the 5' region of P450 db2 from the IID subfamily. We thus sequenced the 608-bp Hind III-Bam HI fragment of LKMC1 and confirmed this identity since it showed complete homology with the rat liver P450 db2 deduced amino acid sequence (14). The restriction map of cDNAs from the clones LKMC3 and LKMC4 was identical to that of the 3' end of the rat P450 db1 form, the other P450 isozyme described in the IID subfamily. Taken together, these results demonstrate that LKMA is directed against cytochromes P450 from the rat IID subfamily. The rat liver isozymes db1 and db2 have 78 and 73% nucleotide and amino acid sequence homology, respectively. Therefore, it is not surprising that LKMA recognizes both db1 and db2 forms. Clones corresponding to

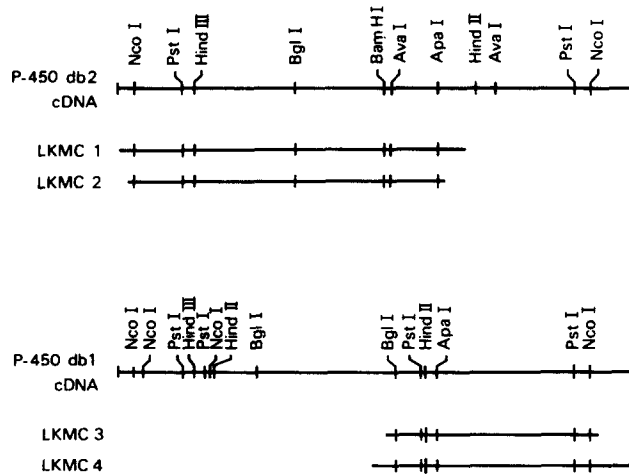


FIGURE 2. Composition of restriction maps of clones LKMC1, LKMC2, LKMC3, and LKMC4, and P450 db2 and db1 (14). The restriction maps of clones LKMC1 and LKMC2 showed a complete identity with P450 db2, 5' region. LKMC3 and LKMC4 restriction maps were identical to the 3' end of P450 db1.

P450 db2 (LKMC1 and LKMC2) and db1 (LKMC3 and LKMC4) overlap on a region of high homology (~90%) of 350 bp, allowing to speculate that the common epitope recognized by LKMA on immunoblots is located in that area coding for 115 amino acids.

In human liver, the only P450 isozyme known to be expressed from the IID subfamily is db1 (15). The human form has 71% amino acid homology with both db1 and db2 from rat liver (16). This suggests that in children with autoimmune chronic hepatitis, LKMA is originally directed against the human P450 db1 form. Indeed, immunoblot analysis of proteins from human liver microsomes shows that LKMA recognizes a band of electrophoretic mobility higher than that of the rat (4). The difference between human and rat antigens  $M_r$  is ~2 kD and corresponds to differences between published rat P450 db1 and db2 and human P450 db1-deduced amino acid sequences.

The production of very high titers of anti-P450 antibodies as a reaction of the immune system against a protein liberated by cellular lysis is intriguing. Most probably another event, for example, a virus infection, could be the initial trigger of the autoimmune process. Recently, several reports showed homologies between antigens recognized by antibodies present in the course of autoimmune disorders and viral proteins (for review, see reference 17). Eventually, B lymphocytes can be boosted by the P450 liberated by the cellular lysis. It was demonstrated in animals having an autoimmune disease that a constant immunization is responsible for the development and maintaining of high titers of autoimmune antibodies (18).

### Summary

Children with autoimmune hepatitis have high serum titers of antibodies directed against a 50-kD protein of rat liver endoplasmic reticulum. Affinity-purified anti-50-kD antibodies were used to screen a rat liver cDNA library in  $\lambda$ GT-11 expression vector. 12 immunopositive clones were obtained. Crossreactivities between fusion proteins of these clones and the 50-kD protein was demonstrated, and four clones

were analyzed by restriction mapping, one of them by nucleotide sequencing. Complete identity was found between the restriction maps of two clones (LKMC1 and LKMC2) and that of the 5' end of the rat cytochrome P450 db2. Sequence of a 608-bp fragment of LKMC1 showed complete homology with the rat P450 db2 form. The restriction map of the other two clones (LKMC3 and LKMC4) was identical to that of rat P450 db1. These results suggest that the antigen recognized by LKMA is a P450 of the IID subfamily.

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