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A HUMAN CYTOCHROME P-450 IS RECOGNIZED BY ANTI-LIVER/KIDNEY MICROSOME ANTIBODIES IN AUTOIMMUNE CHRONIC HEPATITIS[1]

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1- Anti-liver/kidney microsome autoantibodies typel (anti-LKM1), observed in some children with chronic active hepatitis, were used to isolate their antigen in human liver microsomes. A protein, called P-LKM1 was thus purified. This protein was recognized by a rabbit antiserum directed against the related human cytochromes P-450 bufI and P-450 bufII.
2- A human liver microsomal protein immunoprecipitated with anti-LKM1 sera was also recognized by anti cytochromes P-450 bufI/II antibodies.
3- Anti-LKM1 antibodies potently inhibited microsomal bufuralol 1'-hydroxylation.
These results displayed the possible identity between cytochromes P-450 bufI/II and LKM1 antigen. © 1989 Academic Press, Inc.

Circulating antibodies that are directed against cell organelles have been observed in sera of some patients with hepatitis. Anti-liver/kidney microsome (anti-LKM) antibodies can be classified into three groups depending upon their characteristic fluorescence pattern on rat liver endoplasmic reticulum and on kidney tubular cells (1,2): anti-LKM1 are found in some children with autoimmune chronic active hepatitis (3); anti-LKM2 are detected in patients with tienilic acid-induced hepatitis (4) and anti-LKM& in patients with chronic delta hepatitis (5).

A focus of current interest is the nature of the antigen against which the immune response of patients is directed and the mechanism by which this antigenic reaction occur. In this context, it was shown that (i) anti-LKM2

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recognize specifically a human liver cytochrome P-450 (6,7), (ii) anti-LKM1 found in chronic active hepatitis reacte with a rat liver membrane protein of 50 KD (8) which is specific for the endoplasmic reticulum of rat hepatocytes (9). The identity of this 50 KD-LKM1 antigen is still unknown. Identification and characterization of the human antigen against which anti-LKM1 are directed therefore was the aim of the present study. We have observed that the human liver protein recognized by anti-LKM1 seems identical to the recently characterised cytochromes P-450 bufI and bufII (10) and related to P-450 DB (11) or P-450 IID (12). These human cytochromes P-450 isozymes catalyse the hydroxylation of over 20 clinically used drugs including bufuralol (buf) and debrisoquine (DB).

MATERIALS AND METHODS

Human sera. Anti-LKM1 positive sera, showing characteristic immunofluorescence patterns on hepatocytes and on proximal renal tubule cells were obtained from three children (GER,GES,LEF) with a diagnosis of chronic active hepatitis.

Liver samples and preparation of microsomes. Microsomes were prepared from kidney transplant donor livers from the human liver banks established in this laboratory (13) and in the laboratory of URS A. MEYER (10). Microsomal fractions used for purification and immunoblotting were prepared as described by Kremers et al. (13) and for inhibition experiments as described by Meier et al. (10)

Preparation of polyclonal antibodies. Anti human cytochromes P-450 bufI/II serum was kindly provided by U. A. MEYER (10).

SDS PAGE and Immunoblotting. Proteins were separated in the discontinuous Laemmli system (14) containing 9% acrylamide instead of 7,5% and electrotransferred to nitrocellulose sheets according to previously described procedures (15,16). Immunoblots were stained with 3,3'-diaminobenzidine tetrahydrochloride (16).

Purification of LKM1 antigen. All chromatography and dialysis buffer contained 0.05% lubrol PX, 1mM DTT, 1mM EDTA and 20% glycerol. The microsomal pellets were resuspended in 0.2 M phosphate buffer, pH 7.0, in a volume equivalent to the net weight of the liver piece (1ml for 1g) and solubilized with lubrol PX to a final concentration of 0.2%. Proteins were precipitate with solid $(NH_4)_2$ SO_4 to 30-55% saturation. The ammonium sulphate precipitate was subjected to phenyl-Sepharose CL-4B chromatography as described (17). Elution was performed with a linear gradient of lubrol (0.05-1%). An aliquot of every fraction collected was checked on SDS-PAGE for homogeneity and on immunoblots to test the reactivity with anti-LKM1. Only those fractions with highest purity on SDS-PAGE and highest immunoreactivity with anti-LKM1 were pooled. This concept was followed strictly throughout the purification to the last chromatography step. As a result of this procedure a fraction named PS-LKM1 was eluted at 0.8% of Lubrol. The PS-LKM1 pool was applied to a

DEAE-Sepharose CL-6B columm equilibrated with a 25mM phosphate buffer, pH 7.4. After washing the columm, a KCl gradient (0-500 mM) was then added to the buffer and a fraction at about 400 mM of KCl (DS-LKM1) was selected by the same criteria as described. A chromatography of DS-LKM1 on carboxy-methyl cellulose was performed (18). The first protein peak was highly enriched in LKM1 antigen, arbitrarily designated P-LKM1.

Immunoprecipitation. Antisera and 100 µl of solubilized microsomes (5pmoles of cytochrome P-450 in 100 mM phosphate buffer, pH7) were incubated with 100 µl of a 20% suspension of protein-A-Sepahrose CL-4B for 1h at 37°C. The immunocomplexes were centrifuged and washed four times with phosphate buffered saline containing 0,5% Tween 20. The bound protein was released with 6% SDS/100 mM Tris-Hcl (pH 6,8) and submitted to SDS-PAGE for immunoblotting. It is noteworthy that mercaptoethanol was not added and that the samples were not boiled.

Immunoinhibition of bufuralol-1'-hydroxylation. Immunoinhibition assays of bufuralol-1'-hydroxylation were performed as previously described (10).

RESULTS

We subjected human liver microsomes to different chromatographic steps to isolate and identify the macromolecule that could be recognized by the serum from a child (GES) suffering from autoimmune chronic active hepatitis and containing anti-LKM1 by immunofluorescence analysis. By following the reactivity of the purified fractions with anti-LKM1 at each purification step, we could isolate a protein called P-LKM1 which, on immunoblots with the serum GES, had the same mobility as the protein recognized in human liver microsomes (fig.1). All partially purified fractions contained cytochrome P-450 as determined by CO-binding spectra (results not shown). This led to the hypothesis that P-LKM1 possibly was a human P-450 isozyme. For this reason, the protein P-LKM1 was analysed by immunoblotting with various specific antibodies raised in rabbits against some purified microsomal human liver P-450 isozymes. The protein P-LKM1 was recognized only by anti P-450 bufI/II; moreover anti P-450 buf I/II and anti-LKM1 showed the same pattern of recognition in Western blots of human liver microsomes (fig.2).

Two other sera from children with chronic active hepatitis containing anti-LKM1 (LEF and GER) were tested by immunoblotting. They were unable to recognize a band in human liver microsomes. However, these antibodies, when coupled to protein A-Sepahrose were able to bind a microsomal protein which

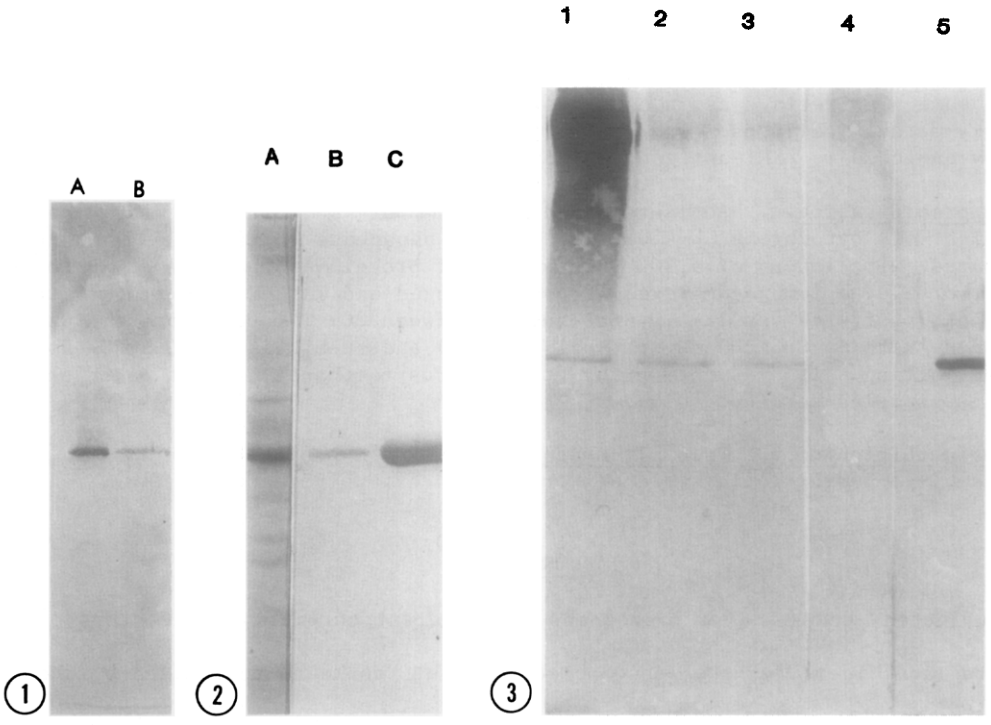

FIG. 1. Immunoblot of human liver microsomal proteins and of LKM1 antigen. Lane A, 15µg of microsomal proteins; 5µg of purified protein. The nitrocellulose sheet was incubated with a 1/400 dilution of anti-LKM1 serum GES.

FIG. 2. Immunoblot of human liver microsomal proteins and of LKM1 antigen. Lanes A and C, 15 µg of microsomal proteins; lane B, 5µg of P-LKM1. The nitrocellulose sheet was incubated with a 1/400 dilution of anti-LKM1 serum GES (lane A) or a 1/200 dilution of rabbit antiserum raised against cytochromes buf I/II (lanes B and C).

FIG. 3. Immunoprecipitation of solubilized human liver microsomes with Protein A - Sepharose CL-4B followed by SDS PAGE and immunoblotting. 15µg of microsomal proteins were immunoprecipitate with 5µl of anti cytochromes P-450 bufI/II (lane 1), 5µl of anti-LKM1 serum GER (lane 2), 5µl of anti-LKM1 serum LEF (lane 3) or 5µl of human control serum (lane 4). Lane 5, 15µg of solubilized microsomal proteins were loaded. The nitrocellulose sheet was incubated with anti cytochromes P-450 bufI/II (1/200 dilution).

reacted with anti P-450 buf I/II; this protein had the same mobility as the protein immunoprecipitated by anti P-450 bufI/II; the control serum failed to react similarly (fig. 3).

As already mentionned , bufuralol-1'-hydroxylation appears to be dependent on two cytochrome P-450 isozymes, P-450 buf I and P-450 buf II, wich have different substrate affinity and stereoselectivity for (+) and (-) bufuralol (10). Serum from patient LEF potently inhibited bufuralol-1'-hydroxylation activities; the inhibition of the hydroxylation

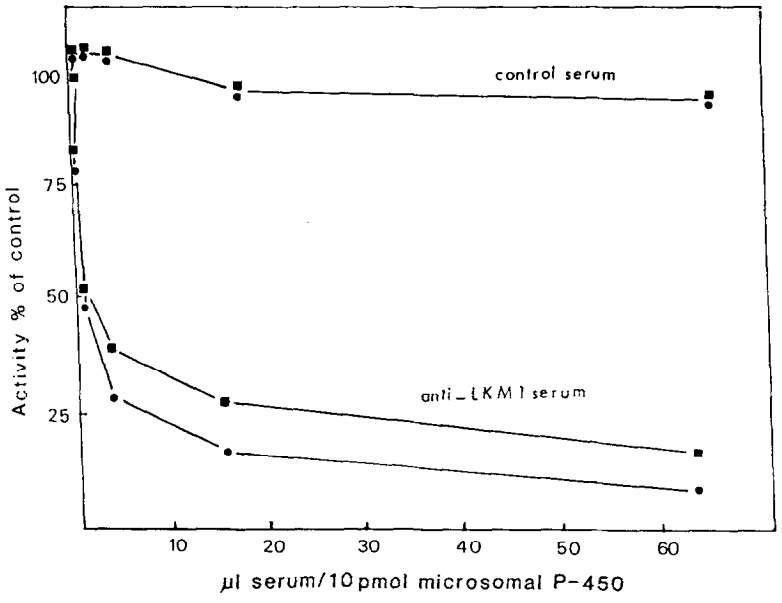

FIG. 4. Inhibition of bufuralol hydroxylase activivities by anti-LKM1 serum LEF. Human microsomes were incubated with various amounts of human control serum or anti-LKM1 serum LEF. The enzymatic activities were determined as previously described (10).
■ ---- ■ (-) bufuralol activity and ● ---- ● (+) bufuralol activity.

of (+) bufuralol being somewhat more pronounced than that of (-) bufuralol (fig. 4).

DISCUSSION

These results show that anti-LKM1 found in children with chronic active hepatitis are directed against a human microsomal protein which shares epitopes with human liver P-450 buf I and/or P-450 buf II. These two P-450 isozymes have an estimated apparent MW of 50 Kd (10). Both isozymes catalyse bufuralol hydroxylation and particularly P-450 buf I can metabolize about 20 other drugs including debrisoquine, sparteine, dextrometorphan, several β-adrenergic blocking drugs and tricyclic antidepressants. Cytochrome P-450 buf II has not been as extensively studied but has a lower affinity for most of these substrates.

The problem that remains to be explained is how a microsomal cytochrome P-450 may function as a target antigen for the immune response in chronic hepatitis. Evidence for the occurrence of circulating antibodies against a cytochrome P-450 has been previously reported (6,7). In this case, a drug

(tienilic acid) is known to be implicated in autoantibody production and it is speculated that the P-450 isozyme covalently modified by a drug metabolite, could migrate to the hepatocyte membrane surface and the modified protein could be recognized by the immune system as antigen. Although no drug is known to be implicated in chronic active hepatitis, the mechanism of production of anti-LKM1 and anti-LKM2 could be quite similar. An other hypothesis is that P-LKM1 and therefore P-450 buf I and/or P-450 bufII share sequence homology with a viral protein against which the antibody was initially directed (19). Obviously, much work is necesary to verify these hypotheses and to elucidate the mechanism of formation of anti-endoplasmic reticulum autoantibodies in chronic active hepatitis.

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