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Study of CYP2D6 gene in children with autoimmune hepatitis and P450 IID6 autoantibodies

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SUMMARY

Cytochrome P450 IID6 is an autoantigen recognized by the sera of children affected with a subtype of autoimmune hepatitis. It was hypothesized that a mutation in the CYP2D6 gene could explain the autoimmune response in these patients. To examine this question, genomic DNA from peripheral lymphocytes ($n=9$) and liver ($n=1$) of 10 patients with anti-LKM-1 antibody was analysed by Southern blot for genetic association studies between a particular CYP2D6 haplotype and autoimmune hepatitis. In addition, a region of CYP2D6, from the same genomic DNA, was amplified by polymerase chain reaction (PCR) and digested by *Bst*NI, in a search for the most prevalent 29B mutation, described in subjects who do not express the P450 IID6. Total RNA and proteins, prepared from the liver of an anti-LKM-1$^+$ patient, were analysed by Northern and Western (immunoblot) blots respectively. Our results do not reveal any major structural change in the DNA of this patient at the CYP2D6 locus that could explain their autoimmune response. Corroborating this observation, no changes were noted either in P450 IID6 mRNA size or in the corresponding protein. However, these data do not exclude the possibility of subtle changes in the protein due to point mutations in critical regions that might trigger an autoimmune response.

Keywords autoimmune hepatitis liver-kidney-microsomes antibodies P450 IID6 protein CYP 2D6 gene

INTRODUCTION

A subtype of autoimmune hepatitis, affecting mostly children, is characterized by the presence in the patients' sera of anti-liver kidney microsome antibody of type 1 (LKM-1). This autoantibody is directed against cytochrome P450 from the IID subfamily [1–4]. The human representative from the IID subfamily, the cytochrome P450 IID6, is involved in the metabolism of debrisoquine among other drugs; its gene is highly polymorphic and several mutations have been described, both in the coding and the non-coding region [5–7]. Five to ten per cent of individuals are poor metabolizers, indicating either that these individuals do not express the P450 IID6 protein or that this protein is not functional. The presence of a non-functional protein could be due to changes either in its structure or in its cellular location. Considering the high frequency of mutations in CYP2D6 (the gene coding for P450 IID6), one might speculate that a mutation in a critical region of the P450 IID6 gene could result in an alteration of the structure of the protein, with repercussions on its cellular location and on the generation of autoantibodies against this protein in patients with autoimmune hepatitis. It was recently shown, however, that all patients with autoimmune hepatitis and anti-LKM-1 antibody, so far tested, are extensive metabolizers, and that the P450 IID6 is present in their liver [8,9]. These results do not exclude the possibility of a mutation in one of the alleles, the other coding for a normal protein. We thus analysed the genomic DNA from patients with anti-LKM-1 antibody, using restriction enzymes known to be informative for several restriction fragment length polymorphisms (RFLP). DNA, RNA and proteins from a patient's liver were also analysed in a search for a gene polymorphism, for the presence for an abnormal RNA or of an abnormal protein. Our results, as reported here, exclude major structural changes in the gene and its products.

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PATIENTS AND METHODS

Patients

Fourteen Caucasian children with autoimmune hepatitis were studied. Sera were analysed using indirect immunofluorescence (Prof. J. C. Homberg, Hôpital St Antoine, Paris), immunoprecipitation [10] and immunoblot [11]. In 10 patients autoimmune hepatitis was associated with liver-kidney microsome antibody type 1 as detected by immunofluorescence; in nine of them

immunoblotting showed the presence of cytochrome P450 IID6 antibody and in another it showed anti-66-kD antibody [12]. Autoimmune hepatitis was associated with anti-smooth muscle antibody in two patients and two others had anti-cytosol antibody [10]. Five healthy unrelated adults were studied as controls.

Mononuclear cells preparation and EBV infection

Peripheral lymphocytes of the 14 children with autoimmune hepatitis and of the five controls were prepared from 10 ml of venous blood, drawn in heparinized tubes. Blood was diluted 1/4 with sterile phosphate-buffered saline (PBS) and 7 ml of the dilution loaded on 3 ml of Lymphoprep (Nycomed, Oslo, Norway) [13]. Tubes were centrifuged at 800 *g* for 20 min. The mononuclear cells at the interface were removed and washed twice with RPMI 1640 culture medium.

Mononuclear peripheral cells (1×10^7) were resuspended in 5 ml RPMI 1640 and mixed with 0·5 ml of B95-8 cell supernate (containing Epstein–Barr virus (EBV)) (kindly provided by Dr M. Masset, Hôpital St. Louis, Paris) [14]. Cyclosporin (Sandoz, Bale, Switzerland) was added, at a final concentration of 0·1 μg/ml, to inhibit T cell cytotoxicity.

DNA preparation

DNA preparation was carried out as described by Marcadet *et al.* [15]. Briefly, $1–3 \times 10^8$ infected B lymphocytes were resuspended in 2 ml of PBS, 13 ml of white cell lysis buffer (10 mM Tris-HCl pH 7·6, 10 mM EDTA, 50 mM NaCl, 0·2% SDS and 200 μg/ml proteinase K) was quickly added and then incubated overnight at 42°C under gentle agitation (50 osc/min in a rotatory shaker). This preparation was extracted twice with a mixture of 1 vol of phenol and 1 vol of chloroform-isoamyl alcohol (24/1). DNA was precipitated with 1 vol of cold 100% isopropanol.

Southern blot analysis

Each patient's DNA was digested with various endonucleases: *Bam*HI, *Hin*dIII, *Eco*RI, *Nco*I and *Xba*I (Boerhinger Mannheim, Meylan, France) following the manufacturer's recommendations. Cytochrome P450 IID6 gene is known to be polymorphic for these restriction enzyme sites [16]. Ten micrograms of digested DNA were separated on a 0·7% or 1% agarose gel, depending on the size range to be separated. Capillary transfer of DNA to Hybond N^+ filters (Amersham, Les Ulis, France) was made under neutral or alkaline conditions. These methods, as well as pre-hybridization and hybridization, were carried out according to McBride *et al.* [17] with minor modifications. Briefly, the composition of the pre-hybridization solution was: 1% SDS, 1 M NaCl, 50 mM Tris-HCl pH 7·5, 5× Denhardt's reagent and 100 μg/ml of denaturated and fragmented salmon sperm DNA. For the hybridization 7% of dextran sulphate and the labelled denatured probe were added to the pre-hybridization solution. The pre-hybridization and hybridization temperature was 65°C and time of hybridization was 16 h. The probe used for hybridization was the complete cDNA of the cytochrome P450 IID6 (kindly provided by Dr U. Meyer, Basel University, Basel, Switzerland), radioactively labelled by random priming with a multiprime DNA labelling systems kit (Amersham). Filters were washed, at 65°C, with 2×SSC (0·15 M NaCl and 0·015 M sodium citrate) containing 1% SDS. After washing, filters were exposed to Fuji X-ray film for autoradiography.

Liver DNA, RNA and protein preparation

The liver of a child with autoimmune hepatitis and end-stage liver cirrhosis was obtained during transplantation. DNA from peripheral lymphocytes was not obtained from this patient. The child's serum displayed LKM-1 antibody as studied by indirect immunofluorescence and anti-P450 IID6 antibodies as studied by ELISA and immunoblot analysis [3]. DNA from this liver was prepared as described by Davis *et al.* [18], and treated with the endonucleases following the manufacturer's instructions. Ten micrograms of each sample were separated on a 0·7% agarose gel for the sample treated with *Xba*I and on a 1% agarose gel for the samples treated with the other endonucleases. Capillary transfer of DNA to Hybond N^+ (Amersham) was carried out under alkaline conditions. Total liver RNA was prepared according to Puissant & Houdebine [19]. Fifteen micrograms of total liver RNA were separated on a 1% agarose-formaldehyde gel. Transfers were made as described by Sambrook *et al.* [20]. Pre-hybridization and hybridization for Southern and Northern blots were made according to McBride *et al.* [17].

Pieces from a normal human liver obtained from a brain-dead donor after reduction for transplantation, as well as the patient's liver, were homogenized and 20 μg of the homogenate proteins from each liver were separated by PAGE (in 0·1% SDS using a running gel of 10%) and processed for immunoblot [21], together with 20 μg of rat liver microsomes [11]. A patient's serum positive for LKM-1 antibody was used as first antibody at a final dilution of 1/1000. The second antibody was a peroxidase-conjugated anti-human IgG rabbit immunoglobulin (Biosys, Compiègne, France) used at a final dilution of 1/1000. The nitrocellulose strips were developed with 3,3′-diaminobenzidine (0·5 mg/ml) in 50 mM Tris HCl pH 7·4 and 0·01% H_2O_2.

*Study of a point mutation on the 29B/*Xbal *band*

The search for the already described 29B mutation [22] was made on DNA samples from the 10 LKM-1^+ patients (nine DNA samples from peripheral lymphocytes and one from patient's liver). Specific polymerase chain reaction (PCR) amplification was performed using Taq DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, CT) and two primers: (i) (TGCCGCCTTCGCCAACCACT) position 1824–1853 from the CAP site; and (ii) (TCGCCCTGCAGAGACTCCTC) position 2113–2132 from the CAP site. The amplified DNA was digested with *Bst*NI endonuclease (Bio-Labs, Beverly, MA) and the fragments obtained were separated on a 7% polyacrylamide gel. The presence of the 29B mutation was demonstrated by loss of the *Bst*NI restriction site [23,24].

RESULTS

Southern blot analysis

Genomic DNA from peripheral mononuclear cells of the 14 children with autoimmune hepatitis was treated with endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Nco*I and *Xba*I) and analysed by Southern blot, together with five control DNAs giving a known profile with the endonucleases used in this experiment. Nine of 14 patients with autoimmune hepatitis had in their sera LKM-1 antibody, as detected by immunofluorescence, that

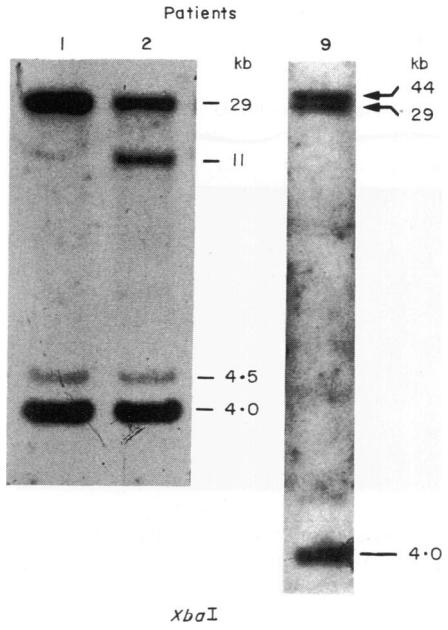

Fig. 1. Southern blot analysis of patients' DNA treated with endonuclease *Xba*I. Three restriction fragment length polymorphisms (RFLPs) generated by *Xba*I were obtained with DNA from nine LKM-1$^+$ children (anti-P450 IID6). Pattern obtained with genomic DNA of patients 1, 2 and 9 are shown.

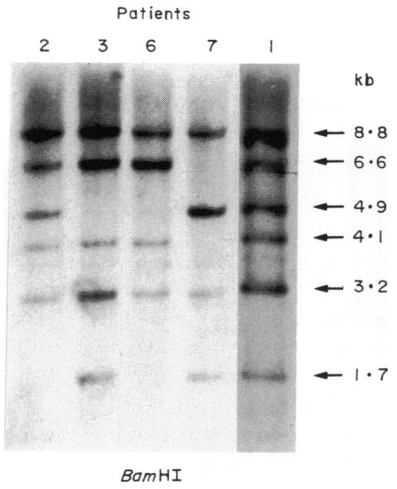

Fig. 2. Southern blot analysis of patients' DNA treated with endonuclease *Bam*HI. Five different patterns of restriction fragment length polymorphism (RFLP) were obtained by digestion with *Bam*HI of DNA from nine children positive for P450 IID6 antibody. Genomic DNA from children 2, 3, 6, 7 and 1 is shown.

recognized P450 IID6 protein in immunoblots (patients 1–9). When *Xba*I endonuclease was used, all nine patients showed bands at 29 kb and 4·0 kb. Additional bands were observed in three different patterns: 4·5 kb (patients 1, 3, 4, 5 and 8); 11 kb and 4·5 kb (patients 2, 6 and 7); 44 kb (patient 9) (Fig. 1). *Bam*HI endonuclease treatment showed in all patients bands at 8·8 kb and 3·2 kb. Additional bands were observed in five different patterns: 6·6 kb, 4·9 kb and 4·1 kb (patient 2); 6·6 kb, 4·1 kb and 1·7 kb (patient 3); 6·6 kb and 4·1 kb (patients 4 and 6); 4·9 kb and 1·7 kb (patients 5 and 7) and 6·6 kb, 4·9 kb, 4·1 kb and 1·7 kb (patients 1, 8 and 9) (Fig. 2). Southern blot analysis of *Nco*I endonuclease-restricted DNAs showed three patterns of bands: 9·9 kb, 8·0 kb, 4·6 kb and 1·6 kb (patient 1), 9·9 kb, 8·0 kb, 6·3 kb and 4·6 kb (patients 4, 6, 8 and 9) and 9·9 kb, 8·0 kb and 6·3 kb (patients 2, 3, 5 and 7) (Fig. 3). No patient displayed the 12·5 kb band with *Nco*I, which is associated to the poor metabolizer phenotype [16]. The same band pattern was observed with all DNAs when endonucleases *Eco*RI and *Hin*dIII were used (results not shown). All restriction profiles were compared with patterns obtained with DNAs from patients with autoimmune hepatitis negative for LKM-1 antibody, from controls, as well as from published results concerning the general population. No difference could be found between children positive for LKM-1 antibody and other subjects for the endonucleases tested. This RFLP showed that patients 2, 6, 7 and 9 are heterozygotes.

Analysis of the liver of a LKM-1$^+$ patient

DNA analysis by the Southern blot technique, after treatment with the same endonucleases, showed patterns identical to those already found in mononuclear cells from the other nine patients (Fig. 4) and in the literature [16].

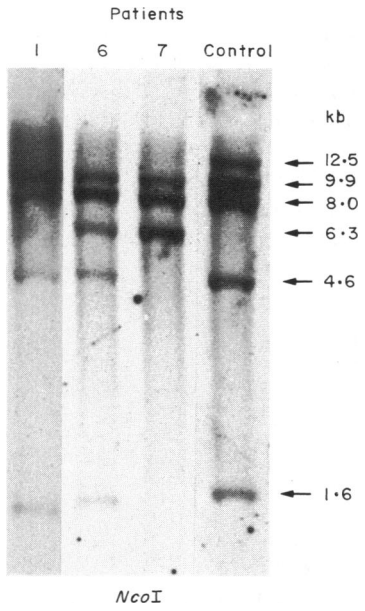

Fig. 3. Southern blot analysis of patients' DNA treated with endonuclease *Nco*I. Three different restriction patterns were obtained with nine patients' DNA treated with endonuclease *Nco*I. Patterns of children 1, 6 and 7 are shown. Control DNA digested with *Nco*I was obtained from a patient with autoimmune hepatitis and anti-cytosol antibody.

Northern blot analysis of total liver RNA showed a band at 1·8 kb, corresponding to the expected size of P450 IID6 mRNA (Fig. 5).

Taking into account that equal amounts of normal and patient total liver homogenate proteins were loaded in each respective line, the patient's liver appears to contain relatively larger amounts of the 48-kD protein (Fig. 5).

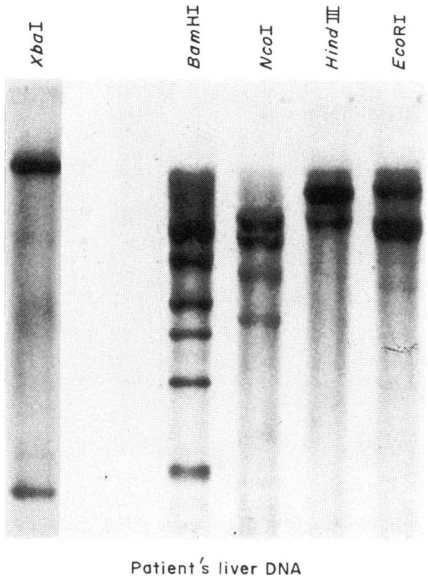

Fig. 4. Southern blot analysis of the patient's liver DNA. DNA extracted from the liver of a child positive for LKM-1 antibody was digested with the following restriction enzymes: *Xba*I, *Bam*HI, *Nco*I, *Hin*dIII and *Eco*RI. Fragments of DNA hybridizing with a P450 IID6 probe were the following, for each enzyme: *Xba*I 29 kb and 4·0 kb; *Bam*HI 8·8 kb, 6·6 kb, 4·9 kb, 4·1 kb, 3·2 kb and 1·7 kb; *Nco*I 9·9 kb, 8·0 kb, 6·3 kb and 4·6 kb; *Hin*dIII 14 kb and 10·5 kb and *Eco*RI 18 kb, 10 kb and 9 kb.

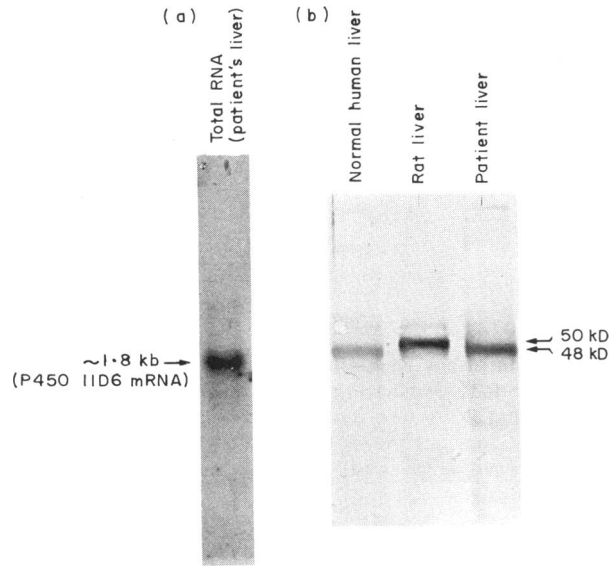

Fig. 5. Northern blot and immunoblot of total RNA and proteins from the liver of a child positive for LKM-1 antibody (a) Northern blot analysis of total liver RNA using a P450 IID6 probe. (b) Immunoblot analysis of proteins from rat liver microsomes, normal human liver and the patient's liver homogenates. The 50-kD band corresponds to rat P450s from the IID subfamily, the 48-kD band corresponds to human P450 IID6.

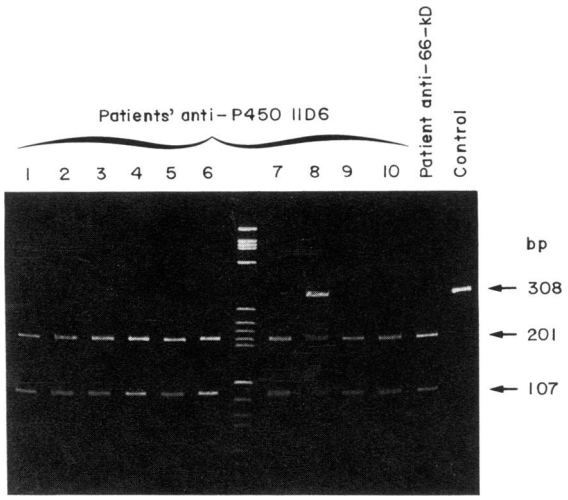

Fig. 6. Polymerase chain reaction (PCR) amplification and *Bst*NI digestion of DNAs from 10 anti-LKM-1$^+$ patients. Control is an undigested amplified DNA fragment of 308 bp. Fragments of 201 and 107 bp are the result of *Bst*NI digestion.

Detection of the 29B mutation

The study of DNAs from 10 children with LKM-1 antibody showed that only one had the 29B mutation (patient 8) in heterozygous state (Fig. 6).

DISCUSSION

While it is now well established that a subgroup of patients with autoimmune hepatitis is characterized by the presence in their serum of autoantibodies directed against cytochrome P450 IID6, the possible role of this antigen–antibody system in the pathogenesis of the liver disease has not been elucidated. Two points are a matter of debate: (i) how would the P450 IID6 molecule become antigenic, and (ii) how would the antigenic P450 IID6 become expressed on the outer part of the hepatocyte cell membrane in order to be the target for a cytolytic immune reaction? The results we report here, concerning the first point, indicate that, under the conditions of this study, the patients' genomic DNA exhibit neither an association with a particular DNA haplotype nor gross structural changes in the CYP2D6 locus as compared with normal subjects and with patients with other types of autoimmune hepatitis. However, these results do not exclude completely the possibility of mutations in a critical region of the protein which modifies its cellular location, triggering the autoimmune response in such patients. Most of the point mutations so far studied in CYP2D6 are associated with the absence of P450 IID6 expression or with the expression of a non-functional protein. One such mutation was described by sequencing a cDNA clone from livers of individuals with more than one mRNA band as detected by Northern blot [5]. This study showed that transcripts from mutated genes can be present in the liver. Translation products from these mRNA were not detected, possibly because the protein was unstable and rapidly degraded. This is why we studied the total RNA prepared from the liver of a patient with LKM-1 antibody looking for the presence of more than one mRNA or for a possible change in the size of the mRNA. No differences were

found when compared with RNAs from normal liver. Immunoblot studies of the patient's liver proteins with another patient's serum showed the presence of only one protein at the expected molecular weight.

In the absence of size abnormality of both mRNA and protein and major structural rearrangements in the gene, we conclude that if ever the CYP2D6 gene is involved in autoimmune response in these patients, it could be only through subtle changes such as microdeletion/insertion or point substitutions which warrant sequencing of the gene.

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