



Cytochrome P450IA2 and aromatic L-amino acid decarboxylase are hepatic autoantigens in autoimmune polyendocrine syndrome type I

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Abstract Autoimmune chronic active hepatitis (AI-CAH) is a feared component of autoimmune polyendocrine syndrome type I (APS I). In this study, immunoreactivity was assessed in sera from eight APS I patients, of whom three had AI-CAH, in an attempt to identify hepatic autoantigens. We performed indirect immunofluorescence staining of human and rat liver sections, Western blots on subcellular fractions of human and rat liver, immunoprecipitations of labelled aromatic L-amino acid decarboxylase (AADC) and cytochrome P450IA2 (CYP IA2) expressed by an *in vitro* transcription and translation system and studies of enzymatic activity. Autoantibodies against AADC were present in sera from all eight APS I patients, while immunoreactivity against CYP IA2 was only found in sera from the three APS I patients with AI-CAH. Enzymatic activity of CYP IA2 was inhibited by sera from APS I patients with AI-CAH but not by control sera. Our results show that CYP IA2 and AADC constitute hepatic autoantigens in patients with APS I and that immunoreactivity against CYP IA2 is associated with the presence of AI-CAH.

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Key words: Autoimmune polyendocrine syndrome type I; Autoimmune chronic active hepatitis; Hepatocyte; Cytochrome P450IA2; Aromatic L-amino acid decarboxylase; Autoantigen

1. Introduction

Autoimmune chronic active hepatitis (AI-CAH) occurs in 11–12% of patients with autoimmune polyendocrine syndrome type I (APS I) [1–3]. Autoantibodies are important diagnostic markers for AI-CAH and at least three subgroups can be distinguished serologically [4,5]. One of these subgroups, AI-CAH type 2, is associated with specific liver–kidney microsomal (LKM) antibodies. Target autoantigens for LKM antibodies have been identified as cytochrome P450 enzymes, the most common being cytochrome P450 IID6 (CYP IID6) [6]. LKM antibodies against cytochrome P450 IIC9 are associated with drug-induced hepatitis [7]. In a case of AI-CAH associated with alopecia, vitiligo and nail dystrophy, LKM antibodies reacting against cytochrome P450IA2 (CYP IA2) were found [8,9]. Although not diagnosed as having APS I,

the child seems to have this syndrome on the clinical description. CYP IA2 is a liver-specific enzyme, located in perivenous hepatocytes [10], and is involved in drug metabolism such as *O*-de-ethylation of phenacetin [11,12].

APS I, also called autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), is characterised by mucocutaneous candidiasis, organ-specific autoimmunity and ectodermal manifestations [1,2]. It is inherited in an autosomal recessive manner. Both cellular and humoral autoimmunity are probably involved in the pathogenesis of this disorder, and the underlying genetic defect, the nature of which is unknown, has been mapped to chromosome 21 [13]. Common endocrine features of this syndrome are hypoparathyroidism, adrenocortical insufficiency, gonadal failure and, more rarely, diabetes mellitus, hypothyroidism and parietal cell atrophy. Additional non-endocrine manifestations are mucocutaneous candidiasis, nail dystrophy, alopecia, vitiligo, intestinal malabsorption and, one of the most serious, autoimmune chronic active hepatitis [1,2]. APS I patients display autoantibodies against several of the organs affected and some of these autoantigens have been identified. The major autoantigen in the adrenal cortex and gonads is P450_{sec} (side-chain cleavage enzyme) [14,15]. In the pancreatic β-cells glutamic acid decarboxylase (GAD) [16,17] and aromatic L-amino acid decarboxylase (AADC) [18] have been identified as autoantigens. AADC is an enzyme known to be involved in the biosynthesis of catecholamine and indolamine neurotransmitters [19]. Besides being present in nervous tissue, AADC is found in large amounts in neuroendocrine cells, the liver and the kidney [20]. Its function in the liver is largely unknown.

Identification of the liver autoantigens in APS I would provide a basis to develop antibody assays to monitor APS I patients with AI-CAH or at risk of developing this disease. Using immunofluorescence staining of tissue sections, Western blots of subcellular fractions and immunoprecipitation assays based on radioactively labelled recombinant proteins, we here describe two liver enzymes, CYP IA2 and AADC, recognised by autoantibodies in APS I patients.

2. Materials and methods

2.1. Subjects

Sera from eight patients with APS I were investigated. All patients have previously been reported on [3,14,15]. Chronic active hepatitis (CAH), as verified by liver biopsy, was present in three of these patients. At the time of our investigations all three patients were having immunosuppressive treatment in the form of azathioprine (25 mg/day), and two of the patients were also receiving synthetic steroids (patient #1: prednisolone 10 mg/day and cortisone 25 mg/day; patient #3: prednisone 5 mg/2 days and cortisone 5 mg/2 days). Further

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediamine-tetracetic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, trishydroxymethylamine methane

clinical details are given in Table 1. Sera from 10 healthy blood donors were used as controls.

2.2. Antibodies

Rabbit anti-rat CYP IA2 and CYP IID6 peptide antibodies were kindly provided by Professor A. Rane. A specific rabbit serum against AADC was a kind gift from Dr. Krieger and was also purchased from Biogenesis (Poole, Dorset, UK). Fluorescein-conjugated and alkaline phosphatase-conjugated species-specific anti-IgG immunoglobulins were obtained from Dakopatts (Glostrup, Denmark).

2.3. Indirect immunofluorescence

Indirect immunofluorescence staining (IF) of unfixed frozen sections of human and rat liver was performed as previously described [21]. All sections were scored positive or negative according to the presence of cytoplasmic IF staining in a blinded fashion by two independent investigators. Randomly selected blood donors and a slide incubated with PBS and secondary antibody were used as negative controls, while specific antisera against CYP IA2 and AADC were used as positive controls.

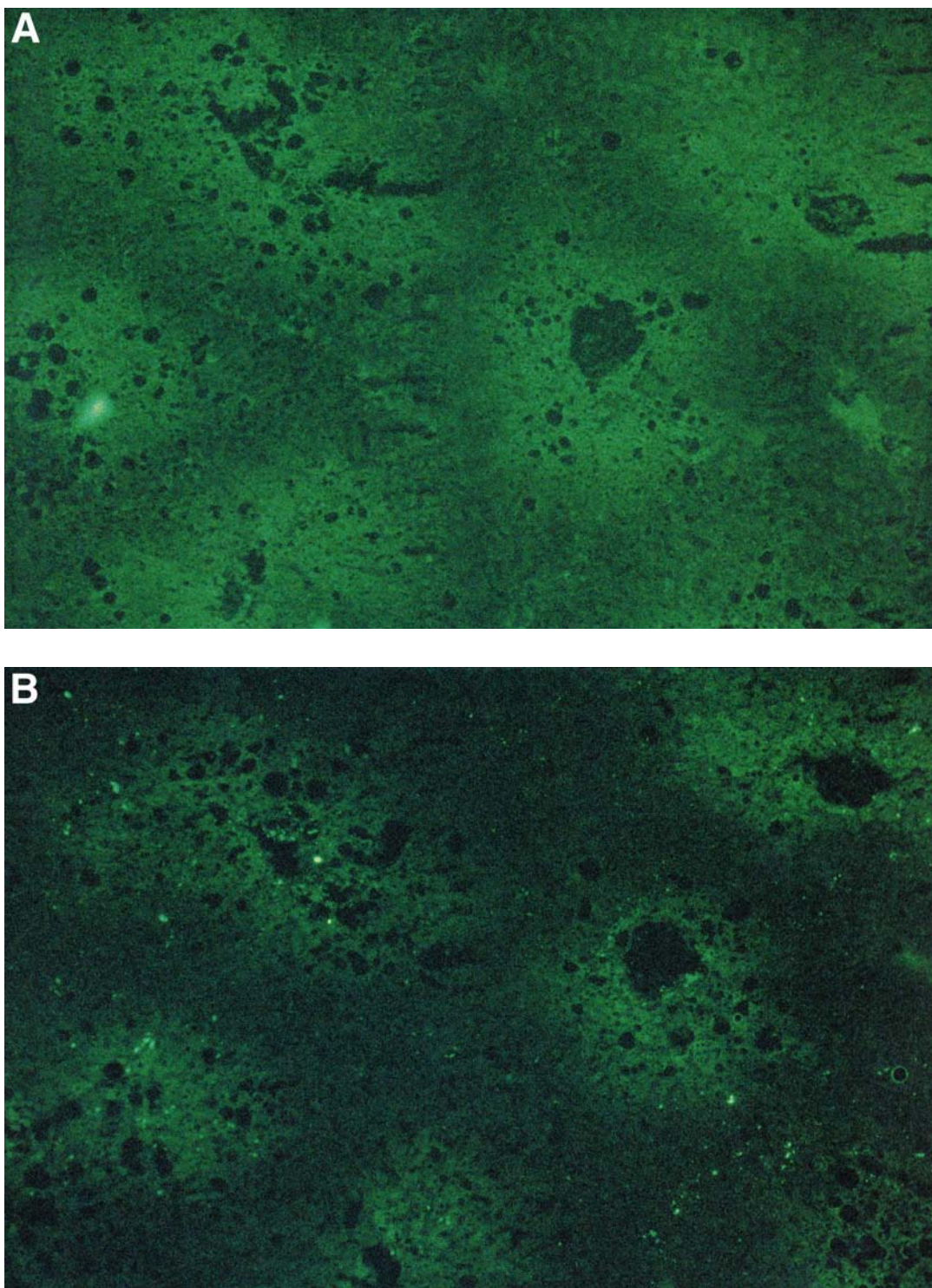


Fig. 1. Indirect immunofluorescence analysis of rat liver sections using specific antisera and patient sera. A: Anti-CYP IA2; B: APS I patient #3; C: healthy control.

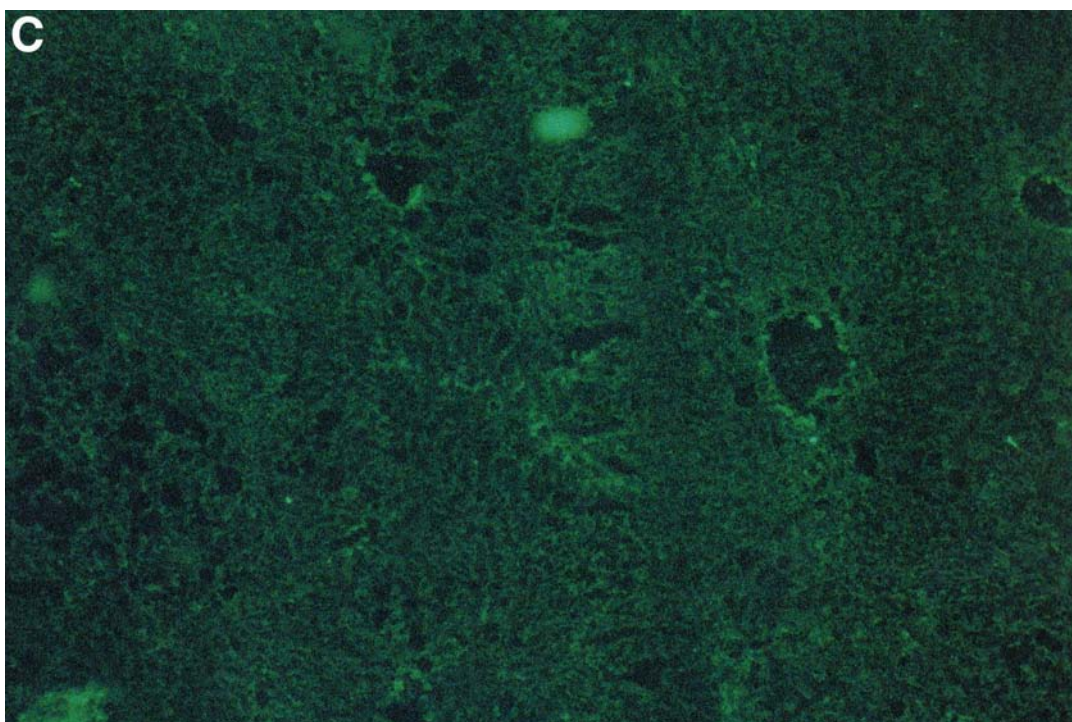


Fig. 1 (continued).

2.4. Preparation of antigen, SDS-PAGE and Western blot

Human liver was obtained from a healthy organ donor. The tissue was removed within 30 min of death and immediately put in liquid nitrogen. Human and rat liver homogenates were prepared by homogenisation in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA and 1 mM PMSF. Cytosol and microsomal fractions were prepared by differential centrifugation as described by Axen et al. [22]. SDS-PAGE [23] and Western blots [14] were

performed as described. Samples of antigen fractions were quantified for their total protein concentration using the *bis*-cinchonic acid (BCA) protein assay method (Pierce Chemical Company, Rockford, IL).

2.5. Synthesis of cytochrome P450 IA2 and AADC by *in vitro* transcription and translation

The full-length CYP IA2 cDNA clone (American Type Culture Collection, Rockville, MD) and the full-length rat cDNA clone encod-

Table 1
Clinical characteristics and experimental results in the eight APS I patients studied

	Patients							
	1	2	3	4	5	6	7	8
Endocrine								
Adrenal insufficiency	+	+	+	+	—	+	+	+
Hypoparathyroidism	—	+	—	+	+	+	+	+
Gonadal insufficiency	—	+	—	—	—	—	+	—
Hypothyroidism	—	—	+	—	—	+	—	—
Non-endocrine								
Candidiasis	+	+	+	+	+	+	+	+
Hepatitis	+	—	+	—	—	—	—	+
Alopecia	+	+	—	—	—	+	—	—
Vitiligo	—	+	—	—	—	+	—	—
Malabsorption	—	—	+	+	+	—	+	+
Investigations								
Liver IF ^a	+	+	+	+	NT	+	+	+
Western blot								
CYP IA2	+	—	+	—	—	—	—	+
AADC	—	+	+	+	+	+	+	+
Immunoprecipitation								
CYP IA2	+	—	+	—	—	—	—	+
AADC	+	+	+	+	+	+	+	+
Enzyme inhibition ^b								
CYP IA2	+	NT	+	NT	NT	NT	NT	+

^aImmunofluorescence as described under Section 2.

^bIn % of control activity (without serum).

+/- denotes presence or absence of the specified manifestation or reactivity. NT, not tested.

ing AADC [18] were used for in vitro transcription and translation using the TNT T7 and T3 coupled reticulocyte lysate system according to the manufacturer's recommendation (Promega, Madison, WI). The [35 S]methionine radiolabelled products were used for immunoprecipitation of patient sera as described below.

2.6. Assay of antibodies against CYP IA2 and AADC

[35 S]Methionine radiolabelled CYP IA2 or AADC (5×10^5 – 1×10^6 cpm/well) and patient sera (dilution 1:10) were incubated overnight at 4°C in buffer A, in a total volume of 50 μ l, containing 150 mM NaCl, 20 mM Tris-HCl, 0.02% NaN₃, 0.1% (w/v) BSA and 0.15% (v/v) Tween-20, pH 8.0 (buffer A). The next day 50 μ l of a 50% (v/v) slurry of protein A–sepharose (Pharmacia, Stockholm, Sweden) was added to each well of a microtitre plate (MABV N12, Millipore Corporation, Bedford, MA). The mixtures of sera and CYP IA2 or AADC respectively were then transferred to the wells, and the plate was shaken on a rotating platform for 45 min at 4°C. The plate was then washed using a vacuum manifold (Millipore) as follows: three washes with buffer A (200 μ l in each well) followed by the addition of 200 μ l buffer A to each well and rotation on the platform for 5 min at 4°C. This cycle was then repeated once, followed by two additional washes with 200 μ l of buffer A. Radioactivity was subsequently counted in a 1450 Micro Beta Trilux (Wallac, Turku, Finland). The results were expressed as a CYP IA2 or AADC index

$$(\text{cpm}_{\text{sample}} - \text{cpm}_{\text{negative control}} / \text{cpm}_{\text{positive control}} - \text{cpm}_{\text{negative control}}) \times 1000$$

A CYP IA2 index of 400 and an AADC index of 200 were chosen as upper normal limits, dividing the cohort of APS I patients into those with elevated indices and those with normal and slightly elevated

indices relative to the healthy controls (controls_{CYP IA2}: -20 ± 360 and controls_{AADC}: 10 ± 70 , mean ± 2 SD). Samples and controls were all analysed in duplicate.

2.7. Enzyme inhibition studies

Inhibition by patient sera of CYP IA2-specific 7-methoxyresorufin *O*-demethylase (MROD) activity was assayed using human liver microsomes from a non-smoking organ donor as the enzyme source [25,26]. Incubations contained 0.66 mg microsomal protein, 1.25 μ M 7-methoxyresorufin dissolved in DMSO (Research Diagnostics Inc., NJ), 50 μ M NADPH and 20 μ l of serum in a total volume of 2 ml with 0.1 M sodium phosphate buffer pH 7.4. Prior to addition of NADPH, the assay mixture was incubated at 37°C for 5 min. The production of resorufin was followed by direct fluorimetry ($\lambda_{\text{ex}} = 535$ nm, $\lambda_{\text{em}} = 585$ nm) on a Perkin-Elmer LS-5 Spectrofluorimeter, and the amount of resorufin produced was quantitated using the addition of a small amount of resorufin (Fluka) as an internal standard in each reaction mixture.

3. Results

3.1. Indirect immunofluorescence

All eight APS I sera stained rat and human liver sections to various degrees with the indirect immunofluorescence method. The sera stained the cytoplasm of hepatocytes, predominantly those located perivenously (Fig. 1A). The same pattern was observed with a specific anti-CYP IA2 antiserum (Fig. 1B).

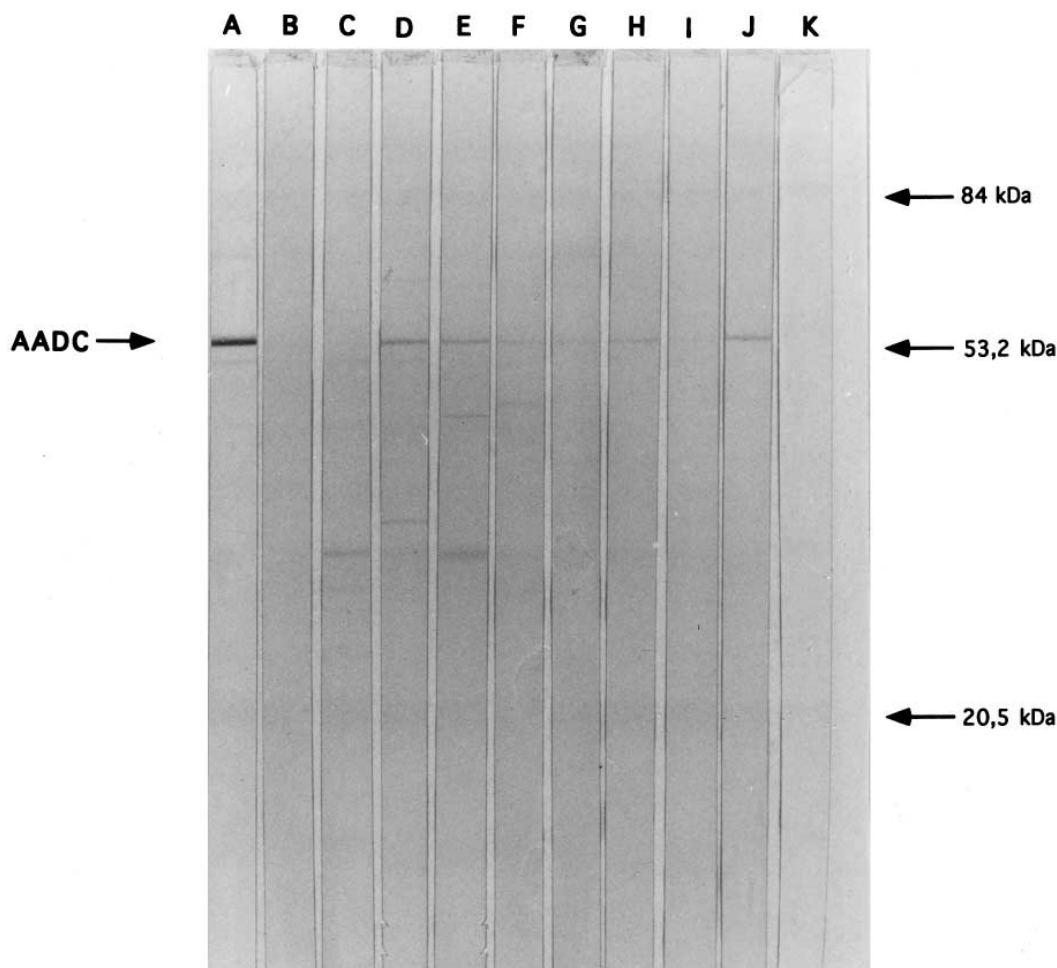


Fig. 2. Western blot of a human liver cytosolic fraction, 30 μ g of total protein/lane, with APS I patient and control sera. Patient sera were incubated at a dilution of 1:100. Lane A: anti-AADC; lane B: anti-CYP IA2; lanes C–J: sera from APS I patients (#1, 6, 2, 7, 8, 3, 4 and 5, respectively); lane K: serum from a healthy control. Arrows to the right indicate the mobilities of prestained molecular weight standards.

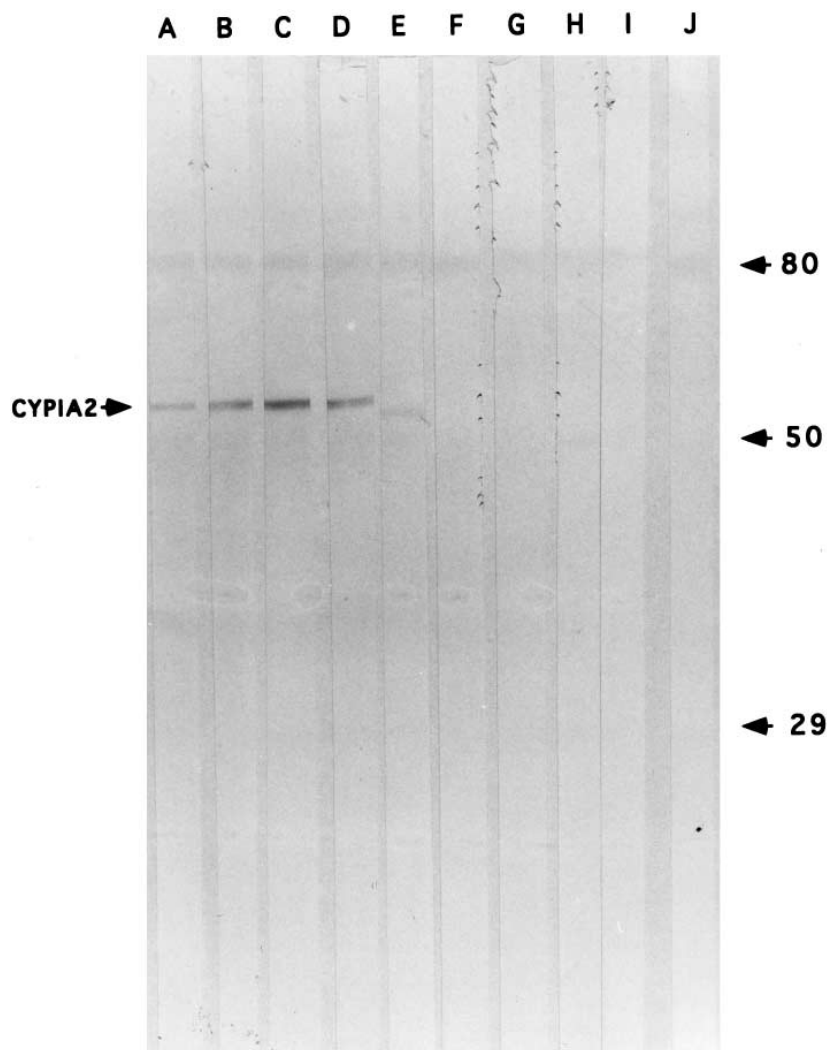


Fig. 3. Western blot of a human liver microsomal fraction, 30 μ g of total protein/lane, with APS I patient and control sera. Patient sera were incubated at a dilution of 1:100. Lane A: anti-CYP IA2; lanes B–I: sera from APS I patients (#1, 3, 8, 2, 4, 5, 6 and 7, respectively); lane J: serum from a healthy control. Arrows to the right indicate the mobilities of prestained molecular weight standards.

Incubation with patient sera containing LKM1 autoantibodies, as well with a specific rabbit antiserum against CYP IID6, resulted in homogeneous cytoplasmic staining of the whole liver lobule (not shown), clearly distinguishable from the pattern obtained with anti-CYP IA2. Incubation with a specific antiserum against AADC yielded a very weak staining of a majority of liver cells, difficult to distinguish from the staining pattern obtained with control sera (data not shown), results in concordance with previous reports [24]. None of the 10 sera from healthy blood donors produced any liver staining (Fig. 1C).

3.2. Western blot analysis of liver fractions

When the reactivities of the eight APS I sera against liver cytosol were analysed, it was found that seven of the eight sera reacted with an antigen with an apparent molecular weight (MW) of approximately 55 kDa corresponding in mobility to AADC (Fig. 2). When the same analysis was performed using liver microsomes, sera from the three patients with AI-CAH reacted with an antigen with the apparent MW of approximately 60 kDa co-migrating with that identified by specific CYP IA2 antisera (Fig. 3). Reactivity with a band

corresponding to AADC was also seen in the microsomal fraction. A specific CYP IID6 antiserum (LKM1) identified a band in the microsomal fraction migrating with an apparent MW of 52 kDa clearly distinguishable from those identified by specific antibodies against CYP IA2, AADC and sera from APS I patients (not shown). No bands in the 50–60 kDa region were identified by sera from 10 healthy blood donors (Figs. 2 and 3).

3.3. *In vitro* transcription and translation of CYP IA2 and AADC

About 0.3% of the [35 S]methionine became incorporated into the CYP IA2 protein. SDS-PAGE analysis of this protein yielded two bands with apparent MW of about 40 and 33 kDa respectively (not shown). Both bands were immunoprecipitated by the specific rabbit anti-CYP IA2 antibody as well as by sera from APS I patients known to contain antibodies against CYP IA2. In the case of AADC, regularly about 5% of the [35 S]methionine became incorporated into the protein and SDS-PAGE analysis of this protein yielded a band corresponding to the full-length AADC molecule (not shown).

3.4. Autoantibodies against CYP IA2 and AADC analysed by immunoprecipitation of the *in vitro* translated proteins

All eight sera contained autoantibodies against AADC, with an AADC index of above 200, while sera from the three patients with AI-CAH contained autoantibodies against CYP IA2, with a CYP IA2 index of above 400 (Fig. 4). No immunoreactivity against CYP IA2 or AADC was recognised by sera from 10 healthy blood donors (Fig. 4).

3.5. Inhibition of CYP IA2 enzyme activity

CYP IA2-mediated MROD activity [25,26] in human liver microsomes was inhibited to 40–50% by two of the three patient sera tested, and to 95% of control activity (no serum added) by the third patient serum. Normal sera from blood donors produced no inhibitory effects (Tables 1 and 2).

4. Discussion

Autoimmune hepatitis is the most feared component of APS I and, together with hypoparathyroidism and the consequences of intestinal malabsorption, requires continuous monitoring. We demonstrate here that CYP IA2 and AADC are major autoantigens in liver tissue, and tests for the presence of autoantibodies against these antigens may have a role in the clinical evaluation of APS I patients.

Subcellular fractions of liver homogenates were used to identify the autoantigens. In the cytoplasmic fraction, reactivity against a protein with mobility identical to that of AADC was found, and in the microsomal fraction, containing endoplasmic reticulum and the Golgi apparatus, a band with mobility matching that of CYP IA2 was detected. In parallel, the reactivity of an LKM-1 standard serum was tested. This serum displayed reactivity against a protein with a mobility that was different from that of both AADC and CYP IA2. Immunofluorescence staining of liver sections revealed a similar pattern for patient sera and anti-CYP IA2 sera, a pattern which was clearly different from that of LKM-1 antibodies, with a typical concentration of staining around central veins, where according to *in situ* hybridization studies CYP IA2 is located [10]. Radioligand assays based on *in vitro* transcribed/translated proteins confirmed these results and provides an easy and reliable assay for AADC and CYP IA2 autoantibodies. The *in vitro* transcription/translation product of CYP IA2 had a shorter length than expected, which implies that a small part of the protein is missing. A perfect correlation was obtained between the results of the radioligand and Western blot assays for both CYP IA2 and AADC.

A 40–95% inhibition of the CYP1A2-specific 7-methoxyre-

Table 2
CYP IA2 enzyme inhibition assay

	Activity ^a (pmol/min/mg protein)	% Remaining activity
Control ^b	12.2	100 ^c
Patient 1	6.9	57
Patient 3	0.6	5
Patient 8	5.7	47
Blood donor 1	12.2	100
Blood donor 2	11.3	93

^aCYP IA2-specific 7-methoxyresorufin *O*-demethylase activity as described in Section 2.

^bIncubation mixture without serum as described in Section 2.

^c100% = activity without serum added.

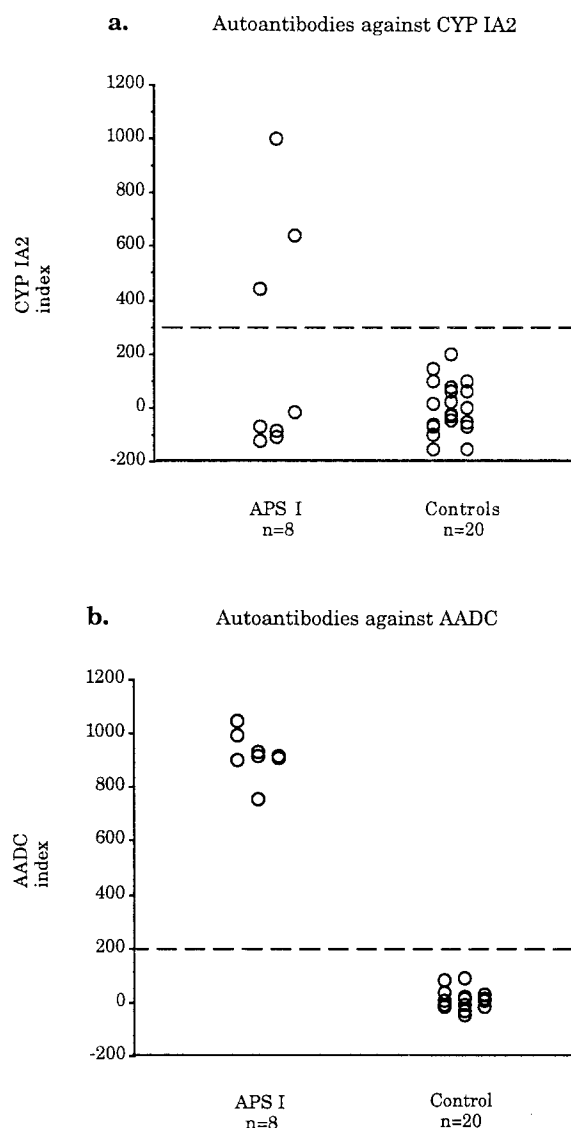


Fig. 4. Autoantibodies against CYP IA2 (a) and AADC (b) in APS I patients and controls measured as CYP IA2 and AADC indices. Immunoprecipitations and calculation of indices were performed as described in Section 2. The horizontal line indicates the upper normal limit, CYP IA2 index 400 (a) and AADC index 200 (b).

sorufin *O*-demethylase (MROD) activity was observed with sera from the three patients suffering from AI-CAH, demonstrating specific inhibitory effects of the CYP1A2 antibodies found in these sera. Due to a rather low specific activity of MROD in the human liver microsomes used as enzyme source, the amount of microsomal protein in each incubation was high, and therefore the serum/microsome ratio was lower than optimal. A stronger inhibitory effect would therefore have been anticipated if more patient serum was available for these analyses. This was reflected in a preparatory trial where increasing the amount of one of the sera from 10 to 20 μ l increased the inhibitory effect from 30 to 43%.

Autoimmune chronic active hepatitis not associated with APS I is a poorly defined entity. Some forms of AI-CAH may represent a component of a systemic disease, e.g. systemic lupus erythematosus (SLE) with anti-nuclear antibodies (ANA). Other forms of AI-CAH may represent viral diseases

for which no specific diagnostic tests are available, although this possibility seems less likely today. The association of AI-CAH with other autoimmune manifestations such as thyroiditis and Sjögren's syndrome, and its association with HLA-B8 [27], clearly suggest an underlying immune reaction.

Cytochromes have previously been identified as autoantigens in AI-CAH and other autoimmune endocrine disorders. In the uncommon type 2 autoimmune hepatitis, which mainly affects children, LKM-1 antibodies recognising CYP IID6 [6] are found. Furthermore, in thienylic acid-induced hepatitis, antibodies against cytochrome P450 IIC9 [7] have been demonstrated. In APS I, the cytochrome P450 cholesterol side-chain cleavage enzyme (SCC) has previously been identified as an adrenal and gonadal autoantigen, and cytochrome P450 21-hydroxylase is the major adrenal autoantigen in Addison's disease. On the basis of a previously reported case of AI-CAH with concomitantly decreased 25-hydroxylation of vitamin D in an APS I patient [28], we previously speculated that a vitamin D 25-hydroxylase could be a candidate as a hepatic autoantigen [14]. However, no results from experiments performed as described in this report have corroborated this theory.

In type 1 autoimmune hepatitis, ANA are found in about half of the patients. No specific nuclear staining patterns are seen and the nuclear antigens have not been defined. Non-organ-specific antibodies against smooth muscle and sometimes against mitochondria are also detected. In this larger group of AI-CAH patients, no specific diagnostic tests are available. It would be of interest to identify subgroups, perhaps with different prognoses and treatment requirements, with more specific tests.

In conclusion, measurements of CYP IA2 and AADC antibodies may be used both to monitor APS I patients with AI-CAH and to identify those at risk of developing this disease. The value of CYP IA2 or AADC antibody testing in isolated AI-CAH remains to be elucidated.

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