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A new antigen recognized by anti-liver-kidney-microsome antibody (LKMA)

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SUMMARY

Sera from 23 children with autoimmune chronic active hepatitis and positive for anti-liver-kidney-microsome antibody (LKMA), as defined by immunofluorescence, were analysed by Western blot (WB) and two-dimensional gel electrophoresis using rat liver microsomes as antigen, and by WB and dot-blot analysis with rat liver microsomal subfractions. Western blot analysis showed three patterns of reactivity: 13 sera recognized a 50 kD polypeptide, six sera a 66 kD polypeptide and four sera both of them. Two-dimensional gel electrophoresis, WB, and dot-blot analysis showed the 66 kD antigen to have a pI of 5·4 and to be located in the smooth domain of the endoplasmic reticulum. Western blot analysis using monospecific antisera against human IgG subclasses showed the LKMA directed against the 66 kD antigen to be mainly of the IgG1 subclass. These results indicate that LKMA associated with a subgroup of autoimmune hepatitis of children react with at least two different microsomal antigens in rat liver: (1) the 50 kD polypeptide, recently shown to be a cytochrome P-450 of the IID subfamily, and (2) a new antigen of 66 kD, the location of which suggests it may also be part of the mono-oxygenase complex.

Keywords anti-liver-kidney-microsome antibody anti-66 kD antibody 66 kD Mr antigen

INTRODUCTION

In children two subgroups of autoimmune hepatitis have been described on the basis of serological and clinical features (Odièvre *et al.*, 1983), depending on the presence in patients' sera of high titres of either anti-smooth muscle antibody (SMA), or anti-liver-kidney microsome antibody (LKMA). The latter is found mainly in very young patients and is frequently associated with extrahepatic manifestations (Maggiore *et al.*, 1986).

By immunofluorescence microscopy, LKMA appears as a cytoplasmic fluorescence evenly distributed in the rat liver lobule and in the kidney cortex, chiefly in the P3 region (Rizzetto, Swana & Doniach, 1973). In rat liver LKMA was shown to react with a 50 kD unglycosylated integral membrane protein of the endoplasmic reticulum (ER) (Alvarez *et al.*, 1985). When analysed on dot-blots without denaturation (Waxman *et al.*, 1988), LKMA cross-reacts with two methylcholantrene-inducible isozymes of cytochrome P450, and it was shown recently that the 50 kD protein is a cytochrome P450 from the IID subfamily (Gueguen *et al.*, 1988).

Using immunoblot techniques, we now show that some of the LKMA-positive sera recognize a 66 kD microsomal protein.

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MATERIALS AND METHODS

Animals

Female rats of the Wistar strain weighing 180–200 g were fasted for 18 h with water ad libitum, before being killed by decapitation.

Sera

Sera were collected before any treatment, or during a relapse, from 23 children with chronic active hepatitis (22 girls and 1 boy), identified as having LKMA (titres ranging from 1:100 to > 1:100 000) by immunofluorescence. These sera were negative for anti-smooth muscle antibodies and anti-nuclear antibody. All sera were stored at −20°C until use. Normal human sera and sera from SMA-positive patients were used as control.

Subcellular fractionation

Rat livers were homogenized in 250 mM sucrose/3 mM imidazole-HCl as described by Amar-Costesec *et al.* (1974). A nuclear fraction and a post-nuclear supernatant were obtained by differential centrifugation. The post-nuclear supernatant was further fractionated into mitochondrial, light mitochondrial, microsomal and cytosolic fractions (Beaufay & Amar-Costesec, 1976).

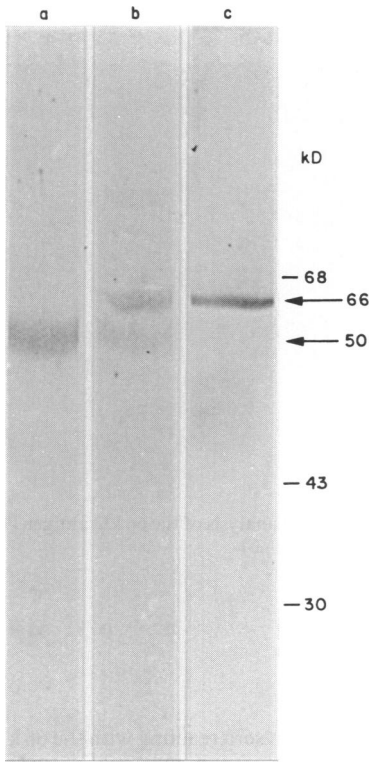

Fig. 1. Different patterns of reactivity of LKMA-positive sera on Western blots. Immunoblot analysis was done with LKMA-positive sera from three different children. An LKMA-positive serum recognized a 50 kD protein (a), another recognized 50 kD and 66 kD proteins (b), and the other a 66 kD protein (c).

Microsomes were subfractionated by isopycnic centrifugation in a linear sucrose gradient (Beaufay *et al.*, 1974). At the end of the run 14 subfractions of about equal volume (3·5 ml) were collected. The subfractions were weighed and their density was measured as described by Beaufay & Amar-Costesec (1976). Rat liver plasma membranes were purified as described by Dorling & Le Page (1973).

Biochemical determinations

NADPH cytochrome c reductase and glucose 6-phosphatase were assayed as described by Beaufay *et al.* (1974). Protein was determined by Lowry's method with bovine serum albumin as standard.

Western blot (WB) analysis

Aliquots (40 μg protein) of the primary rat liver fractions and microsomal gradient subfractions were subjected to SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel. Electroblotting on nitrocellulose paper (Schleicher & Schuell, Dassel, FRG) was made in a Biometra Fast Blot apparatus (ScienceTec, Les Ulis, France). Immunoblot technique was done essentially as described by Towbin, Staehelin & Gordon (1979). All children's sera were used at 1:100 dilution as first antibody. The second antibody was a peroxidase-conjugated $F(ab')_2$ fragment of goat anti-human IgG (Biosys, Compiègne, France) at a final dilution of 1:1 000. 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.

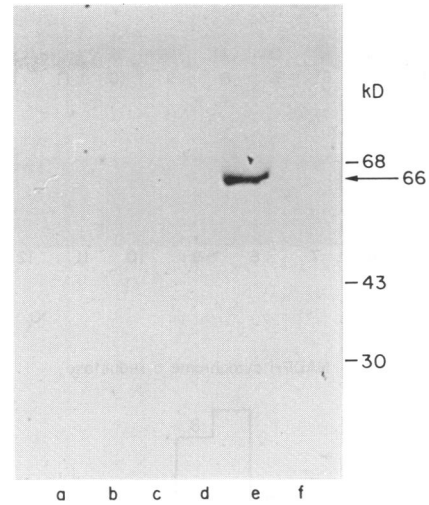

Fig. 2. The 66 kD LKMA antigen is only present in the rat liver microsomal fraction. A strong positive reaction was obtained only in the microsomal fraction (e), very weak positivity was seen in the post nuclear supernatant (a), and this protein was absent from the other cell fractions (b nuclear, c mitochondrial, d light mitochondrial, f cytosol).

Analysis of IgG subclasses

IgG subclasses of LKMA reactivity were investigated in a three-step assay by WB analysis using murine monoclonal antibodies anti-human IgG subclasses (anti-IgG1 (Fc), anti-IgG2 (Fc), anti-IgG3 m(U), anti-IgG4 (Fc)) as second antibody, all provided by the WHO Collaborating Centre of Human Immunoglobulins, Center for Disease Control, Atlanta, USA. Subsequently the nitrocellulose strips were incubated with peroxidase-conjugated immunoglobulin fraction of rabbit anti-mouse-IgG (Byosis, Compiègne, France) at 1:1000 dilution, and developed as described above.

Dot-blot analysis

An aliquot (2 μg protein) from microsomal gradient subfractions was spotted on a nitrocellulose membrane using the hybridot microfiltration apparatus (Bethesda Research Laboratories, Gaithersburg, MD). The nitrocellulose paper was blocked with gelatin 0·02% in Phosphate Buffer Saline pH 7·4 and incubated with LKMA positive serum at a dilution of 1:100 as first antibody. The procedure was completed as described for WB technique.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was essentially done as described (Gorg, Gunther & Weser, 1985). The first dimension was performed in the 2117 Multiphor II Electroforesis Unit (LKB, Bromma, Sweden) using gels 0·50 mm thick. Gel solution consisted of 4% acrylamide/NN′-methylene bisacrylamide (22·2 g/l·4 g), 9 M urea and 2% Nonidet P-40 (NP-40), mixed with ampholines (LKB, Bromma, Sweden), pH 3·5–10 (1·5%) and pH 6–8 (0·5%). Aliquots (3 mg protein/ml) of the microsomal fraction were treated with RNA-ase (Boehringer Mannheim, Penzberg, FRG) as described by Kruppa & Sabatini (1977). After centrifugation the microsomal pellet was resuspended in 0·1 M phosphate buffer, pH 6·8, and 20% glycerol at a protein concentration of 10 mg/ml. The sample was prepared for electrophoresis by adding 2 vol of 10 M urea, 5% β-mercapto

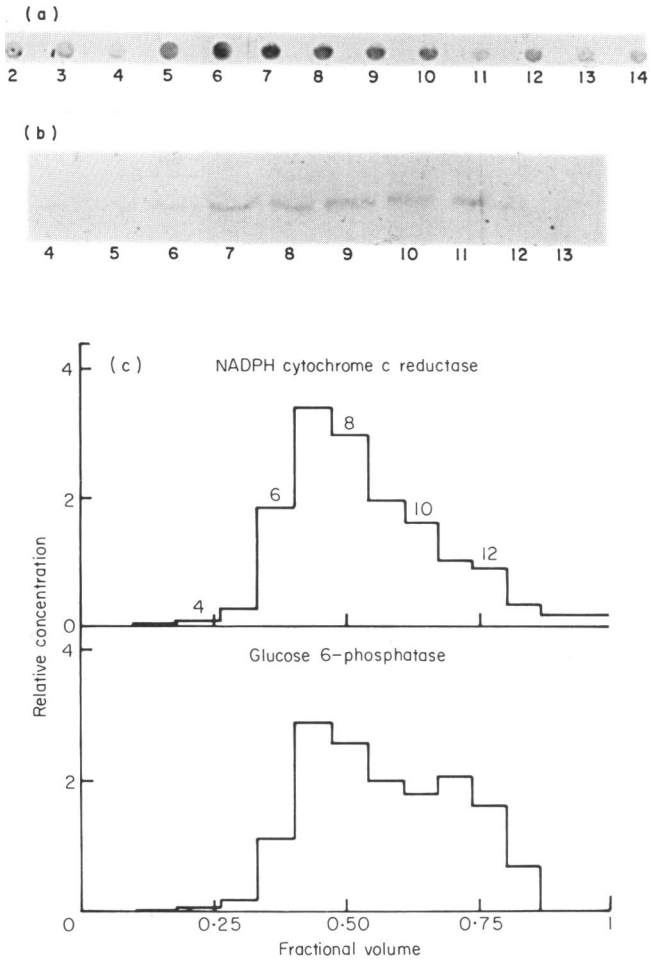

Fig. 3. Organelle localization of 66 kD protein. Dot-blot (a) and WB (b) show a strong positive reaction with subfractions 7 and 8. These fractions correspond with the higher relative activity of NADPH cytochrome c (P-450) reductase (c), indicating that the 66 kD polypeptide is a protein of the smooth domain of the ER.

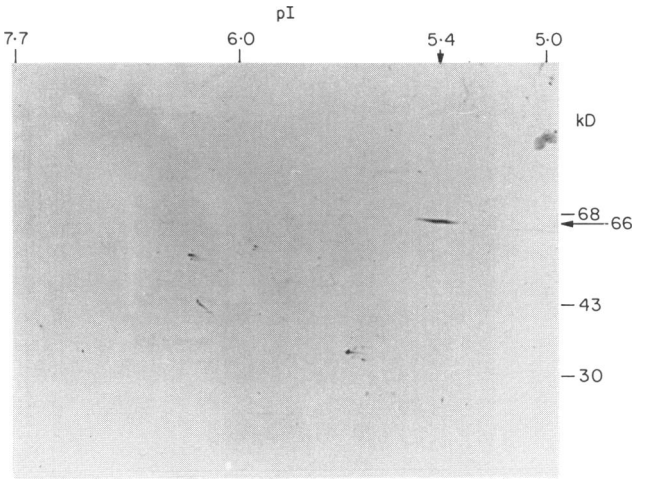

Fig. 4. Two-dimensional analysis of the 66 kD antigen. This antigen was localized at a pI of 5·4.

ethanol, 1·5% pH 3·5–10 ampholines, 0·5% pH 6–8 ampholines, and 2% NP-40. Thirty microlitres of this preparation were run in each line. The second dimension was done in a 10% SDS polyacrylamide gel, followed by WB analysis as described above.

The pH gradient in isoelectric focusing gels was measured by slicing a gel strip containing the same sample into 1 cm fragments and placing them into test tubes with 1 ml of deionized water. After equilibration for 12 h the pH was measured.

RESULTS

Sera positive for LKMA in immunofluorescence, obtained from 23 patients, were analysed by WB, using rat liver microsomes as antigen (Fig. 1). Three patterns of reactivity were distinguished: 13 patients recognized the 50 kD polypeptide already described (Fig. 1, lane a), six patients a 66 kD polypeptide (lane c), and four patients recognized both polypeptides (lane b). These patterns were compared with LKMA titres obtained by immunofluorescence. Patient sera reacting with the 66 kD polypeptide alone had a lower titre (mean 1:600, range 1:100 to 1:2 000) than those reacting with either the 50 kD or with both polypeptides (mean 1:30 000, range 1:1 000 to >1:100 000).

In order to establish the subcellular location of the 66 kD antigen, we tested primary rat liver fractions and microsomal subfractions by immunoblot analysis using sera containing only anti-66 kD protein antibodies. Microsomes showed a strong band of apparent Mr 66 000 (Fig. 2). As expected, the band was also apparent in the post-nuclear supernatant, although to a lesser extent. Other fractions were negative.

Further information was provided by dot-blot and WB analysis of subfractions obtained by isopycnic centrifugation of microsomes on a sucrose gradient. Figure 3(c) shows the density distribution profiles of NADPH cytochrome c reductase and glucose 6-phosphatase, two markers of the b (smooth) and c (luminal) domains of the ER (Amar-Costesec & Beaufay, 1981). The pattern of staining for the anti-66 kD positive serum resembles the distribution pattern of the reductase, with a peak of intensity in subfractions 7 and 8 (densities 1·143 and 1·159, respectively, Fig. 3a, b).

In addition to ER- derived vesicles, microsomes also contain membranes originating from the plasma membranes and Golgi complex. Markers for these two compartments also peak at a density of 1·16. To establish more firmly the association of the 66 kD antigen with the smooth domain of the ER, purified plasma membranes were tested for their reactivity to positive sera. No staining was observed (results not shown).

The isoelectric point (pI) of the 66 kD Mr antigen was shown by two-dimensional gel electrophoresis to be 5·4 (Fig. 4). It has been demonstrated by immunofluorescence that LKMA belongs to the IgG1 subclass (Peakman *et al.*, 1987). Using WB, it was found that the anti-50 kD (data not shown) and anti-66 kD polypeptide antibodies belong to the IgG1 subclass. A very light reactivity was also observed with anti-IgG3 subclass antibody (Fig. 5).

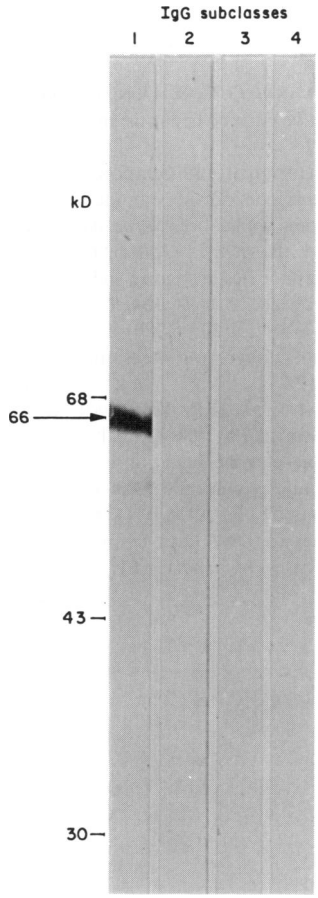

Fig. 5. IgG subclasses of anti-66 kD Mr autoantibody. Lane 1: anti-IgG1 (Fc); lane 2: anti-IgG2 (Fc); lane 3: anti-IgG3 m(U); lane 4: anti-IgG4 (Fc). A strong positive reaction was found with anti-human IgG1 antibody (lane 1).

DISCUSSION

The results reported here indicate that the reactivity of children's sera positive for LKMA, as studied by immunofluorescence, is heterogeneous. Within the limits of the technique used, some sera recognized the rat liver microsomal 50 kD Mr polypeptide, now known as a cytochrome P-450 of the IID subfamily (Gueguen *et al.*, 1988), while others recognized a newly identified antigen, either exclusively or in addition to the 50 kD protein. This antigen appears to be a 66 kD microsomal polypeptide with a pI of 5·4. After isopycnic centrifugation of microsomes, its density distribution is similar to that of the NADPH cytochrome c (P-450) reductase, a marker enzyme of the b (smooth) domain of the ER. Few rat microsomal proteins are located in this pI-Mr region (Vlasuk & Walz, 1980), and this might be an advantage for further procedures of isolation and characterization of the 66 kD polypeptide.

Previous work (Alvarez *et al.*, 1985) using similar techniques and sera from five patients, showed that LKMA was directed against a 50 kD microsomal protein. Testing of a larger number of LKMA-positive sera and using as antigen a double amount of total microsomal proteins (40 μg) allowed us to show this new reactivity with a 66 kD microsomal protein in some of the sera.

The location of the 66 kD antigen in the smooth domain of the ER and the immunofluorescence pattern obtained with the anti-66 kD positive sera are the same as those shown with anti-50 kD antibody. The fact that the 50 kD antigen is a cytochrome P-450, allows us to speculate that the 66 kD polypeptide may, in some way, be related to the mono-oxygenase system.

The nature of the anti-50 kD and anti-66 kD antibodies was studied using WB analysis. These antibodies are mainly of the IgG1 subclass. These results confirm previous studies using immunofluorescence (Peakman *et al.*, 1987). This restriction of autoantibodies to the IgG1 subclass has been reported previously in other autoimmune disorders such as lupus erythematosus (Zouali, Jefferis & Eyguen, 1984) and in islet cell antibody in insulin-dependent diabetics (Millward *et al.*, 1988). The IgG1 subclass is able to fix complement and could play a role in cell lysis during autoimmune hepatitis, provided that the antigen, or a fragment of it, is exposed at the surface of the hepatocyte. It was demonstrated that cytotoxic T lymphocytes can recognize intracellular antigens (Townsend *et al.*, 1986) and a hypothesis was proposed about an intracellular processing of the antigen followed by the exposure of part of it on the surface of the cell (Germain, 1986). Similar mechanisms could be evoked in the case of microsomal proteins.

These are, however, speculative hypotheses, because so far none of the immunological techniques usually used allowed us to conclude on the expression of ER membrane proteins at the surface of the cell.

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