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# Detection of liver–kidney microsomal autoantibodies by radioimmunoassay and their relation to anti-mitochondrial antibodies in inflammatory liver diseases

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## SUMMARY

A radioimmunoassay (RIA) was developed for the detection of liver–kidney microsomal (LKM) autoantibodies. These were detected in four of 62 patients with HBsAg negative chronic active hepatitis (CAH) and in one patient with mixed connective tissue disease (MCTD). LKM antibodies were not detected in other hepatic and non-hepatic diseases. Other autoantibodies, especially anti-mitochondrial ones, do not react in this assay system. Sera positive for LKM antibodies by RIA showed a cytoplasmic staining of hepatocytes and proximal renal tubules by immunofluorescence. The LKM antigen was detected by RIA in microsomes prepared from rat liver, kidney, stomach, heart, lung, and skeletal muscle. It was destroyed after treatment with trypsin and chymotrypsin, but preserved after treatment with RNAase, DNAase and neuraminidase. Upon centrifugation of purified rat liver microsomes in CsCl gradient, LKM reactivity was detected at a density of 1·20 g/ml. In addition, the M2 antigen of the inner mitochondrial membrane specific for primary biliary cirrhosis (PBC) was localized at 1·28 g/ml in these density gradient fractions. The LKM antigen could not be solubilized. The presence of LKM antibodies characterizes a distinct subgroup of HBsAg negative CAH; they do not occur in PBC.

**Keywords** chronic hepatitis liver–kidney microsomal autoantibodies radioimmunoassay autoimmunity

## INTRODUCTION

Chronic active hepatitis (CAH) is a heterogeneous syndrome with a varying aetiology. A determination of the various CAH subgroups is needed, since only some of them seem to benefit from immunosuppressive therapy (Schalm, 1982). In this respect autoantibodies may be useful as diagnostic markers. Anti-nuclear (ANA) and liver membrane antibodies (LMA) are typical for so called lupoid (autoimmune) CAH (Hopf, Meyer zum Büschenfelde & Arnold, 1976). In contrast, these autoantibodies are very rare in hepatitis B virus (HBV)-induced and non-A, non-B type CAH (Meyer zum Büschenfelde & Manns, 1984). Anti-mitochondrial antibodies (AMA), especially the M2 subtype, characterize cholestatic inflammatory liver diseases which can also exhibit the histological picture of CAH (Berg *et al.*, 1980; Manns & Meyer zum Büschenfelde, 1982). Yet

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another autoimmune phenomenon, the appearance of liver–kidney microsomal (LKM) antibodies, was reported in patients with CAH (Smith *et al.*, 1974). In this study we report the development of a specific radioimmunoassay (RIA) for LKM antibodies and describe their clinical relevance.

## MATERIALS AND METHODS

*Patients.* Sera from 114 patients with CAH were included in this study. The diagnosis of CAH was made according to the criteria of DeGroote *et al.* (1968). In 52 patients CAH was due to hepatitis B virus (HBV) infection. The 28 patients with non-A, non-B CAH had a history of blood transfusion or open heart surgery, and they were negative for all autoantibodies, as well as for HBV markers. The infections with herpes simplex, cytomegalo and Epstein–Barr viruses were excluded in these patients. Twenty-seven patients were classified as autoimmune (lupoid) CAH. Three patients with cholestatic inflammatory liver disease had CAH confirmed by liver biopsy and were positive for the M2 subtype of AMA. Four patients with HBsAg negative CAH were LKM antibody positive. These patients had no recent history of surgical intervention, anaesthesia or blood transfusions.

Patients with acute hepatitis consisted of 17 persons with type A, 46 with type B and 18 of them were diagnosed non-A, non-B hepatitis, 40 patients with primary biliary cirrhosis (PBC) had typical histological features comprising different stages of liver tissue destruction and were positive for the M2 subtype of AMA, 12 patients had extrahepatic cholestases of different origin, and 126 patients comprised the group with various types of chronic liver diseases. The latter included: hepatocellular carcinoma, liver metastases, fatty liver, alcohol-induced liver diseases, hemochromatosis and Wilson's disease. Another group of 50 patients with non-hepatic autoimmune diseases consisted of 17 patients with systemic lupus erythematosus (SLE), five patients with rheumatoid arthritis (RA), two with progressive sclerodermia, three with panarteriitis nodosa, 10 with Crohn's disease, two with ulcerative colitis, two with polymyalgia rheumatica, four with thyroiditis and five patients were classified as mixed connective tissue diseases (MCTD, Sharp syndrome).

Two sera were from patients with drug-induced pseudolupus erythematosus syndrome and four had secondary syphilis. Sera from 20 healthy blood donors were included in this study. All sera were heat-inactivated at 56°C for 30 min and stored at −20°C until used.

*Serological tests.* LKM antibodies, AMA, ANA, smooth muscle antibodies (SMA), and parietal cell antibodies (PCA) were detected in patients' sera by indirect immunofluorescence (IF) performed on cryostat sections from rat kidney, liver or stomach, respectively (Rizzetto, Swana & Doniach, 1973; Manns & Meyer zum Büschenfelde, 1982). The presence of AMA against M2 antigen was tested by RIA (Manns & Meyer zum Büschenfelde, 1982). Thyroglobulin antibodies (TGA) and thyroid microsomal antibodies (TMA) were detected by passive haemagglutination (Mastdiagnostica, Hamburg, Federal Republic of Germany); Cardiolipin antibodies were tested by the Venereal Disease Research Laboratory flocculation test for syphilis. Rheumatoid factor was determined by latex fixation test (Behringwerke, Marburg, Federal Republic of Germany). LMA were detected by IF performed on isolated rabbit hepatocytes (Hopf *et al.*, 1976). HBV markers (HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc) were determined by RIA (Abbott Laboratories, Chicago, Illinois, USA).

*Preparation of the microsomal antigen fraction.* The microsomal fraction from rat liver was prepared by several centrifugation steps as described elsewhere (Rizzetto *et al.*, 1973, Rizzetto, Bianchi & Doniach, 1974). Glucose-6-phosphatase activity testing, chosen as endoplasmic reticulum marker, and electron microscopy were used to examine the purity of microsomal fractions.

Aliquots of this preparation were used as microsomal antigen for the detection of LKM antibodies by RIA. Microsomal antigen fractions were also prepared from human, rabbit, and mouse livers, and from various rat organs to study the species and organ specificity of the LKM antigen.

*Solid phase radioimmunoassay for the detection of the LKM antigen–antibody system.* A reference

serum from a patient with HBsAg negative CAH with LKM antibodies detectable by IF, but negative for other autoantibodies, was used to establish the assay.

*Detection of the LKM antigen.* The gammaglobulin fraction of the reference serum was three times precipitated by 1/3 volume of saturated $(NH_4)_2SO_4$. The final precipitate was dialysed against 0·01 M phosphate-buffered saline (PBS), pH 7·5. Test tubes (M 174, Dyna Tech, Plochingen, Federal Republic of Germany) were coated with 75 μl of this gammaglobulin preparation, 0·3 mg/ml protein in PBS, for 4 h at 25°C. After washing with PBS, post-coating was performed with PBS containing 0·1% bovine serum albumin at 4°C overnight. Tubes were then washed three times, before 50 μl aliquots of the microsomal antigen were added; instead of antigen, PBS was taken as a control. After overnight incubation at 4°C and washing, 50 μl of $^{125}$I-labelled antibody (Hunter & Greenwood, 1962; Manns *et al.*, 1980) was added for 4 h at 25°C. Thereafter the tubes were washed five times and counted in a gammacounter. Results were expressed as positive/negative (P/N) ratios; values exceeding 2·1 were regarded as positive.

*Detection of LKM antibodies.* The blocking principle was employed for the detection of LKM antibodies in patients' sera. Fifty microlitres of rat liver microsomal antigen with a protein concentration of 0·6 mg/ml were used as standard antigen. Fifty microlitres of test serum, diluted 1:10 in PBS, were added for 4 h at 25°C to the test tubes before the addition of labelled antibody. Results are expressed as percentage inhibition. A test serum was regarded positive when it inhibited the reaction of reference serum with LKM antigen by more than 40%, as compared with a serum pool from healthy blood donors. To prove the reproducibility of the assay, sera from four patients with LKM antibodies as detected by RIA were assayed on five different days to obtain the inter-assay coefficient of variation. Five serum samples from these four LKM antibody positive sera were assayed within one assay procedure to calculate the intra-assay coefficient of variation.

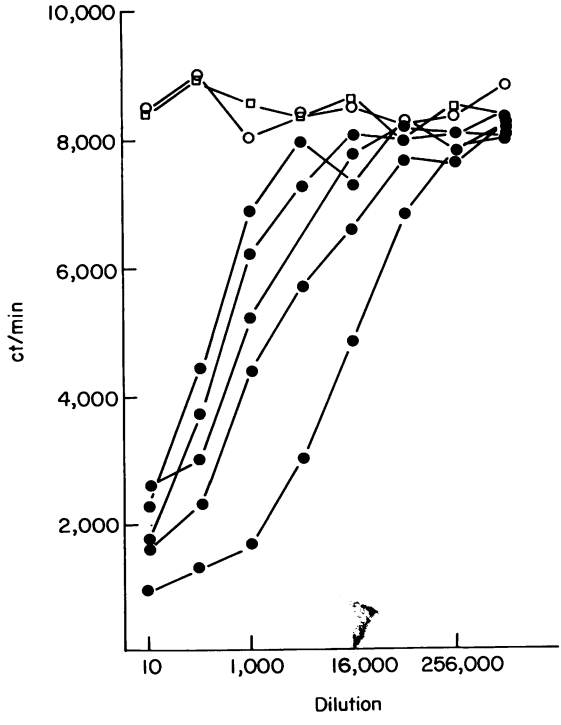


**Fig. 1.** Radioimmunoassay for the detection of LKM antibodies. Titration curves for five LKM antibody positive sera (●——●), an AMA positive serum (○——○), and a serum pool of healthy blood donors (□——□).

*Characterization of LKM antigen.* Rat liver microsomes were treated with trypsin (Serva Feinbiochemica, Heidelberg, Federal Republic of Germany), chymotrypsin (Sigma Chemical Co. St Louis, Missouri, USA), DNAase (Serva), RNAase (Serva) and neuraminidase (Behringwerke), according to Sayers, Binder & Berg, (1979). The microsomal antigen fraction was further treated with sodium dodecylsulphate (SDS) in 0·01, M sodium phosphate buffer, pH 7·5, at the concentrations of 2·0, 1·0, 0·5 and 0·1%. Equal volumes of microsomal antigen and SDS were incubated at 37°C for 2 h. After dialysis, SDS treated microsomes were tested for antigen activity by RIA. Control microsomes underwent the same incubation procedures, but were not exposed to SDS. Rat liver microsomal antigen fraction was also exposed to temperatures of 4°C, 37°C, 45°C, 56°C and 65°C for 5, 15, 30, and 60 min. In separate experiments the antigen preparation was incubated at pH 2, 4, 6, 8, 10 and 12 for 1 h at 37°C. One milligram of rat liver microsomal antigen preparation was submitted to ultracentrifugation in a CsCl density gradient as reported previously (Manns & Meyer zum Büschenfelde, 1982).

## RESULTS

*The assay*

In RIA the LKM reference serum reacted with the purified microsomal antigen fraction with positive/negative (P/N) ratios of 20–25. The autologous and all other LKM antibody positive sera blocked the reaction of reference serum with LKM antigen (Fig. 1). Sera positive for other autoantibodies did not react in this assay system. The assay proved to be reproducible with an inter-assay coefficient of variation from 2·9 to 11·9%, and an intra-assay coefficient of variation was ranging from 1·5 to 6·7%. On electron microscopy, the microsomal fraction containing LKM antigen was rich in membranes of the endoplasmic reticulum (Fig. 2). Glucose-6-phosphatase activity was eight-fold increased when compared with liver homogenate.

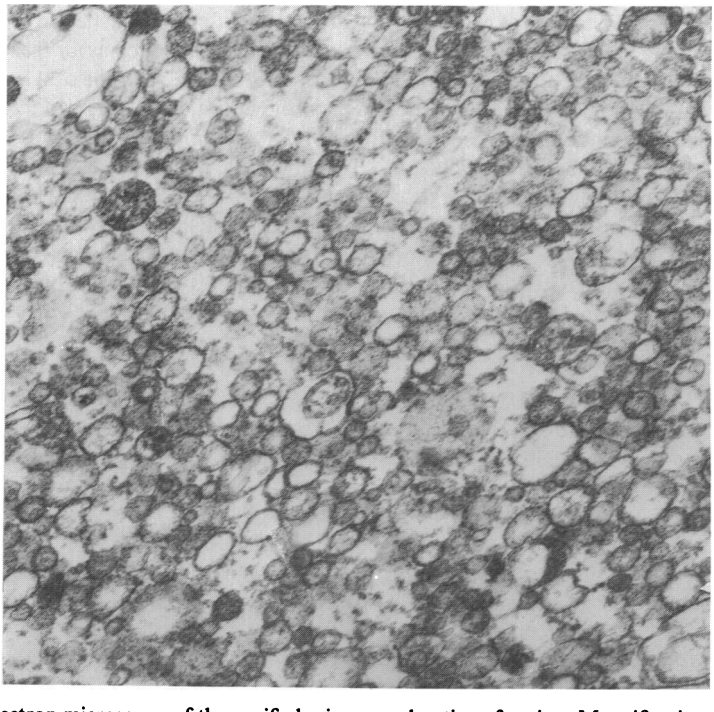

**Fig. 2.** Electron microscopy of the purified microsomal antigen fraction. Magnification × 29,900.

**Table 1.** Incidence of LKM antibodies and AMA in various hepatic and non-hepatic diseases

| Diagnosis | No. of patients | No. of patients positive for LKM | AMA | M2 subtype of AMA |
|---|---|---|---|---|
| CAH–autoimmune | 27 | 0 | 0 | 0 |
| CAH–LKM positive | 4 | 4 | 0 | 0 |
| CAH–cholestatic | 3 | 0 | 3 | 3 |
| CAH–HBsAg positive | 52 | 0 | 0 | 0 |
| CAH–non-A, non-B | 28 | 0 | 0 | 0 |
| Acute viral hepatitis | 81 | 0 | 0 | 0 |
| Primary biliary cirrhosis | 40 | 0 | 40 | 40 |
| Extrahepatic cholestasis | 12 | 0 | 0 | 0 |
| Miscellaneous liver diseases | 126 | 0 | 0 | 0 |
| Non-hepatic autoimmune diseases | 50 | 1 | 2 | 0 |
| Pseudolupus syndrome | 2 | 0 | 2 | 0 |
| Syphilis II | 4 | 0 | 2 | 0 |
| Healthy blood donors | 20 | 0 | 0 | 0 |

*Clinical significance of LKM antibodies detected by RIA and their relation to AMA*

LKM antibodies were detected by RIA and IF in four of 62 patients with HBsAg negative CAH and in one patient with MCTD (Tables 1 & 2). Titres for LKM antibodies by RIA with rat liver microsomes as antigen ranged from 1:100 to 1:16,000 as compared with IF titres between 1:20 and 1:240. No LKM antibodies were detected in other groups of patients nor in healthy controls (Table 1). These sera remained negative when human liver microsomes were used as antigen. Sera from all the patients were also tested for AMA by IF, and by RIA for the M2 subtype of AMA, AMA were detected by IF in 40 patients with PBC, three cases with cholestatic CAH, two patients with the drug-induced pseudolupus syndrome, two with secondary syphilis and two with SLE. The M2 subtype of AMA was only detected in patients with cholestatic inflammatory liver diseases (CAH and PBC). LKM antibodies could not be detected by RIA in cases positive for AMA, independent whether they were M2 antibody positive or negative, nor could the M2 subtype of AMA be detected in LKM antibody positive sera. Clinical, biochemical, and immunological data of LKM antibody positive patients are summarized in Table 2. All four patients with LKM antibody positive CAH were negative for other autoantibodies and they did not possess the HLA phenotype B8, their mean age at onset was $21{\cdot}0 \pm 5{\cdot}4$ years. In two of these patients the illness began as acute hepatitis progressing to CAH. No history of acute hepatitis was reported in the other two patients. The patient with LKM antibodies and MCTD was ANA positive. Liver biopsy from this patient showed non-specific activation of Kupffer cells and an increased iron storage. There were no histological signs for acute or chronic hepatitis. The disease started in this patient at the age of 52 years.

*Characterization of LKM antigen by RIA*

LKM antigen was found to be non-species specific; it was detected in the microsomal antigen fraction prepared from human, rat, rabbit, and mouse livers. Furthermore, LKM antigen is non-organ specific and it was found in the microsomal fraction prepared from rat liver, kidney, stomach, heart, lung, and skeletal muscle (Fig. 3). The antigen is thermolabile, and its activity was destroyed after incubation at 56°C for 30 min. Antigen activity was preserved at pH 7 and 8, while incubation at pH 2, 4, 10, and 12 (37°C) markedly diminished its activity (Table 3). Treatment of the microsomal antigen fraction with different enzymes revealed that LKM antigen is sensitive to

**Table 2.** Clinical, biochemical and immunological data of patients with LKM autoantibodies

| | Patient No. | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Diagnosis | CAH | CAH | CAH | CAH | MCTD |
| Liver biopsy | CAH | CAH | CAH | CAH* | non-specific changes |
| Age of onset | 23 | 24 | 13 | 24 | 52 |
| Sex | F | F | F | F | F |
| IF | 120 | 240 | 240 | 60 | 20 |
| LKM (titre) RIA | 12,500 | 16,000 | 16,000 | 2,500 | 2,500 |
| ANA | − | − | − | − | + |
| TMA | − | + | − | − | − |
| SMA, LMA, AMA, PCA, TGA, RF | − | − | − | − | − |
| HBsAg | − | − | − | − | − |
| AP (units/litre) NR 60–180 | 514 | 220 | 170 | 214 | 160 |
| Bilirubin (mg/dl) NR 0·6–1·0 | 0·6 | 7·5 | 1·1 | 1·0 | 0·8 |
| SGOT (units/litre) NR 5–18 | 1,029 | 365 | 65 | 52 | 20 |
| IgG (mg/dl) NR 800–1800 | 1,191 | 1,251 | 2,100 | 1,250 | 960 |
| IgM (mg/dl) NR 90–350 | 232 | 330 | 68 | 314 | 251 |
| IgA (mg/dl) NR 100–450 | 171 | 152 | 50 | 244 | 128 |

IF = immunofluorescence; RIA = radioimmunoassay; ANA = antinuclear antibodies; SMA = smooth muscle antibodies; LMA = liver membrane autoantibodies; AMA = anti-mitochondrial antibodies; PCA = parietal cell antibodies; TGA = thyroglobulin antibodies; TMA = thyroid microsomal antibodies; RF = rheumatoid factor; HBsAg = hepatitis B virus surface antigen; AP = alcaline phosphatase; NR = normal range; SGOT = serum glutamic oxalo-acetic transaminase.

* This patient already had signs of liver cirrhosis in addition to CAH.

**Table 3.**

(a) Enzymatic degradation of LKM antigen by various enzymes

| *Control* | *Trypsin* | *Chymotrypsin* | *DNAase* | *RNAase* | *Neuraminidase* |
|---|---|---|---|---|---|
| 13·0±3.5* | 1·0±0·3 | 1·0±0·2· | 13·2±3·6 | 12·9±2·5 | 14·6±3·9 |

(b) Influence of different pH on LKM antigen activity

| *2* | *4* | *6* | *7* | *8* | *10* | *12* |
|---|---|---|---|---|---|---|
| 1·2±0·2 | 1·1±0·2 | 2·8±1·9 | 9·8±2·9 | 10·8±4·7 | 1·1±0·4 | 0·9±0·1 |

* LKM antigen activity was measured by RIA, results are given as positive/negative ratios; mean±s.d., $n=3$.

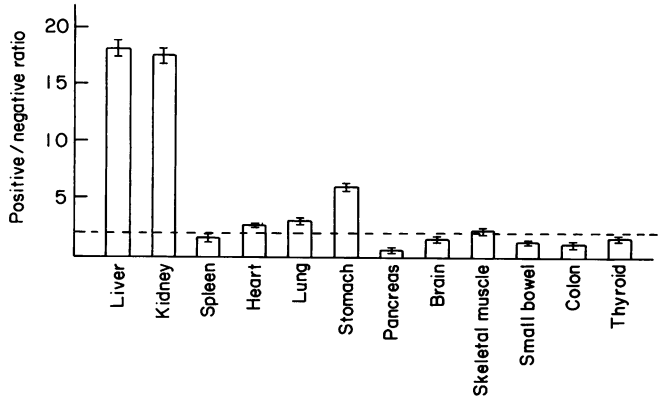


**Fig. 3.** Detection of the LKM antigen by RIA in the microsomal antigen fraction prepared from different rat organs. Protein concentrations = 5 mg/ml. Mean ± s.d., $n = 3$. - - - - = upper limit of normal range.

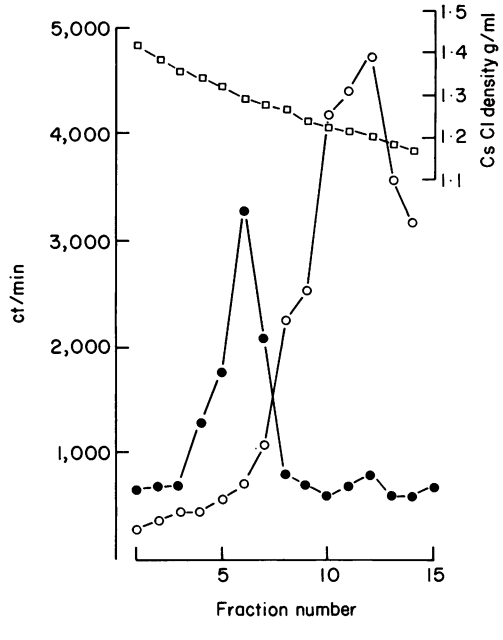


**Fig. 4.** Detection of LKM antigen (○——○) and the mitochondrial PBC specific M2 antigen (●——●) in a CsCl density gradient (□——□) of rat liver microsomes by solid phase RIA.

treatment with trypsin and chymotrypsin, but insensitive to treatment with DNAase, RNAase and neuraminidase (Table 3). The LKM antigen was further destroyed after incubation with SDS at concentrations between 0·1 and 2·0%. In CsCl density gradients of purified rat liver microsomes the antigen was localized at 1·20 g/ml (Fig. 4). These density gradient fractions were also tested for PBC specific M2 antigen by RIA. The M2 antigen was localized at 1·28 g/ml (Fig. 4).

## DISCUSSION

The RIA described in this paper specifically detects LKM antibodies, and the presence of other proteins in antigen preparation does not hamper the specificity of this test system. This is

particularly useful in the detection of antibodies against the LKM antigen which could not be dissociated from the membranes of the endoplasmic reticulum in an immunologically active form. It is difficult to decide by IF whether LKM antibodies occur in addition to AMA in sera from patients with PBC. Using RIA techniques we did not observe a serological overlap between LKM antibody positive CAH and PBC. The reaction of 25% of PBC sera with purified microsomes, as reported by Smith *et al.* (1974), was presumably due to a contamination of microsomes with mitochondrial proteins. A contamination of our microsomal antigen preparation with mitochondrial M2 antigen was proved. Recently, Crivelli *et al.* (1983) described microsomal autoantibodies in a proportion of HBsAg carriers with chronic delta infection. The immunofluorescence staining pattern on kidney tissues was similar to that of LKM antibodies. In contrast to LKM antibodies delta infection associated microsomal autoantibodies (DMA) reacted much stronger with human and chimpanzee than with rabbit and rat tissues. Furthermore, DMA had a wider organ specificity and the reaction of DMA could not be blocked by LKM antibody positive sera and vice versa. This indicates that both autoantibody specificities are immunologically distinct. The RIA described detects LKM antibodies by the blocking principle. Therefore it is unlikely that DMA interfere with the detection of LKM antibodies in this test system. However, the test principle of the RIA described may be used to establish a serological test system for the detection of DMA.

LKM antibodies were found to be rarely present (Smith *et al.*, 1974; Storch, Cossel & Dargel, 1977). Our patients with LKM antibody positive CAH were all young females and were negative for ANA, SMA, and HLA B8. LKM antibodies seem to characterize a subgroup of CAH which differs serologically and clinically from HBV-induced CAH, non-A, non-B CAH and lupoid (autoimmune) CAH. This is in agreement with observations reported by Smith *et al.* (1974) and Odièvre *et al.* (1983). Odièvre *et al.* (1983) could demonstrate that this autoantibody is more frequent in children than in adults.

The characteristics of LKM antigen as determined by RIA are equivalent with those obtained by other techniques apart from its organ specificity (Rizzetto *et al.*, 1973, 1974). By IF LKM antigen can only be detected in liver and kidney tissues, by RIA it is also detectable in microsomes prepared from stomach, heart, lung and skeletal muscle. Thus, LKM antigen is not as organ specific as has been suggested previously (Rizzetto *et al.*, 1974; Smith *et al.*, 1974). However, the reactivity of LKM antibodies with other organs is much less than with liver and kidney microsomes; the reactivity with skeletal muscle microsomes is very weak. The RIA described in this paper may be useful in future studies to further define the antigenic specificities related to LKM autoantibody reactions.

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