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A new anti-liver–kidney microsome antibody (anti-LKM2) in tienilic acid-induced hepatitis

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SUMMARY

The sera of 131 patients with anti-liver–kidney microsome antibodies (anti-LKM) detected between 1973 and 1979 in two different laboratories were re-examined. (1) Eighty-six anti-LKM corresponded to the description given by Rizzetto, Swana & Doniach (1973), with a pattern of fluorescence predominating on the 3rd portion of the proximal tubules (P_3). This group comprised 45 cases of idiopathic chronic hepatitis or idiopathic cirrhosis and one case of halothane-induced hepatitis. (2) Forty-five anti-LKM gave a different pattern on male mouse liver and male rat kidney: (a) fluorescence was greater on centrolobular than on periportal hepatocytes; (b) the first and second portions of proximal tubules (P_1 and P_2) predominated over P_3; (c) P_1 fluorescence was equally intense as P_2 and (d) P_3 cells were heterogeneous with one cell out of 20 more positive than the rest. Absorption tests confirmed that the corresponding antigen was also present in the liver microsomal fraction. A retrospective clinical study discovered tienilic acid-induced hepatitis in all cases. We suggest naming this new antibody 'anti-LKM2'.

Keywords microsomal antibody drug-induced hepatitis tienilic acid

INTRODUCTION

The anti-liver–kidney microsome antibody (anti-LKM) was first described by Rizzetto, Swana & Doniach (1973). This antibody is detected through its characteristic fluorescent pattern, which differs from that of anti-mitochondria antibodies (anti-M). Anti-LKM gives a stronger fluorescence on hepatocytes and on proximal renal tubule cells (P), while anti-M is brighter on distal tubule cells (D). The use of male rat kidney is preferable to that of female rat kidney, since the third portion of renal tubules (P_3) reacts poorly in females (Homberg *et al.*, 1974). Through absorption on organelles (Rizzetto, Bianchi & Doniach, 1974) and ultrastructural localization with peroxidase traced antibody (Rizzetto *et al.*, 1974; Storch, Cossell & Dargel, 1977) and anti-LKM has been shown to recognize liver smooth and rough endoplasmic reticulum and can henceforth be called an anti-endoplasmic reticulum antibody.

Anti-LKM has been found in patients with cryptogenic chronic hepatitis and cryptogenic cirrhosis, especially in children and young adults (Rizzetto *et al.*, 1973; Homberg *et al.*, 1974; Smith *et al.*, 1974; Storch, 1975; Schmidt, Kienle & Wolf, 1978; Volte *et al.*, 1980). A comparison of 206 patients with chronic hepatitis showed that the anti-LKM group was altogether distinct from the group of viral hepatitis, and also from the group of chronic active hepatitis with high titered anti-smooth muscle antibody or lupoid hepatitis (Homberg, Jullien & Abuaf, 1980). Anti-LKM

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have been detected in a few cases of drug-induced hepatitis. Walton *et al.* (1976) discovered anti-LKM with low titres in 25% of cases of halothane-induced hepatitis. One case of hepatitis with methyldopa have been reported by Smith et al. (1974)

Working independently, our two laboratories discovered new criteria that make it possible to distinguish two types of anti-LKM. The new antibody shall be referred to as anti-LKM2 to differentiate it from the classical anti-LKM or anti-LKM1 described by Rizzetto *et al.* (1973) The patients with anti-LKM2 were all suffering from tienilic acid-induced hepatitis.

MATERIALS AND METHODS

Sera with anti-LKM. About 30 hospitals regularly send sera for routine detection of organ specific and non-specific antibodies to our two laboratories. Between 1973 and 1979 approximately 70,000 sera were tested by indirect immunofluorescence on rat liver and kidney. Three hundred and seventy-three samples from 131 patients reacted with hepatocytes and the three portions (P_1, P_2, P_3) of the proximal tubules but not with the distal tubules, thus fulfilling the definition of anti-LKM (Rizzetto *et al.*, 1973, Homberg *et al.*, 1974). However from February 1977 onwards (tienilic acid was put on the market in 1976) we started noticing two different patterns of fluorescence. All sera kept at $-20°C$ since 1973 were later retested for this study.

Both types of anti-LKM were compared to other anti-organelle antibodies, including the anti-ribosome antibodies (anti-R) of systemic lupus erythematous and the six different types of anti-mitochondria antibodies: anti-M1 of syphilis, anti-M2 of primary biliary cirrhosis, anti-M3 of Venocuran-induced pseudolupus, anti-M4 of the mixed form of hepatitis, anti-M5 of lupus-related disorders and anti-M6 of iproniazid-induced hepatitis (see Homberg *et al.*, 1982).

Immunofluorescence technique. The classical Weller and Coons' indirect immunofluorescent method (Roitt & Doniach, 1966) with a 4 μm organ section was used. Since differences in fluorescence intensity on the third portion of proximal tubules have been found with anti-LKM1 (Homberg *et al.*, 1974), more complete tests were carried out with 14 sera on different organs of five male and five female rats (Wistar strain) and five male and five female mice (Swiss strain). The following organs were used: brain, cerebellum, spinal cord, eye, skin, inferior lip, parotid, submaxillary gland, exo-orbitary gland, thyroid, oesophagus, trachea, lung, heart, striated muscle (diaphragm), liver, adrenal gland, kidney, ureter, bladder, ovary, uterus, prostate gland and testis.

After this study the two most demonstrative organs, i.e. male mouse liver and male rat kidney, were singled out for further testing. Four criteria were selected to differentiate anti-LKM2 from anti-LKM1 (see results and Table 2). The 373 sera were retested with cross-reading by several members of the same laboratory and two members of the other laboratory. No major problem was encountered in classifying the samples.

Preparation and purity of subcellular fractions. Rat liver was homogenized in nine times its weight of a solution with saccharose 0·25 M, tris buffer 2 mM, pH 7·4 by a Potter homogenizer. Four subcellular fractions were obtained by centrifugation at different speeds: nuclear fraction at 600*g* for 10 min, mitochondrial fraction at 8,500*g* for 10 min, microsomal and post-microsomal fractions at 105,000*g* for 90 min. These fractions were resuspended or diluted in phosphate buffer solution 0·15 M, pH 7·4 (PBS). Further details have been reported elsewhere (Homberg *et al.*, 1982).

The presence of organelles in these subcellular fraction was verified by electron microscopy and by enzymology (Homberg *et al.*, 1982). The mitochondria specific succinyl dehydrogenase activity was 125 moles of succinate per min per mg protein in the mitochondrial fraction and less than 20 moles in the three other fractions. The microsome specific NADPH cytochrome C reductase activity was 174 moles of cytochrome C per min per mg protein in the microsomal fraction and 5 or less in the other fractions.

Absorption experiments. Two sera of each type (anti-LKM2, anti-LKM1, anti-M2 and anti-R) were diluted to obtain the same final titre of 32. One fifth of a millilitre of each diluted serum was incubated with the same volume of PBS alone or with one of the four subcellular preparations at different concentrations (10, 2 or 0·2 mg/ml protein in PBS) during 48 h at 4°C. After centrifugation

at 5,000*g* for 10 min at 4°C, the supernatants were tested and titred by indirect immunofluorescence on rat tissues for remaining anti-organelle antibodies.

Clinical data. Clinical information on the 131 patients with anti-LKM were gathered from their medical records. A drug inquiry was carried out on all patients: they themselves were reinterrogated, information was sought from their physicians and the records of the Social Security Department where prescriptions are kept were screened.

The responsibility of tienilic acid in causing the cases of hepatitis with anti-LKM2 antibodies was established following the criteria of Daugomau *et al.* (1980). These take into account clinical and biological criteria and literature data where the most important events are: chronology between administration of drug and onset of the side effect, results of drug withdrawal: effects of (involuntary) re-administration, concomitant prescription or not of other drugs. Each case was classified as 'likely', 'possible' or 'dubious'.

RESULTS

Immunofluorescent pattern

The description of the anti-LKM2 fluorescent pattern will be compared to that of LKM1. The two antibodies principally stained two organs.

Liver. To differentiate one type of antibody from the other, male mouse liver must be used. Anti-LKM2 gives a more peculiar pattern of strongly positive areas totally surrounded by areas of weaker intensity (Fig. 1a). Centrolobular hepatocytes appear more positive than periportal ones, with a distinct limit between each type of cell. On the contrary, anti-LKM1 stains periportal and centrolobular hepatocytes with the same intensity (Fig. 1b). No difference between anti-LKM2 and anti-LKM1 could be observed in hepatocytes when using female mouse liver, male and female rat liver, or human liver. The fluorescent pattern on the hepatocyte itself was roughly granular and evenly distributed all over the entire cytoplasm of the cell.

Kidney. Like anti-LKM1, anti-LKM2 is strongly positive on proximal tubules and negative on distal tubules with rat, mouse or man kidneys. To differentiate the two types of antibodies it is necessary to use a rat or a mouse kidney of the male sex. Three differences can be observed. (1) Comparison between the outer cortex with P_1 and P_2 and the inner cortex with P_3: anti-LKM2 appears brighter on the outer cortex (Fig. 1c). On the contrary with anti-LKM1, fluorescence predominates on the inner cortex (Fig. 1d). (2) Comparison between P_1 and P_2: with anti-LKM2 there is no difference between these two portions and the departure of the proximal tubule from its glomerulus is always easily seen (Fig. 2a). With anti-LKM1. P_1 is much weaker than P_2. (3) Homogeneity or heterogeneity in P_3 cells: anti-LKM2 gives a variable intensity amongst P_3 cells and about one cell out of twenty is brighter (Fig. 2b). With anti-LKM1, P_3 tubules appear homogeneous. In the female mouse or rat kidney, P_3 reacts weakly with both antibodies and the differences between criteria No. 1 and 3 are difficult to observe. Human kidney seems less positive in all respects.

Other organs. All the other organs are less sensitive than liver and kidney. Table 1 indicates the positive cells and the difference between anti-LKM1 and anti-LKM2. Anti-LKM2 is always negative on rat and mouse steroid cells and anti-LKM1 gives a strong positivity on Leydig cells of mouse testis and sometimes on rat testis and on mouse or rat ovary. Both types of anti-LKM react with the malpighian epithelium of the oesophagus and cylindrical epithelium of the digestive or respiratory tractus. Anti-LKM2 only stains goblet cells of the trachea and the bronchi while anti-LKM1 stains both ciliated and goblet cells.

Differentiation criteria. The two most relevant organs for detection and typing of anti-LKM antibodies remain male mouse liver and male rat kidney. The four simple characteristics that can be used to differenciate between anti-LKM1 and anti-LKM2 are summarized in Table 2.

Comparison with other anti-organelle antibodies

Anti-LKM2 can quite easily be distinguished from the other anti-mitochondria (anti-M1, anti-M2, anti-M3, anti-M4 and anti-M6) or anti-ribosome antibodies by the fluorescence pattern. The

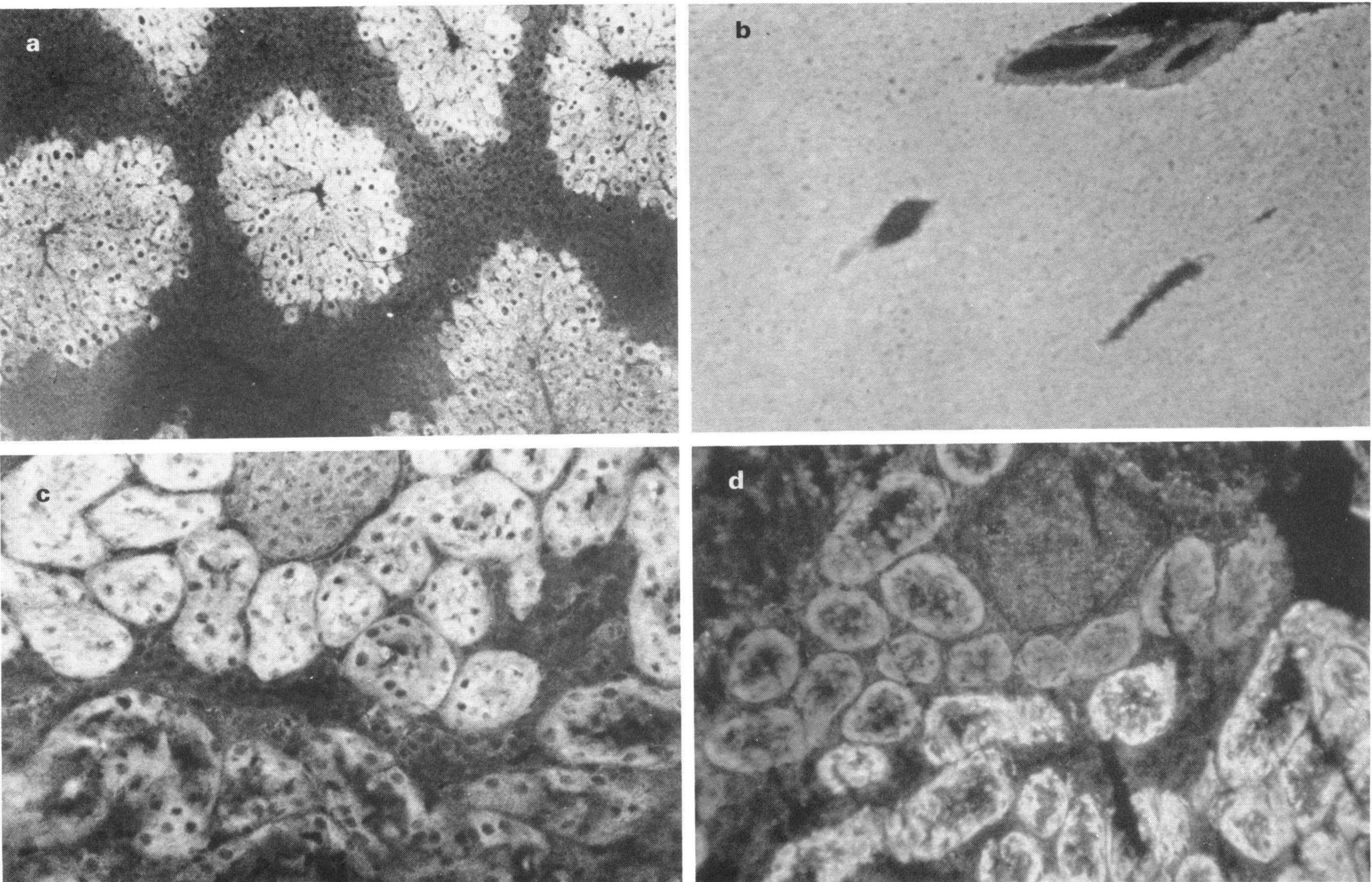

Fig. 1. Two main differences between anti-LKM2 and anti-LKM1. (a) Anti-LKM2 on male mouse liver (× 100): strong positivity of centrolobular hepatocytes and weaker positivity of periportal hepatocytes. (b) Anti-LKM1 on male mouse liver (× 100): homogenity between hepatocytes of different sites. (c) Anti-LKM2 on male rat kidney (× 400): (upper) outer cortex with P_1 and P_2 predominating on (lower) inner cortex with P_3. (d) Anti-LKM1 on male rat kidney (× 400): stronger fluorescence of inner cortex.

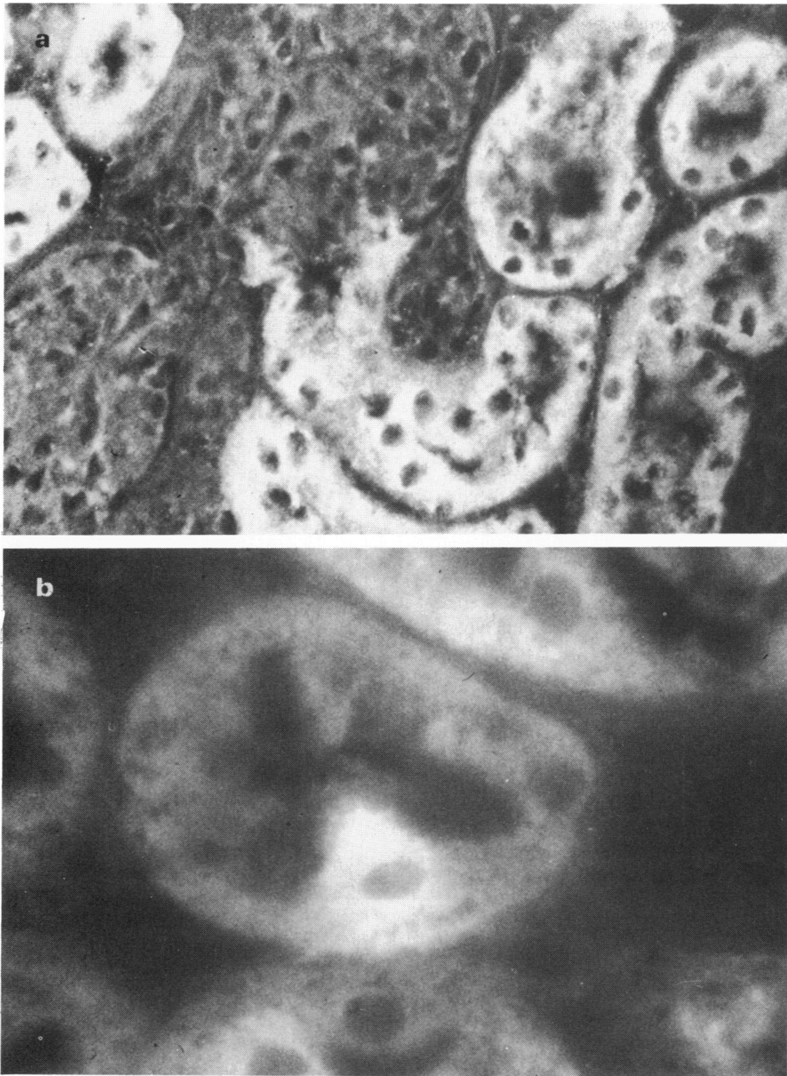

Fig. 2. Two other characteristics of anti-LKM2 on male rat kidney. (a) Strong fluorescence of P_1 (× 400). To detect P_1 we look at 30 renal corpuscules: on the average, a 4 μm kidney section crosses through the central part of the urinary pole for three glomeruli as in this figure; a crescent of P_1 into Bowman's capsule is seen in 11 floculi; for the remaining renal corpuscules, connection between them and proximal tubules is not seen. With anti-LKM1, P_1 with its weak fluorescence is difficult to see (× 400). (b) Heterogeneity between P_3 cells (× 1,000). A difference between P_3 cells demonstrated by anti-LKM2 is at the present time unknown.

intensity of fluorescence on the cells of four rat organs: liver, kidney, stomach and pancreas has been reported previously (Homberg *et al.*, 1982).

Since both anti-LKM2 and anti-M5 antibodies show a predominance of fluorescence on the outer cortex with equal positivity of P_1 and P_2 on rat kidney, these two antibodies can be confused. Furthermore the fluorescence on distal tubules given by anti-M5 can be absent. When there is doubt between anti-LKM2 and anti-M5, the serum should be tested on a male mouse kidney. For anti-LKM2 the positivity remains on P_1 and P_2, and for M5 the fluorescence changes to distal tubules (Table 3).

Table 1. Fluorescence intensity of anti-LKM1 and anti-LKM2 on rat and mouse organs

Rat/mouse organs	Cells	Anti-LKM2	Anti-LKM1
Male mouse liver	Centrolobular hepatocytes	+++	+++
	Periportal hepatocytes	+	+++
Female mouse liver	Centrolobular hepatocytes	+++	+++
Male/female rat liver	Periportal hepatocytes	+++	+++
Male mouse/rat kidney	P_1 and P_2	+++	+
	P_3	+	+++
Female mouse/rat kidney	P_1 and P_2	+++	++
	P_3	+	−
Trachea/bronchus	Ciliated cells	−	++
	Goblet cells	++	++
Oesophagus	Epithelial cells	++	++
Stomach	Mucous cells	+	++
Duodenum	Epithelial cells	++	++
Colon	Epithelial cells	−	+
Mouse testis	Leydig cells	−	+++
Rat testis; rat/mouse ovary	Steroid cells	−	−
Submaxilary/exoorbitary glands	Duct cells	−	− sometimes +
Choroid, plexus	Epithelial cells	−	−
Brain, cerebellum, spinal, cord, eye, skin, thyroid, lung, heart, striated muscle pancreas, adrenal gland, urinary blader, uterus, prostate gland.	Different types	−	−

Table 2. Four relevant differences between anti-LKM1 and anti-LKM2

	Anti-LKM1	Anti-LKM2
Hepatocytes	1 centrolobular = periportal	centrolobular > periportal
Proximal tubules	2 $P_3 > P_1$ and P_2	P_1 and $P_2 > P_3$
	3 $P_1 < P_2$	$P_1 = P_2$
	4 P_3 homogeneous	P_3 heterogeneous

Anti-microsome antibody

The ellipsoid region of rods and cones situated in the retina is very rich in mitochondria and enables recognition of anti-mitochondria antibodies. With the immunofluorescence method, anti-LKM2 were strictly negative on rat and monkey retina and therefore could not be related to this organelle.

Absorption experiments were performed against several rat liver fractions. Anti-LKM2 were absorbed by the microsomal fraction just as easily as anti-LKM1. With a concentration of 0·2 mg protein/ml of this fraction, these antibodies dropped three dilutions; with a concentration of 2 and 10 mg protein/ml, the absorption of antibodies titring 16 was complete. The same results for anti-M2 and anti-M5 were only obtained with a mitochondrial fraction five times more concentrated in proteins.

Human pathology

For most of the patients clinical data could be obtained. Table 4 shows the diagnosis taken into account and demonstrates that the aetiologies of the two antibodies do not overlap. The principal

Table 3. Comparison between anti-LKM2 and anti-M5 on rat and mouse kidney

	Male rat kidney				Male mouse kidney			
	P_1	P_2	P_3	D	P_1	P_2	P_3	D
Anti-LKM2	+ + +	+ + +	+ +	−	+ + +	+ + +	+ +	−
Anti-M5	+ + +	+ + +	+ +	+ or −	+ or −	+ or −	+ or −	+ + +

Table 4. Aetiology of 131 patients with anti-LKM

Aetiology	Anti-LKM1 (86 cases)	Anti-LKM2 (45 cases)
Hepatic disorders		
Virus B	6 (5*)	
Fasciola hepatica	1*	
Alcoholic cirrhosis	2*	
Halothane	1*	
Tienilic acid		43†
Idiopathic disease	45†	
Non-hepatic disorders		
Autoimmune disease	6†	
Bone marrow graft	3†	
Lymphomas	3†	
Miscellaneous	13*	
No or incomplete information	6 (5*)	2†

* = low titre $<\frac{1}{80}$; † = high titre $\geqslant\frac{1}{80}$.

clinical characteristics for the 45 patients with anti-LKM1 suffering from chronic idiopathic hepatitis and for the single case of halothene-induced hepatitis as well as for the 43 patients with anti-LKM2 suffering from tienilic acid-induced hepatitis are as follows.

In chronic hepatitis with anti LKM1, age generally ranged from 2 to 35 years old (40 of 45 cases) with high female predominance (43 of 45 cases). No drug related aetiology was found and none of the patients had taken tienilic acid. HBs antigen was absent. The clinical picture was that of chronic hepatitis. Hypergammaglobulinemia ranged from 20 to 35 g/l yet with a normal or low IgA value. Several autoimmune diseases were associated: vitiligo (three cases). Grave's disease (two cases), pernicious anemia (one case), autoimmune haemolytic anaemia (one case). Progression to cirrhosis was frequent (35 of 45 cases); corticosteroids and immunosuppressor drugs were of questionable value. Only one case of drug-induced hepatitis was discovered among the anti-LKM1: a 47 year old man with hepatitis who relapsed each of the three times he was anesthetized with halothane. Five sera from similar cases were kindly provided by Mrs Doniach and showed the same fluorescence predominance on P_3.

For the patients with anti-LKM2 whose age ranged from 42 to 80 years, there was a female predominance (33 of 43 cases). All 43 patients were suffering from arterial hypertension and received one or more of the following drugs: tienilic acid (43), methyldopa (five), procetophen (four), acebutolol (three), pindolol (two), clonidine (two), dihydroergotamine (two), dihydralazine (two), arniloside (two), amiodarone (two), 15 other drugs (15). All of the patients developed acute onset hepatitis. Tienilic acid therapy generally lasted from 1 to 6 months (range: 10 days and 40

months) before the onset of hepatitis. Average dose was 0·245 g/day (range: 0·065–0·500 g/day) (standard posology is 0·250 g/day). Contrary to the anti-LKM1 group, γ-globulins and IgA were normal. Anti-LKM2 titres ranged from 80 to 3,200. The drug-induced hepatitis regressed completely within 1 to 3 months after cessation of all drugs but five patients died in the acute phase. Anti-LKM2 disappeared within 3 to 9 months. Tienilic acid was involuntarily reintroduced in 18 cases. There was no relapse in one patient receiving corticosteroids and immunosuppressors; for the other 17 cases a second bout of hepatitis occurred with a shorter mean lag time of 30 days (range: 3–52 days) and with average dosage of 0·210 g/day (range: 0·125–0·500 g/day). Clinical and biological symptoms were identical except for the anti-LKM2 antibody titre: first bout of hepatitis: mean titre = 400; second bout: mean titre = 12,800. Mean duration of the second bout was the same as that of the initial episode and two patients died in the acute phase. Four patients had a third bout after reintroduction and one of them even had a fourth which appeared 36 h after intake of a single tablet. Responsibility of tienilic acid was determined in 36 cases by an independent pharmacosurveillance study and was rated as follows: likely = 15, probable = six, dubious = 16 cases. For this drug responsibility determination according to Dangoumau's criteria (see Materials and Methods), the tienilic acid hepatocytotoxicity was considered as unknown and a preference was given to another hepatotoxic drug administered at the same moment. Among the 43 cases mentioned above, 14 cases have been reported in the literature: Poupon *et al.* (1980), Eugène *et al.* (1980), Manigand *et al.* (1980), Mechali *et al.* (1980), Bousquet *et al.* (1980) and Pariente *et al.* (1981). Sera from 25 patients receiving long term tienilic acid treatment (6 months to 3 years) and without liver damage (normal value of seric transaminases) were collected. Anti-LKM2 was absent except in one case with a weak antibody of titre 20.

DISCUSSION

Tienilic acid hepatotoxicity has been recognized ever since the first case was described by Groussin *et al.* (1979). Some 15 other cases were then reported in France and the drug was forbidden in the USA (Ticrynafen recall, 1980). This paper gives the first description of a particular autoantibody associated with this disorder. Anti-LKM2 seems quite specific to tienilic hepatitis, seeing that before this product was put on the market in 1976, no anti-LKM2 could be detected amongst all the anti-LKM stocked in our two laboratories since 1973. The first anti-LKM2 serum is dated February 1977. Furthermore every anti-LKM2 was discovered in the sera of patients who had taken this drug and had developed hepatitis. This is another example of autoantibody related to a drug-induced disease as the anti-M3 and pseudo lupus induced by Venocuran* (Mass & Shubothe, 1972; Grob *et al.*, 1975; Walli, Grob & Muller-Schoop, 1981) or the anti-M6 and iproniazid hepatitis (Homberg *et al.*, 1982).

How can a drug interfere with the immune system and produce such an autoimmune response? Russel (1981) has proposed four main hypotheses. The 'activation of a latent disease is easily refuted here since anti-LKM2 does not seem to appear spontaneously. A 'cross-reaction of the immune response to the drug' can be ruled out since tienilic acid and its three main metabolites did not inhibit the immunofluorescent reaction. Also, such a cross-reaction was also lacking with methyldopa anti-Rhesus antibodies, penicillamine antibodies to the acetylcholine receptor, or with procainamide or hydralazine anti-nuclear factors. Besides, no difference in reactivity was found between the liver of a rat having absorbed tiensilic acid for 8 days and a normal rat liver. However, determining whether or not there is a 'T cell bypass' as has been demonstrated by Allison, Denman & Barnes (1971), Cox & Keast (1973) through the production of autoantibodies by a modified antigen is rather difficult. To do so, one would have to prove that tienilic acid or one of its metabolites had been fixed on the LKM2 substance; unfortunately this antigen has not yet been defined and the endoplasmic reticulum is composed of at least 80 proteins. Finally, a 'direct immunomodulatory effect' of a drug such as that which has been demonstrated for methyldopa would fail to explain why there is such a typical and restricted range of antibodies in drug-induced autoimmune diseases and in tienilic acid-induced hepatitis.

In the book 'Hepatotoxicity', Zimmerman (1978) reviewed the various modes of action of drugs

on the liver. Given the low incidence (estimated at less than 1% of long term treatments) of hepatitis in patients treated by tienilic acid, the possibilities of a metabolic abnormality or of hypersensitivity must be considered. Let us compare the biological data of halothane-induced hepatitis with those of tienilic acid-induced hepatitis. In the former, anti-LKM1 have a low titre and are observed in only 25% of the cases (Walton *et al.*, 1976). The patients' sera react with halothane treated hepatocytes but not with normal hepatocytes (Vergani *et al.*, 1980; Neuberger *et al.*, 1981). An immunoallergic mechanism is highly suspected. In tienilic acid hepatitis, anti-LKM2 have a high titre. This antibody was found in 60% of the sera received for analysis of hepatitis occurring during treatment. Fluorescence on collagenase isolated rat hepatocyte membranes untreated by tienilic acid was obtained with undiluted patient sera. Conversely, direct immunofluorescence on a liver fragment of one patient suffering from hepatitis did not clearly reveal an *in vivo* immunoglobulin fixation on the hepatocyte membrane. Hence it still is difficult to tell whether or not anti-LKM2 is the cause or the consequence of hepatitis and whether this disease is really autoimmune.

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ADDENDUM

While this paper was submitted for publication, Crivelli *et al.* (1983 *Clin. exp. Immunol.* **54,** 232) described a new anti-microsome antibody in hepatitis associated with delta antigen. Opposite to anti-LKM2, this antibody is negative on rat liver and kidney. A numbering scheme was not proposed.

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