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Characterization of anti-liver kidney microsomal antibody in childhood autoimmune chronic active hepatitis: evidence for IgG1 subclass restriction, polyclonality and non cross-reactivity with hepatocyte surface antigens

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SUMMARY

An indirect immunofluorescence technique was used to investigate the immunoglobulin class, IgG subclass, light chain type and complement fixing ability of anti-liver kidney microsomal antibody (anti-LKM) in the sera of six children and five adults with autoimmune chronic active hepatitis (aCAH). Anti-LKM titres ranged from 1/80 to 1/20,480. In the children, the antibody belonged to the IgG1 subclass alone (titre 1/80–1/20,480) and was able to fix complement (titre 1/40–1/5120). In the adult group, antibody belonged to the IgG1 subclass in three cases (titre 1/40–1/640) whilst two belonged to both IgG1 (titre 1/640) and IgG4 (titre 1/40, 1/640). Such subclass restriction is similar to that found in other autoimmune disorders and may be genetically determined. Investigation of the light chain constituent of anti-LKM revealed that the kappa to lambda ratio was consistent with a polyclonal antibody response. To investigate the nature of the antigen to which anti-LKM is directed, the ability of these sera to bind to the surface membrane of isolated human hepatoma cells (Alexander cells) was investigated. Four of the eleven sera showed significant binding activity. Prior incubation of these four sera with Alexander cells abolished their membrane binding activity, but did not alter the anti-LKM titre. These results suggest that anti-LKM binds to cytoplasmic constituents alone and does not cross-react with surface antigens.

Keywords anti-LKM antibody chronic active hepatitis subclass restriction

INTRODUCTION

The anti-LKM antibody was first described by Rizzetto, Swana & Doniach (1973) and defines a subgroup of aCAH in children and young adults with a bad prognosis (Maggiore *et al.*, 1986; Smith *et al.*, 1974). It has a characteristic immunofluorescence pattern, binding to cytoplasmic constituents of hepatocytes and proximal renal tubular cells. Our interest in the nature of anti-LKM has been stimulated by recent evidence that in other autoimmune disorders, autoantibodies exhibit subclass restriction. In Hashimoto's thyroiditis, for example, the anti-microsomal antibody is confined to the IgG1 and IgG4 subclasses, whilst the anti-thyroglobulin antibody is predominantly IgG4 (Parkes *et al.*, 1984). There is preferential production of IgG3 anti-mitochondrial antibodies in primary biliary cirrhosis (Riggione, Stokes & Thompson, 1983). The antibodies to double-

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stranded DNA which are highly specific for the diagnosis of systemic lupus erythematosus are restricted to the IgG1 and IgG3 subclasses (Zouali, Jefferis & Eyquem, 1984). One possible explanation for this subclass restriction would be a clonally restricted B lymphocyte response, in which case antibody production would in addition be restricted to a particular light chain, kappa or lambda. In this study we have characterized anti-LKM antibody in terms of its immunoglobulin class, IgG subclass, light chain expression and complement fixing ability.

The nature and site of the antigen to which anti-LKM is directed and, therefore, the role of the antibody in the pathogenesis of aCAH is unclear. It is well established that the immunofluorescence pattern relates to antigens in the microsomal fraction of the endoplasmic reticulum (Rizzetto, Bianchi & Doniach, 1974), but controversy exists as to whether the antibody also cross-reacts with liver cell membrane antigens, which would indicate a direct role in the mediation of cell damage. Lenzi *et al.* (1984) found that anti-LKM positive sera reacted with antigenic determinants expressed on the surface of isolated mouse hepatocytes. In addition, anti-LKM specificity was absorbed by pre-incubation of the sera with intact mouse liver cells, suggesting that anti-LKM cross-reacts with membrane antigens. In contrast, Alvarez *et al.* (1985) have shown that the major target of anti-LKM is an integral membrane protein of the smooth endoplasmic reticulum of rat hepatocytes which is not expressed on the liver plasma cell membrane. Cell surface binding experiments on normal human hepatocytes are hampered by the difficulty in obtaining normal human liver as substrate. In an attempt to clarify the reactivity of anti-LKM in relation to cell surface antigens, we have investigated whether the antibody is directed against antigens on the human hepatocyte membrane using Alexander cells (PLC/PRF/5), a hepatoma cell line which shares antigens in common with normal human hepatocytes such as determinants present in the LSP (liver specific lipoprotein) complex and LMAg (liver membrane antigen) (Chisari *et al.*, 1981).

MATERIALS AND METHODS

Patients. Anti-LKM positive sera were obtained from six children (one male, five females; median age 12·5 years, range 6 to 19) and five adults (all female; median age 31 years, range 27 to 42) all with a diagnosis of aCAH made according to internationally agreed criteria (Leevy, Popper & Sherlock, 1976). Sera had been stored at −70°C for up to 5 years and were decomplemented by incubating at 56°C for 30 min. In the cases of S.B. and P.H., sera from two different periods of the disease were investigated, separated by 1 and 5 years respectively. Eight patients were on immunosuppressive treatment with prednisolone (0·25–0·5 mg/kg/day) either alone or in combination with azathioprine (1·5 mg/kg/day) or penicillamine (20 mg/kg/day).

Characterization of anti-LKM antibody. Immunoglobulin class, light chain type and complement fixing ability of anti-LKM antibodies were determined by conventional two- or three-step indirect immunofluoresence (IFL) (Thompson, 1981). Briefly, unfixed 5 μm cryostat sections of rat liver, kidney, stomach and pancreas were used as substrate. Fifty microlitres of test sera were incubated with the substrate for 30 min at room temperature at a starting dilution of 1/20 in phosphate-buffered saline (PBS, pH 7·2) and further double diluted to extinction of the characteristic LKM pattern. The titre of anti-LKM was determined by adding 50 μl of fluorescein iso-thiocyanate conjugated (FITC) anti-total human immunoglobulin (Unipath) diluted 1/30 in PBS. The immunoglobulin class and light chain type were determined by using 50 μl of FITC anti-IgG, anti-IgM, anti-IgA (Wellcome), anti-kappa and anti-lambda (Dako) at dilutions of 1/30 in PBS. The ability of anti-LKM to fix complement was determined by incubation at 37°C for 30 min of the substrates, previously incubated with patients' sera, with guinea pig serum diluted 1/5 in PBS as a source of complement. Fixed complement was revealed using FITC goat anti-guinea pig C3 (Cappel). Titration to extinction was performed in all these experiments. Preparations were analysed using a Polyvar fluorescence microscope. As controls the assays were performed in identical fashion but adding to the substrates as first reagent either pooled normal serum or PBS alone.

IgG subclasses of anti-LKM. IgG subclasses were investigated in a similar, three-step indirect IFL assay using murine monoclonal antibodies (MoAb); anti-IgG1 (clone NL16, code BAM15),

anti-IgG2 (clone GOM1, code BAM10), anti-IgG3 (clone ZG4, code BAM08) and anti-IgG4 (clone RJ4, code BAM11; all Unipath). The specificity and reactivity of these MoAb have been evaluated in a large World Health Organization collaborative study (Jefferis *et al.*, 1985) and the MoAb used in this study gave adequate performances. The authors of the study conclude that no MoAb with putative anti-IgG2 specificity is completely satisfactory in all the assays tested. Clone GOM1, however, produced one of the best anti-IgG2 specificities.

To establish the working conditions of the three-step indirect IFL technique and the sensitivity of the MoAb in detecting IgG subclasses in this assay, preliminary experiments were performed using sera from 10 patients with primary biliary cirrhosis with anti-mitochondrial antibody (AMA) which is known to contain all the IgG subclasses (Riggione *et al.*, 1983). Titres of AMA ranged from 1/40 (five patients) to 1/640. Patient sera diluted 1/10 in PBS was incubated with substrate for 30 min followed by the addition of MoAb diluted 1/10 in PBS. After incubation for 30 min, subclasses of AMA were visualized by adding FITC rabbit anti-mouse immunoglobulin (Dakopatts) diluted 1/50 in PBS and incubated for 30 min. All four IgG subclasses were detected in nine patients and IgG1, IgG2 and IgG3 detected in one patient whose AMA titre was 1/40.

The dilution of MoAb to be used in the assay was established by performing block titrations of positive sera using MoAb dilutions of 1/10, 1/25, 1/50 and 1/100, double dilutions of test sera from 1/40 to extinction and a fixed dilution of 1/50 of FITC rabbit anti-mouse immunoglobulin. The dilution of MoAb chosen was 1/25 which was in all cases towards the end-point of the block titration (Johnson, Holborrow & Dorling, 1978).

Hepatoma cell surface binding assay. Cells from the Alexander hepatoma cell line, growing in a monolayer, were isolated by trypsin digestion (0·25%) for 10 min at 37°C and then washed twice in Minimal Essential Medium (MEM). One hundred microlitres of heat-inactivated test sera, diluted 1/160 in MEM, were incubated with 1×10^5 cells at room temperature for 1 h. After two washings, to reveal the presence of antibody bound to the cell membrane, the Alexander cells were incubated at room temperature with 50 μl of I^{125}-Protein A (70–72,000 ct/min) for 45 min. After further washings the radioactivity associated with the cell pellet was determined using a Gamma counter. Controls consisted of Alexander cells incubated with 50 μl of I^{125}-Protein A in the absence of serum or in the presence of serum from normal subjects. To assess whether enzymatic isolation destroys cell surface targets of anti-LKM antibody, the Alexander cell monolayer was mechanically isolated with a rubber policeman. The levels of binding obtained with mechanically or enzymatically isolated cells were similar.

Anti-Alexander cell antibodies detected by indirect IFL. Alexander cells growing in a monolayer and cells held in suspension after trypsin digestion were examined by indirect IFL. In both cases, test sera diluted 1/10 in MEM were added, followed by 30 min incubation at room temperature. Antibody bound to the cell membrane was revealed with FITC anti-total human immunoglobulin (Unipath) diluted 1/30 in MEM.

Absorption of antibody to the liver cell membrane. To absorb liver cell membrane specificities, 100 μl of anti-LKM containing sera, diluted 1/10, positive in the hepatoma cell surface binding assay, were incubated with $0{\cdot}5 \times 10^6$ Alexander cells at 37°C for 30 min. After centifugation, the supernatant was incubated again with $0{\cdot}5 \times 10^6$ Alexander cells at 4°C for 30 min. This manoeuvre allows antibody with optimal reactivity at different temperatures to be removed. The sera were then recovered and tested both in the hepatoma cell surface binding assay and for anti-LKM titre as above.

RESULTS

(See Table 1)

Immunoglobulin classes. Anti-LKM antibody titres as determined by indirect immunofluorescence ranged from 1/80 to 1/20,480. All sera tested had anti-LKM antibody belonging to the IgG class (titre 1/80 to 1/20,480) but not to IgA or IgM. On testing for IgG subclasses, the sera of the six children had anti-LKM of IgG1 (1/80 to 1/20,480) but not of the three other subclasses. Two samples of sera from S.B. obtained at 1 year interval, both during immunosuppressive treatment,

Table 1. Clinical, biochemical and immunological data on the 11 patients with anti-LKM positive autoimmune chronic active hepatitis studied

				Reciprocal anti-LKM titre									Serum levels of:*			
Name	Sex	Age (years)	Immunosuppressive treatment	Total Ig	IgG	IgG1	IgG2	IgG3	IgG4	Complement fixing ability	Light chain Kappa	Light chain Lambda	IgG (g/l)	IgA (g/l)	IgM (g/l)	AST (IU/l)
S.B.	F	15	Prednisolone/ penicillamine	640	640	640	Neg	Neg	Neg	320	160	40	22·6	0·9	8·2	28
S.B.	F	16	Prednisolone/ penicillamine	80	80	80	Neg	Neg	Neg	80	40	40	17·2	0·2	4·3	31
P.H.	M	10	None	2560	1280	1280	Neg	Neg	Neg	1280	640	640	19·5	2·7	1·5	252
P.H.	M	15	Prednisolone	640	640	640	Neg	Neg	Neg	640	320	320	8·6	1·5	1·1	31
M.D.	F	6	None	640	640	640	Neg	Neg	Neg	160	160	160	10·5	1·6	1·7	125
S.P.	F	9	Prednisolone	2560	1280	1280	Neg	Neg	Neg	640	640	320	13·2	1·2	1·9	63
R.P.	F	12	None	20480	10240	10240	Neg	Neg	Neg	5120	5120	5120	32·5	3·5	1·4	1470
G.R.	F	19	Predinisolone	640	640	640	Neg	Neg	Neg	160	320	160	31·0	2·1	0·8	58
J.O.	F	27	Predinisolone/ azathioprine	320	320	320	Neg	Neg	Neg	160	160	80	13·0	1·8	2·3	30
S.H.	F	30	None	640	640	640	Neg	Neg	160	640	320	320	27·8	0·6	3·4	48
S.M.	F	31	Prednisolone/ azathioprine	160	80	80	Neg	Neg	Neg	40	40	40	8·2	1·0	2·0	25
M.G.	F	40	None	160	160	80	Neg	Neg	Neg	40	40	40	11·7	2·6	0·5	241
F.C.	F	42	None	640	640	640	Neg	Neg	40	160	320	160	18·3	1·6	0·4	27

* Normal ranges: IgG 6–16 g/l; IgA 0·8–4·3 g/l; IgM 0·5–2 g/l; AST (aspartate aminotransferase) <40 IU/l.

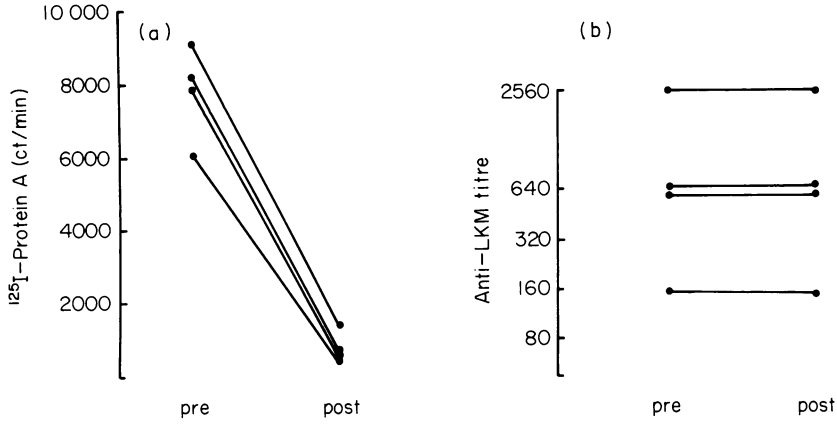

Fig. 1. Absorption of four anti-LKM positive sera with Alexander cells. (a) Binding of antibodies to Alexander cells before and after absorption; (b) anti-LKM titre before and after absorption.

showed an anti-LKM titre of 1/640 and 1/80 but no difference in subclass expression. In the case of P.H., serum obtained at the age of 10 in the absence of treatment had an anti-LKM titre of 1/2560 belonging to the IgG1 subclass only. At the age of 15, during successful immunosuppressive treatment, the anti-LKM titre was 1/640 with no changes in subclass expression. All five of the adult sera were positive for IgG1 (1/40 to 1/640) and, in addition, two showed positivity for IgG4 (1/40, 1/640).

Light chain type. All sera contained anti-LKM of both kappa and lambda light chain type. In six of the 13 sera tested, the titres of anti-LKM antibody of the kappa light chain type were higher than those belonging to lambda.

Complement fixation. All sera tested had anti-LKM antibody capable of fixing complement at titres ranging from 1/40 to 1/5120.

Alexander cell surface binding. Seven anti-LKM positive sera had binding to Alexander cells falling within the normal range (< 2000 ct/min) while four had levels which were significantly increased (all > 8000 ct/min). Of the four sera showing positive cell surface binding, three were children, of whom two (M.D. and S.P.) were receiving immunosuppressive therapy at the time of sampling, whilst R.P. had received no treatment before sampling. The fourth patient, S.M., was an adult receiving immunosuppressive therapy.

Anti-Alexander cell antibodies by IFL. Alexander cells isolated enzymatically and those growing in a monolayer gave a fine granular fluorescence pattern on the hepatocyte surface only when incubated with sera positive in the Alexander cell surface binding assay. Sera positive for anti-LKM antibody but negative in the Alexander cell surface binding assay did not stain live hepatocytes but did stain the cytoplasm of dead hepatocytes.

Absorption tests. Pre-incubation of the four sera positive in the Alexander cell surface binding assay with Alexander cells reduced the binding to background levels but had no effect on anti-LKM titres (Fig. 1). This suggests that the anti-Alexander cell surface reactivity of these sera does not derive from anti-LKM antibody.

DISCUSSION

In this study we have shown that in autoimmune chronic active hepatitis there is IgG subclass restriction in the expression of the anti-LKM antibody. In childhood disease the restriction appears to be confined to the IgG1 subclass whilst in the adults there is expression in both IgG1 and IgG4 subclasses. Such subclass restriction is unlikely to be influenced by either treatment or disease activity since the sera were obtained from untreated as well as treated patients, and similar results were observed in patient P.H. during active disease and whilst on successful immunosuppression.

Subclass restriction of autoantibodies has been described in other autoimmune diseases. The significance of this phenomena is unclear. IgG subclasses are identified on the basis of differing electrophoretic mobilities, antigenic markers and biological properties (Natvig & Kunkel, 1973), suggesting a distinct role for each subclass. Thus they differ in their ability to fix complement (Ishizaki *et al.*, 1967) and to bind to macrophage Fc receptors (Abramson *et al.*, 1970). In addition, response to a number of microbial antigens is IgG subclass restricted. Antibodies to *Staphylococcus aureus* and lipopolysaccharide derived from *Escherichia coli* are mostly IgG2 and those to *Branhamella catarrhalis* and Epstein–Barr virus IgG3 (Walker, Johnson & MacLennan, 1984). Antibody to anti-tetanus toxoid is mostly IgG1 (Yount *et al.*, 1968). In physiological responses, then, the IgG subclasses appear to be as distinct in their roles as the immunoglobulin classes themselves.

In autoimmune disorders, autoantibody production of a particular immunoglobulin subclass in response to an autoantigen could derive from the nature of the antigen itself or from a genetically determined restriction of autoantibody synthesis. The development of aCAH appears to be strongly influenced by genetic factors. The possession of HLA B8/DR3 haplotype confers a greater than 10-fold risk of developing the disease (Mackay & Tait, 1980). The relative risk is further increased if these antigens occur in association with particular Gm allotypes of IgG (Whittingham *et al.*, 1981). IgA deficiency has been noted among these patients (Maggiore *et al.*, 1986) as well as a genetically determined deficiency of the complement component C4 (Vergani *et al.*, 1985). Subclass restriction in the expression of anti-LKM, therefore, may be a further example of the abnormal genetic influences on the development of aCAH, possibly reflecting a defect in the rearrangement of the immunoglobulin heavy chain gene (Flanagan & Rabbitts, 1982).

Although anti-LKM titres are related to disease activity, it is uncertain to what extent if any anti-LKM contributes to pathogenesis. This study provides evidence that anti-LKM antibody may not bind to the human hepatocyte surface. Reactivity to the membrane of Alexander cells was found in only four patients, who did not appear to differ in disease activity, treatment or age from the other seven studied. Absorption of these sera with Alexander cells did not alter anti-LKM titre. This contrasts with the study by Lenzi *et al.* (1984) which showed that sera containing anti-LKM antibodies cross-react with hepatocyte surface antigens, suggesting a direct role for the antibody in cell damage. The discrepancy with these authors might be due to their use of mouse hepatocytes. Murine liver cell membrane may share antigens with microsomes which are not expressed on the surface of human liver cells. In contrast, we have used isolated human hepatocytes, which although from a tumour line, possess antigens of the LSP complex and LMAg (Chisari *et al.*, 1981). It cannot be exluded, however, that the liver kidney microsomal antigen is expressed on the membrane of normal hepatocytes.

In conclusion, anti-LKM antibody in childhood aCAH is restricted to the IgG1 subclass, is able to fix complement, but is unlikely to be directly involved in liver cell damage since it does not react with hepatocyte membrane antigens, such as LSP complex and LMAg which are the likely targets of the immune attack (McFarlane, 1984).

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