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Anti-M4 antibodies in primary biliary cirrhosis react with sulphite oxidase, an enzyme of the mitochondrial inter-membrane space

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SUMMARY

Testing three anti-M2/anti-M4-positive and three anti-M2 positive/anti-M4-negative primary biliary cirrhosis (PBC) marker sera against different mitochondrial enzymes by ELISA it could be shown that only the anti-M4-positive sera reacted with pyruvate dehydrogenase (M2) and sulphite oxidase (SO), an enzyme of the mitochondrial inter-membrane space in parallel. Absorption of these sera with SO abolished completely the anti-M4 antibodies but had no effect on the anti-M2 activity. The specificity of this reaction was also documented by examining 30 anti-M2/anti-M4-positive sera showing that 28 of them were positive with SO. Among ten anti-M2/anti-M8-positive but anti-M4-negative PBC sera, four became positive when tested against SO, indicating a higher sensitivity of SO for the demonstration of anti-M4. Retesting sera from 76 PBC patients with defined anti-mitochondrial antibody (AMA) profiles who had been followed for up to 18 years against SO by ELISA and complement fixation test (CFT), none of 32 patients with profile A/B (positive for anti-M2 and/or anti-M9 by ELISA; benign course) but 33 of 44 patients with profile C/D (anti-M2/anti-M4 and/or anti-M8 positive by CFT and/or ELISA; progressive course) were positive. These data indicate that sulphite oxidase can be used in the ELISA for the detection of anti-M4 antibodies which may be of prognostic relevance.

Keywords anti-mitochondrial antibodies anti-M4 sulphite oxidase primary biliary cirrhosis

INTRODUCTION

Ten years ago we described in anti-M2-positive patients with primary biliary cirrhosis (PBC) a second complement fixing antibody against a trypsin-insensitive antigen (Berg *et al.*, 1980) which was later shown to be associated with outer mitochondrial membranes (Meek *et al.*, 1980; Weber *et al.*, 1986). This antibody type, named anti-M4, was observed preferentially in patients in whom histology revealed features of chronic active hepatitis (CAH) and primary biliary cirrhosis (PBC) in parallel ('mixed form'). Further studies analysing retrospectively 76 PBC patients followed over a period of up to 18 years clearly indicated that the presence of anti-M2 and anti-M4 antibodies in early stages of the disease heralds a more progressive course than the presence of anti-M2 or anti-M9 alone (Klein *et al.*, 1991).

The biochemical nature of the M4 antigen remained, however, obscure. Here we show that anti-M4 antibodies react with sulphite oxidase (SO), an enzyme of the mitochondrial inter-membrane space. The use of this commercially available enzyme in ELISA and complement fixation test (CFT) may facilitate the demonstration of anti-M4 antibodies in sera from patients with PBC.

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PATIENTS AND METHODS

Patients

We tested sera from 40 patients with typical clinical, histological, and biochemical features of PBC all showing anti-M2, anti-M4 and/or anti-M8 antibodies (anti-mitochondrial antibody (AMA) profile C/D: anti-M2/anti-M4/anti-M8 positive: $n=20$; anti-M2/anti-M4 positive/anti-M8 negative: $n=10$; anti-M2 positive/anti-M4 negative/anti-M8 positive: $n=10$). All patients were followed by Prof. J. Eisenburg, Krankenhaus der Barmherzigen Brüder, Munich, Germany.

Furthermore, sera from 76 patients with clinically and histologically defined PBC were included into the study who were in stage I/II at time of first diagnosis and could be followed for up to 18 years (by Dr V. Fintelmann, DRK-Krankenhaus, Hamburg, Germany). They were allocated according to four AMA profiles as described elsewhere (Klein *et al.*, 1991): A, only anti-M9 positive by ELISA ($n=4$); B, anti-M9 and/or anti-M2 positive by ELISA ($n=28$); C, anti-M2, anti-M4 and/or anti-M8 positive by ELISA ($n=26$); and D, anti-M2, anti-M4 and/or anti-M8 positive by ELISA and complement fixation test (CFT) ($n=18$).

Marker sera

Three anti-M2/anti-M4 positive sera (anti-M4 defined by a positive reaction with the M4 fraction in ELISA and CFT) and

three anti-M2 positive/anti-M4 negative PBC sera were used as standard sera.

Tests were also performed with sera from patients with other AMA-positive disorders: syphilis II (anti-M1), pseudolupus syndrome (anti-M3), AMA-positive collagen disorders (anti-M5), iproniazid induced hepatitis (anti-M6), dilated cardiomyopathy and myocarditis (anti-M7) (Berg & Klein, 1989).

Furthermore, we tested sera with different ANA types as well as with antibodies against liver–kidney microsomes (anti-LKM) (Rizzetto, Swana & Doniach, 1973).

Sera from healthy blood donors were used as negative controls.

Antigens

M2, M4, M8, and M9 were prepared as recently described (Klein *et al.*, 1991). Pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase which belong to the alpha–ketoacid–dehydrogenase complex known to constitute the M2 antigen (Gershwin, Coppel & Mackay, 1988; Yeaman *et al.*, 1988) were obtained from Sigma Chemical Co., St Louis, MO.

Different enzymes associated with inner or outer mitochondrial membranes, the matrix or the intermembrane space (obtained from Sigma, or from Boehringer, Mannheim, Germany) were used as antigens in ELISA and Western blotting as previously reported (Klein & Berg, 1990a).

Detection of AMA

CFT, ELISA and Western blot for the detection of AMA were performed as described elsewhere (Klein *et al.*, 1991).

For application of SO in ELISA and CFT, the commercially available SO was first dialysed for 16 h against 0·03 M Tris–HCl buffer (pH 8·0).

Enzymes were applied as antigens in the ELISA at a concentration of 10 μg/ml, patient serum samples were diluted 1/1000.

The optimal antigen concentration and serum dilution for the demonstration of antibodies against SO was 7·5 μg/ml and 1/4000, respectively, as determined by serial dilutions.

For SDS–PAGE, a 10% running and a 4·5% stacking gel were used (Klein & Berg, 1988). Fifty micrograms of total SO as well as of the M4 fraction were applied to the gels. After electrophoresis the proteins were transferred to immobilon sheets (Millipore). The sheets were incubated either with amido black for protein staining or with patients sera at a dilution of 1/50.

Absorption studies

SO from chicken liver was adsorbed to CNBr-activated Sepharose 4B (Pharmacia) at a concentration of 10 mg/ml. After extensive washing, an anti-M2/anti-M4-positive and an anti-M2-positive/anti-M4-negative serum were added at a dilution of 1/10 and allowed to react for 18 h at 4°C on a rotating disk. The absorbed sera were retested by ELISA.

RESULTS

The three anti-M2/anti-M4-positive and the three anti-M2-positive but anti-M4-negative PBC marker sera were tested by ELISA against different enzymes of the inner and outer membrane, the matrix, and the inter-membrane space of mitochondria. Only the three anti-M4-positive marker sera reacted with SO and also with pyruvate dehydrogenase, known to constitute the M2-antigen (Gershwin *et al.*, 1988; Yeaman *et al.*, 1988) In contrast, the three anti-M2-positive/anti-M4-negative marker sera recognized only the M2-related enzymes but not SO.

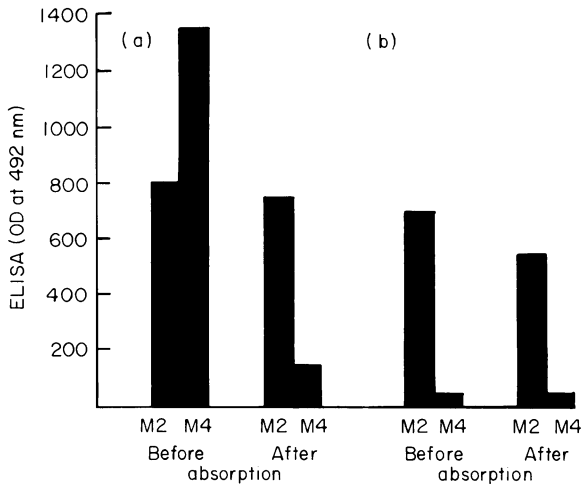

Fig. 1. Absorption of an anti-M2/anti-M4-positive (a) and an anti-M2-positive/anti-M4-negative (b) PBC marker serum with sulphite oxidase (SO) coupled to CNBr-activated Sepharose 4B. SO absorbed completely the anti-M4 antibodies but had no effect on the anti-M2 activity.

Patient sera containing AMA of other subtypes, anti-LKM antibodies or ANA of different specificities were negative when tested against SO.

The specificity of anti-M4 for SO was also proven by absorbing an anti-M2/anti-M4-positive as well as an anti-M2-positive/anti-M4-negative marker serum with SO coupled to Sepharose 4B (Fig. 1). This procedure completely removed anti-M4 but had no effect on the anti-M2 activity.

Protein staining with amido black of SO applied to SDS–PAGE and transferred to immobilon sheets revealed a band at 53 kD which is supposed to be the enzyme itself and a second determinant at 62 kD. Using the purified M4 fraction the same band at 53 kD could be visualized, but other bands were also present at 47 kD and 37 kD.

However, no specific reaction at 53 kD with SO or the M4 fraction was obtained by Western blotting with anti-M4-positive and anti-M4-negative PBC sera.

Testing sera from 40 PBC patients with the AMA profiles C/D against SO and the M4 fraction in parallel by ELISA, a high correlation was found between the antibody titres obtained with the M4 fraction and with SO ($r = 0{\cdot}74$ for anti-M4 of IgG, and $r = 0{\cdot}72$ for anti-M4 of the IgM type; Fig. 2). However, in some instances higher anti-M4 titres where obtained when tested against SO as compared with the M4 fraction. Furthermore, anti-M4-negative/anti-M8-positive sera gave inconsistent results. Thus, four sera being initially clearly anti-M4 negative became positive using SO. In contrast, only two of the 20 sera previously defined to contain anti-M2/anti-M4 and anti-M8 antibodies became negative with SO (Fig. 3).

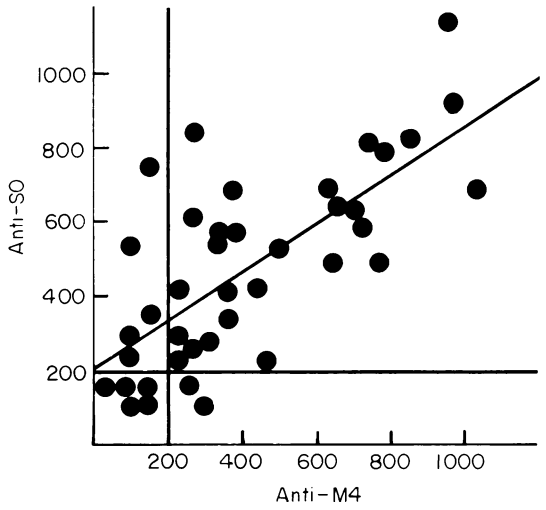

Fig. 2. Correlation between IgG antibody titres obtained by ELISA testing 30 anti-M2/anti-M4-positive and 10 anti-M2-positive/anti-M4-negative/anti-M8-positive PBC sera against the M4 fraction and sulphite oxidase (SO). A good correlation with a correlation coefficient of 0·74 was observed. Horizontal and vertical bars indicate normal values.

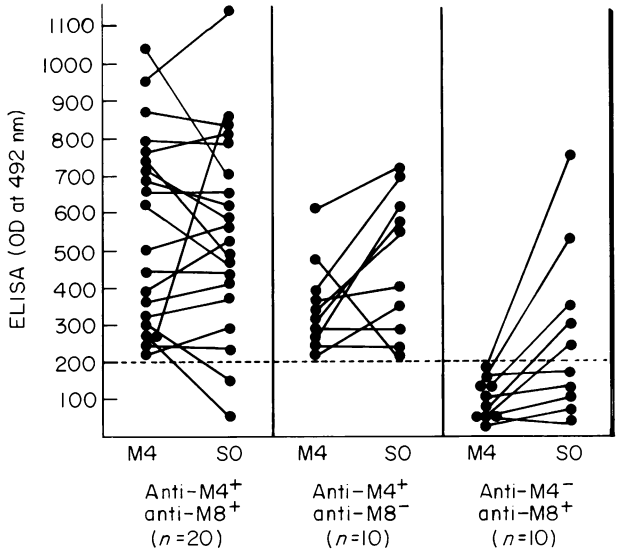

Fig. 3. Comparison of anti-M4 with anti-sulphite oxidase (SO) antibody titres in 30 anti-M2/anti-M4-positive and 10 anti-M2-positive/anti-M4-negative/anti-M8-positive PBC sera. Only two of the 30 anti-M4-positive sera did not react with SO. Five of the 10 anti-M4-negative/anti-M8-positive sera became positive when tested against this enzyme. Dashed line indicates normal value.

Similar results were obtained when sera from 76 PBC patients who had been followed for 6–18 years were tested against SO. Thus, all 18 patients of group D and 15 of the 26 patients of group C were anti-SO positive. In contrast, none of the sera from 32 patients with AMA profile A/B (anti-M4/anti-M8 negative) reacted with this enzyme. All patients who had complement-fixing anti-M4 antibodies also fixed complement with SO, but titres were lower (Table 1).

Analysing the clinical course of these 76 patients in relation to the presence of absence of anti-SO antibodies it could be again established that 82% of the 33 anti-SO positive patients had progressed from stage I/II to stage III/IV during the observation period of 6–18 years, confirming the results obtained with the M4 fraction.

DISCUSSION

In this study it is shown that the use of the enzyme SO in ELISA or CFT allows to detect anti-M4 antibodies in sera from patients with PBC. Thus, testing PBC sera previously defined to be anti-M4 positive against a spectrum of enzymes of the inner or outer membrane, the matrix and the inter-membrane space of mitochondria, a high correlation between anti-M4 titres and titres obtained with SO was obtained. Sera from anti-M2-positive/anti-M4-negative/anti-M8-positive PBC patients as well as from patients with other AMA-positive disorders (anti-M1/anti-M9; Berg & Klein, 1989) or other autoantibodies did not react with this enzyme fraction. Absorption of the anti-M2/anti-M4-positive sera with SO completely abolished the anti-M4 activity but had no effect on the M2 titres.

These data indicate that the commercially available SO fraction prepared from chicken liver contains M4 in high amounts but is devoid of M2 and other mitochondrial antigens including M8.

SO is an enzyme of the inter-membrane space (Johnson &

Table 1. Frequency of anti-M4 and anti-SO antibodies in 76 PBC patients in ELISA and CFT in relation to the AMA profiles

AMA profiles	Number of patients	ELISA		CFT	
		Anti-M4 (*n* positive)	Anti-SO (*n* positive)	Anti-M4 (*n* positive)	Anti-SO (*n* positive)
A (only anti-M9)	4	0	0	0	0
B (anti-M9 and/or anti-M2)	28	0	0	0	0
C (anti-M2/anti-M4 and/or anti-M8, only by ELISA)	26*	13*	15*	0	0
D (anti-M2/anti-M4 and/or anti-M8, by ELISA and CFT)	18	16	18	10	10

*Anti-M2/anti-M4/anti-M8 positive: $n=8$; positive with SO: $n=6$. Anti-M2/anti-M4 positive/anti-M8 negative: $n=5$; positive with SO: $n=5$. Anti-M2 positive/anti-M4 negative/anti-M8 positive: $n=13$; positive with SO: $n=15$.

SO, sulphite oxidase; PBC, primary biliary cirrhosis; CFT, complement-fixing test; AMA, anti-mitochondrial antibody.

Rajagopalan, 1979; Ono & Ito, 1984) which is synthesized in free and microsome-associated ribosomes in the cytoplasm (Kessler & Rajagopalan, 1972). This would agree with our previous findings that the M4 antigen co-purifies with outer mitochondrial membranes and microsomes (Meek *et al.*, 1980; Weber *et al.*, 1986; Berg & Klein, 1989).

SO is known to be a dimeric enzyme which consists of two subunits each of 55–60 kD (Johnson & Rajagopalan, 1979). It is capable of transferring electrons from sulphite to molecular oxygen, ferricyanide, or cytochrom C, utilizing both, a haeme-prosthetic group and a functionally molybdenum (Johnson & Rajagopalan, 1979; Kessler & Rajagopalan, 1972). SO enzyme activity could be determined in the purified M4 fraction (data not shown). Furthermore, amido black staining of the immobilon sheets after immunoblotting with M4 and SO revealed a common band at about 53 kD. However, incubation of the sheets with high-titred anti-M4-positive sera gave no specific reaction. It has, therefore, to be postulated that anti-M4 antibodies are directed against conformational epitopes, and that their reaction depends upon the dimeric structure which is destroyed by SDS treatment involved in Western blotting. From preliminary experiments showing that incubation of anti-M4-positive sera with SO does not effect the SO enzyme activity, it can be concluded that the molybdenum centres within the two subunits of the enzyme which is associated with the enzyme activity, are not part of the antibody binding site.

In previous studies treating sub-mitochondrial particles from rat liver or bovine heart or the purified M4 fraction with trypsin we could show that M4 is not destroyed by this procedure (Berg *et al.*, 1980; Meek *et al.*, 1980; Weber *et al.*, 1986; Berg & Klein, 1989). This observation can be explained by the fact that trypsin treatment of SO leads to cleavage into two fragments at 47·5 kD and 9·5 kD but does not destroy the dimeric structure (Johnson & Rajagopalan, 1977).

As already shown for M2 (alpha-ketoacid dehydrogenase complex; Gershwin *et al.*, 1988; Yeaman *et al.*, 1988) and M9 (glycogen phosphorylase; Klein & Berg, 1990a), SO is an evolutionary highly conserved enzyme which also occurs in bacteria (Toghrol & Southerland, 1983) and is supposed to have a common evolutionary origin with cytochrom b5 (Guiard & Lederer, 1979). As postulated for anti-M2 and anti-M9 one could, therefore, speculate that anti-M4 antibodies are also derived from the pool of naturally occurring antibodies (Klein & Berg, 1990b), and that a cross-reacting epitope may have led to the induction of disease-specific AMA. The variety of different types of PBC-specific antibodies is a further evidence that the hypothetical antigen causing PBC must be very complex being able to stimulate preferentially B cells.

Another major mitochondrial antigen/antibody system in PBC could be characterized. Although these studies do not prove with certainty the identity between M4 and SO, we believe we have shown that the commercially available SO is a useful M4 source, hereby providing a reliable test system for the detection of anti-M4 antibodies. With respect to our observation that these antibodies occur preferentially in patients with a progressive disease, they also may become important in predicting the outcome of PBC at early stages (Klein *et al.*, 1991).

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REFERENCES

BERG, P.A. & KLEIN, R. (1989) Heterogeneity of antimitochondrial antibodies. *Semin. Liver. Dis.* **9,** 103.

BERG, P.A., WIEDMANN, K.H., SAYERS, T., KLÖPPEL, G. & LINDNER, H. (1980) Serological classification of chronic cholestatic liver disease by the use of two different types of antimitochondrial antibodies. *Lancet*, **ii,** 1329.

GERSHWIN, M.E., COPPEL, R.L. & MACKAY, I.R. (1988) Primary biliary cirrhosis and mitochondrial autoantigens-insights from molecular biology. *Hepatology*, **8,** 147.

GUIARD, B. & LEDERER, F. (1979) Amidoacid sequence of the 'b5-like' hemebinding domain from chicken sulphite oxidase. *Eur. J. Biochem.* **100,** 441.

JOHNSON, J.L. & RAJAGOPALAN, K.V. (1977) Tryptic cleavage of rat liver sulphite oxidase. Isolation and characterization of molybdenum and heme domains. *J. biol. Chem.* **252,** 2017.

JOHNSON, J.L. & RAJAGOPALAN, K.V. (1979) The oxidation of sulfite in animal systems. *Ciba Found. Symp.* **72,** 119.

KESSLER, D.L. & RAJAGOPALAN, K.V. (1972) Purification and properties of sulphite oxidase from chicken liver. *J. biol. Chem.* **247,** 6566.

KLEIN, R. & BERG, P.A. (1988) Characterization of a new mitochondrial antigen/antibody system (M9/anti-M9) in patients with anti-M2 positive and anti-M2 negative primary biliary cirrhosis. *Clin. exp. Immunol.* **74,** 68.

KLEIN, R. & BERG, P.A. (1990a) Anti-M9 antibodies in sera from patients with primary biliary cirrhosis recognize an epitope of glycogen phosphorylase. *Clin. exp. Immunol.* **81,** 65.

KLEIN, R. & BERG, P.A. (1990b) Demonstration of naturally occurring mitochondrial antibodies in family members of patients with primary biliary cirrhosis. *Hepatology*, **12,** 335.

KLEIN, R., KLÖPPEL, G., GARBE, W., FINTELMANN, V. & BERG, P.A. (1990) Antimitochondrial antibody profiles can predict the final outcome of primary biliary cirrhosis already at early stages: a retrospective analysis of 76 patients over 6–18 years. *J. Hepatol.* **12,** 21.

MEEK, F., KHOURY, L.E., DONIACH, D. & BAUM, H. (1980) Mitochondrial antibodies in chronic liver diseases and connective tissue disorders: further characterization of the autoantigens. *Clin. exp. Immunol.* **41,** 43.

ONO, H. & ITO, A. (1984) Transport of the precursor for sulfite oxidase into intermembrane space of liver mitochondria—binding of the precursor to outer mitochondrial membrane. *J. Biochem.* **95,** 353.

RIZZETTO, M., SWANA, G. & DONIACH, D. (1973) Microsomal antibodies in chronic active hepatitis and other disorders. *Clin. exp. Immunol.* **15,** 331.

TOGHROL, F. & SOUTHERLAND, W.M. (1983) Purification of Thiobacillus novellus sulfite oxidase. Evidence for the presence of heme and molybdenum. *J. Biol. Chem.* **258,** 6762.

WEBER, P., BRENNER, J., STECHEMESSER, E., KLEIN, R., WECKENMANN, U., KLÖPPEL, G., KIRCHHOF, M., FINTELMANN, V. & BERG, P.A. (1986) Characterization and clinical relevance of a new complement fixing antibody—anti-M8—in patients with primary biliary cirrhosis. *Hepatology*, **6,** 553.

YEAMAN, S.J., DANNER, D.J., MUTIMER, D.J., FUSSEY, S.P.M., JAMES, O.F.W. & BASSENDINE, M.F. (1988) Primary biliary cirrhosis: identification of two major M2 mitochondrial autoantigens. *Lancet*, **i,** 1067.