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Precipitating antibodies to mitochondrial antigens in patients with primary biliary cirrhosis

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SUMMARY

Sera of patients with primary biliary cirrhosis (PBC) were examined for the presence of precipitating antibodies to sonicated rat liver mitochondrial (M) fraction. Three distinct precipitating systems observed in double immunodiffusion were identified and called M-A, M-B and M-C. Unsonicated mitochondria did not form precipitin lines. Precipitating system M-A was found in nineteen of twenty (95%) sera from PBC. The mitochondrial antigen of M-A system had the unusual property of being resistant to enzymatic digestion with deoxyribonuclease (DNase), ribonuclease (RNase) and trypsin under standard conditions. The titres of antibody to M-A antigen correlated ($P<0{\cdot}05$) with titres of mitochondrial immunofluorescence staining on unfixed mouse kidney sections. Precipitating systems M-B and M-C were present in seven of twenty (35%) and five of twenty (25%) patients, respectively. The M-B antigen was sensitive to dexoyribonuclease and trypsin but resistant to ribonuclease indicating that it could be DNA-protein complex. The M-C antigen was destroyed by trypsin suggesting its protein character, but it was difficult to determine if nucleic acids might also be associated with antigenicity. The antibodies to mitochondrial antigens were not present in normals (fifteen healthy adults), systemic lupus erythematosus (forty patients), rheumatoid arthritis (fifteen patients) and chronic liver diseases (fifteen patients). The antibodies did not show identity with antibodies to ribosomal ribonucleoprotein and other known nuclear antigens previously reported. The data confirm previous reports concerning the heterogeneity of mitochondrial antibodies present in sera of patients with PBC. The antibody to M-A antigen appeared to be a diagnostically useful immunological marker since it was present in the majority of patients with PBC.

INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic, slowly progressive disease characterized by non-suppurative destruction of the intrahepatic bile ducts and many serological abnormalities, such as the presence of hypergammaglobulinaemia, various antibodies, complement activation and circulating immune complexes. Many autoantibodies have been described in sera of PBC patients. The most significant of these are antibodies to mitochondria (Walker *et al.*, 1965) which have become the basis of a widely used diagnostic test for PBC. Other antibodies which occur in PBC are directed against nuclei, smooth muscle, bile canaliculi and thyroglobulin (Doniach, 1972).

Anti-mitochondrial antibodies (AMA) are present in the serum of about 84 to 96% of patients with PBC as demonstrated by indirect immunofluorescence or complement fixing test (Doniach *et al.*, 1966; Klatskin & Kantor, 1972; Sherlock & Scheuer, 1973). These antibodies react with the inner membrane of

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mitochondria and perhaps are directed against lipoproteins, with a molecular weight of 180,000 to 200,000 Daltons which have no demonstrable enzyme activity (Ben-Yoseph, Shapira & Doniach, 1974). A heterogeneous group of AMA based on indirect immunofluorescence of different substrates, occurring in a variety of diseases have been reported (Doniach *et al.*, 1970; Wright *et al.*, 1970; Swana *et al.*, 1977; Labro *et al.*, 1978). Their clinical significance is not well understood.

We describe in this paper heterogeneity of AMA present in sera of PBC based on precipitin reaction in immunodiffusion using rat liver mitochondrial fraction. Three different antibodies reacting with mitochondrial fraction were identified which were distinct from antibodies to ribosomal ribonucleoprotein (Schur, Moroz & Kunkel, 1967; Miyachi & Tan, 1979) and other known precipitating antibodies. Antibody to M-A antigen was found in 95% of patients with PBC and may be a diagnostically useful antibody marker.

MATERIALS AND METHODS

Source of sera. Sera used in this study were collected from patients attending Mayo Clinic, Rochester, Minnesota and University of Colorado Medical Center Hospitals, Denver, Colorado. Sera from twenty patients with PBC, forty with systemic lupus erythematosus (SLE), fifteen with rheumatoid arthritis (RA), eight with chronic active hepatitis (CAH), seven with chronic alcoholic hepatitis (AH) and fifteen normal healthy adults were collected and stored at −20°C. The diagnosis of PBC was made if all of the following characteristics were present: (1) evidence of continuous liver disease based on history or biochemical abnormalities for longer than three months; (2) elevation of serum alkaline phosphatase to greater than three times normal; (3) a positive mitochondrial antibody by indirect immunofluorescence; and (4) a liver biopsy characteristic of or compatible with the diagnosis of PBC (Baggenstoss *et al.*, 1964). The diagnosis of chronic active hepatitis was based on the following (Soloway *et al.*, 1972): (1) liver disease was documented by clinical, biochemical and histological abnormalities; (2) chronicity was defined as documented disease, lasting at least ten weeks without clinical or biochemical improvement after the onset; (3) the activity was characterized both biochemically and by biopsy. The diagnosis of chronic alcoholic hepatitis was based on clinical presentation and exclusion of other chronic liver diseases.

Standardized reference sera contained antibodies to Sm antigen (Tan & Kunkel, 1966), nuclear RNP (Sharp *et al.*, 1972), Sjögren's syndrome B antigen (SS-B) (Alspaugh & Tan, 1975), DNA, soluble nucleoprotein (sNP) (Tan, 1967) and ribosomal RNP antigen (Miyachi & Tan, 1979) and had been identified according to studies previously reported. These sera contained monospecific precipitating antibodies and were used in immunodiffusion to characterize identity or non-identity with precipitin lines of other sera being tested.

Rat liver mitochondrial fraction. Cytoplasmic antigens from rat liver were prepared by the method described by Schneider & Hogeboom (1950). In brief, 20 g of fresh rat liver were minced and washed in ice cold 0·25 M sucrose and then homogenized in nine volumes of 0·25 M sucrose with a teflon tissue homogenizer. The homogenate was centrifuged at 700 *g* for 10 min to sediment the nuclei, unbroken liver cells and red blood cells. The sediment was washed once with 0·25 M sucrose. The super natant and washes from the nuclear fractions were centrifuged twice at 5000 *g* for 10 min using 30 rotor of Spinco L type III (Beckman) to sediment the mitochondria. The mitochondrial sediments were combined and washed twice to remove microsomal fractions in the supernatant with 0·25 M sucrose and centrifuged again at 24,000 *g* for 10 min. The mitochondrial sediment thus obtained was washed twice and suspended in 8 ml of 0·25 M sucrose. The protein concentration was measured by the method of Lowry *et al.* (1951) and adjusted to 40 mg/ml.

Sonication of mitochondrial fraction. The mitochondrial fraction (40 mg in 1 ml 0·25 M sucrose) was sonicated in a plastic test tube immersed in ice-alcohol water bath using sonicator model W 220 F and a standard microtip (Heat Systems Ultrasonics, Inc., Plainview, New York). The horn of the sonicator was tuned to vibrate at exactly 20kHz. The mitochondrial fractions were sonicated for different time intervals (30, 45 and 60 sec) and tested as antigens to find out the optimum time of sonication required to give the best precipitin lines with PBC sera.

Preparation of microsomes and other antigens. The microsomal fractions of the rat liver cells were centrifuged at 105,000 *g* for 30 min, and the washed sediments were resuspended in 0·25 M sucrose to obtain a protein concentration of 40 mg/ml (microsomal antigen). A purified ribosomal antigen was obtained by the method of Littlefield *et al.* (1955). A soluble solution of the rabbit thymus extract prepared according to Kurata & Tan (1976) was the source of nuclear antigens: Sm, n-RNP and SS-B. In brief, 10 ml of phosphate buffered saline (PBS), pH 7·4, was added to 750 mg of the lyophilized acetone powder (Pel-Freez Biochemicals, Rogers, Arkansas), stirred at 4°C for 4 hr, and the supernatant obtained by centrifugation at 2000 *g* for 30 min.

Immunodiffusion. The double immunodiffusion method (Ouchterlony) was used to demonstrate and identify the precipitin reaction between soluble antigens and specific antibodies present in sera. Five millilitres of molten 0·4% agarose in PBS (pH 7·4) containing 0·1% sodium azide was poured into 5 cm diameter petri dishes and allowed to gel. Wells, 8 mm in diameter and placed 4 mm apart (circumference to circumference), were filled with the antigens and the sera. The precipitin lines formed between antigens and antibodies were examined up to 72 hr.

Other immunologic techniques. The indirect immunofluorescence technique was performed on cryostat-sectioned unfixed mouse kidney incubated with sera followed by antiserum to human IgG conjugated with fluorescein isothiocyanate (Miyachi

& Tan, 1979). The measurements of antibodies to double-stranded DNA (ds-DNA) and single-stranded DNA (ss-DNA) were done by the millipore filter technique (Picazo & Tan, 1975).

Enzymatic digestion of mitochondrial fraction. Deoxyribonuclease (DNase) I, ribonuclease (RNase) A, trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemicals (Freehold, New Jersey). The enzyme digestion of mitochondrial fraction (40 mg/ml) was done in a water bath at 37°C for 2 hr with the final enzyme concentrations as: DNase 100 μg/ml in 0·01 M PBS, pH 7·4, containing 3 mM $MgCl_2$; RNase 100 μg/ml in PBS without magnesium; and trypsin 500 μg/ml in the same buffer. Trypsin inhibitor was added to inactivate trypsin in the ratio of 1 mg inhibitor to 500 μg of trypsin.

RESULTS

Sonication of mitochondrial fraction in the observance of precipitating antibodies

Since previous work (Berg *et al.*, 1969; Ben-Yoseph *et al.*, 1974) has shown that the antigen to AMA is located in mitochondrial inner membranes, one approach to solubilization of mitochondrial antigens for immunodiffusion studies might be the use of sonication. Sonication of mitochondrial fraction was done at different time intervals to find the optimum sonication time which solubilized mitochondrial antigens and gave the best precipitin lines with mitochondrial antibodies present in serum. Serial dilution of serum of a patient (M.R.) with PBC was used as a source of precipitating mitochondrial antibodies. Unsonicated mitochondrial fraction did not give precipitin lines. When titres of precipitating mitochondrial antibodies were analysed, we found no difference between the activity of mitochondrial fractions sonicated for 30, 45 and 60 sec. For future experiments, therefore, mitochondrial fraction sonicated for 30 sec was used as the standard preparation.

Antigens in mitochondrial fraction

The sonicated mitochondrial fraction was analysed for the presence of nuclear antigens and ribosomal antigens by immunodiffusion against standardized reference sera. The latter sera were in use in our laboratory as standard reagent sera containing high titres of monospecific precipitating antibodies. In this manner, no detectable nuclear antigens such as Sm, nuclear RNP, SS-B, ds-DNA and sNP were present in the mitochondrial fraction. However, ribosomal RNP was present in this fraction and was detectable by antisera when the mitochondrial fraction had a protein concentration of at least 5 mg/ml. By comparison, isolated ribosomal fractions at 0·62 mg protein/ml contained detectable ribosomal RNP antigens (Miyachi & Tan, 1979).

Precipitating antibodies to the mitochondrial fraction

We observed three distinct precipitating antibodies present in sera of twenty patients with PBC. As seen in Fig. 1, two precipitin lines are seen between mitochondrial fraction and the serum MR. The inner precipitin line (closer to antigen well) was seen up to 1:32 dilution and the outer one noted up to 1:8 dilution. The presence of three precipitating systems (M-A, M-B and M-C) in each of twenty sera from patients with PBC was established by the use of prototype sera identified in this manner. The heterogeneity of mitochondrial antibodies present in these sera is exemplified in Fig. 2. The serum E.L. (1:8 dilution) showed three precipitin lines: the inner precipitin line closest to antigen well (M-A); the middle line (M-B); the outer (M-C). The M-C precipitin line was diffuse and faint but was observed to fuse in immunological identity with the stronger precipitin line M-C produced by serum C.W. (1:32 dilution). The serum H.L. (1:8 dilution) and R.H. (1:8 dilution) showed two precipitin lines which were identical with those of the serum E.L. containing antibodies to M-A and M-B antigens. The serum R.P. (1:8 dilution) contained only M-A precipitating system. After testing twenty sera from patients with PBC against mitochondrial fraction, serum E.L. was used as the prototype for the system, M-A and M-B, since it had high titres of the two antibodies. The serum C.W. was selected as the prototype for the system M-C.

The distribution of precipitating antibodies to mitochondrial antigens studied in twenty patients with PBC is presented in Tables 1 and 2. Nine patients had M-A system only (Table 1). Six patients had M-A and M-B systems, three patients had M-A and M-C; one patient (C.W.) had M-C system only, and

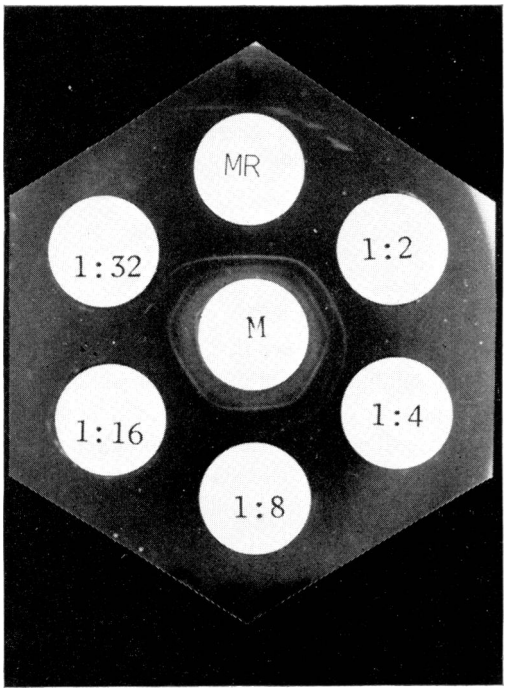

FIG 1

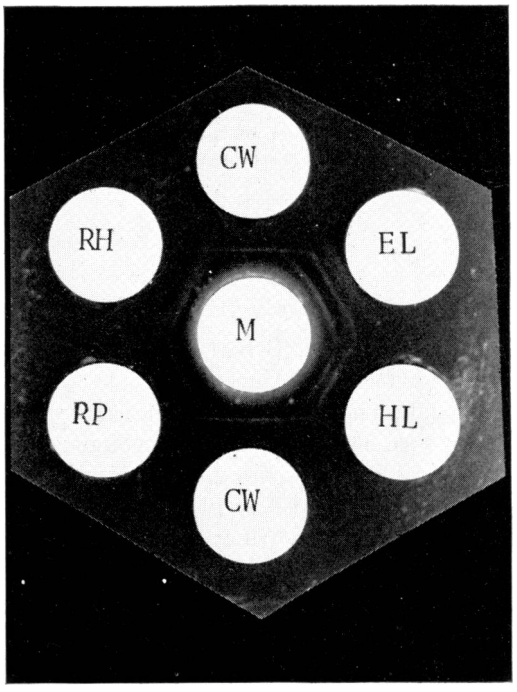

FIG 2

FIG. 1. Immunodiffusion showing precipitin lines between mitochondrial fraction (M) and serially diluted serum from a patient with PBC (M.R.). Inner precipitin line (M–A) is seen up to 1 : 32 dilution and the second precipitin line (M–B) noted up to 1 : 8 dilution.

FIG. 2. Immunodiffusion showing three distinct precipitating systems to mitochondrial fraction (M) in PBC. The serum C.W. (1 : 32 dilution) gave the precipitating system M–C; the serum E.L. (1 : 8), systems M–A, M–B and M–C with M–A nearest to antigen well and M–C farthest from antigen well. Sera with different precipitating antibodies are illustrated: H.L. and R.H. contained antibodies to M–A and M–B, and R.P. only had antibodies to M–A.

TABLE 1. Patients with PBC with the exclusive presence of precipitating antibodies to M–A antigen in their sera

Patients	Titres of antibodies to M–A	Liver pathology		Associated diseases
		Stage*	Inflammation	
R.P.	512	II	Mild	Myxoedema
J.H.	256	IV	Moderate	—
B.H.	128	I	Mild	—
G.G.	64	IV	Moderate	—
A.G.	32	III	Mild	—
W.Z.	32	IV	Severe	Raynaud's, Sjögren's syndrome
K.P.	32	I	Moderate	Hashimoto's' thyroiditis, Raynaud's
D.P.	16	IV	Moderate	Myxoedema
M.H.	8	IV	Mild	—

* Stage I, typical septal and intralobular duct lesions; stage II, ductal proliferation; stage III, periportal and septal fibrosis; and stage IV, cirrhotic stage.

TABLE 2. Patients with PBC with mitochondrial antibodies to M-B and M-C antigens in their sera

Patients	No. of precipitin systems	Titres of antibodies to: M-A	M-B	M-C	Liver pathology Stage*	Inflammation	Associated diseases
R.H.	2	512	32	0	III	Severe	Hashimoto's thyroiditis
V.R.	2	512	16	0	I	Moderate	—
V.G.	2	128	256	0	IV	Severe	—
H.L.	2	128	8	0	III	Moderate	Raynaud's
A.K.	2	32	32	0	III	Moderate	Sjögren's syndrome
R.R.	2	32	8	0	III	Mild	Raynaud's, Sjögren's syndrome
D.P.	2	512	0	64	III	Severe	—
H.N.	2	128	0	64	III	Severe	—
M.R.	2	32	0	8	III	Mild	—
E.L.	3	512	64	8	III	Severe	—
C.W.	1	0	0	512	I	Mild	—

* Stage I, typical septal and intralobular duct lesions; stage II, ductal proliferation; stage III, periportal and septal fibrosis; and stage IV, cirrhotic stage.

another patient (E.L.) had all the three systems (Table 2). The presence of M-B and M-C systems in the absence of M-A was not noted. Thus, M-A system was present in the majority of patients (95%), M-B in seven patients (35%) and M-C in five (25%) patients.

These antibodies were not found in patients with other diseases such as RA, SLE, chronic alcoholic hepatitis and normal healthy controls with the exception of one of the eight patients with chronic active hepatitis who had antibodies to M-A and M-B antigens (Table 3).

Effect of enzyme digestion on the mitochondrial antigens

The effect of enzyme digestion on the mitochondrial antigens reacting with PBC sera were studied using DNase, RNase and trypsin (Table 4). The M-A antigen was not destroyed after DNase or trypsin digestion. The M-B antigen was sensitive to trypsin and DNase but resistant to RNase. The M-C antigen was destroyed by trypsin. The effect of DNase and RNase on the M-C antigen could not be determined since the destructive effect of intrinsic tissue proteases in the antigen preparation could not be inhibited.

TABLE 3. Incidence of precipitating antibodies to mitochondrial antigens (systems M-A, M-B and M-C) in patients with PBC and other diseases

Diseases	No. of patients	No. of positive precipitating systems M-A	M-B	M-C
PBC	20	19	7	5
SLE*	40	0	0	0
RA	15	0	0	0
CAH	8	1	1	0
AH	7	0	0	0
NHS	15	0	0	0

PBC: Primary biliary cirrhosis; RA: rheumatoid arthritis; NHS: normal human serum; SLE: systemic lupus erythematosus; CAH: chronic active hepatitis; AH: chronic alcoholic hepatitis.

* SLE sera contain other types of precipitating antibodies. (See text.)

TABLE 4. The effect of enzyme digestion of mitochondrial antigens on the precipitating anti-mitochondrial antibody systems

Mitochondrial antigens	Effect of enzyme digestion		
	DNase	RNase	Trypsin
M-A	Resistant	Resistant	Resistant
M-B	Sensitive	Resistant	Sensitive
M-C*	Not known	Not known	Sensitive

* The M-C antigen was also destroyed by incubation at 37°C for 2 hr.

Correlation between precipitating mitochondrial antibodies and other serological findings

We found a correlation ($r = 0{\cdot}454$, $P<0{\cdot}05$) between the titres of AMA determined by the indirect immunofluorescence technique and that of precipitating antibodies to M-A antigen.

Anti-nuclear antibodies were positive at 1 : 16 dilution in three of twenty patients with PBC. Antibodies to ds-DNA were not detected, but antibodies to ss-DNA were found in four of twenty patients. We found no relationship between the presence of precipitating mitochondrial antibodies and DNA binding activities. Also, we found no correlation between levels of immune complexes determined by the Raji cell assay (Gupta *et al.*, 1978) and these antibodies.

Absence of correlation between precipitating mitochondrial antibodies and clinical-pathological features of PBC

The presence of various precipitating mitochondrial antibodies did not relate to the pathological stage of the liver of PBC or to the severity of the periportal inflammation. Since patients with PBC are known to have associated diseases, we looked into details of the associated diseases and the presence of specific subtypes of mitochondrial antibodies (Tables 1 and 2). However, no such relations were found.

DISCUSSION

The demonstration of mitochondrial immunofluorescence given by sera of PBC patients (Walker *et al.*, 1965) has established the value of this test in the diagnosis of PBC. These antibodies are found also in 11–28% of chronic active hepatitis, 6–31% of cryptogenic cirrhosis and 2–29% of patients with certain autoimmune diseases (Doniach *et al.*, 1966; Klatskin & Kantor, 1972). Anti-mitochondrial antibodies occurring in PBC have been found to be distributed in all classes of Ig, and the antigens are located in mitochondrial inner membranes (Berg *et al.*, 1969; Bianchi, Penfold & Roitt, 1973; Ben-Yoseph *et al.*, 1974). These antibodies are not observed with cardiolipin, thus differentiating them from other AMA found in secondary syphilis (Wright *et al.*, 1970) and in some patients with monoclonal gammopathies and biological false positive reactions (Doniach *et al.*, 1970). The characterization of the precise nature of the antigen(s) has been difficult since the inner membrane of mitochondria itself is a complex structure composed of mitochondrial DNA, RNA, lipoproteins and basement membrane structural proteins.

In the present study, we demonstrated the presence of heterogeneity of antibodies present in sera of PBC to rat liver mitochondrial antigens by immunodiffusion. Such precipitating mitochondrial antibodies in immunodiffusion have not been demonstrated previously. We found three types of precipitating antibodies to mitochondrial antigens in sera of patients with PBC. Antibodies to M-A antigen were present in 95% of patients with PBC and was not affected by digestion with enzymes DNase, RNase or trypsin. The nature of the antigen is unknown, and the possibility exists that it could comprise lipid or carbohydrate components. M-B antigen activity was abolished by treatment with DNase and trypsin indicating that the antigen might be a DNA-protein complex. However, it is different from antibodies to soluble

deoxyribonucleoprotein present in SLE sera (Tan, 1967), since non-identity was demonstrated in immunodiffusion. The M-C antigen was destroyed by trypsin and may be protein in nature.

The heterogeneous nature of AMA based on indirect immunofluorescence and complement fixation tests in human pathology is well recognized. These mitochondrial antibodies include the antibody which shows fluorescence predominating in renal distal tubules, and the mitochondrial antibody detected only by complement fixation test (Doniach, Lindquist & Berg, 1971). Recently, other types of AMA have been described. Swana *et al.* (1977) described an antibody which had the specificity for human mitochondria but not for that of animal species. Labro *et al.* (1978) described another antibody which showed mitochondrial fluorescence pattern predominantly over the renal proximal tubules. This antibody has not been found in hepatic diseases, but carriers of this antibody either had SLE or autoimmune haemolytic anaemia. Further, the heterogeneity in the mitochondrial antigens is well supported by the work of Sayers, Binder & Berg (1979) who have used purified sonicated rat mitochondrial fractions similar to our study. They demonstrated that the antigen reacting with AMA found in a drug-induced collagen disease-like syndrome (pseudolupus syndrome) could be separated easily from that of PBC by sucrose density gradient fractionation and differential solubilization with salts and enzymes. At present, we do not know whether the three types of mitochondrial antibodies detected by immunodiffusion described in this paper has any relation to any of the above AMA described by other authors.

The significance of the presence of subtypes of mitochondrial antibodies described in this paper is presently unknown, and whether it reflects on clinical or pathological subtypes of PBC, remains to be determined. Patients with PBC are known to have associated diseases with autoimmune features such as Sjögren's syndrome, scleroderma, Raynaud's disease, rheumatoid-like arthritis and Hashimoto's thyroiditis (Sherlock & Scheuer, 1973; Gupta, Dickson & McDuffie, 1977). In our small series of patients we did not find specific distribution of any of the types of mitochondrial antibodies to the subset of PBC having autoimmune diseases. Also, we found no significant distribution of the subtypes of mitochondrial antibodies to early or late stage of the hepatic pathology or other pathological features in the liver. Although two groups of patients with PBC have been described based on circulating IgG complexes (Gupta *et al.*, 1977), subtypes of mitochondrial antibodies did not show specific correlations with the presence and levels of such complexes.

The detection of precipitating mitochondrial antibodies could be a useful method of detecting AMA in patients' sera for the diagnosis of PBC. We have found a significant correlation between M-A system and AMA detected by indirect immunofluorescence technique. However, a larger study needs to be done to compare the precipitating system with detection of AMA by immunofluorescence and complement fixing methods. The precipitating system also could be used to isolate further mitochondrial antigens and identify the nature of these antigens.

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