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The mitochondrial adenine nucleotide translocator is an antigen in primary biliary cirrhosis

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SUMMARY

Circulating antibodies reacting specifically with the adenine nucleotide translocator from liver mitochondria were detected in sera from 12 patients with proven primary biliary cirrhosis (PBC) by a solid phase double antibody immunoradiometric assay (IRMA). Furthermore these antibodies were absorbed with the isolated adenine nucleotide translocator from liver mitochondria. None of the sera from 20 normal individuals, four patients with anti-mitochondrial positive pseudolupus syndrome (PLE) sera (M-3) and three patients with syphilis (anti-M-1) had antibodies directed against this protein from inner mitochondrial membrane. The adenine nucleotide translocator as antigen in PBC could clearly be distinguished from the ATPase associated PBC specific M-2 antigen. With the present study, for the first time, a well characterized protein from inner mitochondrial membrane has been clearly defined as an autoantigen in primary biliary cirrhosis.

Keywords primary biliary cirrhosis anti-mitochondrial antibodies adenine nucleotide translocator

INTRODUCTION

Anti-mitochondrial antibodies (AMA) occurring in primary biliary cirrhosis (PBC) have been found to react with a complement fixing antigen which can be released from inner mitochondrial membrane and co-purifies with the mitochondrial ATPase (Lindenborn-Fotinos, Sayers & Berg, 1982). Further results showed that the ATPase activity can be dissociated from PBC antigenic activity (Sayers *et al.*, 1981). This antigen reacts specifically with sera from patients with PBC and was called M-2 antigen (Berg *et al.*, 1981, 1982). In recent experiments it could be shown that some PBC sera also reacted with inner mitochondrial membrane which were devoid of ATPase antigen. These findings as well as the demonstration of several precipitating antibodies (Myachi *et al.*, 1980), which have been shown by us to react only with antigens associated with the inner mitochondrial membrane (unpublished data) strongly suggest that PBC sera contain mitochondrial antibodies of different specificity.

Schultheiß & Klingenberg (1981) recently isolated the adenine nucleotide translocator (ANT) from mitochondria of calf liver. This protein is the most abundant protein of the inner mitochondrial membrane, at least in heart mitochondria and probably also in liver (Klingenberg, 1976; Klingenberg, Riccio & Aquila, 1978). We were interested to study whether PBC sera contain antibodies also against this important functional component of the inner mitochondrial membrane.

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MATERIALS AND METHODS

Patients. Sera from 12 patients with histologically well defined PBC (Klöppel, Kirchhof & Berg, 1982), four patients with pseudolupus erythematosus syndrome (PLE) known to have antibodies against a trypsin insensitive outer membrane antigen (M-3) (Sayers, Binder & Berg, 1979) and three patients with syphilis (M-1 antigen) (Baum & Berg, 1981) were examined for anti-ANT antibodies. The principal histological features (stage of the disease) and serological findings are given in Table 1.

Preparation of the mitochondria. Mitochondria from liver were prepared according to the method of Berg, Doniach & Roitt (1967).

Preparation of antigen subfractions from the supernatant 40,000 (SN 40). The SN 40 was prepared from fresh liver as described by Berg *et al.* (1967) By gradient centrifugation of the SN 40, as described by Sayers *et al.* (1979), the density gradient fraction 1·10, containing mainly the PLE antigen, and the 1·24 fraction, shown to contain the M-2 complex, were tested in the complement fixaction test (CFT).

Isolation of the mitochondrial ATPase. ATPase was prepared by the chloroform release method of Beechy *et al.* (1975) and used in CFT.

Isolation of the ANT from liver. The solubilization and isolation of the ANT from liver principally followed the same procedure as previously described for the ANT from heart (Riccio, Aquila & Klingenberg, 1975a, 1975b). The greater instability of liver protein necessitated several modifications in the isolation procedure (Schultheiß & Klingenberg, 1982). As usual, mitochondria were first loaded with the tightly bound specific inhibitor ^{3}H-carboxyatratcylate (^{3}H-CAT). Thus the protein is protected against degradation after solubilization by detergent. Furthermore the ^{3}H-CAT binding serves as a marker during the isolation procedure of the protein. After solubilization with Triton X-100 the protein is purified first by HTP chromatography and subsequent gel chromatography (ACA 34). In the final preparation the carrier exists as a mixed protein-detergent micelle. Due to the binding of CAT, the translocator (CAT-protein liver) is fixed in the 'c' conformation which corresponds to the binding centre of the carrier facing the cytosolic side of the inner mitochondrial membrane (Klingenberg 1976).

Solid phase double antibody immunoradiometric assay (IRMA). An indirect solid phase immunoradiometric assay for the ANT according to the method described by Rosenthal, Hayashi & Notkins (1972) and Zollinger, Darlymple & Artennstein (1976) was developed (Schultheiß & Klingenberg, 1982). The test was performed in polyvinyl 'U' form microtitre plates (Dynatech). The antigen (0·5 mg/ml) was incubated in the wells for 4 h at 4°C. After washing, the remaining active binding sites on the plates were blocked by incubation with 3% fetal calf serum (120 μl) for 1 h at 4°C. Then the antigen pre-coated plates were incubated overnight at 4°C with the anti-sera (90 μl) in a dilution of 1:20 with fetal calf serum as diluent. After washing, ^{125}I-protein A was added in order to detect the antibodies to the antigen (Langone, 1978). After 4 h the plates were washed, allowed to dry and counted in a gamma spectrometer. Controls for each test included a 'no-antigen' (filler only), a 'no serum' and a 'control serum' to determine the unspecific binding. All determinations were performed in duplicate.

Immunfluorescence and quantitative CFT. The microtitration method for the CFT and the indirect immunofluorescent test (IFL) were performed as described in the WHO manual by Roitt & Doniach (1969) and according to Berg *et al.* (1967).

Absorption studies. Absorption studies were done according to the method of Kessler (1975). PBC sera were incubated with isolated ANT from liver mitochondria at 4°C for 4 h after pre-absorption of the ANT with 50 μl of a 10% (wt/vol.) suspension of a protein A bearing strain of bacterium *Staphylococcus aureus* (Cowan I) to remove any spontaneously reactive material. Afterwards the protein A bearing staphylococci were added to bind the antigen–antibody complexes. After 15 min incubation the bacterial absorbent was washed twice by centrifugation (2,000 ***g***, 10 min, 4°C) and resuspended. The residual activity of the non-absorbed antibodies in the supernatant was examined in the solid phase IRMA. Control absorption studies performed with the antisera and staphylococcus cells alone did not change the antibody activity in the supernatant after centrifugation of the bacterial cells.

Table 1. Histological and serological findings in PBC and PLE patients

Patient	Stage of disease	IFL	ATPase*	SN 40*	Outer Membrane	Sucrose gradient fraction 1·10†	Sucrose gradient fraction 1·24†
B.K.	PBC I	AMA†	128	1,024	—	0	512
G.J.	PBC IV	AMA†	1,024	1,024	—	0	1,024
G.E.	PBC I	AMA†	2,048	4,096	—	0	1,024
H.H.	PBC I	AMA†	1,096	2,048	—	0	2,048
K.J.	PBC III	AMA†SMA†	512	512	—	0	128
K.O.	PBC I	AMA†	512	512	—	0	256
P.M.	PBC I	AMA†SMA†	2,048	8,192	128	256	1,024
R.E.	PBC III	AMA†SMA†	512	1,024	—	0	512
S.D.	PBC IV	AMA†SMA	2,048	4,096	—	—	2,048
V.J.	PBC III	AMA†	4,096	4,096	256	512	2,048
W.L.	PBC II	AMA†SMA†	512	2,048	—	—	256
Z.A.	PBC II	AMA†SMA†	512	1,024	—	—	512
G.H.	PLE	AMA†	0	256	512	256	0
K.A.	PLE	AMA†	0	256	512	512	0
K.R.	PLE	AMA†	0	4,096	2,048	512	0
Z.A.	PLE	AMA†	0	2,048	2,048	512	0

Mitochondrial antibody in patients with PBC ($n = 12$) and PLE ($n = 4$) tested in IFL and CFT*. For methodical details see Materials and Methods.

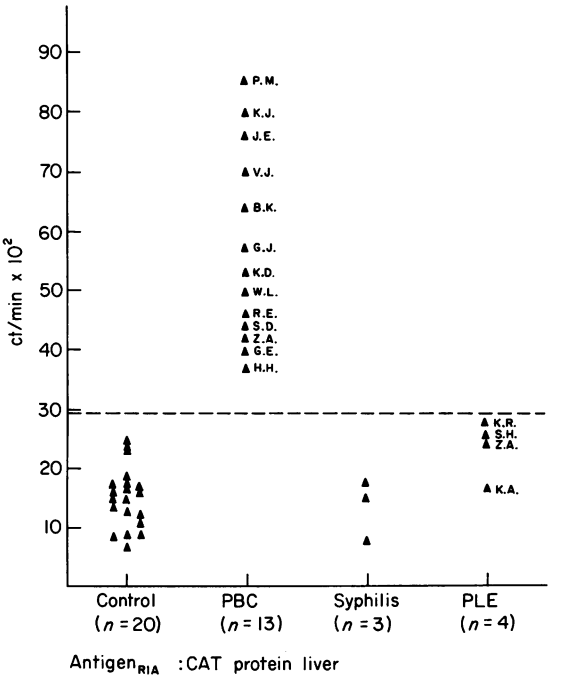

Fig. 1. The binding of AMA to the ANT from liver in the 'c' conformation (CAT protein liver) in the solid phase IPMA from patients with PBC, syphilis, PLE and 20 healthy control persons. Fifty microlitres serum (1/20 dilution in 3% fetal calf serum) were added to the ANT coated wells of a microtitre plate. After a 12 h incubation at 4°C, ^{125}I-protein A (80 μl) was incubated in the well (100,000 ct/min/well) for 4 h at 4°C. All samples were tested in duplicate.

RESULTS

Sera from 12 patients with PBC and four patients with PLE were tested. Diagnosis of PBC and PLE were obtained by clinical, biochemical, immunological and histological examinations. The characteristics of the patients with PBC and PLE studied are in Table 1.

The reaction of the 12 PBC sera with the ANT protein isolated from the liver mitochondria in the solid phase IRMA, is shown in Fig. 1. The amount of antibody binding was, in all 12 PBC sera; significantly greater than the amount found in the four AMA positive PLE sera, three AMA positive syphilis sera and 20 control sera. In these control sera no anti-mitochondiral antibody activity was detected by IFL.

To control the specificity of the antibody binding, all 12 PBC sera were absorbed with the isolated ANT from liver mitochondira and the non-absorbed antibodies were tested in the solid phase IRMA with the liver ANT as antigen. In Fig. 2 it can be seen that there was no residual antibody activity after incubation of the PBC sera with the antigen and the precipitation of the antigen–antibody complexes with protein A bearing staphylococci. An unspecific reaction of the antisera with bacterial absorbent was excluded. The intactness of the ANT under the chosen experimental conditions was proved by the stability of the CAT binding.

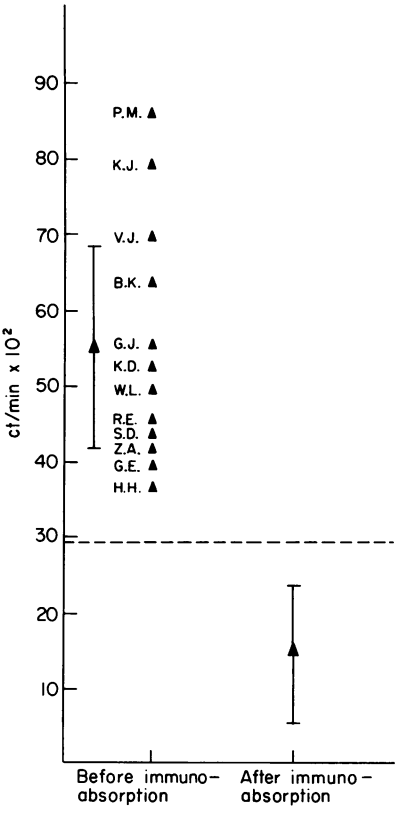

Fig. 2. The binding of AMA from patients with PBC to the ANT from liver (ANT liver) before and after immunoabsorption on the isolated carrier protein. After pre-coating with the antigen (ANT liver) 90 μl of unabsorbed (before immunoabsorption) or absorbed (after immunoabsorption) antiserum (1/20 dilution in 3% fetal calf serum) were added to the wells of a microtitre plate. After a 12 h incubation at 4°C, ^{125}I-protein A (80 μl) was incubated in the well (100,000 ct/min/well) at 4°C for 4 h. All samples were tested in duplicate.

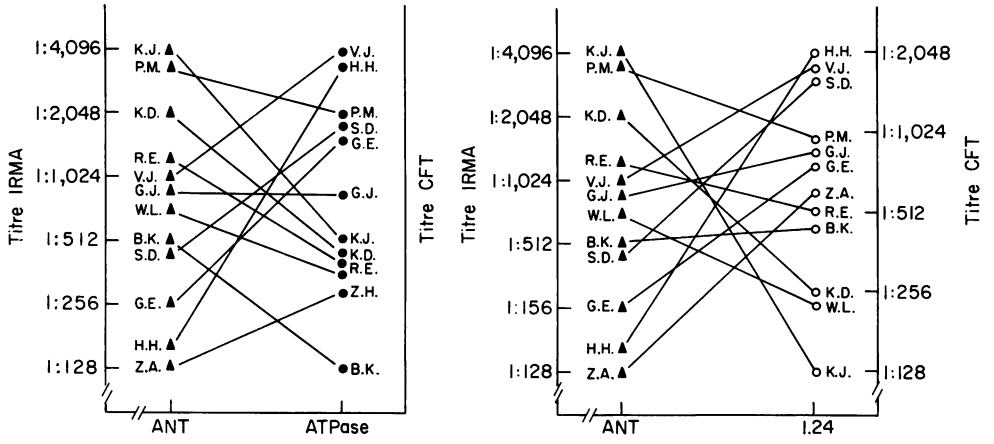

Fig. 3. Comparison of the titres against the CAT protein liver obtained in the solid phase IRMA and those against the M-2 antigen and the 1·24 sucrose density gradient fraction obtained in CFT. For methodical details see Materials and Methods and legend Fig. 1. All samples were tested in duplicate.

In Fig. 3 the anti-ANT titre measured in the solid phase IRMA is compared with the CFT titre of the M-2 and the density gradient fraction 1·24. The different titres of the antisera show that there was no correlation between the different antigen preparations indicating that the complement fixing antigens are not identical with the ANT. In some sera (H.H., K.D., K.J., S.D.) the discrepancy of the measured titres was quite striking.

DISCUSSION

In the present study we have demonstrated that ANT is a new mitochondrial antigen in PBC. This well defined antigen could clearly be distinguished from the M-2 antigen (Berg & Baum, 1980). In contrast to the M-2 antigen protein associated with the ATPase, which can be easily released from beef heart mitochondria by chloroform treatment and from liver mitochondria by sonication—the ANT is an integral membrane protein which can only be solubilized by the use of non-ionic detergents such as Triton X-100 (Klingenberg, 1976). The ANT is isolated from liver mitochondria in a defined conformational state using the non-covalent ligand CAT, which protects the protein against denaturation (Klingenberg *et al.*, 1978). Reconstitution experiments proved that the isolated carrier represented the complete transport system including the regulatory function (Krämer & Klingenberg, 1980, 1982). Therefore it can be concluded that antigenic determinants were not altered by the isolation procedure. Although we have tested only 12 PBC sera, the anti-ANT antibodies occur in high frequency in patients with PBC. Thus all PBC sera showed a significant binding while sera from patients with syphilis and with pseudolupus syndrome (Mass & Schubothe, 1973; Berg, Traunecker & Märker, 1973), an AMA positive drug-induced systemic disorder (Grob *et al.*, 1975), did not bind in the solid phase IRMA with the ANT. The specificity of the antigen–antibody reaction was also shown by the absorption studies which were performed with all PBC sera, abolishing completely the anti-ANT activity.

Indirect evidence for different antigenic components between the ANT and the previously purified complement fixing antigens from inner mitochondrial membrane was obtained by comparing the CFT titres against the M-2 and the antigen of the density gradient 1·24—previously shown to contain the PBC specific marker antigen—and the ANT titres. The fact that ANT is a water insoluble intrinsic membrane protein also makes it unlikely that anti-ANT antibodies are identical with the precipitating antibodies against water soluble proteins in the immunodiffusion test by Miyachi *et al.* (1980).

These findings favour the view that PBC sera may have mitochondrial antibodies which react

with different antigenic determinants within the inner mitochondrial membrane. With the present study for the first time a well characterized mitochondrial protein has been clearly defined as a target antigen to which AMA are directed in PBC. Although an interpretation of those findings in relation to the aetiology and pathogenesis of PBC is not yet possible, the detection of this new characterized autoantibody clearly fits into the concept that PBC is a disease with severe alteration of the immune regulatory functions leading to autoimmunity (Bahn *et al.*, 1982; James et al., 1980; Thomas & Epstein, 1980).

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