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Inhibition of the adenine nucleotide translocator by organ specific autoantibodies in primary biliary cirrhosis

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SUMMARY

Sera from 13 patients with proven primary biliary cirrhosis (PBC) were studied for the capacity to bind to the adenine nucleotide translocator (ANT) isolated from heart, kidney and liver mitochondria. Antibodies against the ANT from liver were detected in the serum of all PBC patients, while 10 of 13 sera were negative when tested with the ANT from heart. None of the sera showed a significant binding to the ANT from kidney. The specific binding and the organ specificity of the autoantibodies against the ANT from liver were also confirmed by immunoabsorption studies on the isolated proteins. To distinguish between antibody titre and antibody activity, we measured the ability of the antisera to inhibit the adenine nucleotide transport across inner mitochondrial membrane using isolated mitochondria from heart, kidney and liver. Six of 13 patient sera tested inhibited the adenine nucleotide transport from liver mitochondria, however, none of the sera inhibited the transport from heart or kidney mitochondria again indicating the organ specificity of the antigen and the autoantibodies.

Keywords adenine nucleotide translocator autoantibodies primary biliary cirrhosis organ specificity

INTRODUCTION

Primary biliary cirrhosis (PBC) is a progressive destructive liver disease of unknown cause (Thomas & Epstein, 1980; Popper & Pornetto, 1980). It is serologically characterized by the presence of anti-mitochondrial antibodies (AMA) (Berg & Baum, 1980). These antibodies are present in a very high proportion of cases (Munoz *et al.*, 1981; Berg *et al.*, 1981) and include several classes of immunoglobulins and more than one antigenic specificity (Miyachi *et al.*, 1980).

Recently, we isolated the adenine nucleotide translocator (ANT) from liver (Schultheiss & Klingenberg, 1984) and showed that it reacts specifically with sera from patients with PBC (Schultheiss, Berg & Klingenberg, 1983). The ANT, which is the most abundant protein of mitochondria, is an intrinsic, hydrophobic protein located at the inner mitochondrial membrane (for review see Klingenberg, 1976). It facilitates the transport of ATP from the mitochondria to the energy consuming processes in the cytosol and the return of ADP to the inner mitochondrial space for regeneration by oxidative phosphorylation (Klingenberg & Heldt, 1982).

By immunizing rabbits with the ANT from heart and kidney, we could demonstrate the existence of organ specific, non-cross-reacting antigenic determinants on the ANT from heart,

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kidney and liver (Schultheiss & Klingenberg, 1981a, 1982). In view of this organ specificity it was of great interest to examine whether autoantibodies in patients with PBC might also be specific for these organ specific antigenic determinants of the ANT from liver. Moreover, we measured the ability of the antisera to block the adenine nucleotide transport across the inner mitochondrial membrane.

MATERIALS AND METHODS

Patients. Thirteen female patients (mean age 53 years, range 36–70) with PBC were investigated. All of them had elevated alkaline phosphatase and IgM levels. The diagnosis and state of the disease was established by liver biopsy (Klöppel, Kirchhof & Berg, 1982). AMA were detected in all patients displaying anti-M2 specificity (Berg & Baum, 1980). Two of them had anti-M4 antibodies in addition (VI and PM) which recently have been shown to react with a trypsin insensitive antigen of the outer mitochondrial membrane (Berg *et al.*, 1980b). As controls seven AMA positive sera were used, four from patients with pseudolupus syndrome (PLE) known to have mitochondrial antibodies of the IgG type reacting exclusively with a trypsin insensitive antigen of the outer membrane (anti-M3), and three from patients with syphilis (anti-M1) reacting with cardiolipin, an inner membrane component (Berg & Baum, 1980). Sera from 20 blood donors served as negative controls.

Methods. The solubilization and isolation of the ANT from the three organs principally followed the same procedure as previously described for the ANT from heart (Riccio, Aquila & Klingenberg, 1975a, 1975b). However, the greater instability of the kidney and liver protein as indicated by an irreversible release of the specific ligand carboxyatractylate (CAT) necessitated several modifications of the isolation procedure as described elsewhere (Schultheiss & Klingenberg, 1984).

The solid phase double antibody immunoradiometric assay (IRMA) was performed by the following procedure on flexible polyvinyl U shaped microtitre plates. The antigen (100 μl) at a protein concentration of 0·5 mg/ml was incubated in the wells for 4 h at 4°C. After aspiration of the antigen solution and washing three times, the remaining active binding sites were blocked by a filler (3% fetal calf serum). After a further washing, 90 μl of the diluted sera (1:20) were transferred to the antigen coated plates. After binding of these primary antibodies to the antigen overnight at 4°C, the plate was washed three times with filler. ^{125}I-protein A diluted with the filler (100,000 ct/min/well) was then placed in each well for 4 h at 4°C. Afterwards, the plates were washed three times with the filler, allowed to dry and counted. All determinations were performed in duplicate. Controls for each test included a 'no-antigen' (filler only), a 'no-serum' and a 'control serum' to determine the unspecific binding.

The determination of the ADP/ATP exchange rate was based on the 'inhibitor stop method' combined with the 'back-exchange' (Pfaff & Klingenberg, 1968). The adenine nucleotide translocation as a 1:1 exchange between intra- and extramitochondrial nucleotides is calculated as percentage exchange of the total intramitochondrial content, according to the equation

$$\% \text{ exchange} = 100 \times \frac{\text{ct/min}_{\text{assay}} - \text{ct/min}_{\text{control}}}{\text{ct/min}_{\text{total}} - \text{ct/min}_{\text{control}}}.$$

The inhibitory activity of the sera from patients with PBC was tested by the 'back-exchange' after incubation of mitochondria with equilibrated sera at 4°C.

For immunoabsorption studies, the Staphylococcal protein A antibody adsorbent was used (Kessler, 1975). After pre-absorption of the ANT with 50 μl of 10% (wt/vol.) suspension of a protein A bearing strain of the bacterium *Staphylococcus aureus* (Cowan I) to remove any spontaneously reactive material, the CAT–protein complex from heart, kidney and liver was incubated with the antisera at 4°C for 4 h. Then the antigen–antibody complexes were bound to the *S. aureus* by addition of 150 μl of a 10% (wt/vol.) Cowan I suspension. After incubation for 30 min, the bacterial adsorbent was washed twice by centrifugation (2,000*g*, 6 min, 4°C). The supernatants containing

the free antibodies were collected and the residual antibody activity against the ANT from liver was determined with the IRMA. Control absorption studies performed with the antisera and *S. aureus* cells alone did not change the antibody activity in the supernatant after centrifugation of the bacterial cells.

Mitochondria from beef heart, calf kidney and calf liver were prepared according to the method of Smith (1967).

Outer and inner mitochondrial membranes were prepared by the method of Schnaitmen & Greenawalt (1968).

Submitochondrial particles (SMP) from beef heart and rat liver mitochondria were prepared according to the method of Beyer (1967). SMP were treated with Nagarse, a protease from *Bacillus subtilis* at a concentration of 25 μg Nagarse/mg protein.

The supernatant 40 (SN40) was prepared from fresh rat kidney mitochondria as described by Berg, Doniach & Roitt (1967). Then the supernatant 40 was centrifuged by discontinuous sucrose density yielding two fractions discriminating between the PLE (1·10) and the PBC specific antigen (1·24).

Mitochondrial ATPase was isolated from beef heart and rat liver following chloroform release method of Beechey *et al.* (1975).

Table 1. Histological and serological findings in patients with PBC, PLE and syphilis

Patient	Antibody specificity	Diagnosis and stage of disease	IFL	SN 40*	ATPase*	Outer membrane fraction*	Sucrose gradient fraction*		ANT liver†
							1·10	1·24	
BK	Anti-M2	PBC I	AMA+	1,024	128	—	—	512	512
GI	Anti-M2	PBC IV	AMA+	1,024	1,024	—	—	1,024	1,024
GE	Anti-M2	PBC I	AMA+	4,096	2,048	—	—	1,024	256
HH	Anti-M2	PBC I	AMA+	2,048	1,024	—	—	2,048	128
JE	Anti-M2	PBCII	AMA+	2,048	2,048	—	—	1,024	4,096
KI	Anti-M2	PBC III	AMA+SMA+	512	512	—	—	128	4,096
KD	Anti-M2	PBC I	AMA+	512	512	—	—	256	2,048
RE	Anti-M2	PBC III	AMA+SMA+	1,024	512	—	—	512	1,024
SD	Anti-M2	PBC IV	AMA+SMA+	4,096	2,048	—	—	2,048	512
WL	Anti-M2	PBC II	AMA+SMA	2,048	512	—	—	256	1,024
ZA	Anti-M2	PBC II	AMA+SMA	1,024	512	—	—	512	128
PM	Anti-M2 +anti-M4	PBC I	AMA+SMA	8,192	2,048	128	256	1,024	4,096
VI	Anti-M2 +anti-M4	PBC IV	AMA+	4,096	4,096	2,048	512	2,048	1,024
GH	Anti-M3	PLE	AMA+	256	—	512	256	—	—
KA	Anti-M3	PLE	AMA+	256	—	512	512	—	—
KR	Anti-M3	PLE	AMA+	4,096	—	2,048	512	—	—
ZA	Anti-M3	PLE	AMA+	2,048	—	2,048	512	—	—
FS	Anti-M1	Syphilis	AMA+	128	—	—	not tested		—
AT	Anti-M1	Syphilis	AMA+	512	—	—	not tested		—
WB	Anti-M1	Syphilis	AMA+	256	—	—	not tested		—

Mitochondrial antibody pattern and titres are given as reciprocal of serum dilution in patients with PBC ($n=13$), PLE ($n=4$) and syphilis ($n=3$) tested in the IFL, CFT* and IRMA.† For details see Materials and Methods.

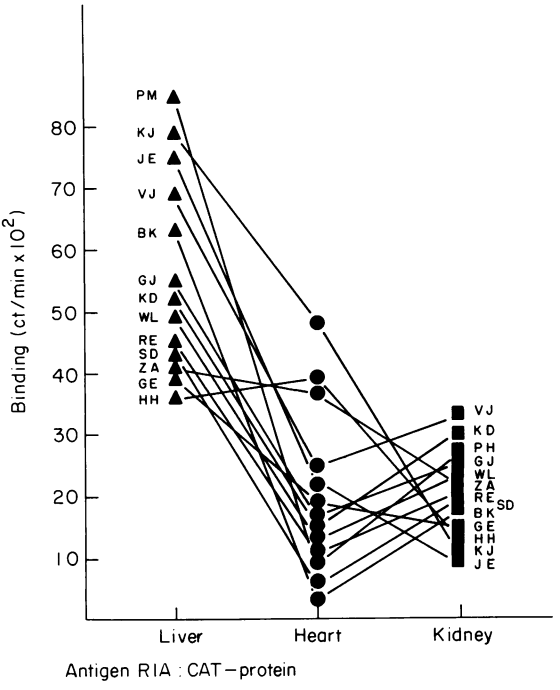

Fig. 1. The binding of autoantibodies from patients with PBC to the ANT from liver, heart and kidney. For details see Materials and Methods.

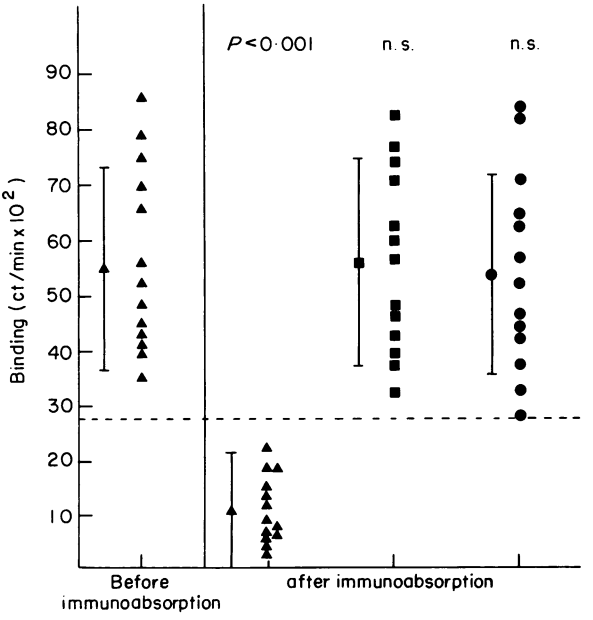

Fig. 2. The binding of AMA from patients with PBC to the ANT from liver before and after immunoabsorption on the isolated ANT from liver (▲), kidney (■) and heart (●). After pre-coating with the antigen (ANT liver), 90 μl of unabsorbed or absorbed antiserum (1:20 dilution in 3% fetal calf serum) were 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 wells (100,000 ct/min per well) at 4°C for 4 h. All samples were tested in duplicate.

RESULTS

For comparison, the antisera were first tested in various assays hitherto used for classifying the different AMA antibody populations. The results of this pre-screening are summarized in Table 1. While all PBC patients reacted with the M2 marker antigen, the patients PM and VI had in addition antibodies of the anti-M4 specificity. The PLE sera reacted only with the outer membrane fraction (anti-M3) and the three patients with syphilis (anti-M1) did not fix complement with any of the marker antigens. When these sera were tested in the solid phase IRMA against the ANT from liver, a significant binding was only found with the sera from patients with PBC while the binding activity for the PLE (anti-M3) and for syphilis (anti-M1) sera was in the range of the control sera (Table 1).

As shown in Fig. 1, 10 of 13 PBC sera were negative when tested with the ANT from heart and none of the PBC sera showed a significant binding to the ANT isolated from kidney. Two of the heart positive PBC sera (ZA and KJ) showed a lower antibody activity with the heart protein than with the liver protein and only one PBC serum showed about the same binding activity to the heart protein in comparison to the liver protein (HH).

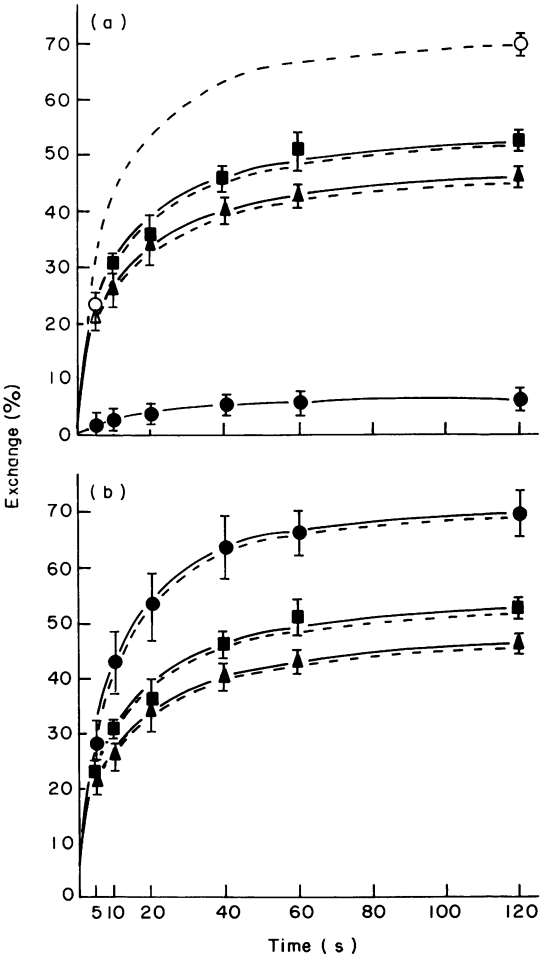

Fig. 3. Influence of PBC sera on the exchange of ADP. Mitochondria loaded with ^{14}C-ADP were incubated with PBC sera (1:10 dilution) at 4°C for 30 min. The percentage exchange was calculated from the radioactivity released in the supernatant and was measured as 'back exchange' by CAT stop. Exchange time was 5, 10, 20, 40, 60 and 120 s at 8°C. The leakage of labelled adenine nucleotides into the supernatant was measured in parallel samples and was less than 10%. —=PBC sera (a=type I; b=type II); - - -=control sera; ▲=kidney; ■=heart; ●=liver.

The specific binding of the autoantibodies to the ANT isolated from liver and the organ specificity of this reaction were also confirmed by immunoabsorption studies on the isolated translocator protein. Only the liver protein completely abolished the antibody activity while the isolated translocator proteins from heart and kidney did not significantly change the antibody activity against the liver protein (Fig. 2) as indicated by the unchanged rest activity in the supernatant after immunoabsorption.

To test a possible inhibitory activity of the antisera, the nucleotide transport across the inner mitochondrial membrane was measured. None of the control sera, the PLE sera and the syphilis sera produced a significant blockade of the nucleotide transport. In contrast, six of 13 sera from patients with PBC tested (KJ, JE, BK, KD, GE and HH) caused a significant inhibition of the nucleotide transport in liver mitochondria (Fig. 3a). A serial dilution of the serum samples resulted in a progressive decrease of the inhibitory activity of the antisera. The nucleotide transport from heart and kidney mitochondria was not influenced by any of those sera which inhibited the transport in liver mitochondria again indicating the organ specificity of the antigen and the autoantibodies (Fig. 3b).

DISCUSSION

The results demonstrate for the first time the existence of organ specific and functional active autoantibodies against a biochemically and immunochemically characterized and in its function well known protein (the ANT) of the inner mitochondrial membrane in sera from patients with PBC. The ANT with about 12% of total membrane protein represents the major single membrane protein in mitochondria. Moreoever, the ANT has been shown to be the most abundant protein on the outer surface of the inner mitochondrial membrane using membrane surface labelling experiments with lactoperoxidase catalysed iodine incorporation (Boxer, Feckl & Klingenberg, 1976). Therefore, it seems to be understandable that the ANT serves as a mitochondrial autoantigen.

The ANT is isolated from mitochondria as a CAT–protein complex. Thus it is protected against denaturation and maintained in a defined conformation ('c' conformation) (Klingenberg, 1976). Reconstitution experiments proved that the isolated carrier represents the complete transport system including the regulatory function (Krämer & Klingenberg, 1982). Therefore, it can be concluded that the protein structure, function and antigenicity is not destroyed by isolation procedure—an important prerequisite for interpretation of these immunological studies.

In this paper, it could be shown that PBC sera contain antibodies which react preferentially with organ specific determinants of the ANT isolated from liver mitochondria. These findings verify previous experiments in which organ specific determinants on the ANT isolated from heart, kidney and liver were demonstrated (Schultheiss & Klingenberg, 1981b, 1981c). The specificity of the antibodies exclusively for the liver protein in 10 out of 13 PBC sera seems, however, to be surprising as a partial cross-reactivity between the ANT from heart, kidney and liver had been observed.

Besides the organ specific binding in some patients these antibodies have been shown to have a functional effect inhibiting the adenine nucleotide transport. From the fact that only some of the PBC sera inhibited the transport, it can be concluded that at least two types of antibodies against the ANT exist in PBC: a blocking antibody (type I) which combines with the ANT at or near the translocation site and inhibits the transport reaction, and a non-blocking antibody (type II) which reacts with the ANT at a sight distant from the translocation site. The specific inhibition of the nucleotide transport only from liver mitochondria again agrees with the existence of organ specific autoantibodies against the native carrier protein.

The studies of the blockade of the nucleotide transport show that there is no correlation between the antibody titre and the inhibition cavity of the sera or the clinical state. This observation could be explained by the heterogeneity of the anti-ANT antibodies reacting with different antigenic determinants and a different avidity of the antibodies to the different antigenic determinantes. Furthermore, the cold temperature and short incubation time required for the transport studies on mitochondria to prevent unspecific leakage may lead to incomplete binding of the antibodies to the ANT causing this discrepancy.

Follow-up studies will have to prove whether the antibody titre or the inhibition activity may be

taken as an indicator for the prognosis or progression of the disease. If the antibody interferes not only *in vitro* but also *in vivo* with the function of the ANT, the existence of blocking autoantibodies in PBC might indicate a novel mechanism of immunologically mediated damage and/or dysfunction of the liver cell.

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