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Detection of anti-mitochondrial antibodies by ELISA and Western-blot techniques and identification by one and two-dimensional gel electrophoresis of M2 target antigens

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SUMMARY

Seven hundred and eleven sera were simultaneously studied by immunofluorescence (IF), complement fixation test (CFT) and ELISA for the detection of anti-mitochondrial antibodies (AMA). One hundred and nineteen of these sera were also studied by Western-blot techniques, while some of them were examined by two-dimensional gel electrophoresis so as to identify the polypeptides recognized by M2 antibodies. The results indicated that: (1) ELISA is a more sensitive technique for detecting type M2 AMA (27 scored positive in 27 primary biliary cirrhosis (PBC), as compared to 21/27 by IF and 16/27 by CFT). (2) Although ELISA appeared to be a promising screening method, some false positive results were observed that necessitated a double confirmation of positive sera by another technique. (3) Western-blot experiments with rat mitochondrial purified preparation indicated that sera from AMA type 2 could recognize eight different polypeptides and that most of them identified 63–60, 48, 44, and 35–33 kD polypeptides, whereas the 54 and 27 kD were less frequently recognized. A trypsin treatment of antigens confirmed the enzyme sensitivity of most of these antigens. These results suggested some heterogeneity among M2 AMA, though this series of PBC was not large enough to relate the heterogeneous pattern noticed in Western-blot to the clinical and histological patterns observed in PBC.

Keywords M2 AMA ELISA immunoblot

INTRODUCTION

Anti-mitochondrial antibodies (AMA), routinely detected by indirect immunofluorescence (IF) (Walker *et al.*, 1965), are present in about 90% of patients with primary biliary cirrhosis (PBC), and

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Abbreviations used in the text. IF, immunofluorescence; CFT, complement fixation test; ELISA, enzyme-linked immunosorbent assay; AMA, anti-mitochondrial antibodies; PBC, primary biliary cirrhosis; CAH, chronic active hepatitis; FIAX, fluorimetric immunoassay; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; HBsAg, hepatitis B surface antigen; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; LKM, liver kidney microsomal antibodies; ANA, anti-nuclear antibodies; dsDNA, double-stranded deoxyribonucleic acid; ssDNA, single stranded deoxyribonucleic acid; SMA, smooth muscle antibodies; PBS, phosphate-buffered saline; PBS-T, PBS containing Tween-20; PBS-T-G, PBS containing Tween-20 and gelatin; Ig, immunoglobulin; MBI, mitochondrial binding index; OD, optical density; PSS, primary systemic sclerosis.

in less than 1% of the general population (Doniach *et al.*, 1966). In the recent past, considerable evidence has accumulated favouring the presence of different types of AMA. Thus, nine types of reactivity (M1 to M9) according to the immunofluorescence and complement fixation test (CFT) have now been defined (Wright *et al.*, 1970; Labro *et al.*, 1978; Sayers, Binder & Berg, 1979; Homberg *et al.*, 1982; Klein *et al.*, 1985). The AMA reactivity found in the case of PBC is directed against a trypsin-sensitive protein of the inner mitochondrial membranes, termed M2, but other reactivities detected by CFT (M4, M8, M9) can also coexist with M2 (Berg *et al.*, 1986).

The development of new immunological methods like ELISA (Kenna *et al.*, 1984) and FIAX (Klein *et al.*, 1983) have enhanced sensitivity of detection but immunoblotting techniques offer a more interesting approach since they help to define at the molecular level the antigen(s) implicated in the reaction. Thus, this approach has shown the presence of antigenic determinants of 80, 63, 56, 46, 43 and 36 kD (Lindenborn-Fotinos, Baum & Berg, 1985), of 70 and 45 kD (Frazer *et al.*, 1985) and 70 and 60 kD (Mendel-Hartvig *et al.*, 1985) in the case of PBC. However, clearly defined antigenic determinants implicated in the reaction remained to be revealed until now.

In this work, we have tried to compare and evaluate the results of IF, CFT with ELISA and Immunoblot techniques. Double-dimension gel electrophoresis was also used to define better the antigens reacting with M2 antibodies.

MATERIALS AND METHODS

Mitochondrial preparations. A mitochondrial suspension employed for CFT (Diagnostics Pasteur, France) was the antigen source. This preparation is obtained from Lewis rat kidneys according to a classical procedure. The different proteins contained in this lyophilized preparation were analysed by 12·5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions (Fig. 1. Track B).

Sera. Four groups of sera were studied. *Group 1:* (normal control group) contained 76 sera from Blood Donors of the Blood Bank at the Pasteur Institute. *Group 2:* (pathological control group) comprised 32 hepatitis B surface antigen (HBs) positive sera without any sign of CAH or PBC, 15 sera from patients with active systemic lupus erythematosus (SLE), 15 sera from rheumatoid arthritis (RA) patients in active phase, and 23 sera from patients with VDRL positive reaction. *Group 3:* 27 sera supplied by the 'Laboratoire Central d'Immunologie et d'Hématologie' from Saint-Antoine Hospital, Paris (Professor J.C. Homberg). These sera originated from 12 PBC cases, two sera from patients with an AMA type 5 and respectively exhibiting an Evans syndrome and leukopenia, six sera from patients exhibiting chronic hepatitis (displaying high titres of anti-smooth muscle antibodies in four cases and high titres of anti-liver kidney microsomal (LKM) type 1 (Homberg *et al.*, 1984) in one case and another case of hepatitis in an HBs Ag and delta (δ) antigen carrier with δ antigen-associated microsomal autoantibodies (Crivelli *et al.*, 1983)). Two sera were from patients suffering from chronic graft-versus host disease, one of which had hepatitis with high titres of anti-LKM1 antibodies. The remaining sera originated from cases of different pathological conditions. These sera were studied blindly without any knowledge of diagnosis or immunofluorescent results obtained by the Saint-Antoine Laboratory. All these sera were tested for AMA, antinuclear antibody (ANA), anti-smooth muscle antibody (SMA), anti-microsomal antibodies (LKM) by using 6 μm frozen sections of rat kidney, liver and stomach composite blocks. They were also tested against double and single stranded DNA (dsDNA and ssDNA), by ELISA methods as previously described (Dighiero *et al.*, 1986). *Group 4:* 523 sera referred to the Laboratoire d'Immuno-Pathologie of Institut Pasteur for screening of AMA.

Complement fixation test (CFT). CFT was performed by using sheep red blood cells sensitized by rabbit anti-sheep antibody as the haemolytic detector and commercial guinea-pig sera as the source of complement (Walker *et al.*, 1965). The antigen source was the same as that used for ELISA and Immunoblot.

Enzyme-linked immunosorbent assays (ELISA). Polystyrene microtitre plates (NUNC, Denmark) were coated with 100 μl of the mitochondrial antigen described above at a protein concentration of 10 μg/ml in Mayer Buffer pH 7·3 overnight at 4°C. Antigen-coated plates were

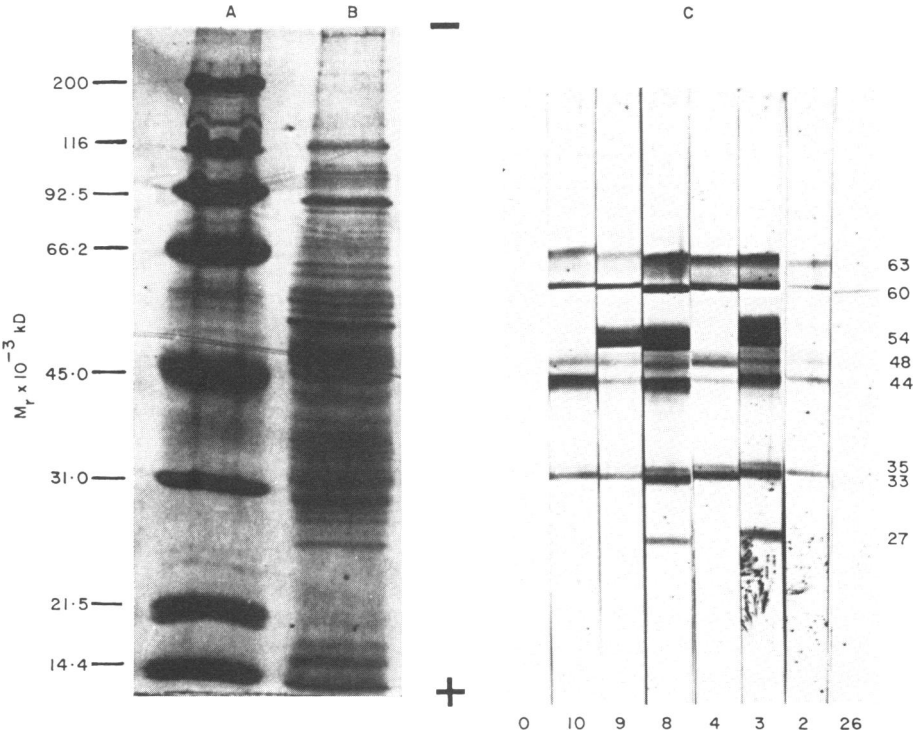

Fig. 1. Coomassie stained SDS-page of the mitochondrial preparation, and Western-blot pattern after staining with diaminobenzidine. Track A, Coomassie display of molecular weight markers (200,000, 116,000, 92,500, 66,200, 45,000, 31,000, 21,500 and 14,000 daltons; Bio-Rad, California, USA). Track B Coomassie display of the mitochondrial preparation. Track C Western-blot pattern of PBC sera (No 10, 9, 8, 4, 3, 2, 26) of the third group and one control serum (sample 0). Sera No 3 and 8 displayed broad reactivity with 63–60, 54, 48, 44, 35–33 and 27 kD polypeptides, whereas sera Nos 2, 4, 9 and 10 bound to 63–60, 48, 44 and 35–33 kD and serum No 26 corresponding to a PBC with an atypical pattern of AMA showed slight binding with 63–60 and 35–33 kD polypeptides. Lane 0 shows normal control serum.

thoroughly washed with phosphate-buffered saline (PBS) containing 0·1% Tween-20 (PBS-T) and were incubated with each serum at 1/200 in PBS-T containing 0·5% gelatin (PBS-T-G) overnight at 4°C. A positive serum at 1/200, 1/400 and 1/800 dilutions and a negative serum at 1/200 dilutions were introduced in each plate as controls. After washings with PBS-T, the plates were incubated for 2 h at 37°C with β-glactosidase labelled sheep anti-human Ig antibody (Biosys, France) at 1·25 μg/ml in PBS-T-G. An *o*-nitrophenyl β-D-galactopyranoside substrate (Sigma Chemical Co, Saint-Louis, MO) was added after washings and optical densities were measured by a Titerteck Multiskan (Dighiero *et al.*, 1986).

SDS-PAGE, two-dimensional gel electrophoresis and immunoblotting of mitochondrial polypeptides. PAGE was carried out on 1 mm thick slab gels in 0·1% SDS by using a 4% stacking gel and 12·5% resolving gel. The mitochondrial antigens at a 4 mg/ml protein concentration, were analysed in SDS-PAGE under reduced conditions according to the procedure described by Laemli (1970). In some cases, two-dimensional gel electrophoresis according to the procedure described by O'Farrell (1975), was performed.

Serum reactivity with mitochondrial polypeptides. Blotting of SDS-PAGE gel, after two-dimensional gel electrophoresis onto nitrocellulose papers, was carried out as previously described (Dighiero *et al.*, 1983). Sera were diluted at 1/100 in PBS-T-G. The second antibody was peroxidase labelled anti-human Ig (Biosys, France) at a final concentration of 2·5 μg/ml. The activity was revealed by diaminobenzidine substrate.

Table 1. Results observed with control series in IF, CFT and ELISA

		AMA determined by						
				ELISA (MBI%)				
Sera	No.	IF	CFT	≤20%	20–40%	40–60%	60–80%	>80%
Blood donors	76	0	0	71	5	0	0	0
HBs positive	32	0	0	29	2	1	0	0
VDRL positive	23	0	0	19	4	0	0	0
SLE	15	0	0	6	1	5	1	2
RA	15	0	0	11	1	2	1	0

Trypsin treatment of mitochondria. The antigen preparation adjusted to a concentration of 4 mg/ml was incubated for 1 min at 37°C in presence of a 0·01% trypsin solution (porcine pancreas trypsin type IX, SIGMA Chemical Co, St Louis, MO) in Sörensen buffer pH 7·3. After denaturation for 2 min at 100°C the preparation was migrated in SDS-PAGE, blotted on nitrocellulose and incubated with sera as described above.

RESULTS

Control group sera. All these sera were studied by IF, CFT and ELISA for the presence of AMA and results are summarized in Table 1. An optimal antigen concentration of 10 μg/ml of protein content was previously established and routinely used in ELISA tests. Results are expressed as mitochondrial binding index (MBI) calculated as follows:

$$\frac{\text{OD test serum} - \text{OD of pool control sera}}{\text{OD positive control} - \text{OD of pool control sera}} \times 100$$

OD ranging from 0·010 to 0·180 were observed for the 76 negative control sera; as compared to OD of about 0·700 for known positive controls. As none of the negative normal control sera exceeded an OD of 0·180 and an MBI >40%, we adopted this threshold to score a serum as positive, and included as negative control a pool of normal sera giving an OD of approximately 0·060. Only one of the 32 HBs positive sera exhibited an MBI between 40 and 60%, whereas none of the 23 sera from patients with syphilis showed an MBI >40% (Table 1). Eight of the 15 sera from SLE patients and three of the 15 sera from RA patients showed an MBI >40%. Thirty out of the 76 normal control sera, 10 sera from the HBs positive sera, five from the syphilis series, 10 from the SLE and five from the RA series were studied by immunoblot. All sera exhibiting an MBI >40% were included. Normal control sera, as well as the HBs and syphilis sera failed to display any significant binding. Two RA sera displaying an MBI of 78% and 53% reacted with the 48 and 44 kD polypeptides whereas three out of the eight SLE sera with a high MBI slightly bound to 54, 42, and 38 kD polypeptides.

Group 3 sera. The 12 PBC sera displayed an MBI >40% (>80% for eight out of 12). Three sera exhibiting lower binding (40–60%), showed an atypical IF pattern, defined by staining of the cytoplasm of distal kidney tubules, thin limb of the loop of Henle and collecting ducts (Table 2). The two sera with anti-M5 reactivity as well as the five sera with other diseases were found to be negative in ELISA, whereas two of the four patients with autoimmune CAH, displaying high levels of anti-SMA (1/500) showed an MBI >40% (54 and 55%). Four sera with high levels of anti-LKM antibodies did not react in ELISA. All the 12 PBC sera reacted with mitochondrial polypeptides by Western-Blot (Table 2). Three sera, showing an atypical pattern in IF, bound to a 44 kD polypeptide in one case, 54 kD in another and 63–60 and 35–33 kD in the third. Nine other sera bound to 63–60 and 35–33 kD, and eight out of these nine sera to 48 and 44 kD polypeptides. On the contrary, the 54 kD polypeptide was only recognized by four sera and the 27 kD by two sera. Figure

Table 2. Results observed with the 12 PBC sera of group 3 in ELISA expressed in MBI, IF, CFT and Western-blot as well as other immunological studies performed with the sera

	AMA determined by											
				Immunoblot (kD)						IF		
Serum number	ELISA (MBI)	CFT	IF	63–60	54	48	44	35–33	27	SMA	ANA	Anti-dsDNA
1	67%	1/16	1/200	+	–	–	–	+	–	1/50	–	–
2	96%	–	1/500	++	–	+	+	+	–	1/50	–	–
3	81%	1/256	1/500	+++	+++	+	+++	++	+++	1/50	–	–
4*	91%	–	1/500	+++	–	++	+	++	–	1/50	1/40	–
5	98%	–	1/500	+	–	±	±	+	–	–	–	–
8	87%	> 1/256	1/1000	+++	+++	+	+++	++	+++	1/20	1/50	–
9	108%	1/256	1/1000	++	+++	+	+	+	–	–	–	–
10†	103%	1/32	1/100	++	–	+	+++	+	–	–	1/1000	–
13	101%	1/8	1/200	++	–	+	+	+	–	–	–	–
7‡	47%	–	1/100	–	–	–	+	–	–	ND	ND	–
11‡	53%	–	1/500	–	+	–	–	–	–	1/10	–	–
26‡	49%	–	1/1000	+	–	+	–	+	–	1/10	–	–

* Associated primary systemic sclerosis (PSS).
† Associated PSS and anti-RNP antibodies.
‡ Atypical pattern of AMA in IF.

1 shows the Coomassie display of the mitochondrial preparation and the results observed by Western-blot for seven of these sera.

As M2 antigen has been defined to be trypsin-sensitive and M4 to be resistant to this enzyme, our mitochondrial preparation was treated with 0·01% trypsin. The results observed with serum No 8 are shown in Fig. 2, where it can be seen that after trypsin treatment, only two bands of 38 and 33 kD are noticed as compared to the reactivity with 63–60, 54, 48, 44, 35–33 and 27 kD bands observed before this treatment. Furthermore, similar results were obtained for serum No 10.

A further characterization of the polypeptides involved in the reactions, was obtained by immunoblot experiments after two-dimensional gel electrophoresis (Fig. 3). Briefly, these experiments showed that all polypeptides evident on SDS-PAGE were recognized by PBC sera, and that the isoelectric differences within some of these polypeptides probably reflected glycosylation differences between them. CFT was performed with the 27 sera: six out of the 12 PBC were found to be positive, while the test was always negative for all sera not belonging to the PBC group. All the three cases giving an atypical IF pattern failed to display reactivity on CFT (Table 2). Finally, anti-dsDNA autoantibodies were not detected in the 27 sera.

Group 4 sera. Four hundred and eighty-four out of the 523 sera of this group were negative for AMA by ELISA, CFT and IF. Thirty-nine sera displayed an MBI > 40% in ELISA. Only 13 out of these 39 sera were found to be positive by IF and/or CFT. Immunoblot could be performed for 32 out of 39 sera. Three out of the 17 sera with an MBI between 40 and 60%, five out of seven with an MBI between 60 and 80% and seven out of eight with an MBI > 80% displayed a typical pattern on Western-blot (63–60, 48–44, 35–33 kD). The results observed with these 15 sera displaying a typical pattern on immunoblot are summarized in Table 3.

DISCUSSION

Immunofluorescence is a routinely used technique to detect AMA. It requires particular skill in the observer to distinguish the characteristic staining pattern of these autoantibodies. Other methods

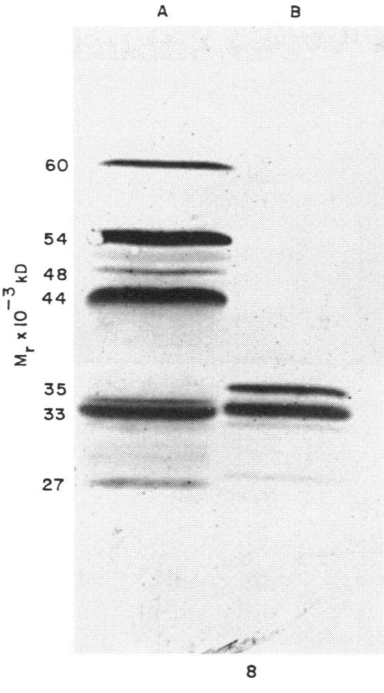

Fig. 2. Western-blot pattern with serum No 8 (A) before and (B) after treatment of mitochondrial preparation with trypsin. After enzyme treatment, two polypeptides of 38 and 33 kD are seen, as compared to the pattern (63–60, 54, 48, 44, 35–33 and 27 kD) obtained before treatment.

like CFT (Walker *et al.*, 1965), FIAX (Klein *et al.*, 1983), ELISA (Kenna *et al.*, 1984) and Western-blot (Lindenborn-Fotinos *et al.*, 1985; Frazer *et al.*, 1985; Mendel-Hartvig *et al.*, 1985), have also been proposed. In the present work, we have compared and evaluated results obtained from IF, CFT, ELISA and Immunoblot techniques. Furthermore we employed the last technique along with SDS-PAGE and two-dimensional gel electrophoresis, to define better the antigen recognized by PBC sera. Results observed with IF in a small number of PBC patients agree with other reports (Berg *et al.*, 1986) but the results with CFT are lower than the 81% reported by Berg *et al.* (1986). However, these data are too limited to allow any definite conclusion. Interestingly, the ELISA technique developed in this work showed positive results with all the sera displaying a positive pattern on IF and/or CFT. These results should be compared to those from Berg *et al.* (1986) indicating a 96% positivity of ELISA, in 757 sera from PBC patients.

An interesting point arising from our results is the discrepancy observed between ELISA and traditional methods like IF and CFT. Five of the 22 sera from Group 4, which were negative on IF and CFT but displayed an MBI >40%, exhibited significant binding with mitochondrial polypeptides in immunoblot. Of 17 other sera, 12 possessed anti-cytoskeleton antibodies (anti-actin, anti-tubulin and/or anti-myosin antibodies; data not shown). The two CAH as well as the HBs carrier, which had an MBI between 40 and 60% but were negative in IF and CFT, did not react with mitochondrial polypeptides in Western-blot. Two out of the three RA sera and three of the eight SLE with an increased MBI, reacted with mitochondrial polypeptides in immunoblot, but none of them displayed the typical pattern found in PBC (63–60 and 35–33 kD). These results should be compared to a recent report (Mouritsen *et al.*, 1986) in which a high prevalence of AMA was found in connective tissue diseases.

Taken together, these results suggest that ELISA appears to be the most sensitive technique for the diagnosis of AMA (27 out of 27 PBC confirmed by Western-blot as compared to 21/27 by IF and

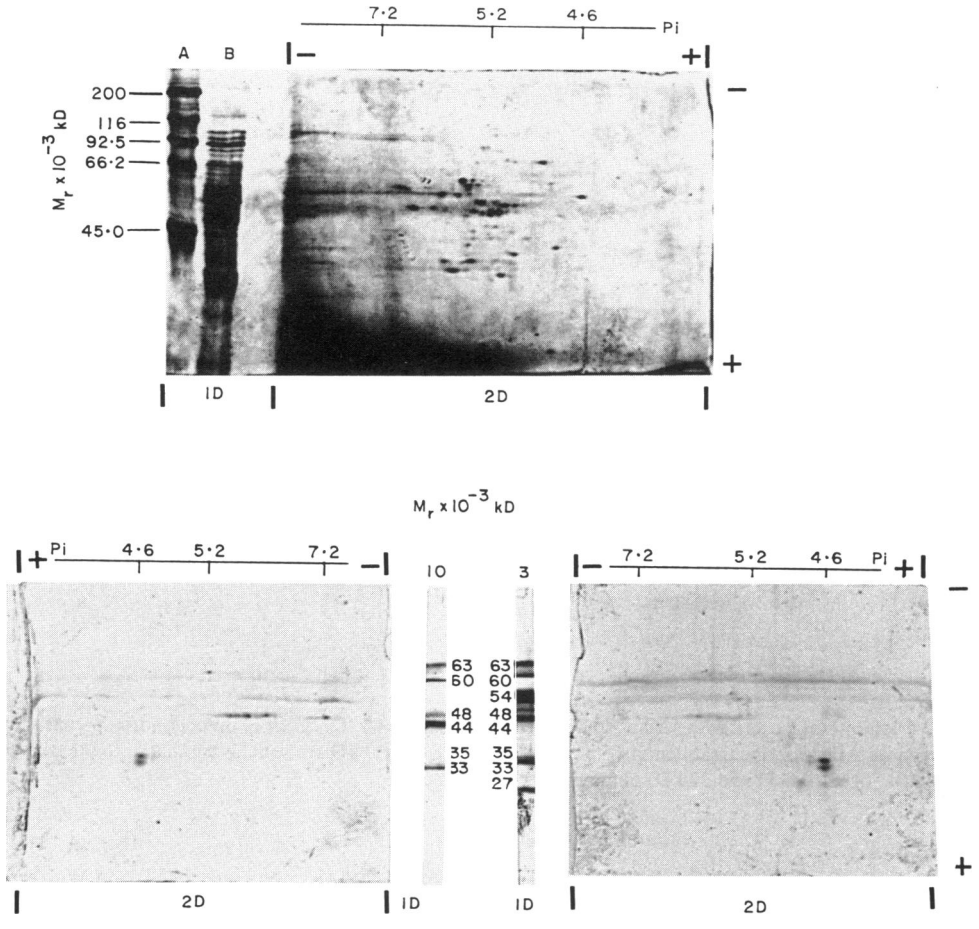

Fig. 3. This picture represents the Coomassie stained two-dimensional gel electrophoresis of mitochondrial polypeptides (top) as well as the results obtained by Western-blot in these conditions with sera Nos 3 and 10 of the third group (bottom). Both the 63 and 60 kD antigens appeared to relate to an isoelectric point of 5·2, the 54 kD to two polypeptides of 5·3 and 5·5 isoelectric point, both reacting with positive PBC sera; the 48 and 44 kD to four different polypeptides of 5·3, 5·4, 5·8 and 7·2 isoelectric point and also in this case, these four polypeptides bound to positive sera; the 35 and 33 kD to a single polypeptide of isoelectric point 4·6 and the 27 kD to three close isoelectric point polypeptides (4·5, 4·6 and 4·7) which all reacted with positive sera. The slightly different isoelectric points found for these polypeptides as well as their constant binding with positive sera suggest some glycosylation differences between them.

16/27 by CFT) and confirm previous results from other groups (Kaplan *et al.*, 1984; Berg *et al.*, 1986). However, the two cases displaying an M5 pattern could not be detected by ELISA. The claimed phospholipid nature of this antigen (Merroni *et al.*, 1986; Norberg *et al.*, 1984) might explain the negative results because of the systematic use of detergents in the ELISA methods.

Although, ELISA is very sensitive and capable of detecting true AMA that are not detected by IF and CFT, yet it showed false positive results with our purified rat kidney mitochondrial preparation. Some of these false positive results could be because of contamination by microsomal fractions, but this appears unlikely, since all the four sera with high levels of anti-LKM antibodies were negative in ELISA. Although only eight of the 15 SLE sera with high levels of anti-dsDNA antibodies reacted in ELISA, the possibility that this binding could correspond to reaction with DNA contaminating our mitochondrial preparation could not be completely ruled out.

Table 3. Results observed with 15 out of the 39 sera from group 4 exhibiting an MBI > 40% and displaying an immunoblot pattern compatible with an M2 antibody

Serum number	ELISA (MBI)	AMA determined by		Immunoblot (kD)					
		CFT	IF	63–60	54	48	44	35–33	27
1	56%	−	−	++	−	−	±	++	−
2	43%	1/32	−	++	−	+	++	++	−
3	46%	−	−	+++	±	+	++	++	−
4	62%	−	−	+	+++	−	−	++	−
5	77%	1/256	1/300	+++	−	++	++	++	−
6	72%	−	−	+	−	+	+	+	−
7	70%	1/256	1/300	+++	−	+	++	++	−
8	77%	1/256	1/600	+++	+++	+	++	++	+
9	90%	−	−	+++	−	++	++	++	−
10	90%	1/128	1/100	+++	+	±	++	++	−
11	85%	1/256	1/100	+	−	−	±	+	−
12	105%	1/256	1/300	+++	+++	+	++	++	+
13	100%	1/128	1/100	+++	±	+	++	++	−
14	99%	1/32	1/100	+++	−	++	++	++	−
15	96%	1/256	1/300	+++	±	+	++	++	−

Another important aim of the present work was to define better the antigenic determinants implicated in AMA type M2 by Western-blot techniques. Our results indicate that when purified rat kidney mitochondrial polypeptides were employed, polypeptides of 63–60 and 35–33 kD were consistently found in the nine typical cases of PBC and polypeptides of 48 and 44 kD in eight out of nine. The 54 kD polypeptide was also found in four cases, while the 27 kD polypeptide in only two cases. However, two out of the three PBC sera exhibiting an atypical immunofluorescence pattern did not bind to the 63–60, 35–33 kD polypeptides but to the 54 and 44 kD polypeptides. Taken together, these results indicate some heterogeneity, as far as mitochondrial polypeptides recognized by PBC sera are concerned. This relative heterogeneity is close to the results observed by Lindenborn-Fotinos *et al.* (1985) and differs from the results of Frazer *et al.* (1985) (70 and 45 kD) and Mendel-Hartvig *et al.* (1985). It is unlikely that the use of different mitochondrial preparations could explain these differences since Lindenborn-Fotinos *et al.* and Mendel-Hartvig *et al.* used beef heart mitochondrial preparations. M2 specificity is directed against a trypsin-sensitive protein of the inner mitochondrial membrane. Our results indicate that most of the polypeptides recognized by PBC sera were sensitive to this enzymatic treatment. The 33 kD polypeptide alone seemed to be insensitive to trypsin, yet the possibility that it might be a degradation product remains to be ruled out. Indeed, outer purified mitochondrial membrane polypeptides are needed to determine the relationship of this 33 kD polypeptide and M4 specificity.

M2 has been claimed previously to relate to two mitochondrial proteins, the β sub-unit of the proton pump dependent mitochondrial ATPase (Lindenborn-Fotinos, Sayers & Berg, 1982) and the 30 kD adenine translocator protein (Schulteiss, Berg & Klingenberg, 1983). The frequent binding with 48, 44 and 33 kD, could correspond to recognition of β and γ sub-units of H^+ATPase, whereas the less frequently found 54 and 27 kD could indicate binding with α sub-units of H^+ATPase and adenine translocator protein. However, Lindenborn-Fotinos *et al.* (1985) and Frazer *et al.* (1985) with Western-blot techniques have excluded such a possibility. PBC is considered by some as an organ specific autoimmune disease; others regard AMA as a secondary autoantibody. Our results with purified rat mitochondrial preparation indicate that the antigen is not liver specific. They also show that there is a wide range of reactivity with the different polypeptides. Whether this heterogeneous reactivity could be related to the different clinical and

histological pictures of PBC is an important question that cannot be answered by the data on the limited number of patients examined here.

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