Clin. exp. Immunol. (1987) 67, 484–491

Anti-mitochondrial type M5 and anti-cardiolipin antibodies in autoimmune disorders: studies on their association and cross-reactivity

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(Accepted for publication 6 October 1986)

SUMMARY

In a series of 42 positive sera, anti-mitochondrial type M5 antibodies (AMA-M5) were found most frequently in patients with SLE (24) and SLE-like syndromes (10). Patients with AMA-M5 displayed a higher prevalence of thrombocytopenia, thrombosis, biological false positive seroreactions for syphilis, lupus-like anticoagulant activity and anti-cardiolipin antibodies in comparison with a group of 43 SLE AMA-M5 negative patients. The strong association between anti-phospholipid and AMA-M5 antibodies cannot be explained entirely by cross-reactivity between these two groups of antibodies, as indicated by absorption experiments and studies using affinity purified antibody preparations. However, cardiolipin liposomes were able to reduce partially the titres of AMA-M5 sera, suggesting that a small population of AMA-M5 antibodies exists that cross-reacts with cardiolipin. The existence of this population was further substantiated by our demonstration that an IgM monoclonal antibody, from a patient with Waldenström's macroglobulinaemia, displayed both anti-cardiolipin and AMA-M5 activity, and AMA-M5 activity was completely inhibited by cardiolipin.

Keywords anti-mitochondrial antibodies anti-cardiolipin antibodies systemic lupus erythematosus

INTRODUCTION

Anti-mitochondrial autoantibodies (AMA) have been recognized as an heterogeneous group of antibodies occurring in a variety of diseases (Meek *et al.*, 1980). Seven different types of AMA have been identified, according to antigen specificity and their association with different clinical syndromes (Meek *et al.*, 1980; Homberg *et al.*, 1982; Klein *et al.*, 1984). After the first description by Labro *et al.* (1978), the anti-mitochondrial type M5 antibody (AMA-M5) has been recently reinvestigated and identified in a few patients with systemic connective tissue diseases and in Lupus-like syndromes (Norberg *et al.*, 1984; Tincani *et al.*, 1985). We have recently demonstrated, in a group of patients with systemic lupus erythematosus (SLE), a strong association between AMA-M5 and the presence of anti-phospholipid antibodies (Tincani *et al.*, 1985).

In this report we describe the clinical and laboratory features of a larger group of AMA-M5 positive patients, collected for routine testing of various autoantibodies in our laboratory. We have

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confirmed the association between AMA-M5 and anti-phospholipid antibodies, and we also report the results of absorption experiments indicating that this association is not entirely due to cross-reacting antibodies, even if a subpopulation of AMA-M5 does seem to bind cardiolipin.

MATERIALS AND METHODS

Patients. We studied sera from 42 patients displaying AMA-M5 immunofluorescence (38 females and four males; mean age ± s.d.: 30·5 ± 13·4 years) collected from sera sent to our laboratories for routine testing for autoantibodies.

As a control group we studied AMA-M5 negative sera from 43 SLE patients, who satisfied the revised American Rheumatism Association classification for SLE (Tan *et al.*, 1982). This group consisted of 39 females and four males; mean age ± s.d.: 33·5 ± 11·6 years.

Immunofluorescence technique. Patients' sera were tested by a standard indirect immunofluorescence technique (IFL) on 5 μm thick unfixed frozen sections of human stomach, pancreas, rat liver and kidney, as previously described (Meroni *et al.*, 1984).

The following sera were used as control: (1) AMA-M5. Mitochondrial autoantibodies type M5 were identified by their characteristic pattern on rat kidney, in which proximal tubules in the outer cortex were brighter than distal tubules, and by the lack of staining on gastric parietal cells (Labro *et al.*, 1978; Meek *et al.*, 1980). An AMA-M5 positive serum, kindly provided by Professor D. Doniach and Dr G. F. Bottazzo, was used as reference. (2) AMA-M2. The sera were chosen from typical primary biliary cirrhosis cases. They gave strong fluorescence on renal distal tubules, weak fluorescence on proximal tubules and stained gastric parietal cells (Meek *et al.*, 1980). The mitochondrial autoantibodies type M2 are directed against an antigen present in the chloroform ATPase preparations (Ben-Yoseph, Shapira & Doniach, 1974; Sayers & Baum 1976). (3) AMA-M1. The sera were from patients with secondary syphilis and contained cardiolipin fluorescent antibodies as described by Wright *et al.* (1970) and Doniach *et al.* (1971). The fluorescence was similar to the M2 type and was easily absorbed by cardiolipin as previously reported (Wright *et al.*, 1970; Meek *et al.*, 1980).

Anti-cardiolipin antibodies. Anti-cardiolipin antibodies (ACA) were performed according to Harris *et al.* (1983) with minor modifications. Briefly, cardiolipin (Sigma Chemicals, MO, USA; 25 μl, 50 μg/ml) was coated on polystyrene microtitre wells (Indywell, Greiner, Germany) by evaporation under nitrogen. The plates were blocked for 2 h and washed three times with 10% fetal calf serum in phosphate buffered saline (FCS-PBS). The sera, diluted 1/50 in FCS-PBS, were added to plates and incubated for 4 h at room temperature. After three washes with FCS-PBS, affinity purified ^{125}I-labelled rabbit antibodies against human IgG or IgM were added. The plates were incubated overnight, washed three times, and then the wells were counted. Results were expressed in ng of anti-human IgG or IgM bound. Values greater than three standard deviations above the mean of 40 normal sera were considered raised: namely 3 ng bound for IgG and 4·2 ng bound for IgM. The pool as well as three out of these 40 normal sera were included in each run as reference.

Antibodies to double stranded DNA (ds-DNA). Anti-DNA antibodies were detected by the Farr assay as previously described (Tincani *et al.*, 1985). Results were given as percentage of antigen bound; the upper limit of normal (mean + 3 s.d.) was 25%.

Venereal disease reference laboratory test. This was performed by the commercial slide flocculation test (Wellcome Foundation Limited, UK).

Lupus like anticoagulant activity. This was detected as previously described (Tincani *et al.*, 1985).

Mitochondrial preparations. Rat liver mitochondria were prepared by gradient centrifugation with modifications of the technique of Gaja *et al.* (1973) as previously described (Meroni *et al.*, 1984). The mitochondria were finally suspended in PBS and disrupted by extensive sonication to expose the inner membranes. Protein content was measured by the method of Lowry *et al.* (1951) and contamination by non-mitochondrial subcellular structures was checked by electron microscopy.

Mitochondrial phospholipid preparations. Phospholipids were extracted from mitochondria

using the Folch technique (Folch, Lees & Stanley, 1957) with minor modifications. A 3 ml suspension of mitochondria, prepared as described above, was first sonicated for 2 min twice in an ice-bath. The sonicated preparation was treated with 5 ml of methanol for 5 min; then 10 ml chloroform was added, the mixture was vortex-mixed for 2 min, and placed on an end-over-side mixer for 1 h at room temperature.

Subsequently, the mixture was centrifuged at 2,000 *g* for 10 min, and the supernatant decanted. The extraction cycle was repeated three times and the supernatants pooled. The pooled supernatants were evaporated to dryness under nitrogen. The dried phospholipids were resuspended in 1 ml 0·15 M NaCl and sonicated for 2 min twice in an ice-bath. The phosphorus content was measured according to Bartlett (1959), and expressed as micromoles.

Absorption experiments. Absorption experiments were performed using the following antigens: (a) rat liver mitochondria preparations at protein concentrations ranging from 0·31 to 10 mg/ml; (b) cardiolipin liposomes prepared according to Harris *et al.* (1985 b, c) at concentrations ranging from 0·39 to 25 mg/ml in 0·15 M NaCl; (c) mitochondrial phospholipid liposomes at phosphorus concentrations ranging from 511·4 μmol to 31·9 μmol.

Briefly, the sera were mixed with the different antigens, incubated 1 h at 37°C and overnight at 4°C. The mixtures were then centrifuged at 30,000 *g* for 15 min at 4°C, and the supernatants kept as absorbed sera.

Affinity purified anti-cardiolipin antibodies. Affinity purified ACA were prepared from patient sera using cardiolipin liposomes as previously described (Harris *et al.*, 1985c).

Statistical analysis. Statistical analysis was by the chi-square test with Yates' correction.

RESULTS

AMA-M5: clinical and laboratory associations. Forty-two AMA-M5 positive patients were studied (titres ranging from 1/10 up 1/640). Twenty-four were diagnosed as SLE, according to the revised ARA criteria (Tan *et al.*, 1982), and 10 patients, presenting with fewer than four clinical and laboratory features of SLE, as 'Lupus-like' syndromes. In the remaining patients the following diagnoses were made: two rheumatoid arthritis, one thyroiditis, two recurrent fetal loss, one drug addict without liver disease, one Waldenström's macroglobulinaemia, and one autoimmune haemolytic anaemia.

Since the majority of AMA-M5 positive patients were classified as SLE or 'Lupus-like' syndrome, we compared them to a control group of 43 SLE patients without AMA-M5. As shown in Table 1, the patients with anti-mitochondrial antibodies displayed a higher prevalence of thrombocytopenia and thrombosis. It is noteworthy too that AMA-M5 positive women with a

Table 1. Relationship between AMA M5 and clinical features

	AMA M5 positive		AMA M5 negative		
	Proportion	%	Proportion	%	*P*
History of thrombosis	15/33	45·4	9/39	23.1	*
Thrombocytopenia	12/31	38·7	4/39	10·3	**
Skin involvement	10/25	40·0	30/39	76·9	***
Renal disease	8/26	30·8	17/39	43·6	NS
CNS involvement	12/24	50·0	16/38	42·1	NS
Obstetric problems	9/17	52·9	9/23	39·1	NS

Proportion, affected patients/investigated patients.
* $0·05 < P < 0·1$; **: $P < 0·05$; ***: $P < 0·01$.
NS, Not significant.

Table 2. Previous obstetric histories of nine AMA M5 positive women

Patient	Diagnosis	Pregnancies	Spontaneous abortions (at < 12 wk)	Fetal deaths (at > 12 wk)	Eclampsia Eclampsia	Live-born infants	ACA	LLAC	VDRL
F.M.	SLE-LS	2	1	1	—	0	neg	neg	neg
C.A.	SLE	2	0	0	1	2	pos	pos	ND
C.C.	SLE	4	1	0	—	3	neg	pos	neg
B.E.	SLE	5	1	3	—	1*	pos	pos	pos
B.T.	SLE	7	0	6	—	1	pos	pos	pos
K.N.	SLE	2	1	1	—	0	pos	pos	pos
E.D.	RFL	2	1	1	—	0	pos	pos	ND
R.G.	RFL	3	0	2	—	1*	pos	pos	pos
R.I.	SLE-LS	2	0	1	—	1†	pos	pos	pos

SLE, Systemic lupus erythematosus; SLE-LS, SLE-like syndrome; RFL, recurrent fetal loss; ND, not determined.

* On therapy with prednisone and azathioprine.

† Small for gestational age, dead 1 week after the delivery.

history of pregnancy had an increased prevalence of serious obstetrical problems compared to the SLE AMA-M5 negative group (see Table 1 and Table 2). On the other hand skin involvement was less frequent in the AMA-M5 positive patients (see Table 1).

In Table 3 we report the association between AMA-M5 and the other autoantibodies. It can be seen that AMA-M5 were strongly associated with the presence of anti-phospholipid antibodies, namely ACA, LLAC, and a biological false positive seroreaction for syphilis.

Fourteen out of 39 AMA-M5 positive sera did not display anti-cardiolipin activity. Furthermore, using class specific anti-human Ig FITC labelled antisera, we did not find the same Ig-class pattern in 12 out of 19 AMA-M5 and ACA positive sera (see Table 4). These observations suggested that there might be differences between anti-cardiolipin antibodies detected by the radio-immuno assay and the antibodies responsible for the AMA-M5 immunofluorescence pattern. To examine this question further, absorption experiments were performed with different antigens.

Absorption experiments. Six ACA and AMA-M5 positive sera were absorbed with sonicated rat liver mitochondria (protein concentration 10 mg/ml). In all the samples the cytoplasmic fluorescent staining was completely absorbed, while the nuclear fluorescence, present in three sera, was not

Table 3. Relationship between AMA M5 and other autoantibodies

	AMA M5 positive		AMA M5 negative		
	proportion	%	proportion	%	*P*
LLAC	19/25	76·0	4/22	18·2	**
VDRL	19/31	61·3	6/36	16·7	**
ACA (IgG and/or IgM)	25/39	64·1	17/43	39·5	*
Anti ds-DNA	23/34	67·6	25/43	58·1	NS
Positive direct Coombs' test	12/23	52·2	8/16	50·0	NS

Proportion, number of positive sera/number of tested sera.

* $P<0·05$; ** $P<0·01$.

NS, Not significant.

Table 4. Pattern of isotypes of AMA-M5 and ACA in AMA-M5 and ACA positive sera

No. of patients	AMA-M5		ACA		
	IgM	IgG	IgM	IgG	
5	+	+	+	+	
1	–	+	–	+	Same isotype
1	+	–	+	–	
6	+	+	–	+	
2	+	+	+	–	
2	–	+	+	+	Different isotype
1	+	–	–	+	
1	–	+	+	–	

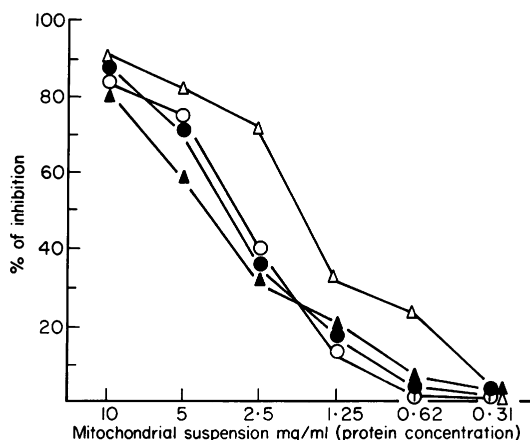

Fig. 1. Absorption of the anti-cardiolipin activity with a mitochondrial suspension in four ACA and AMA-M5 positive sera. (△) Patient M.F., IgG ACA; (○) patient S.M., IgG ACA; (●) patient D.I., IgM ACA; (▲) patient H.I., IgM ACA.

affected (data not shown). The same mitochondrial suspension was also able to reduce, in a dose-dependent manner, the anti-cardiolipin activity (Fig. 1).

In order to investigate whether the phospholipids present in the mitochondrial suspension were responsible for this inhibitory activity, the sera were absorbed with a phospholipid suspension obtained by chloroform-methanol extraction from rat liver mitochondria. The mitochondrial phospholipids had no effect on the AMA-M5 fluorescence, but inhibited the anti-cardiolipin activity in a dose-dependent manner (Fig. 2).

Since contrasting observations have been reported in the literature (Norberg *et al.*, 1984; Tincani *et al.*, 1985) about the ability of cardiolipin to inhibit AMA-M5 fluorescence, further absorption experiments were performed using cardiolipin liposome suspensions at different concentrations. The cardiolipin liposomes displayed only a partial inhibition of the AMA-M5 fluorescent staining, even at the highest cardiolipin concentrations (Fig. 3).

The specificity of the absorption test was supported by the fact that no cardiolipin concentration affected either high titres of anti-nuclear antibodies present in the AMA-M5 sera tested or the anti-mitochondrial type M2 fluorescence in two control sera from patients with primary biliary cirrhosis (data not shown). In addition, the lowest cardiolipin concentrations completely inhibited anti-

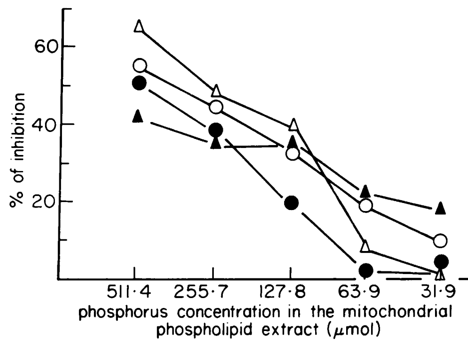

Fig. 2. Absorption of the anti-cardiolipin activity with mitochondrial phospholipid extract in four ACA and AMA M5 positive sera. Symbols as Fig. 1.

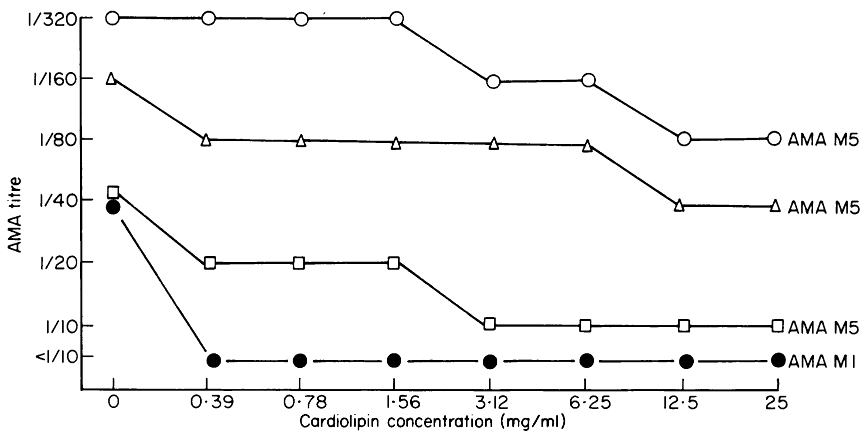

Fig. 3. Absorption of three AMA M5 and ACA positive SLE sera (○, △, □) and one AMA M1 positive serum (●), from a patient with secondary syphilis, with increasing cardiolipin liposome concentrations.

mitochondrial type M1 antibodies (Fig. 3), which are known to be directed against cardiolipin (Meek *et al.*, 1980).

Studies with monoclonal anti-cardiolipin antibodies. We tested by indirect immunofluorescence two monoclonal IgM anti-cardiolipin antibodies, one from a patient with Waldenström's macroglobulinaemia and another from a patient with hairy cell leukaemia. Only the IgM K Waldenström macroglobulin was able to stain the kidney sections, when tested by anti-μ or anti-k specific FITC labelled antisera. This cross-reactivity was further confirmed by the following experiments: (a) affinity purified IgM Waldenström anti-cardiolipin macroglobulin still reacted with mitochondria when tested by immunofluorescence; (b) cardiolipin lipsomes (3 mg/ml) completely inhibited either ACA activity or the AMA-M5 fluorescence.

DISCUSSION

Our results confirm and extend in a larger series of patients the association between AMA-M5 and SLE-like syndromes (Norberg *et al.*, 1984; Tincani *et al.*, 1985). Twenty-four of 42 AMA-M5 positive patients satisfied the ARA criteria for diagnosis of SLE, and 10 subjects presented a 'Lupus-like' syndrome.

When compared to a control group of AMA-M5 negative SLE, these AMA-M5 autoantibodies identified a population of patients characterized by a higher prevalence of thrombocytopenia and thrombosis, and less frequent skin involvement. It was interesting too that nine out of 17 AMA-M5 positive women had had obstetric problems during pregnancy. This suggests that the anti-mitochondrial type M5 antibodies, like the LLAC and ACA, may identify women prone to obstetric complications. It was noteworthy that one AMA-M5 positive patient, F.M., had a poor obstetrical history although her sera did not contain LLAC and ACA (Table 2).

Anti-phospholipid antibodies, in particular antibodies with LLAC and ACA activities, have been shown in studies from several centres to be associated with thrombosis, fetal loss and thrombocytopenia (Harris *et al.*, 1985a). The clinical features of AMA-M5 positive patients described in this study closely resemble those with anti-phospholipid antibodies, strongly suggesting an association between the occurrence of these two groups of autoantibodies.

The question arises whether this association could be explained by cross-reactivity between anti-phospholipid and AMA-M5 antibodies, particularly since it is known that mitochondria contain cardiolipin (Ernster & Schatz, 1981).

It should be noted that different Ig isotypes were found in some ACA and AMA-M5 positive sera. This finding may suggest a difference between the two antibody groups, even if the assay systems used (RIA and IFL) could not be comparable with regard to the affinity and the avidity of the detected antibodies.

To investigate the possible cross-reactivity we performed absorption experiments with different antigens. We found that mitochondrial membranes were able to absorb AMA-M5 fluorescence completely and were also able to inhibit, in a dose-dependent manner, anti-cardiolipin activity. On the other hand, a phospholipid extract of mitochondria did not inhibit AMA-M5 immunofluorescence, although this extract was able to inhibit anti-cardiolipin activity in a dose dependent manner. This suggests that the AMA-M5 antigen may not be phospholipid. Comparable results were also reported by Meek *et al.* (1980) who were unable to absorb anti-mitochondrial type M5 fluorescence using chloroform/ATPase preparations from sonicated bovine heart mitochondria.

In view of the above results, we undertook further absorption experiments to determine whether cardiolipin liposomes might affect AMA-M5 fluorescence. We found only a partial reduction of the AMA-M5 titre in sera absorbed with cardiolipin liposomes. In addition, increased cardiolipin concentrations did not result in a clear dose-dependent loss of anti-mitochondrial activity, suggesting that only a small subpopulation of AMA-M5 may cross-react with cardiolipin.

In contrast, the mitochondrial fluorescence AMA-M1, associated with secondary syphilis and believed to be due to antibodies directed against cardiolipin (Meek *et al.*, 1980) were completely inhibited by low cardiolipin concentrations. The specificity of this mention was also apparent by our finding that cardiolipin had no effect on antinuclear fluorescence.

That a small subpopulation of AMA-M5 antibodies bind cardiolipin was confirmed by our finding of a monoclonal IgM antibody from a patient with Waldenström's macroglobulinaemia which displayed complete cross-reactivity between cardiolipin and mitochondria. Affinity purified preparations of this monoclonal IgM antibody using cardiolipin liposomes (Harris *et al.*, 1985c) gave an AMA-M5 immunofluorescence pattern. Both ACA activity and AMA-M5 immunofluorescence were inhibited by low concentrations of cardiolipin.

On the other hand another monoclonal IgM anti-cardiolipin antibody from a patient with hairy cell leukaemia did not give anti-mitochondrial fluorescence, suggesting that the cross-reactivity is not necessarily common to paraproteins with anti-cardiolipin activity.

Taken altogether our data suggest that the clinical and laboratory association between AMA-M5 and anti-phospholipid antibodies is not due to cross-reactivity, even though it is likely that a subpopulation of AMA-M5 antibodies can bind cardiolipin. Why the AMA-M5 antibodies occur in association with anti-phospholipid antibodies, particularly in patients with thrombosis, fetal loss and thrombocytopenia is the subject of ongoing studies in our laboratory.

This work was in part supported by grants from Piano Finalizzato per la Ricerca Biomedica della Regione Lombardia. The authors wish to thank Dr A. E. Gharavi for his helpful comments, Dr C. Colombi for the electron microscopic studies, Dr F. Allegri for the determinations of phosphorus concentrations and Miss A. Cercone for her excellent technical assistance.

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