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Demonstration of organ specific antibodies against heart mitochondria (anti-M7) in sera from patients with some forms of heart diseases

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SUMMARY

Using submitochondrial particles (SMP) from beef heart, pig kidney and rat liver in the ELISA, we detected partial organ specific anti-mitochondrial antibodies (AMA) against heart and kidney SMP in sera from patients with different forms of cardiomyopathies. Serum samples from 50 of 159 patients with congestive or hypertrophic cardiomyopathy (31%) and from two of 15 patients with acute myocarditis (13%) were AMA positive. These AMA could be clearly differentiated from other known AMA (anti-M1–M6) and were therefore named anti-M7. Thirteen of the 52 sera (25%) reacted only with heart SMP (type a) and 39 showed a cross-reaction with kidney, lung and pancreas mitochondria (type b). However, using liver SMP, no positive reaction was found. The anti-M7 type a and b activity was abolished completely by absorption with heart SMP. The anti-M7 antibodies were directed against an antigen which co-purified with the inner mitochondrial membrane and had a molecular weight of 67,000–72,000. They seem to be confined to some forms of cardiomyopathies and myocarditis of unknown aetiology and were not detected in sera from patients with other diseases.

Keywords heart diseases anti-mitochondrial antibodies organ specificity anti-M7

INTRODUCTION

In recent years there is growing evidence that the heart may be affected by autoimmune processes which are directed against autoantigens like heart valve glycoproteins, intercalated discs and several other myocardial antigens (Chang, Friedman & Goldberg, 1979).

A preferential immunofluorescence staining of heart and skeletal muscle was also found in sera from patients with different forms of cardiomyopathies indicating that autoimmune processes may play a role in these diseases (Maisch *et al.*, 1983a).

Schultheiß *et al.* (1983) was able to detect antibodies against the adenine nucleotide translocator (ANT) isolated from beef heart mitochondria in sera from patients with acute myocarditis and congestive cardiomyopathy.

Screening sera from patients with various disorders for anti-M2 antibodies, we were recently able to demonstrate in some sera from patients with cardiomyopathies a partial organ specific reaction with heart mitochondria in the fluorometric immunoassay (Berg *et al.*, 1982; Klein, Lindenborn-Fotinos & Berg, 1983).

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We were, therefore, interested to study in greater detail the nature of this reaction in order to find out whether these patients have in their sera a mitochondrial antibody which can be differentiated from the previously described anti-M1–M6 antibodies (Berg *et al.*, 1981). It will be shown that this heart specific antibody recognizes antigenic determinants present either only on heart mitochondria or on mitochondria from different organs except for liver, skeletal muscle, spleen, and brown fat. These partial organ specific antibodies will, therefore, be named anti-M7 type a and b.

MATERIALS AND METHODS

Patients. Sera from 107 patients with dilated and 52 patients with hypertrophic cardiomyopathy as well as from 125 patients with other cardiac diseases (see Table 1) were examined.

All patients with dilated cardiomyopathy (DC) had undergone routine heart catheterization to exclude coronary artery disease. An increased left ventricular end diastolic volume (LVEDV 100 ml/m^2) was regularly found and consequently pulmonary wedge pressure was increased in 70% of patients (congestion). Their ejection fraction was less than 60%.

Hypertrophic cardiomyopathy (HCM) was diagnosed on the basis of asymmetrical septal hypertrophy either with or without outflow obstruction as detected either by echocardiography and/or heart catheterization. Patients with valvular heart disease and arterial hypertension were excluded.

Acute myocarditis ($n=15$) was diagnosed either by biopsy proven cellular infiltration of the myocardium or pericardial effusion and arrhythmia or a syndrome of global or segmental hypokinesia (echocardiography/angiocardiography) and arrhythmia in the context of a viral infection (Maisch *et al.*, 1982b, 1983).

Patients with postpericardiotomy syndrome ($n=25$) had fever, transient signs of pericarditis, leucocytosis and circulating anti-sarcolemmal antibodies (Maisch, Berg & Kochsiek, 1979).

Similar symptoms were found in patients with post-myocardial infarction syndrome ($n=25$) as in patients with postpericardiotomy syndrome.

Sera from 100 blood donors and from patients with non cardiac disorders served as controls as follows: acute and chronic hepatitis ($n=50$), cryptogenic cirrhosis and alcoholic liver disease ($n=50$), Crohn's disease and ulcerative colitis ($n=20$), collagen disorders ($n=117$), organ and non-organ specific autoimmune diseases ($n=107$), other diseases ($n=85$).

The anti-M7 antibody specificity was analysed using two sera with high anti-M7 type a and four sera with high anti-M7 type b activity (sera from three patients with dilated and from three patients with hypertrophic cardiomyopathy, Fig. 1), and compared with the specificity of the previously described AMA types anti-M1–M6 (Berg *et al.*, 1981) using defined marker sera.

For the detection of anti-M1–M6 the following antigens were used: highly purified cardiolipin (M1) obtained from Serva, Heidelberg (Wright *et al.*, 1970), the ATPase associated antigen (M2; Lindenborn-Fotinos, Sayers & Berg, 1982), outer mitochondrial membranes from rat liver (M3, M4 & M6; Sayers, Binder & Berg, 1979; Berg *et al.*, 1980; Homberg *et al.*, 1982), and inner mitochondrial membranes (M5; Labro *et al.*, 1978).

Anti-M5 and anti-M6 sera were kindly provided by Dr Homberg (Hôpital Saint Antoine, Paris).

To test the purity of subcellular fractions a serum with antibodies directed against liver and kidney microsomes (LKM) was used (Rizzetto, Swana & Doniach, 1973).

Preparation of mitochondrial antigens. Mitochondria, SMP and the supernatant (SN) from SMP of beef heart, pig kidney and rat liver were isolated according to Smith (1976) and Berg *et al.* (1967).

Outer and inner mitochondrial membranes and microsomes were prepared from beef heart, rat liver and pig kidney following the method of Schnaitman & Greenawalt (1968).

A fraction yielding mainly plasma membranes and outer mitochondrial membranes free of inner mitochondrial membranes was isolated from pig kidney according to the method described for the isolation of plasma membranes from rat liver by Behrens & Hollander (1976) and Ray (1970).

In order to test the organ specificity of anti-M7 antibodies cytoplasmic extracts and mitochondria from brown fat of rabbits, beef pancreas, rat lung, stomach, spleen and skeletal muscle were prepared following the method of Berg *et al.* (1967).

Cytoplasmic extracts from heart and kidney were fractionated by isopycnic sucrose density gradient centrifugation as described by Sayers *et al.* (1979).

The different subcellular fractions were tested for purity using marker enzymes for microsomes, outer and inner mitochondrial membranes, and plasma membranes. Succinate–cytochrome C reductase, a marker for inner mitochondrial membranes, rotenone–insensitive NADH–cytochrome C reductase, a marker for outer mitochondrial membranes (Sottocasa *et al.*, 1967), as well as glucose-6-phosphatase, a marker for microsomes (Baginski, Foa & Zak, 1974) and 5′nucleotidase, a marker for plasma membranes (Mitchell & Hawthorne, 1965), were estimated. The subfractions were also tested against the defined mitochondrial marker sera: anti-M2 sera for inner membranes, anti-M3 sera for outer mitochondrial membranes and anti-LKM sera for microsomes.

Treatment of SMP with nagarse and mersalyl. SMP from beef heart, rat liver and pig kidney were treated with nagarse, a protease from *Bacillus subtilis*, at a concentration of 25 μg/ml protein. After incubation for 30 min at 30°C in a waterbath they were centrifuged at 100,000***g*** to remove the enzyme. The pelleted SMP were resuspended and washed three times. As a control, buffer was added instead of nagarse.

Mersalyl was added at a concentration of 1 μm/mg protein according to Sayers *et al.* (1979). After incubation for 20 min, the SMP were washed and reisolated.

Detection of anti M7 antibodies. **ELISA** Microtitre plates (immunoplates) were incubated with different subcellular and submitochondrial antigens in concentrations in the range of 5 and 50 μg/ml (diluted with 0·2 M hydrogencarbonate buffer, pH 9·6) for 16 h at 4°C. The plates were washed with phosphate-buffered saline (PBS, 60 mM, pH 7·4) containing 0·5% bovine serum albumin (BSA) to saturate free binding sites, and then incubated with patient's serum samples at a dilution of 1:500 for 90 min at room temperature. After washing with PBS containing 0·5% BSA and 0·2% Triton-X 100 (used to prevent non-specific protein interactions), monovalent peroxidase conjugated goat anti-human IgG, IgM and IgA antibodies (Fa. Medac, Hamburg, FRG) were added in parallel at a dilution of 1:2,000. After incubation for 60 min and washing with PBS (+0·5% BSA+0·2% Triton-X 100) substrate (*o*-phenylendiamine) was added in a concentration of 0·5 mg/ml. It was diluted in 0·1 M citrate buffer, pH 5·0, containing 0·01% H_2O_2. The enzyme reaction was stopped after 20 min using H_2SO_4 (25%) and the absorption was measured in the Microelisa® Auto reader.

Absorption studies. The six marker sera with anti-M7 specificity type a and b (see patients) were incubated with SMP from beef heart, rat liver and pig kidney, and microsomes from beef heart in parallel at a concentration of 25–30 μg protein/ml serum and with buffer as a control for 24 h at 4°C. After centrifugation with 100,000***g*** to remove the SMP the absorption was repeated twice. After the third absorption (dilution 1:40) the sera were tested in the ELISA against the different submitochondrial fractions at a final dilution of 1:500.

Western blot. Three anti-M7 positive sera (type a: $n=1$; type b: $n=2$) were tested in the Western blot against the supernatant of beef heart, pig kidney and rat liver SMP following the method of Towbin, Staehelin & Gordon (1979) in a modification by Lindenborn-Fotinos, Baum & Berg (submitted to *Clin. exp. Immunol.*).

Immunofluorescence and complement fixation test. All sera from 159 patients with cardiomyopathies were examined for anti-interfibrillary antibodies (IFA) on heart and skeletal muscle and isolated acetone fixed cardiocytes in the immunofluorescence test (Maisch *et al.*, 1980) as well as in the complement fixation test (CFT) using cytoplasmic extract and SMP from beef heart (Mackay & Ritts, 1979).

Immunodiffusion. All anti-M7 positive sera from patients with cardiomyopathies were examined for precipitating antibodies by immunodiffusion using SMP from beef heart and pig kidney at a concentration of 40 mg/ml. Agarose was used at a concentration of 0·4% following the method of Miyachi *et al.* (1980).

Protein estimation. The protein concentrations of the different antigen fractions were estimated as described by Bradford (1976).

RESULTS

Prevalence and specificity of anti-M7

Only sera from patients with cardiomyopathies and acute myocarditis had antibodies against heart mitochondria. Positive results were obtained in 33 of the 107 patients with dilated cardiomyopathy (31%), 17 of the 52 patients with hypertrophic cardiomyopathy (33%) and two of the 15 patients with acute myocarditis (13%) (Table 1). The antibody activities are shown in Fig. 1.

Two different reactions could be observed: 13 sera reacted exclusively with beef heart mitochondria (anti-M7 type a) and 39 sera with SMP from beef heart, pig kidney, beef pancreas and rat lung in parallel (anti-M7 type b). Both anti-M7 types were completely negative when tested

Table 1. Prevalence of anti-M7 antibodies in heart and other diseases

Diagnosis	No. patients	No. positive
Heart diseases (n = 284)		
dilated cardiomyopathy (DC)	107	33 (31%)*
hypertrophic cardiomyopathy (HCM)	52	17 (33%)*
acute myocarditis	15	2 (13%)*
postpericardial injury syndromes (postpericardiotomy and postmyocardial infarction syndrome)	50	0
congestive heart failure	20	0
ischemic heart disease and myocardial infarction	40	0
Other diseases (n = 429)		
(1) Organ and non-organ specific autoimmune diseases		
thyroiditis and myxedema	20	0
Addison's disease	12	0
juvenile diabetes mellitus (islet cell antibody positive)	10	0
Myasthenia gravis	30	0
Pemphigus vulgaris	10	0
autoimmune hemolytic anaemia	25	0
(2) Collagen diseases		
systemic lupus erythematosus (ANA/DNA positive)	50	0
progressive systemic sclerosis	34	0
rheumatoid arthritis (seropositive)	20	0
Sjögren's syndrome (anti-Ro, anti-La positive)	7	0
Polymyalgia rheumatica	6	0
(3) Disorders of the gastrointestinal tract		
HBsAg positive and negative acute and chronic hepatitis	50	0
cryptogenic cirrhosis	25	0
alcoholic cirrhosis	25	0
Crohn's disease, ulcerative colitis	20	0
(4) Epithelial tumors and haematological diseases		
colon cancer	15	0
lymphomas and multiple myeloma	30	0
(5) sensorineural deafness	40	0
Blood donors	100	0

* DC = anti-M7—type a: $n = 11$; type b: $n = 22$. HCM = anti-M7—type a: $n = 1$; type b: $n = 16$. Acute myocarditis—type a: $n = 1$; type b: $n = 1$.

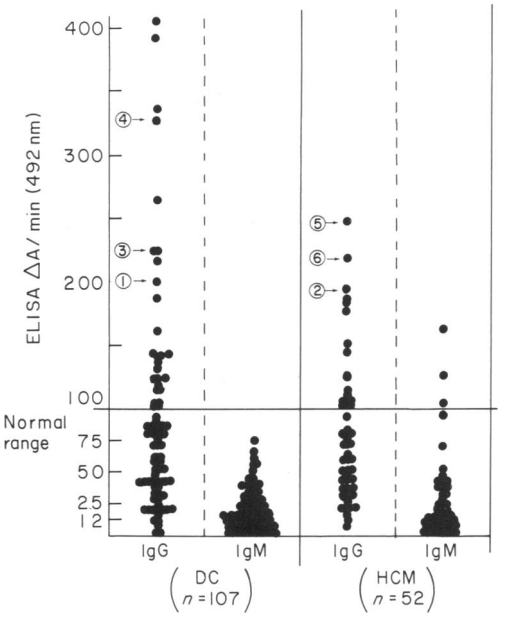

Fig. 1. Anti-M7 antibody activity in sera from 159 patients with dilated and hypertrophic cardiomyopathy tested against beef heart SMP in the ELISA. O→ = sera used for absorption studies (see Figs 2 & 3).

against mitochondria prepared from rat liver, stomach, spleen and skeletal muscle, brown fat of rabbits.

In order to find out whether anti-M7 antibodies may be associated with non-organ specific AMA two sera with anti-M7 type a and four sera with anti-M7 type b were absorbed with SMP from beef heart, pig kidney and rat liver and tested in the ELISA against SMP from beef heart and pig kidney (Figs 2 & 3). Absorption of type a sera with beef heart SMP completely abolished the anti-M7 activity while absorption with kidney of liver SMP did not influence the initial antibody activity. When type b sera were absorbed with beef heart mitochondria, no antibody activity could be observed either with heart or with kidney, lung or pancreas, indicating that similar antigenic determinants were recognized by anti-M7 type b sera on these different tissue mitochondria.

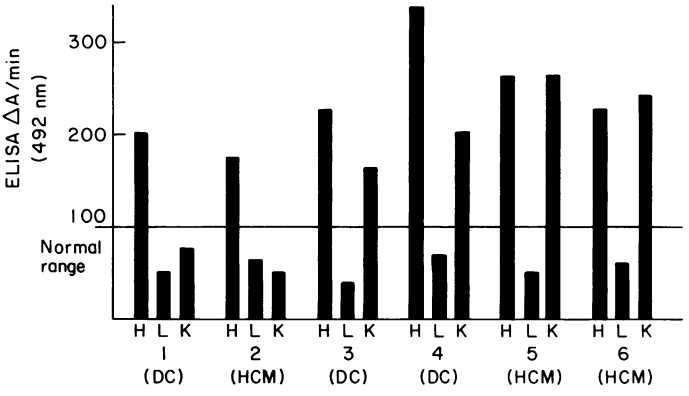

Fig. 2. Organ specificity of anti-M7. Sera from six patients with cardiomyopathy were tested in the ELISA against SMP from beef heart (H), rat liver (L), and pig kidney (K). Anti-M7 antibodies reacted either exclusively with heart SMP (type a sera Nos 1 & 2) or simultaneously with heart and kidney SMP (type b—sera Nos 3–6).

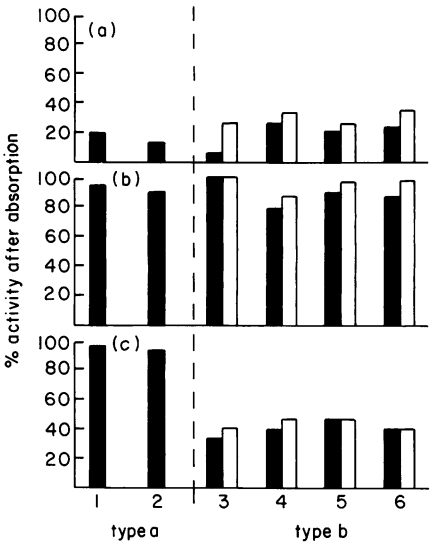

Fig. 3. Absorption of two anti-M7 sera type a and four anti-M7 type b with SMP prepared from beef heart (a), rat liver (b) and pig kidney (c). The absorbed sera were retested in the ELISA against heart (■) and kidney (□) SMP. Only heart SMP absorbed completely anti-M7 antibodies type a while the anti-M7 type b activity was absorbed by heart and kidney SMP in parallel.

Absorption with liver SMP did not diminish the anti-M7 antibody activity type a or b again demonstrating that heart and liver mitochondria differ considerably in their antigenic structure.

Localization and characterization of M7

In these studies the six anti-M7 marker sera were tested against inner and outer mitochondrial membranes as well as plasma membranes prepared from pig kidney and against microsomes from beef heart and pig kidney. None of the four sera with anti-M7 type b reacted with the plasma membranes prepared from pig kidney shown to be contaminated to a large extent with outer mitochondrial membranes and microsomes. The four anti-M7 type b and the two anti-M7 type a sera also failed to react with microsomes from heart or kidney containing outer mitochondrial membranes, and the anti-M7 activity could not be absorbed with these fractions (data not shown). However, all six sera yielded positive results when tested against fractions which could be shown by enzyme marker and marker sera to contain inner mitochondrial membranes (Table 2).

Three of the six marker sera were also analysed in the Western blot. A single band with heart and kidney SMP-SN was observed with the two type b sera at about 70,000 mol. wt (Fig. 3), while the type a marker serum gave only a faint band with heart SMP-SN in the same region as the type b sera. The bands at about 94,000 were also observed in normal sera (not shown).

All sera from 159 patients with cardiomyopathies were also examined in the immunofluorescence test for the presence of IFA which have been previously described in these patients. No correlation was found between anti-M7 and IFA and only 37% of the anti-M7 positive sera were IFA positive while in 52% of the anti-M7 negative sera IFA could be detected.

Anti-M7 antibodies did not fix complement. Thus, all 50 *anti-M7 positive sera were negative* when tested in the CFT against SMP or cytoplasmic extract from beef heart.

No precipitating antibodies against heart or kidney mitochondria could be observed by immunodiffusion.

Anti-M7 antibodies are not identical with the previously described anti-M1–M6 antibodies (Table 3). The antigen was destroyed by nagarse and mersalyl while M1, M3, M4 and M6 are nagarse and mersalyl insensitive antigens.

Table 2. Subcellular localization of the M7 antigen using anti-M7 antibodies type b

Fractions from beef heart and pig kidney	Reaction of the various enzyme markers with membrane fractions				Serological characterization of subcellular membranes using marker sera for			Anti-M7 type b activity
	1	2	3	4*	inner membranes (anti-M2)	outer membranes (anti-M3)	microsomes (anti-LKM)	
Plasma membranes from kidney	+	+	+	neg.	neg.	+	+	neg.
Microsomes from heart and kidney	neg.	+	+	neg.	neg.	+	+	neg.
Outer mitochondrial membranes from kidney	neg.	+	+	+	+	+	+	+
Submitochondrial particles from heart and kidney	neg.	+	+	+	+	+	+	+
Inner mitochondrial membranes from kidney	neg.	neg.	+	+	+	+	neg.	+

* (1) Enzyme marker for plasma membranes: 5′-nucleotidase; (2) enzyme marker for microsomes: Glucose-6-phosphatase; (3) enzyme marker for outer mitochondrial membranes: rotenone insensitive NADH–cytochrome C reductase; (4) enzyme marker for inner mitochondrial membranes: succinate–cytochrome C reductase.

As it was shown for anti-M1–M6 except for anti-M3 antibodies, anti-M7 type a and b can belong to the IgG and/or IgM class.

In the density gradients M7 was found between 1·04 and 1·12. This observation also clearly differentiates M7 from the M2 antigen.

DISCUSSION

A new AMA type which occurs only in patients with heart diseases is described. A high prevalence was found for patients with dilated and hypertrophic cardiomyopathy and acute myocarditis: 52 of the 174 patients were positive. These antibodies either reacted only with heart mitochondria, or were able to detect in addition similar antigenic determinants on pig kidney, beef pancreas and rat lung. Since absorption with heart SMP completely abolished the anti-M7 activity in all instances some degree of organ specificity can be postulated for both anti-M7 antibody types a and b.

Anti-M7 antibodies clearly differ from the previously described anti-M1–M6 antibodies. They are also not identical with the organ specific antibodies against the adenine nucleotide translocator (ANT) from heart mitochondria recently described by Schultheiß *et al.* (1983). Although anti-ANT antibodies have been found in patients with congestive cardiomyopathy and acute myocarditis, the mol. wt of the ANT (30,000) differs considerably from that of M7 which was found to be around 70,000.

The fact, that anti-M7 antibodies could not be detected by CFT or immunodiffusion—in contrast to anti-M2 antibodies which can be detected by both test systems in most instances of patients with primary biliary cirrhosis (PBC)—can be explained by the low titre of anti-M7 antibodies.

They are also not identical with the IFA detected in the immunofluorescence test and were detected only in a low incidence in IFA positive sera (unpublished observation).

Although the clinical relevance of anti-M7 antibodies remains quite unclear their exclusive

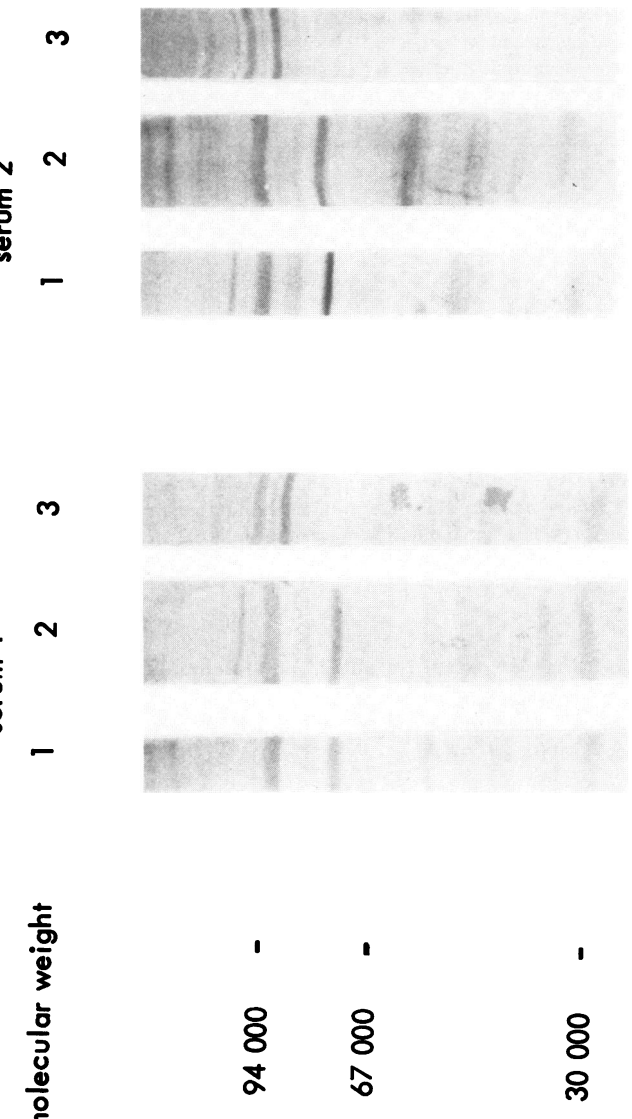

Fig. 4. Demonstration of M7 in the Western blot using SMP-SN from (1) pig kidney, (2) beef heart and (3) rat liver. Using anti-M7 antibodies type b a single band at about 70,000 is observed with SN of beef heart and pig kidney SMP as antigens. Anti-M7 antibodies type a gave only a very faint band with the supernatant of beef heart SMP (not shown). No reaction was obtained using the SN of rat liver SMP as antigen. The bands at about 94,000 are non-specific.

Table 3. Comparison of anti-M7 with the antibody specificities of anti-M1–M6

	Antibody activity demonstrated in the ELISA against						
AMA type*	heart	liver	kidney	outer membranes liver/kidney	marker antigens M1	M2	Ig class of AMA
anti-M1	+	+	+	−/−	+	−	IgG, IgM
anti-M2	+	+	+	−/−	−	+	IgG, IgM, IgA
anti-M3	+	+	+	+/+	−	−	IgG
anti-M4†	+	+	+	+/+	−	−	IgG, IgM
anti-M5	+	+	+	−/−	−	−	IgG, IgM
anti-M6	−	+	+	+/+	−	−	IgG, IgM
anti-M7							
type a	+	−	−	−/−	−	−	IgG, IgM
type b	+	−	+	−/−	−	−	IgG, IgM

* Reacting either with inner membrane antigens (anti-M1, M2, M5, M7) or outer membranes (anti-M3, M4, M6). Anti-M1–M6 can be detected by IFL, CFT and ELISA, anti-M2 also by ID.

† Only in association with anti-M2.

occurrence in heart muscle disease of unknown cause must be taken as evidence that they may be related to an immunopathological mechanism which may be operative in at least some forms of cardiomyopathies or myocarditis.

It remains to be seen whether anti-M7 antibodies are induced by cross reacting antigens leading to myocarditis or cardiomyopathies of unknown aetiology.

Further characterization of the M7 antigen could help to clarify the role of immune mechanisms which seem to be operative in at least some forms of heart diseases.

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