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Anti-mitochondrial antibodies (anti-M7) in heart diseases recognize epitopes on bacterial and mammalian sarcosine dehydrogenase

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SUMMARY

The anti-mitochondrial antibody (AMA) anti-M7 has been shown to occur exclusively in sera from patients with acute and chronic myocarditis. Applying different enzymes of the inner mitochondrial membrane to ELISA, anti-M7-positive sera reacted only with sarcosine dehydrogenase (SD) from *Pseudomonas aeruginosa*. Testing these sera in the Western blot against a commercially available SD as well as against SD prepared from rat liver mitochondria, a determinant at 42 kD and 90 kD, respectively, was visualized. Using submitochondrial particles (SMP) from bovine heart and rat liver another major determinant at 64 kD could be observed with both antigen fractions. Liver SMP also expressed the SD-related, 90-kD epitope. Sera from patients with other AMA-positive and AMA-negative autoimmune diseases were negative with these different determinants. The identity of the 64-kD epitope on heart and liver SMP as well as the 42-kD polypeptide of bacterial SD and the 90-kD epitope on mammalian SD was proven by absorption studies and by elution of antibodies from the antigen bound to the immobilon sheets after immunoblotting. The SD enzyme activity was not affected by anti-64-kD and anti-42-kD antibodies *in vitro*. It is concluded that anti-M7 antibodies may be stimulated by an antigen expressed on cardiocytes during an infection which shares epitopes with SD, an evolutionary highly conserved protein. SD-sensitized B cell clones could therefore be triggered by the M7-antigen which shows homology to SD.

Keywords anti-mitochondrial antibodies anti-M7 myocarditis dilated cardiomyopathy sarcosine dehydrogenase

INTRODUCTION

Several types of autoantibodies against heart muscle and other autoantigens have been described in some forms of cardiac diseases indicating that autoimmune processes may play a role in those diseases (Maisch *et al.*, 1983; Neu *et al.*, 1987; Schultheiss, 1987; Autore *et al.*, 1988; Rose *et al.*, 1988).

Recently, an anti-mitochondrial antibody (AMA) was detected by ELISA which reacted preferentially with submitochondrial particles (SMP) from bovine heart (Klein *et al.*, 1984, 1987) and was named anti-M7. The corresponding M7 antigen was found to be tightly bound to the inner membrane of bovine heart mitochondria. It was not released into the supernatant by sonication and only partially by chloroform treatment (Klein *et al.*, 1984).

These anti-M7 antibodies could be detected in 60% of patients with acute myocarditis of known and unknown aetiology and 33% of patients with dilated cardiomyopathy (Przechera *et al.*, 1989).

Here we show that the M7 antigen is an epitope of sarcosine dehydrogenase (SD), an evolutionary highly conserved enzyme of the inner mitochondrial membrane which is also present in bacteria (Wittwer & Wagner, 1981; Kvalnes-Krick, Schuman & Jorns, 1987).

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PATIENTS AND METHODS

Patients

For the characterization of the M7 antigen we used as marker sera one serum from a patient with acute myocarditis of unknown aetiology and two sera from patients with dilated cardiomyopathy showing high anti-M7 titres of the IgG type as defined by ELISA, applying submitochondrial particles from bovine heart.

Sera from 60 anti-M7-positive patients with aetiologically undefined acute ($n=36$) and chronic heart disease including dilated cardiomyopathy ($n=24$), as previously described (Klein *et al.*, 1987), were examined.

As controls we used AMA-positive sera expressing different antibody specificities (anti-M1–anti-M9) (Berg & Klein, 1989). In addition, sera from 50 patients with organ and non-organ-specific autoimmune diseases and from 50 blood donors were included in the study.

All sera were stored at −20°C.

Antigens

Human heart and liver tissue was obtained from a 42-year-old man who had died from cerebral aneurysma bleeding. Mitochondria and SMP were prepared from these tissues as well as from rat liver as described by Berg, Doniach & Roitt (1967) and from bovine heart according to Smith (1976).

Different mitochondrial enzymes known to be associated with inner or outer membranes or the matrix of mitochondria (obtained from Sigma, St Louis, MO) were used as antigens.

Inner-membrane-associated enzymes were: beta-hydroxybutyrate dehydrogenase; isocitric dehydrogenase; alpha-glycerophosphate dehydrogenase; L-glutamic dehydrogenase; alpha-ketoglutarate dehydrogenase; glutamic-oxalacetic transaminase; sulphite oxidase; nucleoside-5′-diphosphate kinase; malic dehydrogenase; sarcosine dehydrogenase; aldehyde dehydrogenase; fumarase; pyruvate dehydrogenase; aconitase; myokinase; creatine phosphokinase; and cytochrome oxidase.

Matrix-associated enzymes were: beta-hydroxy-coenzyme A (CoA) dehydrogenase; lipoamide dehydrogenase; citrate synthase; carnitine acetyltransferase; S-acetyl CoA synthetase; pyruvate carboxylase; succinic thiokinase; and beta-hydroxyacyl-CoA dehydrogenase.

Outer-membrane-associated enzymes were: monoamine oxidase; CoA synthetase; and phospholipase A2.

The commercially available SD (Sigma) was derived from *Pseudomonas aeruginosa*. We therefore prepared SD also from rat liver mitochondria according to the method described by Cook & Wagner (1986). In each antigen fraction obtained during the purification procedure, SD enzyme activity was determined (Cook & Wagner, 1986) in order to evaluate the purity.

Absorption studies

Anti-M7-positive sera were absorbed with bovine heart SMP and SD from *Ps. aeruginosa*. Undiluted sera were incubated with SMP at a concentration of 25–30 mg protein/ml serum and with buffer as a control for 24 h at 4°C. After centrifugation at 100 000 ***g*** to remove the SMP, the absorption was repeated twice.

SD was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) and also incubated with patients' sera for 24 h at 4°C on a rotating disk. The supernatant was retested for autoantibodies.

Antibodies that were bound to the enzyme coupled to the gel were eluted using 0·2 M glycine buffer, pH 2·6. The pH of the eluate was immediately neutralized (pH 7·4).

Detection of antibodies

ELISA. The ELISA method for the detection of anti-M7 was performed as previously described (Klein *et al.*, 1984). SMP from rat liver were used at a concentration of 30 μg/ml, and SMP from bovine heart at a concentration of 50 μg/ml.

The various enzymes (16 inner-membrane-associated enzymes, eight enzymes of the matrix, and three outer-membrane-associated enzymes) were applied at a concentration of 10 μg/ml. Patients' sera were diluted 1/500.

Peroxidase-conjugated monovalent goat anti-human IgG and IgM antibodies (Medac, Hamburg, FRG) were diluted 1/2000. Monoclonal murine anti-human IgG1, IgG2, IgG3 and IgG4 antibodies (Sigma) were diluted 1/500 and the peroxidase-conjugated anti-mouse antibody from rabbit (Medac) was diluted 1/1000.

Western blot. SDS–PAGE was performed according to Towbin, Staehlin & Gordon (1979). A 4·5% stacking gel and a 10% running gel were used. Eighty micrograms of antigens were applied to the gel. The following immunoblot was carried out as described by Laemmli (1970) using immobilon membranes (Millipore, Bedford, MA). Patients' sera were diluted 1/50.

Antibodies that had bound to a defined antigenic determinant were eluted by cutting out the band from the immobilon sheet and dipping it in 3 ml of 0·2 M glycine buffer, pH 2·6, which was neutralized after 15 min. Eluted antibodies were retested by Western blotting against the different antigens.

Determination of the effect of patients' sera on SD enzyme activity

Patients' serum samples were examined for their ability to inhibit or stimulate the enzyme activity. As marker sera we used an anti-M7 positive serum and as control an anti-M2 positive serum from a patient with primary biliary cirrhosis as well as antibody-negative serum.

In addition, anti-M7-specific antibodies that had been eluted from the 64-kD band from bovine heart SMP and the 42-kD band from bacterial SD after immunoblotting were incubated with bacterial SD.

Varying amounts of patients' sera as well as of purified anti-M7 antibodies (0–200 μl) were pre-incubated at 30°C with 200 μl of SD from *Ps. aeruginosa* (Sigma); then total SD activity was examined according to Cook & Wagner (1986).

RESULTS

Definition of the antigenic specificity of anti-M7 as tested by ELISA using different mitochondrial enzymes

Testing the three marker sera with high anti-M7 titres against the different enzymes associated either with the matrix, the inner or the outer membrane of mitochondria by ELISA gave a positive reaction only with SD from *Ps. aeruginosa*. These results could be confirmed preparing SD from rat liver. The enzyme activity in this preparation was high, measuring 150 nmol/min per mg as compared with the starting material, a mitochondrial extract, showing a specific activity of 1·9 nmol/min per mg.

Furthermore, all sera from 60 anti-M7-positive patients with acute and chronic heart diseases reacted with SD derived from *Ps. aeruginosa* or rat liver mitochondria. There was a good correlation between the anti-M7 titres as defined by the reaction with bovine heart SMP in the ELISA and the titres obtained with SD from *Ps. aeruginosa* ($r = 0{\cdot}89$ for IgG and 0·85 for IgM antibodies).

Sera with AMA of other specificities (anti-M1–anti-M9) as well as from patients with different autoimmune disorders and from healthy controls were negative by ELISA using either bacterial or mammalian SD.

Immunoglobulin classes of anti-M7

Testing the sera from these 60 patients for the immunoglobulin subclasses of anti-M7 by ELISA using bovine heart SMP and SD from *Ps. aeruginosa* showed that 51% had anti-M7 of the

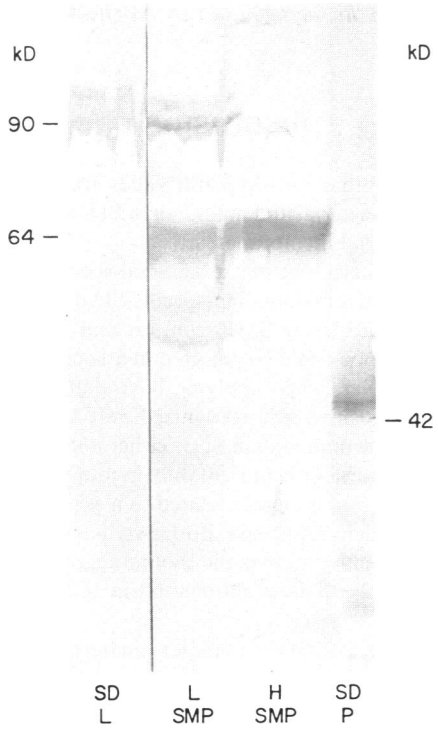

Fig. 1. Demonstration of M7-specific determinants by Western blotting, testing an anti-M7-positive serum from a patient with dilated cardiomyopathy against submitochondrial particles (SMP) from rat liver (L) and bovine heart (H) as well as against sarcosine dehydrogenase (SD). The serum recognizes one determinant on liver and heart SMP at 64 kD and an additional band at 90 kD on liver SMP as well as an epitope on SD at 42 kD.

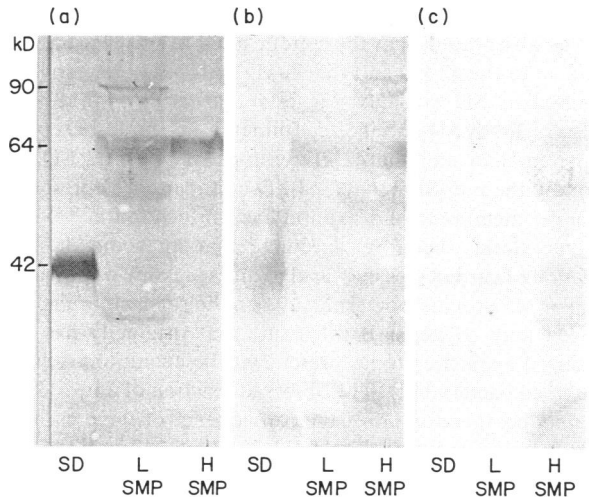

Fig. 2. Demonstration of the identity of the 64-kD epitope on bovine heart (H) and rat liver (L) submitochondrial particles (SMP) with the 42-kD determinant on sarcosine dehydrogenase (SD) by absorption studies. An anti-M7-positive serum was tested by Western blotting against the three antigen fractions (a). After absorption with heart SMP (b) as well as with SD (c) the reaction with the 64-kD determinant on heart and liver SMP and with the 42-kD band on SD was completely abolished.

IgG1, 10% of the IgG2, 33% of the IgG3, and 43% of the IgM class. None had anti-M7 of the IgG4 type.

Characterization of M7 by Western blotting

The three anti-M7-positive marker sera recognized a 42-kD epitope on SD from *Ps. aeruginosa* and a 90-kD epitope of SD prepared from rat liver. Protein staining of the immobilon sheets after blotting showed two protein bands with SD from *Ps. aeruginosa* at 90 and 42 kD and a single band at 90 kD with SD from rat liver (not shown), thus excluding the possibility of a major contamination of both antigen preparations with other proteins.

Using SMP from bovine heart in the Western blot, the marker sera only reacted with a determinant at 64 kD. When applying liver SMP, these sera also recognized the 64-kD determinant and in addition a band at 90 kD that corresponded to the 90-kD epitope of rat liver SD (Fig. 1). The same results were obtained using SMP from human heart and liver. Two additional strong bands were visualized at 75 kD and 45 kD. By incubating the immobilon sheets only with anti-human immunoglobulins it could be shown that this reaction was due to a contamination of the SMP derived from human tissues with immunoglobulins.

Testing the sera from 60 anti-M7-positive patients against the bacterial and mammalian SD as well as against SMP from rat liver and bovine heart gave identical results.

Anti-M7 antibodies of the IgG and IgM type always reacted with the same antigenic determinants.

None of the sera from patients with other AMA-positive and AMA-negative autoimmune disorders, and none of those from healthy controls were positive with bacterial or mammalian SD.

Absorption studies

The three anti-M7 positive marker sera were absorbed with SD from *Ps. aeruginosa* coupled to CNBr-activated Sepharose 4B as well as with bovine heart SMP. No M7-specific band could be detected after absorption with both antigens as shown by Western blotting using bacterial SD, bovine heart and rat liver SMP (Fig. 2).

Elution of antibodies bound to bacterial SD again revealed a positive reaction with bacterial SD at 42 kD, with bovine heart SMP at 64 kD, and with rat liver SMP at 64 and 90 kD.

Antibodies were also eluted from the 42-kD band of bacterial SD on the immobilon sheets. After retesting by Western blotting they reacted not only with the 42-kD but also with the 64-kD and 90-kD determinants (Fig. 3a). Antibodies that had been eluted from the 64-kD band of heart SMP again reacted with epitopes present on the 42-kD and the 90-kD polypeptide of bacterial and mammalian SD, respectively (Fig. 3b). These data strongly suggest that common epitopes are shared by bacterial and mammalian SD as well as the 64-kD polypeptide of rat liver and beef heart SMP.

Influence of anti-M7 on SD enzyme activity

SD enzyme activity was inhibited by incubation with an anti-M7-positive marker serum but also by incubation with an anti-M2-positive and a normal control serum, indicating a non-specific reaction.

Incubation of SD with antibodies that had been eluted from the 64-kD band of bovine heart SMP and the 42-kD determi-

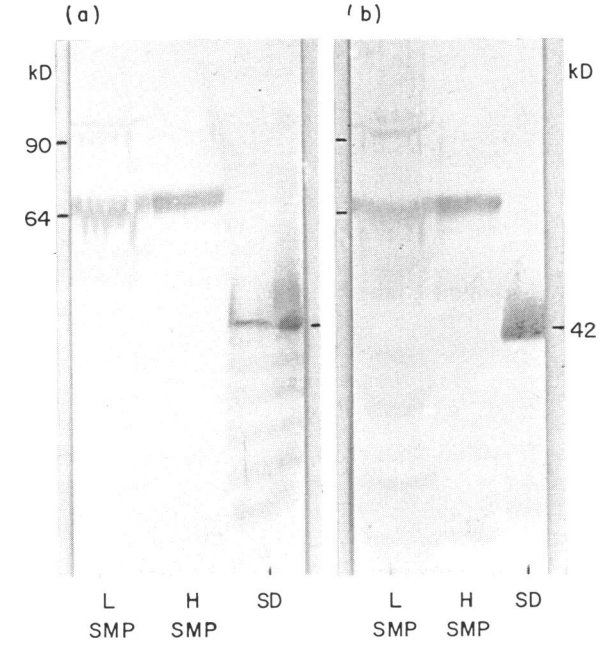

Fig. 3. Demonstration of the identity of the 64-kD epitope on bovine heart (H) and rat liver (L) submitochondrial particles (SMP) with the 42-kD determinant of sarcosine dehydrogenase (SD) by eluting antibodies which had bound to these polypeptides from the immobilon sheet after immunoblotting. Antibodies that had been eluted from the 64-kD determinant of heart SMP were retested against the three antigen fractions. They again reacted with the 64-kD band of heart and liver SMP and the 42-kD determinant of SD (a). The same result was obtained with antibodies that had been eluted from the 42-kD epitope of SD (b).

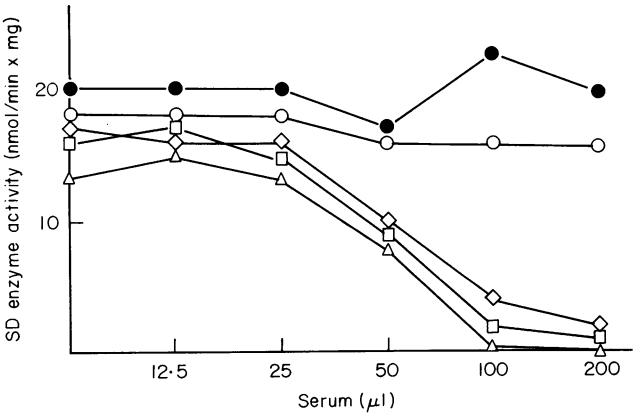

Fig. 4. Effect of anti-M7 antibodies on sarcosine dehydrogenase (SD) activity. Anti-42-kD (●) and anti-64-kD (○) antibodies that had been eluted from immobilon sheets after immunoblotting were incubated with SD from *Pseudomonas aeruginosa* in varying amounts (0–200 μl). They had no effect on the specific enzyme activity, while a whole anti-M7-positive serum inhibited non-specifically the enzyme activity (□) as shown by the inhibitory effect of an anti-M2 positive PBC-serum (△) and a normal serum (◇).

nant of SD from *Ps. aeruginosa* had no effect on the enzyme activity (Fig. 4).

DISCUSSION

We have shown that anti-M7 antibodies recognize epitopes associated with bacterial and mammalian SD, an enzyme of the inner mitochondrial membrane.

Thus, by Western blotting, a bacterial-specific SD determinant at 42 kD and a mammalian-specific SD determinant at 90 kD was visualized. Using SMP from rat and human liver the same 90-kD epitope could be detected in association with a 64-kD band. However, when applying bovine and human heart SMP, anti-M7-positive sera recognized only the 64-kD band, indicating that the mammalian SD is either not present in heart muscle mitochondria or is present only in minute amounts. SD is a flavoprotein that is closely related to a second enzyme, the dimethylglycine dehydrogenase. Both enzymes perform sequential oxidative demethylation in the choline degradation pathway which occurs only in liver mitochondria (Cook & Wagner, 1986).

Bacterial SD exhibits a complex quaterny structure and consists of four non-identical subunits at molecular weights 100, 42, 20 and 6 kD, containing two non-equivalent flavins. The 42-kD epitope against which anti-M7 antibodies are directed is known to be the binding site for flavins (Cook, Misono & Wagner, 1985; Kvalnes-Krick *et al.*, 1987). In contrast, mammalian SD is a much simpler protein containing only one subunit with a molecular weight of 90–105 kD and one flavin (Wittwer & Wagner, 1981; Cook & Wagner, 1986). Despite these structural differences there seems to be an homology between the amino acid sequences of SD from bacteria and those of SD from rat liver (Frisell & Mackenzie, 1970). The reaction of anti-M7 with both antigens and the elution experiments presented in this paper are further arguments in favour of the similarities between bacterial and mammalian SD.

The observation that the epitope at 64 kD on heart SMP is identical to the 42-kD and the 90-kD epitopes of bacterial and mammalian SD strongly suggests that anti-M7 antibodies recognize firstly SD-specific determinants, i.e. the 90-kD epitope of mammalian and the 42-kD epitope of bacterial SD, and secondly the non-SD-related 64-kD epitope that is present on the inner membrane of heart and liver mitochondria.

One could, therefore, speculate that an acute or latent infection of cardiocytes may lead to an expression of previously hidden (M7-specific?) determinants on the cell membrane. This antigen may trigger a B cell clone that originally has been sensitized against epitopes present on the evolutionary highly conserved bacterial SD. The observed reaction of anti-M7 with SD may be, therefore, only the consequence of the presence of cross-reacting epitopes. The failure of anti-M7 antibodies to inhibit SD activity could be taken as further evidence for this hypothesis.

The question therefore arises whether the anti-M7 antibodies may have been derived from the pool of naturally occurring antibodies. These have been shown to belong preferentially to the IgM and IgG3 class (Bussard, Vinit & Pages, 1977; Guilbert, Dighiero & Avrameas, 1982; Hayakawa *et al.*, 1984; Holmberg *et al.*, 1984; Avrameas, 1986; Schwartz, 1986; Stall *et al.*, 1986; Klein & Berg, 1990), and our observation that

part of the anti-M7 antibodies also express these immunoglobulin subtypes fits into this concept.

Anti-M7 antibodies can be detected in only 60% of patients with acute myocarditis of known and unknown aetiology and in 30% of patients with dilated cardiomyopathy (Przechera *et al.*, 1989) indicating that the M7-specific determinants are probably not always expressed on cardiocytes during infectious myocarditis. However, the detection of the 64-kD epitope in association with the 48-kD determinant of bacterial SD by Western blotting is absolutely relevant for the diagnosis of an inflammatory process of the heart muscle, as it will be shown in another report.

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