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Autoantibodies to mitochondrial 2-oxo-acid dehydrogenase complexes in localized scleroderma

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SUMMARY

Sera from patients with localized scleroderma frequently produce cytoplasmic staining by indirect immunofluorescence, although the antigen remains to be determined. We studied the prevalence, antigen specificity and associated clinical characteristics of anti-cytoplasmic antibodies in localized scleroderma. Serum samples from 60 patients with localized scleroderma were examined by indirect immunofluorescence analysis and immunoblotting. By immunofluorescence analysis on HEp-2 cell substrate, seven of 60 (12%) patients were shown to be positive for anti-cytoplasmic antibodies. Among these, six patients with generalized morphea had anti-mitochondrial antibodies as shown by immunoblotting: they showed reactivity with the E2 component of pyruvate dehydrogenase complex (PDC), with protein X, and with the E2 component of α-oxo-glutarate dehydrogenase complex, while two of them showed reactivity with PDC-E1α. One of these patients who was positive for anti-PDC-E1α antibody showed laboratory abnormalities, suggesting the presence of primary biliary cirrhosis. The age of disease onset was significantly higher in these six patients than in those without anti-mitochondrial antibodies. Furthermore, five of them were classified into generalized morphea with multiple plaque lesions but without linear lesions (multiple plaque type). These observations suggest that major antigens for anti-cytoplasmic antibodies in patients with localized scleroderma are mitochondrial enzymes, 2-oxo-acid dehydrogenase complexes. Patients with anti-mitochondrial antibodies may comprise a unique subset of localized scleroderma designated multiple plaque type of generalized morphea of older onset.

Keywords anti-mitochondrial antibodies pyruvate dehydrogenase complex

INTRODUCTION

Scleroderma is a chronic disease which has been proposed to have an autoimmune mechanism, and is divided into two types: localized scleroderma and systemic sclerosis. In localized scleroderma, the lesions are limited to the skin and the subcutaneous tissues underlying the cutaneous lesions. The absence of Raynaud's phenomenon, acrosclerosis, and internal involvement differentiates localized scleroderma from systemic sclerosis. Furthermore, the onset of systemic sclerosis is usually in middle age, while localized scleroderma tends to affect children or young adults [1–3].

Morphologically, localized scleroderma is classified into three variants; morphea, linear scleroderma, and generalized morphea [1]. Morphea is usually characterized by one or a few circumscribed sclerotic plaques with an ivory-coloured centre and a surrounding violaceous halo. Linear scleroderma appears in a linear, band-like distribution, and often involves the muscles underlying the skin lesions. Generalized morphea (GM) is considered to be a severe form of localized scleroderma characterized by widespread skin involvement, with multiple lesions. However, the diagnostic criteria for GM differ among investigators. In a narrower sense, patients having multiple plaque (morphea) lesions without linear lesions are classified into GM; while, in a broader sense, those who have both linear and plaque lesions are also diagnosed as having GM.

Localized scleroderma, especially generalized morphea, has been reported to be accompanied by various immunologic abnormalities including anti-nuclear antibody, anti-single-stranded DNA (ssDNA) antibody, rheumatoid factor (RF), and lupus erythematosus cell phenomenon [2–8]. In our survey of anti-nuclear antibodies in sera of patients with localized scleroderma by indirect immunofluorescence, we noted that patients whose sera produced cytoplasmic staining constituted a characteristic subset, showing the distribution of elderly onset of the disease. Therefore, in this study, we attempted to clarify the prevalence, antigen specificity

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and clinical significance of anti-cytoplasmic antibodies in localized scleroderma.

PATIENTS AND METHODS

Patients and sera

Serum samples from 60 patients with localized scleroderma were examined. Fifteen patients were males and 45 were females. Their ages ranged from 4 years to 67 years, with a mean of 26·1 years. Patients were classified, according to Tuffanelli & Winkelmann's criteria [1], into one of the following three groups: GM ($n = 18$), linear scleroderma ($n = 26$), and morphea ($n = 16$). Furthermore, patients with GM were divided into two types: eight patients with multiple plaque type (i.e. GM in 'narrower' sense) and 10 with linear and plaque type (i.e. GM in 'broader' sense). As controls, serum samples from 30 patients with systemic lupus erythematosus (SLE), 20 with discoid lupus erythematosus (DLE), 20 with dermatomyositis (DM), 100 with other skin diseases who did not have any signs or symptoms of connective tissue diseases, and 50 healthy individuals were assessed. In ELISA, 20 healthy individuals were used as controls.

Immunofluorescence analysis

Anti-nuclear antibodies (ANA) and anti-cytoplasmic antibodies were detected by indirect immunofluorescence (IIF), on HEp-2 cell substrate, as described previously [3,7].

Immunoblotting for antibodies to 2-oxo acid dehydrogenase complexes

Pyruvate dehydrogenase complex (PDC) (Sigma, St Louis, MO) or α-oxo-glutarate dehydrogenase complex (OGDC) (Sigma) were subjected to electrophoresis on 10/20% gradient SDS–polyacrylamide slab gels, and then electrotransferred from the gels onto nitrocellulose sheets as described elsewhere [7,9]. Nitrocellulose sheets were cut into strips and were incubated overnight with serum samples diluted 1 : 50. The strips were then incubated for 1·5 h with alkaline phosphatase- conjugated goat anti-human IgG antibody (Cappel, Durham, NC), and colour was developed with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitroblue tetrazolium (Sigma).

Absorption of 2-oxo-acid dehydrogenase activity

Six serum samples positive for anti-PDC and anti-OGDC antibodies by immunoblotting were assessed by absorption tests as described previously, with some modifications [10]. Serum samples diluted 1 : 100 were incubated with 500 μg/ml PDC and 1 mg/ml OGDC as absorbent for 2 h at room temperature. After centrifugation at 8000 ***g*** for 20 min, ANA on HEp-2 cells was determined by IIF as described above. Two serum samples positive for anti-U1RNP antibody or antiribosomal P protein antibody, and one sample from a normal individual were used as controls. A preliminary absorption test with various concentrations (1 μg/ml, 10 μg/ml, 100 μg/ml, 500 μg/ml, 1 mg/ml and 5 mg/ml) of PDC and OGDC showed 500 μg/ml and 1 mg/ml to be the optimal respective concentrations (data not shown).

ELISA for anti-histone antibodies

The ELISA was performed as described previously [7]. Briefly, microtitre plates (Corning, Corning, NY) were coated with total histones (Sigma) at 5 μg/ml, and serum samples (diluted 1 : 100) were added. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG or IgM antibodies (Cappel). Absorbance values greater than the mean +3 s.d. were considered positive.

ELISA for anti-ssDNA antibody

Wells were pretreated for 1 h with 0·1% protamine sulphate (grade X; Sigma). Then calf thymus ssDNA was added at 1 μg/ml. The ELISA was performed as described above.

Measurement of RF

The IgM RF was tested using a latex agglutination slide test (Eiken, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Student's *t*-test for comparison of means and Fisher's exact probability test for analysis of frequencies. $P < 0{\cdot}05$ was considered significant.

RESULTS

Immunofluorescence analysis

By the IIF method, cytoplasmic staining was observed in seven of 50 (12%) patients with localized scleroderma (Table 1). The most common pattern of cytoplasmic immunofluorescence was reticular, suggesting the presence of anti-mitochondrial antibodies (AMA). No patients had anti-topoisomerase I, anti-centromere, or anti-U1RNP antibodies.

Table 1. Frequency of autoantibodies to 2-oxo-acid dehydrogenase complexes in patients with localized scleroderma (LS) and other diseases

		IIF†	Immunoblotting‡		
Groups*		Cytoplasm	PDC	OGDC	PDC or OGDC
Localized scleroderma					
	($n = 60$)	7 (12)	6 (10)	6 (10)	6 (10)
GM	($n = 18$)	6 (33)	6 (33)	6 (33)	6 (33)
MP	($n = 8$)	5 (63)	5 (63)	5 (63)	5 (63)
LP	($n = 10$)	1 (10)	1 (10)	1 (10)	1 (10)
LS	($n = 26$)	1 (4)	0 (0)	0 (0)	0 (0)
Morphea	($n = 16$)	0 (0)	0 (0)	0 (0)	0 (0)
SLE	($n = 30$)	8 (27)	1 (3)	1 (3)	1 (3)
DLE	($n = 20$)	1 (5)	0 (0)	0 (0)	0 (0)
DM	($n = 20$)	8 (40)	0 (0)	0 (0)	0 (0)
Other skin diseases					
	($n = 100$)	5 (5)	0 (0)	0 (0)	0 (0)
Normal	($n = 50$)	2 (4)	0 (0)	0 (0)	0 (0)

Values are the number (%) of positive cases.

* GM, Generalized morphea; MP, multiple plaque subtype; LP, linear and plaque subtype; LS, linear scleroderma; SLE, systemic lupus erythematosus; DLE, discoid lupus erythematosus; DM, dermatomyositis.

† IIF, Indirect immunofluorescence; cytoplasm, anti-cytoplasmic antibodies.

‡ The sera which showed cytoplasmic staining in IIF were assessed by immunoblotting. PDC, Antibodies to pyruvate dehydrogenase complex; OGDC, antibodies to α-oxo-glutarate dehydrogenase complex.

Immunoblotting for 2-oxo-acid dehydrogenase complexes

To determine reactivity with mitochondrial antigen, seven serum samples positive for cytoplasmic immunofluorescence analyses were assessed. Table 1 shows the results of immunoblotting; six serum samples showed reactivity with PDC (Fig.1). These six samples reacted with the 70-kD ED component of PDC and 54-kD protein X, while only two reacted with E1α subunit of PDC. These six sera also reacted with E2 component of OGDC (Fig. 2). All six patients positive for anti-PDC and OGDC antibodies were classified into generalized morphea.

Absorption of 2-oxo-acid dehydrogenase activity

To confirm that AMA produce cytoplasmic staining by immunofluorescence, an absorption test was performed. In all six of the above serum samples, immunofluorescence staining in the cytoplasm disappeared completely with preabsorption with PDC and OGDC (Fig. 3). Absorption with PDC and OGDC did not alter the titre of immunofluorescence in either control serum sample positive for anti-U1RNP or anti-ribosomal P protein antibodies. Antibodies to nuclei or cytoplasm remained negative after the same treatment in a serum from a normal individual.

Clinical correlations

All six patients positive for AMA had GM (Table 1). Among GM patients, the frequency of positive AMA was significantly higher in multiple plaque type than in linear and plaque type ($P < 0{\cdot}05$). Table 2 shows the correlations of clinical and laboratory findings with AMA in patients with GM. The age of disease onset in patients positive for AMA was significantly higher than that of those who did not have AMA. Figure 4 shows the distribution of the ages of disease onset. At the initial consultation, no patients showed symptoms or laboratory abnormalities suggesting primary biliary cirrhosis (PBC). However, one patient (lane 1 in Figs 1 and 2) later showed elevation of alkaline phosphatase and gamma glutamyl transpeptidase, which suggested the presence of PBC.

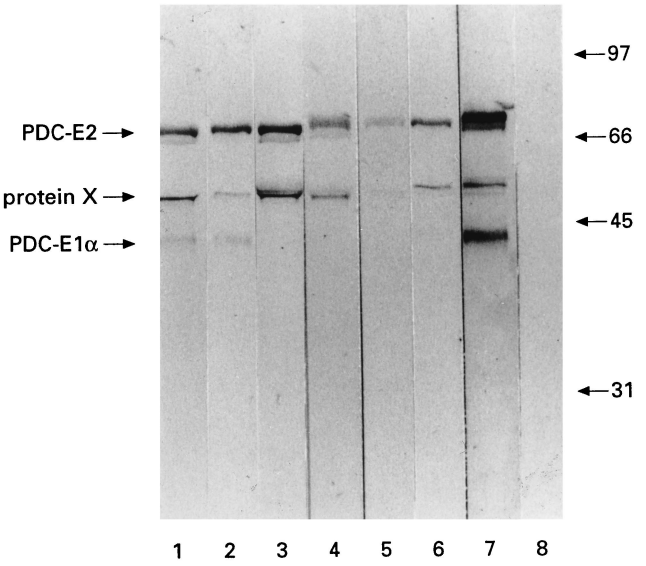

Fig. 1. Immunoblotting of pyruvate dehydrogenase complex (PDC) with sera from patients with localized scleroderma. Lanes 1–6, sera from patients with localized scleroderma, showing reactivity with PDC-E2 and protein X but not with PDC-E1α; lane 7, serum from a patient with primary biliary cirrhosis; lane 8, normal human serum. Molecular weights are shown on the right.

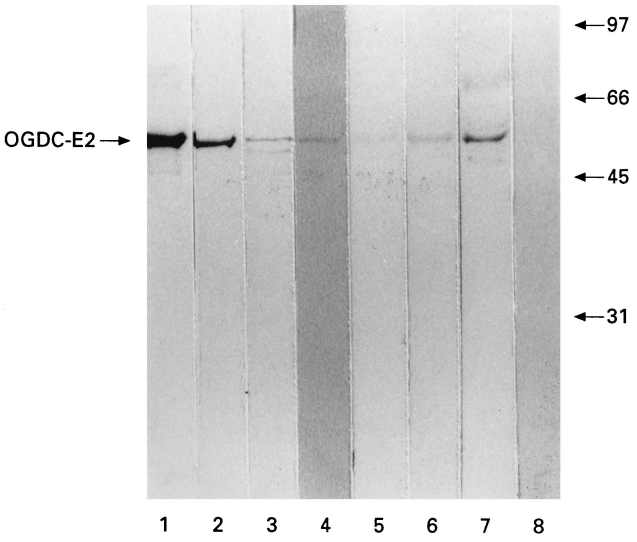

Fig. 2. Immunoblotting of α-oxo-glutarate dehydrogenase complex (OGDC) with sera from patients with localized scleroderma. Lanes 1–6, sera from patients with localized scleroderma, showing reactivity with OGDC-E2; lane 7, serum from a patient with primary biliary cirrhosis; lane 8, normal human serum. Molecular weights are shown on the right.

DISCUSSION

AMA are present in the sera of over 95% of patients with PBC, and are considered to be a hallmark of this disease [11]. The target autoantigens for the AMA were recently identified as three closely related metabolic enzymes located in the mitochondria, termed 2-oxo-acid dehydrogenase complexes: PDC, OGDC, and the branched-chain α-oxo-acid dehydrogenase complex (BCODC) [12–16]. These three enzymes consists of three subunits, E1, E2, and E3, each of which is nuclear-coded. The E1 subunit of PDC and BCODC exists as two forms: E1α and E1β. After synthesis on ribosomes, the enzyme subunits are separately imported into

Table 2. Correlation of anti-mitochondrial antibodies (AMA) with clinical and laboratorial features in 18 patients with generalized morphea

	AMA$^+$ ($n = 6$)	AMA$^-$ ($n = 12$)	*P*
Mean age at onset (years)	44·3	12·6	<0·001
Sex (M/F)	1/5	4/8	NS
Disease type	5/1	3/9	<0·05
(Multiple plaque/linear and plaque)			
No. of lesions	7·4	7·5	NS
Muscle involvement	0	1	NS
Positive AHA	3	7	NS
Positive anti-ssDNA	3	9	NS
Positive RF	2	8	NS

AHA, anti-histone antibodies; anti-ssDNA, anti-single-stranded DNA antibodies; RF, rheumatoid factor.

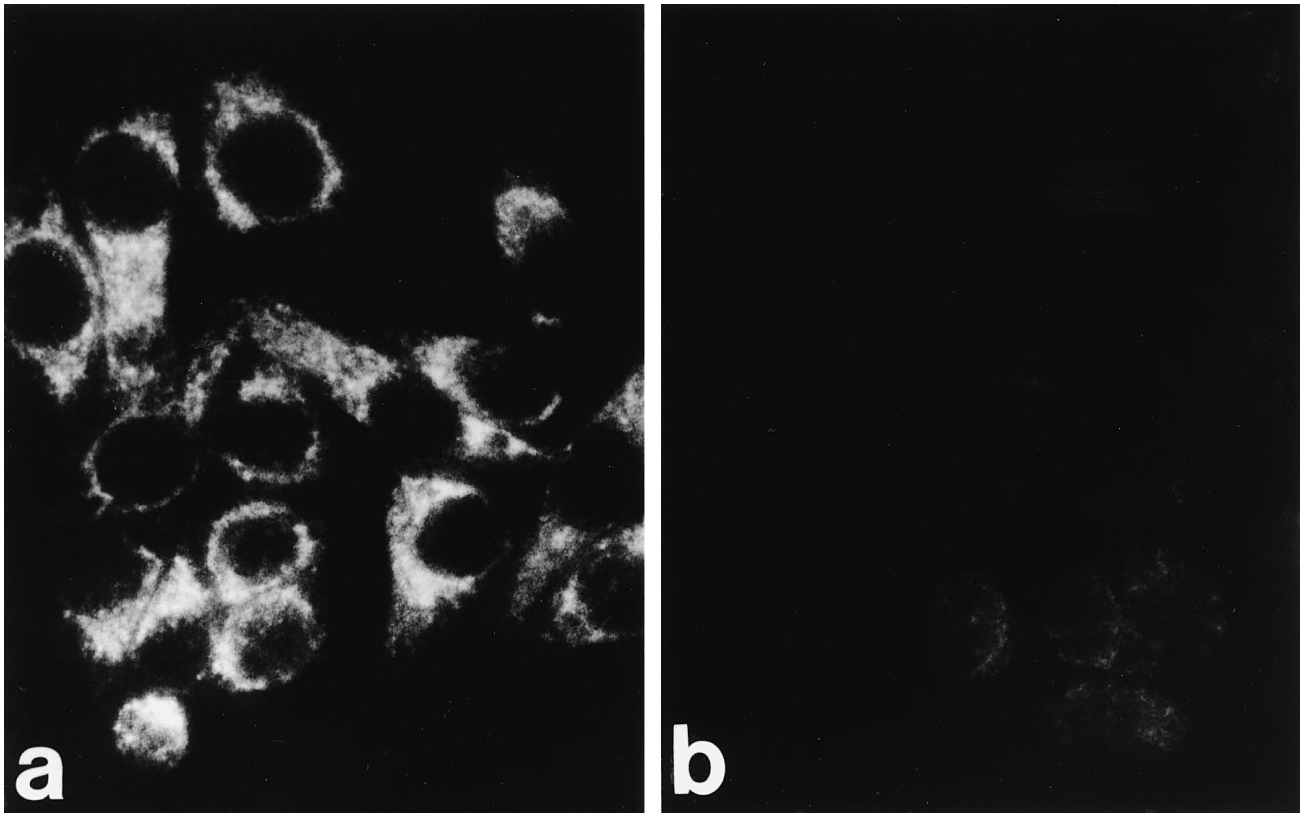

Fig. 3. Absorption of 2-oxo-acid dehydrogenase activity with a representative serum sample positive for anti-mitochondrial antibodies. Reticular staining in the cytoplasm was observed before absorption (a), while cytoplasmic staining disappeared after absorption (b).

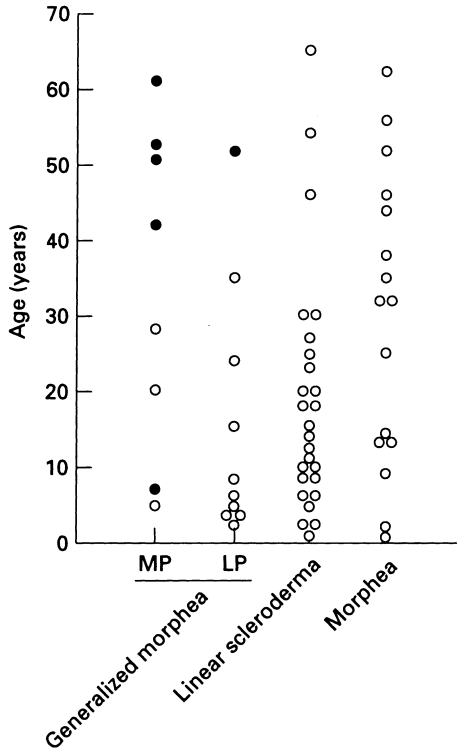

Fig. 4. Distribution of the ages of disease onset. MP, Multiple plaque subtype; LP, linear and plaque subtype. ●, Positive for anti- mitochondrial antibodies (AMA); ○, negative for AMA.

mitochondria and assembled on the inner membrane, where they are packaged as high-molecular-weight multimers [12].

Although most characteristic of PBC, AMA has also been detected in a minority of patients with other autoimmune diseases [17,18]. A high prevalence of AMA has been reported in patients with systemic sclerosis, especially in limited type (CREST variant) [9,17–20]. However, there have been no studies on the presence of AMA in patients with localized scleroderma. In the present study, AMA was detected in 10% of patients with localized scleroderma, and more significantly in 33% of patients with GM, which is almost equivalent to that reported in systemic sclerosis [9,17–20].

In this study, AMA was positive in patients with GM who shared two features. First, most of them were classified into multiple plaque type of GM. Second, these patients tended to be affected in middle age. Thus, although the number of patients was rather small, our findings suggest that AMA may be a serological marker for a subset of GM in the narrower sense, showing older onset. As localized scleroderma usually affects children and young adults, this subset stands out as different.

Localized scleroderma is generally considered to be different from systemic sclerosis, although these two diseases have a number of features in common, and some investigators have suggested that they represent two ends of a spectrum of disease [21,22]. We showed that, in patients with localized scleroderma, AMA was positive exclusively in patients with GM, the severest form of this disease. A review of the literature [22] showed that patients with localized scleroderma who progressed to systemic sclerosis were often classified into this subset, although none of our patients has shown any signs or symptoms suggestive of

systemic sclerosis. Furthermore, our AMA-positive patients showed a distribution of older onset, which is again similar to systemic sclerosis. It is interesting that the milder form of systemic sclerosis and the severest form of localized scleroderma share a common serological abnormality, and these findings may suggest that patients with GM who are positive for AMA are close to systemic sclerosis in the disease spectrum.

In the present study, reactivity with PDC-E2, protein X, and OGDC-E2 was observed in all six patients positive for AMA, while anti-PDC-E1α antibody was positive in only two patients among them. Recently, we reported that anti-PDC-E1α antibody may be a serological indicator of the occurrence of PBC in patients with systemic sclerosis who are positive for AMA [9]; patients positive for anti-PDC-E1α antibody showed clinical evidence of PBC, while those without anti-PDC-E1α antibody did not have PBC, although they were positive for anti-PDC-E2 antibody. In this study of localized scleroderma, one patient positive for PDC-E1α antibody showed laboratory abnormalities suggestive of PBC, whereas the other did not develop PBC. On the other hand, none of those who were negative for anti-PDC-E1α antibody developed PBC. Hence, our findings in systemic sclerosis may be true for localized scleroderma as well.

Our findings suggest that the major antigens for anti-cytoplasmic antibodies in patients with localized scleroderma are mitochondrial enzymes, 2-oxo-acid dehydrogenase complexes including PDC and OGDC, and that patients positive for AMA may comprise a unique subset of GM. Although the mechanism of induction of these autoantibodies in localized scleroderma is not clear, studying the antigen specificity and clinical relevance will be important for elucidation of the pathogenesis of localized scleroderma.

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