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Clinical features of anti-chromo antibodies associated with anti-centromere antibodies

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SUMMARY

Anti-chromo antibodies (AChA) are autoantibodies accompanying anti-centromere antibodies (ACA). We determined the frequency and clinical significance of AChA in autoimmune rheumatic diseases. Serum samples from 252 patients with rheumatic diseases were examined by immunoblotting with HeLa nuclear extract and with recombinant N-terminus of 25-kD chromo protein (p25). AChA were detected in 28 (36%) of 77 sera with ACA. AChA were found only in ACA-positive sera. Twenty-two (79%) of 28 recognized a recombinant N-terminal portion of p25, including the chromo domain which is conserved among species. AChA were related to leucopenia, thrombocytopenia, elevated erythrocyte sedimentation rate, and existence of Sjögren's syndrome (SS). In ACA-positive patients, AChA might be a serologic indicator of systemic sclerosis (SSc), having features of systemic lupus erythematosus and/or SS or diseases other than SSc.

Keywords anti-centromere antibodies anti-chromo antibodies rheumatic diseases chromo domain autoepitope

INTRODUCTION

Anti-centromere antibodies (ACA) have been found in 20–50% of patients with systemic sclerosis (SSc) by indirect immunofluorescence (IIF) [1–4]. Although ACA have been considered a serological marker of CREST variant of SSc [5], they have also been detected in patients with various rheumatic diseases [6]. ACA commonly recognize the three chromosomal autoantigens 17 kD (CENP-A), 80 kD (CENP-B), and 140 kD (CENP-C) in immunoblotting analysis [6,7]. cDNA for these three antigens have been cloned [8–11], and of these, autoimmune responses against CENP-B have been studied most extensively [12–16].

Sera from some patients with ACA also recognize a group of several nuclear proteins with molecular size of 23–25 kD [7,17–20]. Autoantibodies against these proteins did not cross-react to centromere antigens [8,20], were inactive in IIF to intact human cell, and failed to bind to centromere [17,18,20]. Therefore these antigens were classified as non-centromeric. Recently, it has been shown that affinity-purified autoantibodies from blotted 25-kD protein are concentrated in pericentromeric heterochromatin by *in situ* immunolabelling [21]. Anti-23-kD protein (p23) and -25-kD protein (p25) antibodies have cross-reactivity [20]. Saunders *et al.* [22] have cloned the p25 and found that, from DNA sequence analysis, this autoantigen is a human homologue of heterochromatin protein 1 (HP1), which is a non-histone chromosomal protein in *Drosophila melanogaster* with a dose-dependent effect on heterochromatin-mediated gene silencing [23,24], and named this protein 'HP1$^{HS\alpha}$'. HP1 has an evolutionarily conserved amino acid sequence in the N-terminal half called the 'chromo domain' (*chr*omatin *o*rganization *mo*difier) [25], and this domain is recognized by human autoantibodies in the epitope mapping [22]. These autoantibodies therefore are termed 'anti-chromo antibodies' (AChA) [22].

In this study, we determined the frequency and clinical significance of AChA in rheumatic disease patients using immunoblotting, and investigated whether AChA from various patients could recognize bacterial recombinant protein of the N-terminal portion of p25 peptide including the chromo domain, which is conserved across the animal and plant kingdoms [26,27].

PATIENTS AND METHODS

Patient population

Sera from a total of 252 Japanese patients with various rheumatic diseases were analysed. They included 101 patients with SSc, 66 with systemic lupus erythematosus (SLE), 28 with dermatomyositis (DM), 17 with primary Sjögren's syndrome (pSS), seven with mixed connective tissue disease (MCTD), five with discoid lupus erythematosus (DLE), three with rheumatoid arthritis (RA), three with Raynaud's disease, four with overlap syndromes (one

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SSc + SLE, one SSc + RA, one SSc + DM, one SLE + RA), and 18 with other rheumatic diseases.

SLE and RA were diagnosed by the revised criteria for SLE [28] and RA [29] proposed by the American College of Rheumatology (ACR; formerly the American Rheumatism Association). DM was diagnosed by the Bohan and Peter's criteria [30]. SS and MCTD were defined by Japanese diagnosis criteria for SS [31] and MCTD [32]. Fifty SSc patients were diagnosed by 1980 ACR criteria [33] and the other 51 SSc patients had sclerodactyly and at least one other feature of CREST syndrome (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, telangiectasia).

Laboratory analysis

Laboratory studies included determination of the complete blood cell count, blood chemistry profile, complement level, urinalysis, and erythrocyte sedimentation rate (ESR). Rheumatoid factor (RF) was determined by the latex agglutination with RA-E kit (MBL, Nagoya, Japan). ACA were detected by IIF using HEp-2 cells (MBL) and/or HeLa cells as described previously [6].

Preparation of the recombinant p25 antigen

The human cDNA partially coding for p25 antigen was cloned from λgt11 cDNA library by immunoscreening [34] with a patient's serum containing ACA and AChA (K. Sugimoto, unpublished results). The obtained 0·5-kb cDNA (λ50–1) was identical to the reported one [22] from the position of base 2–451 except for one G insertion at the position between 9 G and 10 C in the 5′ noncoding region. The cDNA fragment was inserted into the EcoRI-NotI site of an expression vector (pGEX4T-3) (Pharmacia, Uppsala, Sweden), so as to express 106 N-terminal amino acids of p25 as a fusion protein with glutathione S-transferase (GST; 26 kD). The expression construct was transformed into *Escherichia coli* strain JM109 for fusion protein expression. Bacterial fusion protein was induced by adding isopropyl thio-β-D-galactoside (IPTG) to a growing culture. Induced cell pellets were sonicated in Laemmli's sample buffer [35].

Immunoblotting

Nuclear extract of HeLa cells was obtained by the method described previously [6]. SDS–PAGE and transfer of proteins (cell extract or bacterial fusion protein) from 17·5% polyacrylamide gel onto polyvinyldene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) was performed as described previously [6]. Strips of membrane were incubated with sample serum at 1 : 200 dilution. The antibody–antigen complexes were detected with horseradish peroxidase-conjugated goat anti-human IgG, IgM, or IgA (Dako, Glostrup, Denmark) at 1:1000 dilution. Colour development was carried out with Konica Immunostain (Konica Co. Ltd, Tokyo, Japan).

Statistical analysis

For statistical analysis, Fisher's exact probability test was employed for analysis of frequencies. Student's *t*-test was used for analysis of mean ± s.d. $P < 0{\cdot}05$ was considered significant.

RESULTS

Profile of anti-chromo reactivity

Of sera from 252 patients, ACA were detected in sera of 77: 49 SSc, nine SLE, eight SS, three Raynaud's disease, two DM, two DLE, one overlapping SLE with RA, one RA, and two other rheumatic diseases by IIF and immunoblotting (Table 1). By the immunoblotting method with nuclear extract of HeLa cells, IgG-AChA reacting with both 23- and 25-kD proteins were detected in 28 of 77 ACA-positive sera (36%) (Fig. 1a), whereas AChA could not be found in any ACA-negative serum. The diagnoses of AChA-positive patients were 14 SSc, five SS, four SLE, two DLE, one SLE + RA, one DM, one Raynaud's disease (Table 1). In the patients with AChA, 57% had SS (five pSS and 11 secondary SS), while, in the ACA-positive patients without AChA, 35% had SS (three pSS and 14 secondary SS) ($P < 0{\cdot}05$).

For all AChA-positive sera, immunoreactivity of the recombinant protein of p25 was investigated by immunoblotting method (Fig. 1b). Recombinant fusion protein was detected with anti-GST MoAbs (Pharmacia) at a size of 50 kD (data not shown). This size was about the same as the predicted size (45 kD) of the protein which was composed of GST (26 kD) + 5′ region (44 amino acids) + the N-terminus of p25 (106 amino acids) + linker portions (20 amino acids). Of 28 AChA-positive sera, 22 (79%) recognized

Table 1. Clinical diagnosis in anti-centromere antibody-positive patients with or without anti-chromo antibodies (AChA)

Diagnosis	AChA-positive patients ($n = 28$)	AChA-negative patients ($n = 49$)
Systemic sclerosis	14	35
Systemic lupus erythematosus (SLE)	4	5
Primary Sjögren's syndrome	5	3
Raynaud's disease	1	2
Discoid lupus erythematosus	2	0
Dermatomyositis	1	1
Rheumatoid arthritis (RA)	0	1
Overlap syndrome (SLE + RA)	1	0
Others	0	2
Sjögren's syndrome (primary and secondary)	16*	17

*$P < 0{\cdot}05$ *versus* AChA-negative patients.

fusion protein. A few dozen AChA-negative patient sera did not recognize it. Twenty-two positive sera produced no bands around 50 kD in the control experiments with extract from bacteria which produced GST only (data not shown).

So far, IgM or IgA class AChA have not been reported. In our experiments, although IgA class AChA were not found, IgM-AChA were detected in four of 28 AChA-positive sera with HeLa nuclear extract (Fig. 2, lane N). With fusion protein (Fig. 2, lane F), 12 IgM-AChA were found, but IgA-AChA were not.

Laboratory data of AChA-positive patients

Table 2 shows the laboratorial correlations of AChA in ACA-positive patients. Not only in all ACA-positive patients, but also in ACA-positive SSc patients, even those who did not fulfil the SLE criteria, frequencies of leucopenia, thrombocytopenia, and elevated ESR were higher than in AChA-negative patients with ACA. Although the frequency of hypocomplementaemia was not significantly different between the two groups, the levels of CH_{50} were lower in AChA-positive patients than in AChA-negative patients, and in AChA-positive SSc patients than in AChA-negative SSc patients ($31{\cdot}5 \pm 12{\cdot}5$ *versus* $39{\cdot}7 \pm 6{\cdot}36$, $P < 0{\cdot}005$; $26{\cdot}1 \pm 15{\cdot}6$ *versus* $40{\cdot}1 \pm 4{\cdot}90$, $P < 0{\cdot}005$, respectively). Seven (25%) of 28 AChA-positive patients had RF, while eight (16%) of 49 AChA-negative patients with ACA were positive for RF ($P > 0{\cdot}1$, not significant). Of seven RF-positive patients with AChA, five patients had primary or secondary SS with no evidence of RA, and only one patient had erosive arthritis. Liver dysfunction was found in 29% of AChA-positive patients and in 27% of AChA-negative patients with ACA ($P > 0{\cdot}2$, not significant).

Longitudinal study of AChA

We had paired serum samples collected 5 years apart from five patients with AChA. By immunoblotting with HeLa nuclear extract, the first samples from three patients who already had ACA recognized neither 23- nor 25-kD proteins, while the second samples from these patients obtained after 5 years

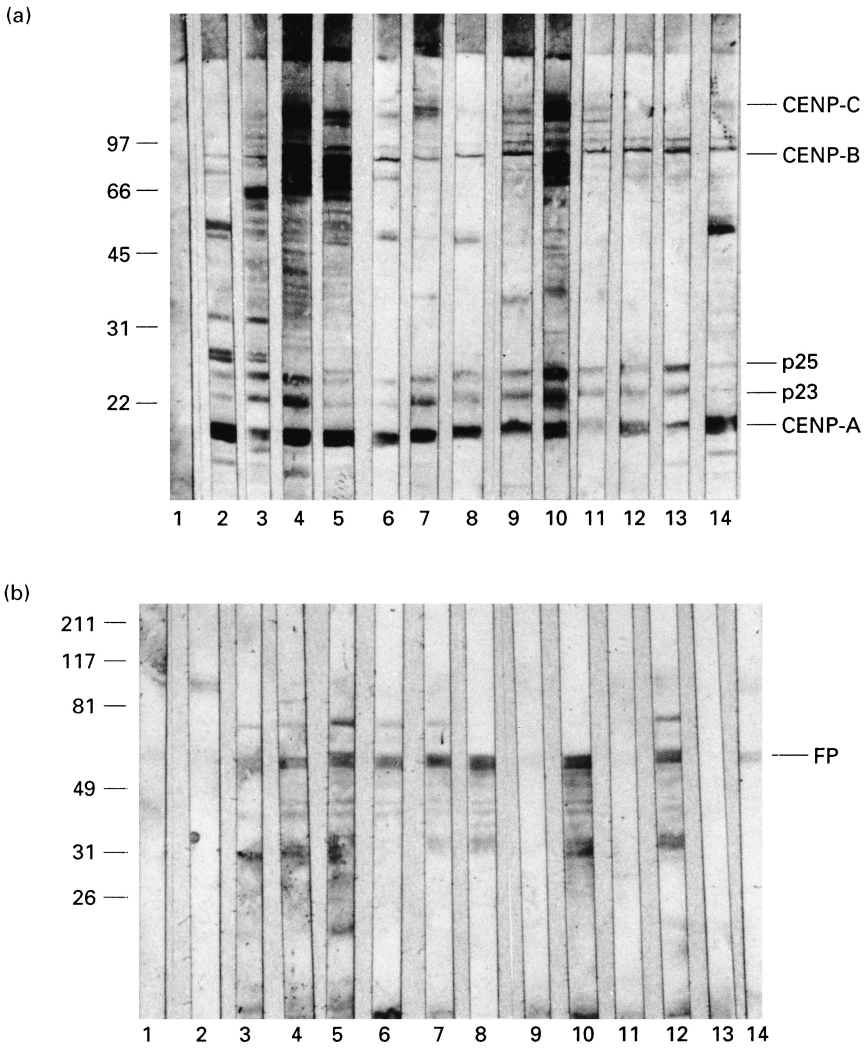

Fig. 1. Immunoblotting of anti-chromo antibody (AChA)-positive sera using HeLa nuclear extract (a) and bacterial fusion protein of the N-terminus of p25 which is one of the chromo-antigens (b). The lane numbers correspond to the same patients in a and b. Lane 1, a healthy blood donor; lanes 2–14, reactivity of IgG-AChA reacting with the 23- and 25-kD bands (a); lanes 2–14, also anti-centromere antibody-positive and all recognizing at least one of the CENP antigens. It is shown (b) that lanes 3–8, 10 and 12 recognize the fusion protein while the other four sera (lanes 2, 9, 11 and 13) do not recognize it. Molecular weight standards are shown on the left of the blots. FP shows the position of fusion protein.

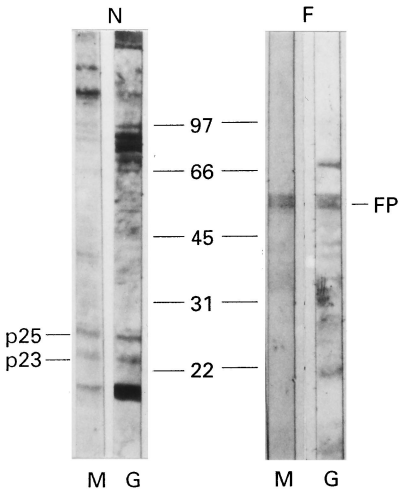

Fig. 2. Immunoglobulin isotype analysis of anti-chromo antibodies (AChA) in immunoblotting with nuclear extract of HeLa cells (N) and fusion protein of the N-terminus of p25 (F). Lanes M, detected with an anti-IgM conjugate; lanes G, detected with an anti-IgG conjugate. The serum of patient SA was positive for both IgM and IgG class AChA and also recognized the fusion protein by both IgM and IgG class. Anti-CENP-C antibodies were strongly positive in IgM class, while anti-CENP-A and -B antibodies were IgG class dominant. Molecular weight standards are shown between the blots. FP denotes fusion protein.

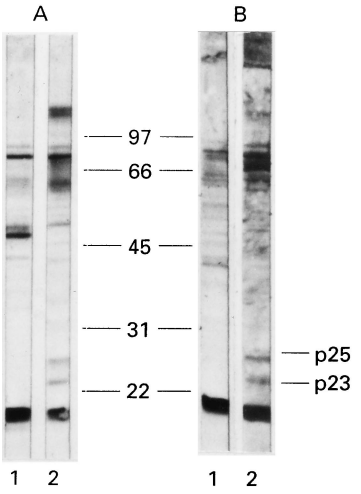

Fig. 3. Longitudinal study of anti-chromo antibodies (AChA). Lanes A, sera from patient MI/SA; lanes B, sera from patient 1. Sera of lanes 2 were obtained 5 years after taking sera of lanes 1. For both patients, in lanes 1 AChA were negative, while in lanes 2 AChA were positive by immunoblotting with HeLa nuclear extract and an anti-IgG conjugate. Anti-CENP-C antibodies changed positive in lanes 2 for both patients, and an unidentified 46-kD band disappeared in lane 2 of patient MI.

recognized 23- and 25-kD proteins (Fig. 3). The first sera of all three patients did not recognize CENP-C, but the second sera recognized CENP-C. There was no apparent change in the pattern of disease over this time. We experienced the disappearance of AChA after corticosteroid and cyclophosphamide therapy in one patient with overlapping SLE and RA. ACA titres of this patient in IIF and immunoblotting became lower after therapy (data not shown).

DISCUSSION

Guldner *et al.* first described autoantibodies which recognized a group of antigens with molecular weight of 23 and 25·5 kD in five of 18 ACA-positive sera [17]. Earnshaw *et al.* reported similar autoantibodies in four of 39 ACA-positive sera [7], and another group showed autoantibodies named 'anti-chromo antibodies' in three of 32 sera with ACA [20]. In our analysis, AChA were detected in 36% of ACA-positive Japanese patients. This prevalence of AChA is higher than in the previous reports, and this might be caused by differences in experimental conditions and/or in races. Our results as well as other reports have shown that AChA have been detected only in patients with ACA. This finding, that AChA were a part of the response in ACA-positive patients, may suggest that AChA reflect an immune response different from classical CREST and that AChA-positive patients form a subset of ACA-positive patients. The frequency of AChA in non-SSc

Table 2. Comparison of laboratory data between patients with anti-chromo antibody (AChA)-positive and AChA-negative patients in all anti-centromere antibody (ACA)-positive patients and ACA-positive systemic sclerosis (SSc) patients

	All patients with ACA ($n = 77$)		SSc patients with ACA ($n = 49$)	
	AChA (+) ($n = 28$)	AChA (−) ($n = 49$)	AChA (+) ($n = 14$)	AChA (−) ($n = 35$)
Age, mean	59·1*	51·5	60·7*	53·7
Duration (years), mean	10·1	9·19	12·6	9·40
Male : female	3 : 25	6 : 43	1 : 13	5 : 30
Leucocytes $<4000/mm^3$	11/28 (39%)*	6/49 (12%)	5/14 (36%)	4/35 (11%)
Platelet $<160\,000/mm^3$	8/28 (29%)	7/49 (14%)	6/14 (43%)*	5/35 (14%)
ESR >20 mm/h	12/22 (55%)*	10/38 (26%)	5/11 (45%)	8/29 (28%)
Hypocomplementaemia†	6/24 (25%)	8/47 (17%)	1/13 (7·7%)	4/34 (12%)

*$P < 0{\cdot}05$ *versus* AChA (−).
† C3 < 60 mg/dl, C4 < 10 mg/dl or CH_{50} < 30 U/ml.
ESR, Erythrocyte sedimentation rate.

patients with ACA was significantly higher than that in SSc patients with ACA (50% *versus* 29%; $P = 0{\cdot}035$). Moreover, in 14 patients with SSc with AChA, only one patient had simple SSc, which had neither any aspect of SLE nor overlapping with SS, and the other 13 patients had some features of SLE (leucopenia, thrombocytopenia, hypocomplementaemia) and/or SS. On the other hand, of 35 ACA-positive SSc patients without AChA, 16 patients had simple SSc ($P = 0{\cdot}0088$).

Soriano *et al.* [20] reported that two of three patients who were positive for AChA had limited cutaneous SSc and erosive arthritis. Although, in this study, there was only one AChA-positive case of SSc overlapping with RA, it is suggested that AChA are not specific for a particular disease, and that SSc patients with AChA are likely to have characteristics of other rheumatic diseases such as SLE, SS, etc. This finding that AChA were detected more frequently in SLE and/or SS-like patients might be a reflection of polyclonal B cell activation. When encountering patients with AChA, we should investigate possible overlapping with SLE, sicca, and diseases other than SSc, which might lead to the requirement of corticosteroid therapy. Interestingly, in one patient with SLE + RA, AChA titres were reduced when disease activity decreased after immnosuppressive therapy.

cDNA of p25, one of the antigens recognized by AChA, was cloned and, from sequence analysis, p25 contains two conserved domains located at N- and C-termini [22]. A highly conserved 37-amino acid region known as the 'chromo domain' was found at amino acid resides 20–56 in p25. This domain shares >70% sequence identity with protein in Drosophila and mouse [26]. Recently it was shown that the HP1 chromo domain has chromosome binding activity and that proteins with this domain are recruited to their distinct chromosomal binding sites, probably due to protein–protein interaction [36]. It is generally acknowledged that autoepitopes on nuclear antigens are conserved and functional domain regions [5,37,38]. In this study, 79% of AChA-positive sera recognized our fusion protein with N-terminus of p25 containing the chromo domain by immunoblotting method. Since fusion partner and/or translated 5′ non-coding region might influence the result, further experiments using C-terminus of p25 and also p23 clones will be required to confirm the data. Nonetheless, one-fifth of AChA-positive sera did not recognize the chromo domain, and probably react to C-terminus of p25, which also shares >50% sequence homology to proteins in Drosophila and mouse [22]. Interestingly, Powers *et al.* identified two functional domains in C-terminal HP1: a nuclear localization domain and a heterochromatin binding domain [39]. Although the chromo domain is one of the major epitopes of AChA, the term 'anti-chromo antibodies' might not be suitable for all AChA. By immunoblotting with HeLa nuclear extract, IgM-AChA were positive in only four patients, but with fusion protein, 12 patients were positive for IgM-AChA. We thought that this discrepancy might be caused by differences in detection sensitivity. Fusion protein as an antigen source appears to increase the sensitivity in immunoblotting. ELISA with highly purified recombinant protein will solve such a problem.

Many autoantigens are functional macromolecular complexes involved in fundamental cell functions [5,37,38]. Although interactions of chromo antigens to CENP antigens are still unknown, AChA-positive sera without ACA have not been reported. Three patients with ACA developed AChA-positive and in another report [20] a similar finding was observed. Moreover, the age of AChA-positive patients was higher and disease duration slightly longer than those of AChA-negative patients with ACA in our study. It is suggested that in some patients with ACA, immune responses against centromeric complex (possibly including chromo antigens) will progress and might be related to the symptoms seen in SLE, SS, and/or other diseases.

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