

Frequency and clinical associations of anti-chromo antibodies in connective tissue disease

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Abstract

Objectives—To assess the frequency and clinical associations of anti-chromo antibodies in connective tissue disease and to study their relationship to anti-centromere autoreactivity.

Methods—Anti-chromo and anti-centromere antibodies (ACA) were measured by immunoblotting in 50 patients with systemic sclerosis (SSc) and 58 connective tissue disease controls. Common epitopes on centromere and chromo antigens were sought using affinity-purified antibodies from immunoblots.

Results—Anti-chromo antibodies were detected in three of 32 sera with ACA and no controls. The three patients with anti-chromo antibodies had limited cutaneous SSc, and two had erosive arthritis. No cross-reactivity between chromo or centromere-related proteins was demonstrated.

Conclusions—Anti-chromo antibodies form a rare autoantibody specificity that may identify an overlap group of patients with SSc and arthritis. Chromo and centromere antigens are associated but immunologically distinct autoimmune targets. Whether they localise to the same chromosomal site remains unknown.

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Anti-centromere antibodies (ACA) detected by indirect immunofluorescence (IF) are found in 25-55% of patients with systemic sclerosis (SSc),^{1,2} especially those with limited cutaneous disease.^{3,4} ACA are highly specific for SSc, although they have been reported infrequently in rheumatoid arthritis,⁵ in systemic lupus erythematosus (SLE)⁶ and in 19-21% of patients with idiopathic Raynaud's phenomenon.⁷⁻⁹ Several polypeptides extracted from human cell lines and chromosomal preparations are detected by ACA using immunoblotting techniques. The three proteins most commonly recognised by ACA are CENP-A (17 kD), CENP-B (80 kD) and CENP-C (140 kD).¹⁰ Two other proteins, CENP-D (50 kD)¹¹ and CENP-F (400 kD)¹² are much less commonly recognised by ACA. All these proteins have been distinctly located within the kinetochore region of the centromere or in the immediately adjacent heterochromatin throughout the cell cycle, except for CENP-F which localises to the kinetochore in a cell-cycle dependent manner.

Another group of proteins usually visualised as a doublet between 23 and 26 kD are occasionally recognised by ACA on immunoblotting.^{6,10,13,14} Although the autoantibodies that recognise these proteins have been detected exclusively in sera containing ACA, elution experiments have suggested that the proteins may not locate to the centromere.¹³ One of the proteins has recently been cloned and has sequence homology to chromosomal proteins that suppress gene expression in *Drosophila*.¹⁵ Consequently the antibodies that recognise the 23/26 kD proteins have been termed anti-chromo antibodies. We have determined the frequency and clinical associations of anti-chromo antibodies in a variety of connective tissue disorders, and have studied their potential cross-reactivity with centromere-related autoantigens.

Methods

PATIENTS AND CONTROLS

Thirty two patients with ACA detected by indirect immunofluorescence (IF) using Hep-2 cells were studied. Seventy six other patients attending the same clinic (35 SLE, 28 SSc, 9 primary Sjögren's syndrome and 14 with undifferentiated connective tissue disease), and 45 patients with rheumatoid arthritis (32 seropositive for rheumatoid factor) attending a general rheumatology clinic were also studied. Clinical, radiological and serological features were documented using a predefined protocol as previously described.⁶ Limited skin involvement was defined as cutaneous sclerosis not extending proximal to the elbows or the knees, with or without facial involvement.

SEROLOGY

Indirect immunofluorescence

Sera were screened at a dilution of 1/40 for antinuclear antibodies (ANA) by IF using Hep-2 cells (Biodiagnostic Ltd, Worcs, UK) and FITC-conjugated polyvalent anti-human immunoglobulin.

Immunoblotting

A nuclear-enriched sonicate of K562 cells was prepared as the antigen source for immunoblotting as previously described.⁶ The nuclear protein was diluted with an equal volume of sodium dodecyl sulphate (SDS) sample buffer (62 mM Tris-HCL, pH 6.8, 0.2% SDS, 50 mM dithiothreitol and 10% glycerol). Proteins in the nuclear-enriched extract were separated by SDS-polyacrylamide

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gel electrophoresis (SDS-PAGE)¹⁶ in 12.5% polyacrylamide and transferred to nitrocellulose as described¹⁷ except the transfer buffer contained 0.1% SDS. The nitrocellulose was blocked in 5% dried milk powder in phosphate-buffered saline (PBS). The strips were probed with serum samples (1/100 in PBS/milk powder) and then with goat-anti-human alkaline phosphatase conjugate directed against IgG and IgM isotypes (Sigma, St Louis, MO). Bands were visualised using nitro-tetrazolium blue and 3-bromo-2-chloro-5-indoyl phosphate as substrates.

Affinity-elution of antibody fractions from nitrocellulose

Antibodies were affinity-purified from antigens immobilised on nitrocellulose blots using a modification of Olmsted's strategy.¹⁸ A 1 cm strip was excised from each side of the blot, incubated with a 1/100 dilution of patient serum for 2 hours at room temperature, and bands visualised as above. The remaining sheet of nitrocellulose was blocked with PBS/milk powder for 1 hour. The nitrocellulose sheet and probed strips were aligned and regions on the sheet containing the appropriate antigen were excised, minced and placed in 25 ml tubes. An equivalent amount of nitrocellulose containing no antigen was used as a negative control. The minced blot was preincubated with 25 ml PBS/milk powder for 1 hour then serum added to a dilution of 1/100 and incubated at 4°C for 18 hours with shaking. The nitrocellulose was transferred to 10 ml plastic columns and washed with 40 ml of PBS containing 0.05% Tween-20 and 40 ml of PBS. A further wash with PBS containing increasing salt concentrations (0.25 M, 1 M, 2 M and 4 M KCl) (10 ml) was used to minimise non-specific interactions. Specifically bound antibody was removed from the nitrocellulose with elution buffer (5 ml, 0.2 M glycine, pH 2.8 containing 1 mg/ml BSA, 2 min incubation) and the pH of the eluate was quickly restored by the addition of 150 µl of 1 M Tris-HCl, pH 8.0. Samples were dialysed against 3 L of 1 mM phosphate buffer (pH 7.4) and concentrated to less than 1 ml by placing them in polyethylene glycol crystals for 30–60 minutes. The eluted antibodies were tested undiluted by IF on HEp-2 cells, by immunoblotting against the nuclear extract and by a solid-phase ELISA for detection of anti-CENP-B antibodies.

ELISA for detection of anti-CENP-B antibodies

Ninety six well polyvinyl microtitre plates (Nunc Immunosorp) were coated overnight at 4°C with 100 µl/well of 10 µg/ml of cloned CENP-B. The CENP-B clone comprised the 147 amino-acids of the C-terminal region of CENP-B fused to β-galactosidase (gift of W C Earnshaw).¹⁹ The plates were washed with PBS/Tween, blocked with PBS containing 2% fetal calf serum (PBS/FCS) (150 µl/well for 1 h at room temperature) and incubated with patient and control sera diluted 1/1000 in PBS/FCS. After washing three times with PBS/Tween, 100 mls of alkaline phosphatase

labelled goat anti-human IgG (Sigma) diluted 1/1000 in PBS/FCS was placed in each well for one hour at room temperature. Plates were washed as before and substrate (10 mg/ml para-nitrophenyl phosphate in carbonate buffer pH 9.6) was added for colour development. The plates were read after 30 minutes at 412 nm on an ELISA reader (Dynatech). The results were expressed as a percentage optical density of the highest standard. Samples were considered positive if readings were greater than three standard deviations above the mean of 60 healthy controls.

Results

ACA AND CLINICAL FEATURES

The 32 sera selected for ACA all showed a characteristic staining pattern for ACA on IF. Twenty eight of the 32 recognised both CENP-C and CENP-A on immunoblotting, of which 14 also recognised CENP-B (fig 1). Of the other four sera positive for ACA on IF, two recognised CENP-C alone (fig 1, lanes 13 and 16) and two recognised CENP-A alone (fig 1, lane 17). Twenty six of the 32 sera were from patients who fulfilled criteria for SSc²⁰ of whom all had limited cutaneous involvement, three sera were from patients with Raynaud's phenomenon and three sera were from patients with SLE. Of the 26 patients with SSc and ACA, three had primary biliary cirrhosis in addition. The other clinical features in association with ACA in some of these patients have been reported previously.⁶

ANTI-CHROMO ANTIBODIES AND CLINICAL FEATURES

Three of the 32 sera with ACA had anti-chromo antibodies (fig 1, lanes 22–24). The chromo antigens were detected by IgG antibodies and not by IgM antibodies. Anti-chromo antibodies were not present in any of the control sera. All three patients with anti-chromo antibodies had limited cutaneous SSc with disease duration of 14, nine and three years respectively. Two of these three patients had an erosive arthritis, were seropositive for rheumatoid factor and fulfilled

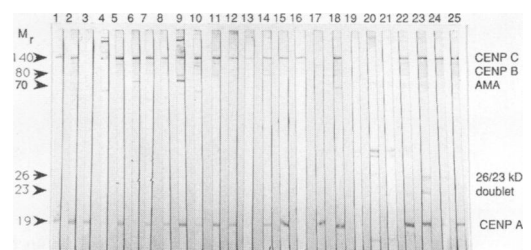


Figure 1 K562 cell polypeptides recognised by sera with ACA after separation on a 12.5% SDS-polyacrylamide gel and transfer to nitrocellulose. Lanes 19 to 21 are control sera positive for anti-Sm that recognise B'/B proteins. Lanes 1–18 and lanes 21–25 are sera with ACA and all recognise at least one of the CENP proteins. The serum in lane 17 recognises CENP-A alone and sera in lanes 13 and 16 recognise CENP-C alone. In addition to centromere proteins, sera in lanes 6 and 9 recognise mitochondrial antigens and sera in lanes 22 to 24 recognise chromo antigens (p23 and p25).

ACR criteria for RA.²¹ In contrast, only three of the 29 patients with ACA but without anti-chromo antibodies fulfilled criteria for RA (one seropositive for rheumatoid factor). There were no other differences between the two groups.

DEVELOPMENT OF ANTI-CHROMO ANTIBODIES

In one of the patients with anti-chromo antibodies on whom we had stored samples of sera, we were able to demonstrate the development of anti-chromo antibodies over time. The patient first presented in 1985 with arthritis and Raynaud's phenomenon. At that time her serum recognised centromere polypeptides alone (fig 2, lane 5). Over the following 12 months she developed strong anti-chromo reactivity, which was present in all later samples. (fig 2, lanes 6–11). There was no apparent change in the pattern of disease or other serological features over this time.

RELATIONSHIP BETWEEN ACA AND ANTI-CHROMO ANTIBODIES

Sera from two of the three patients with anti-chromo antibodies were used to affinity-purify antibodies to specific antigens. Results obtained with both sera were the same. Affinity-purified antibodies were prepared from CENP-C, CENP-A, the 23 kD component of the chromo antigen (p23¹⁵), the 26 kD component of the chromo antigen (p25¹⁵), and from a region of the blot where no bound antibody was detected. Antibodies were not eluted from the CENP-B antigen, due to the generally weaker recognition of this antigen (fig 1). However, an ELISA for recombinant CENP-B was used to study whether affinity-eluted antibodies recognised this polypeptide.

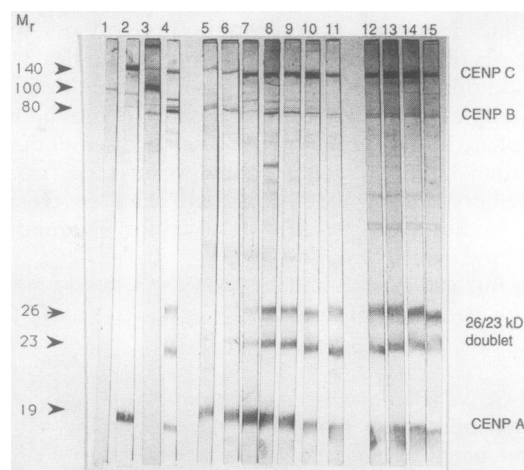


Figure 2 K562 cell polypeptides recognised by serial samples of sera from patients with anti-chromo antibodies after separation on a 12.5% SDS-polyacrylamide gel and transfer to nitrocellulose. Lane 1, normal serum. Lane 2, ACA positive control. Lane 3, anti-topoisomerase-1 antibody control. Lane 4 serum with ACA and anti-chromo antibodies in first patient. Lanes 5–11, sera taken from second patient at yearly intervals from 1985–1992 showing the development of anti-chromo antibodies over time in contrast to more long-standing anti-centromere reactivity, especially to CENP-A. Lanes 12 to 15, sera from third patient taken at two yearly intervals from 1980–86 showing constant levels of ACA and anti-chromo antibodies.

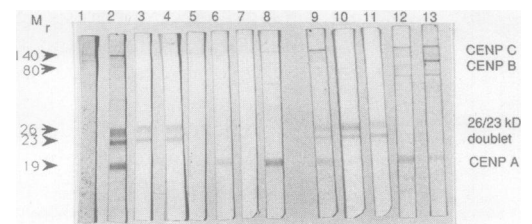


Figure 3 K562 cell polypeptides recognised by anti-chromo antibodies from whole serum and by serum affinity-purified from proteins on nitrocellulose. Lane one, normal serum. Lane 2, whole serum from a patient with anti-chromo antibodies. Lane 3, anti-26 kD (p25) affinity-purified antibodies from serum in lane 2 recognise both p23 and p25. Lane 4, anti-23 kD (p23) affinity-purified antibodies from serum in lane 2 recognise both p23 and p25. Lane 5, anti-CENP-C affinity-purified antibodies from serum in lane 2 recognise CENP-C alone. Lane 6, anti-CENP-A affinity-purified antibodies from serum in lane 2 recognise CENP-A alone. Lanes 8, 12, 13, ACA control sera. Lane 9, whole serum from a second patient with anti-chromo antibodies. Lane 10, anti-26 kD (p25) affinity-purified antibodies from serum in lane 9 recognise both p23 and p25. Lane 11, anti-23 kD (p23) affinity-purified antibodies from serum in lane 9 recognise both p23 and p25.

Affinity-purified anti-CENP-A and anti-CENP-C antibodies recognised their respective antigens on reprobing immunoblots but showed no cross-reactivity with each other (fig 3 lanes 5 and 6) or with chromo antigens. Affinity-purified anti-chromo antibodies did not recognise centromere antigens (fig 3 lanes 3, 4, 10 and 11). There were therefore no cross-reactive epitopes shared between centromere polypeptides and chromo antigens. However, affinity-purified anti-p25 recognised both 26 kD and 23 kD bands on reprobing (fig 3 lanes 3 and 10) as did affinity-purified anti-p23 (fig 3 lanes 4 and 11). There appears therefore to be at least one epitope shared between these chromo antigen components. No affinity-purified antibodies recognised the carboxyl-terminal fragment of CENP-B. Whereas both affinity-purified CENP-A and CENP-C stained the centromere on IF of HEP-2 cells, antibodies eluted from chromo antigens were consistently negative on IF.

Discussion

The centromere is the primary constriction site of eukaryotic chromosomes where the two sister chromatids are tightly paired. Sera containing ACA give a discrete speckled pattern on IF due to ACA recognising antigens in the region of the centromere.²² Closely adjacent to the centromeric DNA is the kinetochore which acts as the point of microtubule attachment for chromosomes before cell division. A family of centromere-related proteins localised to distinct regions of the kinetochore and underlying DNA have been characterised using ACA.²³ As reported in previous work^{3 4 6} and confirmed in the current study, sera containing ACA are very likely to have been obtained from patients with a limited cutaneous form of SSc and only very occasionally from patients with other connective tissue diseases. Why the cluster of proteins around the centromere should be a target for autoantibodies from such a defined group of patients is unknown.

Cox *et al*²⁴ described several proteins (14, 20, 23 and 34 kD) recognised by sera from patients with limited cutaneous sclerosis. Earnshaw *et al* suggested that the 14 kD protein corresponds to CENP-A, and subsequently has cloned cDNAs for the CENP-B¹⁹ and CENP-C²⁵ antigens. The CENPs are structurally, immunologically and most likely functionally distinct, although there is at least one cross-reactive epitope on CENP-B.²⁶ In the present study we were unable to demonstrate any cross-reactivity between the CENP-A, CENP-C and a carboxyl terminal fragment of CENP-B. The existence of a few ACA positive sera that did not recognise one either CENP-A, -B or -C also confirms that these proteins must have at least some independent epitopes. The existence of multiple independent epitopes associated with the kinetochore complex is consistent with the widely held notion that autoantibody generation is driven by the autoantigen particle itself.

A group of proteins with an apparent molecular mass between 23 and 26 kD are recognised by 10–15% of sera containing ACA.^{10 13 14} The antigens have been termed p23–25 and the autoantibodies anti-chromo.¹⁵ The p25 antigen has been cloned and found to have sequence homology with a heterochromatin protein of *Drosophila*, HP1.¹⁵ Anti-chromo antibodies are thought to target a structural motif shared by HP1 and a number of other chromosomal proteins that produce stable alterations in chromatin structure. Guldner *et al* reported two proteins of 23 and 25.5 kD immunoreactive with five of 18 sera positive for ACA, all of which recognised CENP-A.¹³ However, antibodies eluted from the 23 and 25.5 kD proteins failed to bind to the centromere. We have confirmed these findings and shown that p23 and p25 share a cross-reactive epitope with each other but not with any centromere antigens. These findings are consistent with the lack of primary sequence homology between the CENPs and the p25 antigen. Because the chromo antigenicity appears to be masked in tissue culture cells used for IF experiments, it is not clear whether the chromo antigens localise to centromeric heterochromatin or other chromosomal sites.

Anti-chromo antibodies were present in three of 32 sera positive for ACA (9.4%) which is similar to previous reports and confirms the rare occurrence of this class of autoantibodies. The antibodies were found only in sera containing ACA. It is of interest that two of the three patients with anti-chromo antibodies had an erosive arthritis and positive rheumatoid factor in addition to limited cutaneous scleroderma. Therefore anti-chromo antibodies may be a marker for a group of patients with an overlap syndrome of scleroderma and rheumatoid arthritis, but larger numbers are needed to confirm this.

In one patient studied, longitudinally anti-chromo antibodies developed after ACA. An ordered development of autoantibody responses to different proteins on the same complex has been observed with other auto-

antigens such as the U1 RNP particle²⁷ consistent with the process of affinity maturation of an antigen driven immune response. It remains to be determined whether the chromo antigens form part of a complex that includes the CENPs. More certainly, anti-chromo antibodies will prove further examples of how naturally occurring autoantibodies from patients with connective tissue disease are valuable tools for studying cell biology.

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