Characterisation of centromere (kinetochore) antigen reactive with sera of patients with a scleroderma variant (CREST syndrome)

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SUMMARY Anticentromere (kinetochore) antibody is the marker antibody in CREST syndrome, but the precise molecular composition of the partner antigen has been poorly defined. This report describes for the first time a procedure for the successful extraction and biochemical characterisation of the centromere antigen molecule. The centromere antigen was extracted with 4M NaCl solution. The molecular weight of the partner antigen of the centromere antibody was determined to be 70 000 daltons by the SDS-PAGE and immunoblotting methods. A Sephacryl S-300 column experiment confirmed these results. Centromere antigenic activity was preserved at pHs between 3 and 11 and was resistant to three enzymes, trypsin, RNase, and DNase.

Key words: antinuclear factors, autoantibody, connective tissue disease, collagen disease, antigens.

CREST syndrome is a variant of scleroderma characterised by calcinosis, Raynaud's phenomenon, oesophageal hypomotility, sclerodactyly, and telangiectasia. Recently Moroi and his coworkers^{1,2} reported a new autoantibody, the anticentromere (kinetochore) antibody, which is found mainly in the sera of patients with CREST syndrome. Because the centromere antigen is unextractable by physiological saline solution, its extraction and characterisation have been unsuccessful. We describe here for the first time a successful extraction procedure and the biochemical characterisation of the partner antigen of the centromere antibody.

Materials and methods

Patients' sera. Three reference sera containing anticentromere antibody were kindly provided by Dr Y. Moroi from among the sera of the patients with CREST syndrome who visited the Second Tokyo National Hospital. It was confirmed that anticentromere antibodies are also found in our 16 Japanese

patients with incomplete CREST syndrome or other connective tissue diseases with Raynaud's phenomenon (Table 1) as described in Caucasian population.²⁻⁷ Patients with incomplete CREST syndrome were defined as those who fulfil three or four of the five symptoms or findings

Table 1 *Diagnoses of 16 patients with anticentromere antibody*

Patient no.	Sex	Age	Diagnosis
1	M	32	Incomplete CREST syndrome
2	F	39	Incomplete CREST syndrome
3	F	47	Incomplete CREST syndrome
4	M	56	Incomplete CREST syndrome
5	F	68	Incomplete CREST syndrome
6	F	73	Incomplete CREST syndrome
7	F	71	Incomplete CREST syndrome
8	F	30	Incomplete CREST syndrome
9	F	74	Raynaud's and Sjögren's
10	F	64	Raynaud's and Sjögren's
11	F	55	Raynaud's and Sjögren's
12	F	59	Raynaud's and RA
13	F	54	Raynaud's and SLE [†]
14	F	56	Raynaud's alone
15	F	70	RA and Sjögren's
16	F	47	SLE

* Rheumatoid arthritis

† Systemic lupus erythematosus

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mentioned above. Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) were diagnosed according to the criteria of the American Rheumatism Association.^{8,9} Sjögren's syndrome was diagnosed by the criteria of the Research Committee for Sjögren's Syndrome of the Ministry of Health and Welfare, Japan,¹⁰ which are the modified criteria by Shearn.¹¹

Immunofluorescence study. The anticomere antibodies in the patients' sera were detected by an indirect immunofluorescence technique using cultured human epithelial cell lines (HEp-2 cells) (Kallstad, Chaska, Mn, USA) as substrate.

Preparation of crude centromere antigen. Acetone-treated rabbit or calf thymus powder (Pel-Freeze, Rogers, AR, USA) was used as the source of the centromere antigen. The extraction of the centromere antigen from 5 g of the thymus powder was carried out with 20 volumes of 4 M NaCl-0.01 M sodium phosphate buffer at pH 7.4 for 16 hours at 4° C with gentle stirring with a magnet stirrer. This 4 M NaCl extract was dialysed overnight against 20 volumes of 0.01 M phosphate buffered saline (PBS) at pH 7.4, the PBS being changed three times. After the dialysis this material was centrifuged with a Hitachi RPR-12 Rotor (Hitachi Koki, Tokyo, Japan) at 10 000 rpm for 30 min at 4° C. The supernatant was concentrated up to 50 mg protein/ml with an Amicon Hollow Fiber Concentrator DC2 and a Minicon B15 concentrator (Amicon, Lexington, Mass, USA). Extraction of the centromere antigen with isotonic saline solution, 6 M urea and 1% sodium dodecylsulphate (SDS) was also tried.

Absorption study. 0.1 ml of a 1:400 dilution of the anticomere antibody-positive serum was mixed with 4, 2, 1, 0.5, 0.1 mg of the crude centromere antigen and PBS and incubated for 48 hours at 4° C. After centrifugation of the solutions for 30 min at 10 000 rpm at 4° C the supernatant was tested for remaining anticomere activity by an indirect immunofluorescence test employing HEp-2 cells.

Purification of centromere antigen. The immunoaffinity column method was used for this purpose. Normal human IgG and IgG containing anticomere antibody were prepared with a DEAE-cellulose column from 20 ml of pooled normal human sera and from 20 ml of serum from a patient with CREST syndrome respectively. The patient's serum did not contain any other immunofluorescent antibodies in routine tests using rat liver cells as substrate or any precipitating antibodies using the double immunodiffusion technique. Each IgG was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden) as described previously.¹² The crude centromere antigen was loaded and eluted with PBS

through a normal human IgG coupled CNBr-activated Sepharose column to remove non-specifically adsorbed antigens which have affinity for normal human IgG. The eluate was poured onto the anticomere column and the column was eluted with PBS at 4° C. The adsorbed centromere antigen was detached with 6 M urea, which does not inactivate the centromere antigen. The eluate was dialysed against PBS and concentrated.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out as described previously.¹² Beta-galactosidase (130 000), bovine serum albumin (69 000), bovine gamma globulin (50 000 and 25 000), trypsin (24 000), and sperm whale myoglobin (17 200) (all from Sigma, Saint Louis, Mo, USA) were used as markers for the molecular weights, which are expressed in daltons.

Immunoblotting study. The immunoblotting procedures were carried out according to the method of Towbin *et al.*¹³ Bovine IgG was used as the marker for molecular weight.

Gel chromatography. Sephacryl S-300 Superfine (Pharmacia, Uppsala, Sweden) was packed in a Pharmacia KP column (2 × 90 cm). Blue dextran (2 000 000), thyroglobulin (640 000), bovine IgG (150 000), sperm whale myoglobin (17 200) (all from Sigma, Saint Louis, Mo, USA) were used as markers for molecular weight. 50 mg of the crude centromere antigen protein was fractionated with a Pharmacia Fraction Collector FRAC-300 (Pharmacia, Uppsala, Sweden). Each separated eluate sample (5 ml) was concentrated to 0.2 ml with the Minicon concentrator, and the antigenic activity of the centromere antigen was tested by the immunoblotting procedure.

Enzyme treatment. The enzyme treatment of the centromere antigen with trypsin, RNase A, DNase I (all from Sigma, Saint Louis, Mo, USA) was carried out as described previously.¹² Each enzyme substrate ratio was 1:5 by weight. Incubation was performed for 1 h at 37° C.

pH stability. pH stability was tested between pH 3 and pH 11. 0.1 M acetate buffer was used at pH 3, 4, and 5; 0.1 M sodium phosphate buffer was used at pH 6, 7 and 8; and 0.1 M bicarbonate buffer was used at pH 9, 10 and 11. 1 ml of crude centromere antigen (20 mg protein/ml) was dialysed overnight against each buffer at 4° C. Each antigen was redialysed against PBS pH 7.4, and after centrifugation the antigenic activity of the supernatants was tested with the immunoblotting technique.

Species specificity. In order to study the species specificity of the centromere antigen, the crude calf and rabbit thymus antigens were compared.

Double immunodiffusion. This was performed as described previously.¹²

Protein concentration. The Biuret reaction was used for the estimation of the protein concentration.

Results

Extraction of the centromere antigen. The PBS, 6 M urea, 1% SDS, and 4 M NaCl solutions were tested for extraction of the centromere antigen, and it was demonstrated that only the 4 M NaCl solution successfully extracted this antigen. The 4 M NaCl solution showed better extraction capacity than 2 M, 1 M, or 0.5 M NaCl solution. Preservation of the antigenic activity of the centromere antigen after exposure to 4 M NaCl solution was demonstrated by the absorption test (Fig. 1) and subsequent immunoblotting tests (Figs. 3, 4).

Characterisation of the centromere antigen. The immunoaffinity column-purified centromere antigen appeared as a 70 000 dalton protein band (CREST-70) by SDS-PAGE in the presence of 2-mercaptoethanol (Fig. 2), and this band showed centromere-antigenic activity in the immunoblotting experiment (Fig. 4). This result was confirmed with sera from three different patients which contained anticentromere antibody. The Sephacryl S-300 column experiment also showed that the molecular weight of the centromere antigen is around 70 000 daltons after 4 M NaCl treatment of the antigen (Fig. 3).

Enzyme treatment of the centromere (CREST-70) antigen. The RNase and DNase did not affect the CREST-70-antigenic activity in the immunoblotting experiments. Trypsin degraded the CREST-70 antigen molecule to 24 000 daltons, but the antigenic activity was unaffected. These results mean that the CREST-70 antigen does contain protein as a part of its molecular structure, as suggested previously,¹ but

Fig. 2 Coomassie brilliant blue staining patterns in SDS-PAGE. Lane 1: marker proteins. Lane 2: crude centromere antigen. Lane 3: purified centromere antigen. Lane 4: bovine IgG.

that the true antigenic site may not reside in the trypsin-sensitive protein portion.

pH stability. The antigenic activity in immunoblotting tests of the CREST-70 antigen was equally great at pH values between 3 and 11.

Species specificity. The CREST-70 antigenic activity was seen in the 70 000 daltons protein band in both the calf and rabbit antigens.

Fig. 1 (a) Anticentromere staining pattern (control). (b) Partially absorbed anticentromere staining. (c) Complete absorption of the anticentromere staining.

Fig. 3 (a) Sephacryl S-300 gel chromatogram of the crude centromere antigen in the absence of SDS and 2-mercaptoethanol. (b) SDS-PAGE results with concentrated materials in tubes No. 30 to 60 in Fig. 3a. Coomassie brilliant blue G-250 was used for the staining of the proteins. (c) Immunoblotting experiment using the gel under the same conditions as in Fig. 3b. The anticentromere antigenic activity is shown at the position of around MW 70 000 in the Sephacryl S-300 column chromatogram (Fig. 3a), showing that the molecular weight of the CREST-70 antigen is unchanged in the presence and absence of SDS and 2-mercaptoethanol.

Discussion

Since the first description of the anticentromere antibody in 1980¹ it has been established with some exceptions that this antibody is the marker antibody of the CREST variant of scleroderma. In the main this was confirmed in our Japanese patients with positive anticentromere antibody. Although it had been demonstrated by the indirect immunofluorescence method¹ that the partner antigen is located in the centromere (kinetochore) portion of the chromosome, the precise molecular components of the centromere antigen have been poorly defined. The reason for this was the difficulty in the extraction of the centromere antigen. This report

describes for the first time a newly developed procedure for the successful extraction and partial characterisation of the centromere antigen molecule (CREST-70).

The CREST-70 antigen showed the same molecular weight as the Scl-70,¹⁴ which is an additional scleroderma-related autoantigen. But it is clear that the CREST-70 antigen is different from the Scl-70 antigen in immunofluorescent staining pattern, reaction to enzymes, solubility, and pH stability. McKeon *et al.*¹⁵ recently described an autoantibody which specifically recognised the nuclear envelope of Chinese hamster ovary (CHO) cells in the serum of a patient with linear scleroderma. Interestingly, this antibody bound the 70 000 daltons nuclear

Fig. 4 Lane 1: crude centromere antigen in SDS-PAGE. Lane 2: immunoblotting experiment using the gel under the same conditions as in lane 1. The arrow denotes the antigenic activity of the centromere antigen (CREST-70).

lamin protein. It may be necessary further to test its identity with the CREST-70 antigen, because Moroi *et al.*¹⁶ have already reported that in interphase nuclei of CHO or Ramos cells the centromere-antigenic foci were usually associated with the inner surfaces of the nuclear envelope.

Recently Habets and his coworkers¹⁷ reported an additional antigen of molecular weight 70 000 (MCTD-70) that is reactive mainly with the sera of patients with mixed connective tissue disease (MCTD). The MCTD-70 antigen is also unextractable with PBS and is similar in this respect to the

CREST-70 antigen. The CREST-70 antigen, however, appears to be different from the MCTD-70 antigen, because the MCTD-70 antigen is reported to be found mainly in MCTD patients, and the antigen is sensitive to protease.

The CREST-70 antigen molecule is unique in respect of its toughness. All of the autoantigens described recently, such as Sm,¹⁸ nuclear RNP,¹⁹ Ro/SSA,^{20, 21} La/SSB,^{21, 22} Mu/tRNP,^{23, 24} Sci-70,¹⁴ PM-1,²⁵ MA,²⁶ Jo-1,¹² Mi-1,²⁷ Ku,²⁸ and Ki²⁹ are sensitive to trypsin or RNase. In contrast, the CREST-70-antigenic site is resistant to all of the three enzymes, trypsin, DNase and RNase, though its molecular size is degraded by trypsin. Further study is needed to define more precisely the nature of the CREST-70 antigenic site. In addition the CREST-70 antigenic activity was preserved at pH 3 and pH 11. This characteristic is also unique because all of the soluble antigens mentioned above lose their antigenic activity at pH 3 and pH 11.

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