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Autoantibody to centromere (kinetochore) in scleroderma sera

(antinuclear antibody/chromosome)

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ABSTRACT **Sera from patients with scleroderma contained several autoantibodies to nuclear antigens which were distinguished by different patterns of nuclear immunofluorescence staining. One of these autoantibodies reacted with centromeric regions of chromosomes. In chromosome spreads, the staining appeared as two small spheres at the centromere, resembling kinetochores. The antigenic determinant appeared to be a protein or polypeptide tightly bound to DNA. The autoantibody was reactive with centromeres of cells derived from humans, mice, and Chinese hamsters. The autoantibody was present in high frequency in the calcinosis/Raynaud's phenomenon/esophageal dysmotility/sclerodactyly/telangiectasia variant (CREST) of scleroderma.**

Progressive systemic sclerosis, also known as scleroderma, is a chronic systemic rheumatic disease that can affect many organ systems including the skin and subcutaneous tissues, the gastrointestinal tract, the heart, the lungs, and the kidneys. The etiology of the disease is unknown but much information has been accumulated on the clinical, pathogenetic, and serological abnormalities associated with it. Sera of patients with scleroderma have been shown to contain autoantibodies to nucleolar and other intranuclear components. Generally, these autoantibodies have been detected by the immunofluorescence technique. By using tissue sections as substrate, autoantibodies have demonstrated different patterns of nuclear staining which have been described variously as nucleolar, speckled, and homogeneous (1, 2).

Some of the intranuclear antigens reacting with autoantibodies in scleroderma sera have been elucidated. There is a 4S–6S RNA, isolated from liver nucleoli, that specifically precipitates with serum autoantibody (3). Recently, another nuclear antigen has been identified and termed Scl-70 (4). This was shown to be a nonhistone nuclear protein of approximately 70,000 daltons. By polyacrylamide gel electrophoresis, Scl-70 was identified as a distinct protein band closely associated with but clearly separable from histone fraction H1.

In this report, we describe an autoantibody in scleroderma sera which reacts with the centromere (kinetochore) of chromosomes. This autoantibody was initially observed to give speckled nuclear staining on substrates consisting of organ sections. When tissue culture cells of different origins were used as substrates, the speckled staining was clearly observed to be associated with the centromeres of chromosomes. Further analysis suggested that the centromere antigen might be protein or polypeptide components tightly bound to the centromeric DNA of chromosomes.

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MATERIALS AND METHODS

Serum. Sera from 32 patients with scleroderma (25 females and 7 males) were studied. Twenty-three healthy persons (15 females and 8 males) were used as controls. All sera were heat-inactivated at 56°C for 30 min prior to use.

Immunofluorescence Studies. The indirect fluorescent antibody technique (5) was used to determine the nuclear staining patterns produced by the sera under study. The substrates consisted of snap-frozen 4-μm mouse kidney sections and three tissue culture cell lines. One was a human B lymphoid cell line (Ramos) originally propagated from American Burkitt lymphoma (6) and maintained in RPMI-1640 medium (Associated Biomedic System, Buffalo, NY). In contrast to most human B lymphoid cell lines, Ramos cells have been shown not to contain the Epstein–Barr virus genome (6). We ascertained that the cells were negative for Epstein–Barr virus-associated nuclear antigen by the anticomplement immunofluorescent technique (7) before using them as substrates for detection of antinuclear antibodies. The second cell substrate was Ehrlich mouse ascitic tumor cells grown in the peritoneal cavity of Swiss Webster female mice by weekly passage of about 10^7 cells. Both these cell lines were washed three times in RPMI-1640, and 0.1-ml aliquot of suspensions (2×10^6 cells per ml) were sedimented on to glass slides (8) by using a Cytospin (Shandon Southern Instruments, Sewickley, PA). The third cell line, HEp-2 (from human laryngeal carcinoma), was obtained from Antibodies Inc. (Davis, CA). These were adherent cells grown on glass slides and were used in the form supplied by the manufacturers.

For fixation of cells to be used in immunofluorescence studies, a modification of a periodate/lysine/paraformaldehyde fixative (PLP) was used (9). Experiments showed that PLP did not alter the antigenicity of nuclear components and, at the same time, nuclear antigens were retained in nuclei when cell smears were allowed to react with or were rinsed in physiological buffer solutions. Fixation with acetone, ethanol, methanol, or other agents either altered antigenicity of nuclear antigens or did not prevent loss of nuclear antigens by spontaneous solubilization in buffer solutions. PLP was prepared as described (9), but the final solution contained only 0.5% paraformaldehyde in 0.01 M $NaIO_4$/0.075 M lysine·HCl/0.0375 M sodium phosphate, pH 7.4. The slides of mouse kidney sections, Ehrlich cells, and Ramos cells were placed in a dry oven

Abbreviations: PLP, periodate/lysine/paraformaldehyde fixative; MNase, micrococcal nuclease.

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at 37°C for 30 min, fixed for 10 min in PLP at 4°C, and washed in balanced salt solution for 5 min. Slides were not allowed to dry but were used immediately for antinuclear antibody testing. They could also be kept, after fixation in PLP, in a balanced salt solution/glycerol mixture, 1:2 (vol/vol), at −20°C at least for 1 week. Balanced salt solution was used for serum dilution and rinsing of slides; it contained 137 mM NaCl, 1.2 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.4 mM KH_2PO_4, and 6.4 mM Na_2HPO_4 and was adjusted to pH 7.4.

Enzymatic Digestion of Cell Smear. Information concerning the nature of nuclear antigens in cells was obtained by pretreatment with enzymes or chemical reagents to determine whether or not such treatment destroyed reactivity with antisera. After PLP fixation, Ramos cells were treated with enzymes prior to reaction with sera containing autoantibodies. DNase I, RNase A, micrococcal nuclease (MNase), trypsin, and α-chymotrypsin were obtained from Worthington. Proteinase K was obtained from E. Merck (Darmstadt, W. Germany). Concentrations of enzymes were: DNase I and RNase A, 40 μg/ml; MNase, 3 μg/ml; trypsin, 0.5–50 μg/ml; α-chymotrypsin, 2.5–100 μg/ml; and proteinase K, 0.5–40 μg/ml. All were diluted with balanced salt solution except that DNase was diluted with balanced salt solution containing 6 mM $MgSO_4$. Cell smears were treated in Coplin jars containing 40 ml of enzyme solution at 37°C for 30 min with DNase I, RNase A, and MNase and at room temperature for 10 min with trypsin, α-chymotrypsin, and proteinase K. After this, slides were washed three times for 5 min each in balanced salt solution and used as substrates for antinuclear antibody testing.

In the case of the proteolytic enzymes trypsin, α-chymotrypsin, and proteinase K, concentrations higher than ≈2 μg/ml caused destruction or detachment of cells from slides, and this made it difficult to judge the susceptibility of nuclear antigens to these proteolytic enzymes. In this situation, celloidin (Parlodion, Mallinckrodt) was used to prevent the detachment of cells (10). Celloidin was dissolved at 20 μg/ml in ethanol/ether, 1:1 (vol/vol). PLP-fixed cell smears were dipped in celloidin solution at 4°C for 30 sec and dried for 10 min at room temperature. This made it possible to use up to 50–100 μg of proteolytic enzyme per ml without detachment of cells. At the same time, this did not hinder reaction between proteolytic enzymes or antibodies and cells. Control slides without enzymes were always tested in the same manner and were compared to digested samples. Susceptibility of nuclear antigen to enzyme digestion was judged by absence or significant decrease of staining intensity.

Other assays to determine the nature of nuclear antigens included treatment with acid solution, high molarity salt, sodium metaperiodate, sodium deoxycholate, and $NaDodSO_4$ under conditions shown in Table 1. Enzyme digestions were always performed in conjunction with known reference sera to determine the effectiveness of enzyme digestion of the respective substrates. For example, when DNase was used to digest DNA, the reference serum was from a patient with systemic lupus erythematosus and contained antibody to DNA. This serum was positive for immunofluorescent nuclear staining on nondigested cells but was negative on DNase-digested cells. When RNase was tested, serum from a patient with mixed connective tissue disease was used; it contained antibody to nuclear ribonucleoprotein. When proteolytic enzymes were tested, serum from a patient with systemic lupus erythematosus was used; it contained antibodies to nuclear histones. These sera had been characterized for immunospecificities of antinuclear antibodies by described methods (11, 12).

Preparation of Chromosomal Spreads. Colcemid (final concentration, 0.05 μg/ml) was added to culture media of Ramos cells and Chinese hamster ovary cells (kindly provided by F. T. Kao, Eleanor Roosevelt Institute for Cancer Research, University of Colorado); after incubation for 4 hr, cells were harvested. Also, Ehrlich cell-bearing mice were injected with 2 μg of Colcemid intraperitoneally and cells were collected 4 hr later. After washing in RPMI-1640, cells were incubated in 0.075 M KCl at room temperature for 15 min, sedimented on to slides by Cytospin, heated at 37°C for 30 min, and fixed with PLP. After the procedure of antinuclear antibody staining, cell smears were counterstained for 5 min with ethidium bromide at 10 μg/ml in balanced salt solution.

Table 1. Characteristics of centromere antigen

Treatment of cell smears*	Conditions °C; min	Staining with anticentromere antibody†
1. DNase I (40 μg/ml)	37; 30	−
2. RNase A (40 μg/ml)	37; 30	+
3. MNase (3 μg/ml)	37; 30	−
4. Trypsin (50 μg/ml)	23; 10	+
5. α-Chymotrypsin (100 μg/ml)	23; 10	+
6. Proteinase K (40 μg/ml)	23; 10	+
7. Trypsin (40 μg/ml) in 6 M urea	23; 10	+
8. Trypsin (5–40 μg/ml) in 0.01% $NaDodSO_4$	23; 10	−
9. α-Chymotrypsin (30–80 μg/ml) in 0.01% $NaDodSO_4$	23; 10	−
10. Proteinase K (2–40 μg/ml) in 0.01% $NaDodSO_4$	23; 10	−
11. HCl (0.1–0.2 M)	23; 30	+
12. NaCl (2 M)	23; 30	+
13. $NaIO_4$ (0.2 M)	23; 30	+
14. HCl (0.1 M) → $NaIO_4$ (0.2 M)	23; 30	+
15. $NaDodSO_4$ (1%)	23; 30	+
16. Sodium deoxycholate (1%)	23; 60	+
17. Balanced salt solution	37 or 60; on.‡	+

* Ramos cell smears were treated with the reagents listed prior to reaction with serum containing anticentromere antibody.

† +, Staining present; −, staining absent.

‡ Overnight treatment.

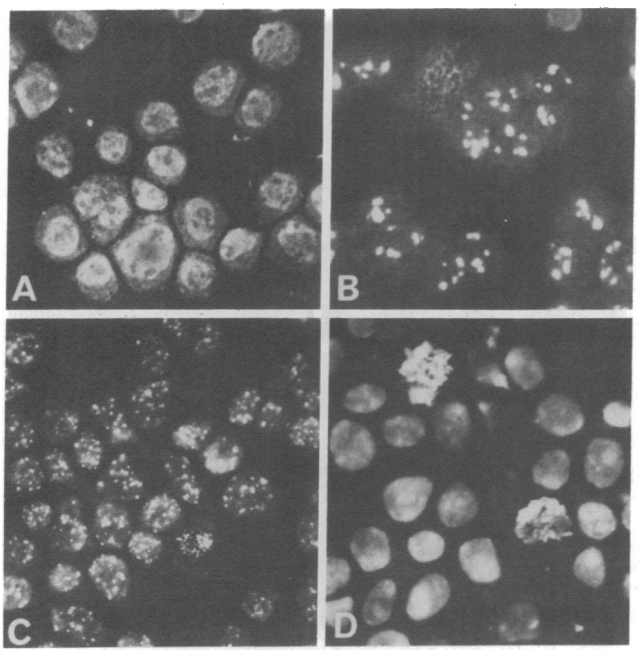

FIG. 1. Four patterns of nuclear staining produced by scleroderma sera on Ramos cells: diffuse granular (*A*), nucleolar (*B*), discrete speckled (*C*), and homogeneous (*D*). (×330.)

RESULTS

Patterns of Nuclear Staining Produced by Scleroderma Sera. In general, four major patterns of nuclear staining were produced by scleroderma sera (Fig. 1). The indirect immunofluorescence technique was used and the conjugate was anti-human Ig conjugated with fluorescein isothiocyanate. These patterns of staining were produced by different sera and are representative examples. Many sera showed combinations of these patterns of staining. The Ramos and HEp-2 cells and the mouse Ehrlich ascites cells showed comparable patterns of staining. However, with mouse kidney sections as substrate, nucleolar staining was much less obvious and the discrete speckled nuclear staining (Fig. 1*C*) could not be easily differentiated from the diffuse granular nuclear staining (Fig. 1*A*). On further careful inspection of several different sera giving the discrete speckled nuclear staining, it became apparent that this pattern of staining was associated with chromosomes (Fig. 2). In Fig. 2*A*, in a cell in late prophase the speckled staining is seen to be segregated in the center of the nucleus in a ring-like arrangement. In a metaphase cell (Fig. 2*B*, short arrows), the speckled staining is lined up on a metaphase plate. In an anaphase cell (Fig. 2*B*, long arrow; Fig. 2*C*, arrow), the speckled staining appears to be associated with chromosomes on the dividing cells.

Discrete Speckled Nuclear Staining is Associated with Centromeres of Chromosomes. Chromosomal spreads were prepared from Ramos cells, and sera producing the large speckled nuclear staining were studied further. The staining of the chromosomes was specifically restricted to the centromeric region of the chromosomes (Fig. 3). Each centromeric region could be clearly seen under the fluorescence microscope to consist of two small spheres of staining, resembling kinetochores. The arms of the chromosomes were completely negative for staining, suggesting a strict specificity of the antibody for the centromeric region. Centromere staining could be demonstrated not only on chromosome spreads from Ramos cell line but also on chromosomes of mouse Ehrlich ascites cells and Chinese hamster ovary cells. Therefore, this autoantibody from scleroderma sera was not limited in species specificity to human cells.

Nature of Centromere Antigen. In other studies characterizing autoantibodies to nuclear antigens, it has been possible to solubilize nuclear antigens and to identify the antibody in precipitating systems. However, we were not successful in developing an immunodiffusion precipitating system for the centromere–anti-centromere system in spite of attempts to solubilize the centromere antigen with different reagents. Therefore, the centromere antigen was analyzed by using the immunofluorescence technique to determine whether or not the antigenicity would be affected by various enzymes and chemical reagents. After such treatment, the cell smears (Ramos) were allowed to react with serum containing anti-centromere antibody followed by fluorescein-conjugated antiserum to human Ig. The centromeric antigen (in Ramos cells) was destroyed by DNase and trypsin with 0.01% $NaDodSO_4$ but not by RNase (Fig. 4). The effects of other enzymes and chemical reagents are shown in Table 1. MNase also destroyed the antigenicity of the centromere. Proteases such as trypsin, α-chymotrypsin, and proteinase K did not destroy centromere antigenicity. It required a combination of 0.01% $NaDodSO_4$ and proteolytic enzymes to destroy antigenicity. $NaDodSO_4$ or sodium deoxycholate alone did not remove antigenicity. When purified calf thymus DNA was used to repeatedly absorb

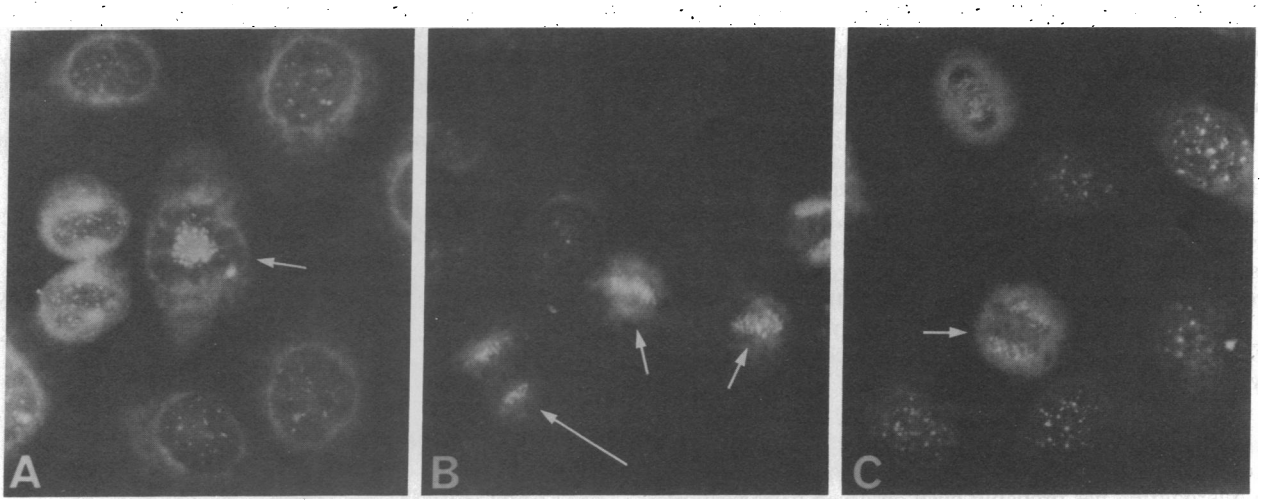

FIG. 2. Centromere staining on HEp-2 cells. (*A*) On interphase cells, discrete speckles are evenly distributed on whole nuclei. In late prophase (arrow), there is a ring-like arrangement of speckled staining. (*B*) In metaphase (short arrows), the speckled staining is lined up on metaphase plates. (*C*) In anaphase (arrow; also, long arrow in *B*), the speckled staining is associated with dividing chromosomes. (×440.)

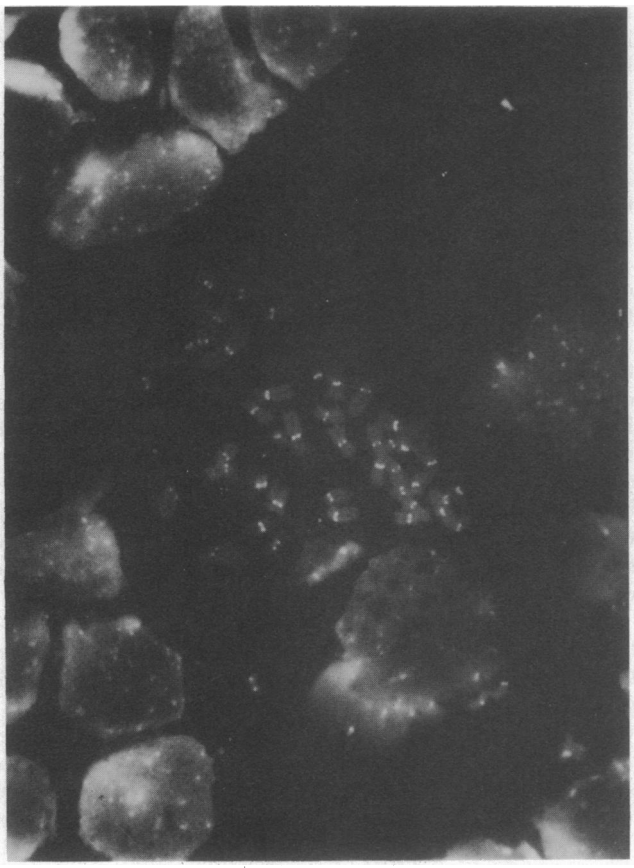

FIG. 3. Centromere staining on chromosomal spread of Ramos cells. Immunofluorescent staining is located only on centromeric region of each chromosome, and each centromeric region showed two small spheres of staining resembling kinetochores. (Counterstained with ethidium bromide; ×800.)

antibodies for DNA that might be present in the scleroderma sera, the centromere staining was not diminished or removed. On the other hand, this absorption procedure was effective in removing DNA antibody from systemic lupus erythematosus sera known to contain this antibody.

Clinical Correlation of Anticentromere Antibody. We examined the sera of 32 patients with scleroderma and observed that 10 (31%) contained antibody to centromere. Some of the features of patients with and without anticentromere antibody in scleroderma are presented in Table 2. The mean duration of disease was longer in those patients with anticentromere antibody than in those without it. The patients with anticentromere antibody appeared to fall into a subgroup of patients with scleroderma who have less extensive involvement of the skin than do those with diffuse scleroderma. This group of patients generally do not have sclerodermatous involvement of the trunk and the proximal portions of the extremities. However, their disease is characterized by prominence of calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia (a constellation of symptoms that has been described as CREST). One patient with the CREST syndrome did not have the anticentromere antibody.

DISCUSSION

The centromere staining produced by sera from some patients with scleroderma has probably been observed previously by other investigators but this type of staining may have been included among speckled nuclear staining patterns. In the studies

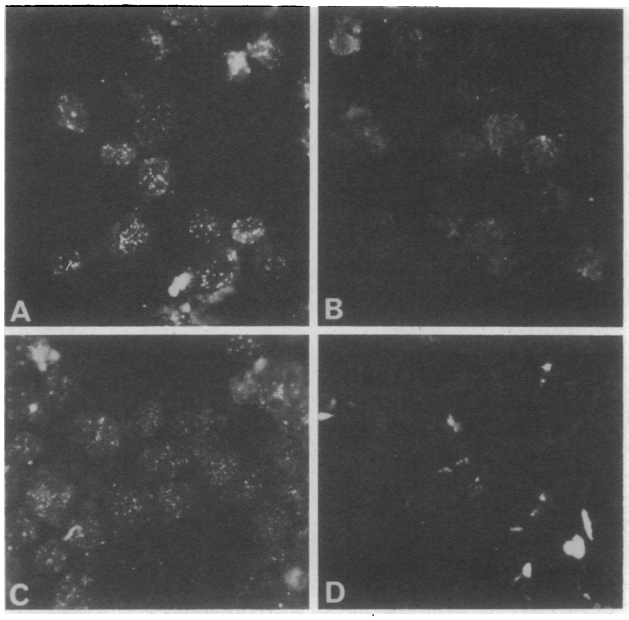

FIG. 4. Enzyme digestion of centromere staining shows removal of centromere staining after digestion with DNase I (*B*) or trypsin in 0.01% $NaDodSO_4$ (*D*). Digestion with RNase A (*C*) showed no changes compared to control (*A*). All enzymes were at 40 μg/ml. (×330.)

by Burnham *et al.* (13), Rothfield and Rodnan (2), and Parker and Kerby (14), different subtypes of speckled staining were described. It is not surprising that centromere staining could have been described as speckled staining, because the speckled staining pattern can be produced by antibodies to many nonhistone nuclear protein antigens. These include the Sm nuclear antigen, nuclear ribonucleoprotein, the SS-B antigen (Sjogren's syndrome-B), and the Scl-70 (scleroderma-70) antigens.

There are some interesting features concerning the reaction of centromere antigen with scleroderma sera. It appeared that the centromeric antigen remained antigenically unchanged in interphase cells and was present as large discrete speckles in the nucleus. On chromosomal spreads, the centromere staining could be distinguished as two small spheres, presumably one on each chromatid and located in regions associated with kinetochores. It was noted that the standard method for preparing chromosomal spreads by fixation in methanol/acetic acid caused a marked decrease of immunofluorescent staining, probably due to denaturation of the centromere antigen under these conditions. In these studies, we have demonstrated that the antibody in scleroderma sera was capable of reacting with centromere antigens in tissue culture cells or organ sections derived from humans, mice, and Chinese hamsters. Therefore, the centromere antigen is a highly conserved nuclear component.

Table 2. Comparison of patients with and without anticentromere antibody

	Anticentromere antibody	
	Positive	Negative
Cases, no.	10	22
Females, no.	10	15
Males, no.	0	7
Mean age, yr	56	48
Mean duration of disease, yr	15	7
CREST or CRST*	7	1

* C, calcinosis; R, Raynaud's phenomenon; E, esophageal dysmotility; S, sclerodactyly; T, telangiectasia.

The nature of the centromeric antigen that reacts with antibody can only be surmised from our studies. The antigen was resistant to digestion with trypsin and trypsin in 6 M urea. It has been reported (15, 16) that the histones in the nucleosome are resistant to trypsin digestion but susceptible to trypsin in 6 M urea. It would appear, therefore, that the centromeric antigen is not a histone protein. This is also supported by the experiments which showed that the centromeric antigen was not removed by treatment with 0.1 M HCl. Previous studies (12) showed that such treatment was effective in removing histones from tissue sections. The centromere antigen was sensitive to proteolytic enzymes in the presence of $NaDodSO_4$. $NaDodSO_4$ alone did not destroy antigenicity, and thus it required a combination of this protein denaturing agent with a proteolytic enzyme to remove the antigenicity. The property of the antigen suggests that it might be a tightly bound protein or polypeptide associated with the centromere. Indeed, some investigators (17–19) have demonstrated that some protein present in the centromeric region is tightly bound and is not removed by treatment with acid or other reagents that remove histones from isolated chromosomes. This might also explain why the antigenicity of the centromere could be removed by either DNase or MNase. Proteins tightly bound to DNA at this region might be removed en bloc with digested nucleotides. An alternative explanation would be that the antigenic determinant is formed by the total complex of centromeric DNA and associated proteins. At the present time, we have no evidence to favor one or the other hypothesis.

In the connective tissue or "autoimmune" diseases, autoantibodies have been detected that have been shown to be highly specific in their reactions with intranuclear antigens. Many of these autoantibodies are also highly specific in their disease relationships. For example, antibody to the Sm antigen appears to be segregated in patients with systemic lupus erythematosus (11, 20). Antibody to the Scl-70 antigen is seen primarily in patients with scleroderma (11). The anticentromere antibody is also highly specific for patients with scleroderma. Also of interest is the observation that the anticentromere antibody is present primarily in a subset of patients with scleroderma who have the CREST syndrome. Thus, these autoantibodies have been extremely useful in clinical situations as serological markers for certain diseases. In the broader context of biomedical research, these autoantibodies can be useful reagents for isolation of intranuclear proteins and nucleic acid–protein complexes.

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