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Fine Specificity Mapping of Autoantigens Targeted by Anti-Centromere Autoantibodies

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Summary

Autoantibodies to centromeric proteins are commonly found in sera of limited scleroderma and other rheumatic disease patients. To better understand the inciting events and possible pathogenic mechanisms of these autoimmune responses, this study identified the common antigenic targets of CENP-A in scleroderma patient sera. Utilizing samples from 263 anti-centromere immunofluorescence positive patients, 93.5% were found to have anti-CENP-A reactivity and 95.4% had anti-CENP-B reactivity by ELISA. Very few patient samples exclusively targeted CENP-A (2.7%) or CENP-B (4.2%). Select patient sera were tested for reactivity with solid phase overlapping decapeptides of CENP-A. Four distinct epitopes of CENP-A were identified. Epitopes 2 and 3 were confirmed by additional testing of 263 patient sera by ELISA for reactivity with these sequences constructed as multiple antigenic peptides. Inhibition CENP-A Western blots also confirmed the specificity of these humoral peptide immune responses in a subset of patient sera. The first three arginine residues (aa 4-6) of CENP-A appear essential for antibody recognition, as replacing these arginines with glycine residues reduced antibody binding to the expressed CENP-A protein by an average of 93.2% (range 80-100%). In selected patients with serial samples spanning nearly a decade, humoral epitope binding patterns were quite stable and showed no epitope spreading over time. This epitope mapping study identifies key antigenic targets of the anti-centromere response and establishes that the majority of the responses depend on key amino-terminal residues.

Keywords

anti-centromere autoantibodies; epitope mapping; scleroderma

Introduction

Systemic sclerosis (SSc), or scleroderma, is a connective tissue disorder characterized by vascular abnormalities and fibrosis of the skin and other internal organs. Often this disease is

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divided into two major clinical variants, classified as limited or diffuse, based upon the extent of skin involvement. To date, a full understanding of the underlying etiology and pathogenesis of these disorders remains to be elucidated. Although no direct pathogenic role of autoantibodies in scleroderma has been demonstrated, more than 95% of patients with SSc have antinuclear antibodies [1]. These autoantibodies are directed against a variety of antigens, including centromere-associated proteins.

In 1980, Moroi *et al* first identified anti-centromere antibodies (ACA) by means of indirect immunofluorescence [2]. Since then, ACA have been shown to be highly specific for scleroderma in general and are most commonly present in the patient subset with limited skin disease. These autoantibodies can also be detected in sera from patients with primary Raynaud's phenomenon and occasionally in other rheumatic diseases such as Sjögren's syndrome, systemic lupus erythematosus, rheumatoid arthritis [3-6] and very rarely in normal individuals [4,7]. The presence of ACA in patient sera generally correlates with a lesser risk of major organ involvement and, hence, a relatively better prognosis than many other autoantibodies associated with scleroderma [3]. The frequency of ACA varies depending on race and sex, being more prevalent in white females than in blacks or males [8].

The major centromere proteins bound by SSc patient sera are CENP-A (17kDa), CENP-B (80kDa), and CENP-C (140kDa) as first described by Earnshaw *et al* [9,10]. More than 90% of ACA positive sera recognize these 3 major antigens [9]. However, a small percentage of ACA positive sera contain autoantibodies directed against CENP-D (50 kDa) [11,12], CENP-E (312 kDa) [13], CENP-F (400 kDa) [14], and CENP-G (95 kDa) [15].

Although indirect immunofluorescence historically has been used for detecting ACA in the sera of patients with SSc and remains the gold standard, commercial antibody screening kits are now available. Developed by Rothfield *et al* [17], the first enzyme-linked immunosorbent assay (ELISA) using a cloned fusion protein, CENP-B [16], proved more sensitive in detecting ACA in sera of patients with SSc or Raynaud's disease than immunofluorescence techniques. This result has been confirmed by other groups [18-22]. ELISA tests for the presence of anti-CENP-A antibodies showed similar sensitivity and specificity to indirect immunofluorescence for detecting ACA [20-22].

Since CENP-A and CENP-B represent the major targets of the immune response in patients with SSc, several studies have examined the target epitopes of these autoantibodies. The autoimmune response to CENP-B has been extensively studied, and several major and minor epitopes have been characterized [16,18,23-25]. These identified antigenic regions have been shown to correspond to biologically functional regions.

The autoimmune response to CENP-A has been previously reported to be restricted to the CENP-A N-terminus [26-28]. Two major antigenic regions that both contain the core linear motif G/A-P-R/S-R-R have been further identified as prime targets of the anti-CENP-A response.

This study elucidates the sequential antigenic regions of the CENP-A protein that are recognized by anti-CENP-A autoantibodies. This is accomplished using overlapping decapeptides constructed on solid-phase peptide supports. Confirmatory studies with inhibition, affinity purification and protein mutagenesis are presented. By examining both the prevalence and the fine specificity of these antibodies in a large cohort of anti-centromere positive patients, we seek to better understand the origin and effects of the anti-centromere response in scleroderma.

Materials and methods

Sera selection and autoantibody screening

De-identified serum samples were obtained from the Oklahoma Clinical Immunology Serum Repository at the Oklahoma Medical Research Foundation. Anti-centromere positive sera were selected based upon having a titer of 1:360 or greater by Hep-2 cell staining (INOVA Diagnostics, San Diego, CA). Patient samples were excluded if their ANA displayed a pseudocentromere pattern, multiple immunofluorescent staining patterns, anti-dsDNA antibodies and/or precipitating levels of antibodies to select extractable nuclear antigens (Ro, La, Sm, nRNP, Jo-1 and P).

Antibodies to dsDNA were tested by immunofluorescence with *Critidia luciliae* in patients with sufficient sera as previously described [29-30]. Precipitating levels of other autoantibodies such as Ro, La, Sm, nRNP, and ribosomal P were detected by immunodiffusion against calf thymus extract. Antibodies representing previously unidentified lines (UIL) were observed in 24 of 263 patients (9%). Of the patients with serum samples from multiple dates, two went from UIL to negative and four went from negative to UIL. Based upon these selection criteria, 348 unique samples from 263 patients were obtained. Also, sera for two or more serial dates were obtained in 59 patients. Thirty-one frequency matched normal individuals (matched for age, gender and ethnicity) were used as controls.

ELISAs—Commercially available ELISA kits coated with either CENP-B (Helix Diagnostics, West Sacramento, CA) or recombinantly derived CENP-A and B antigens (INOVA Diagnostics, San Diego, CA) were used according to manufacturer instructions. Plates coated exclusively with CENP-A antigen were specially prepared for this study by INOVA. The positive and negative controls used in the INOVA CENP-A and B kit were also used for the CENP-A plates.

Solid phase peptide synthesis and autoantibody assays

The 66 decapeptides overlapping by two amino acids of CENP-A were synthesized on the rounded ends of radiation derivatized polyethylene pins using solid-phase peptide chemistry as previously described [31-33]. Assay steps were performed by lowering the solid phase supports into microtiter plate wells. Peptides were blocked with 3% low-fat milk in PBS for 1 hour at room temperature and then incubated in 1:100 serum dilutions in 3% milk/PBS with 0.05% Tween (PBST) for 2 1/2 hours at room temperature in sealed, humidified, plastic containers. The peptide blocks were then washed four times, with PBST for 8 min each, using vigorous agitation. Each solid phase peptide was then incubated with anti-human IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:10,000 dilution. Para-nitrophenyl phosphate disodium was used as a substrate, and plates were read at 405 nm with a microelisa reader (Dynatech, Alexandria, VA). Results for each plate were standardized by normalization to positive control responses to a set peptides synthesized on every plate.

Expression of CENP-A

A CENP-A construct was generated by using the forward primer 5'ACG CGT CGA CAT GGG CCC GCG CCG CCG GAG 3' and the reverse primer 5' ATA AGA ATG CGG CCG CTC AGC CGA GTC CCT CCT CAA GG' (Molecular Biology Resource Facility, Oklahoma City, OK) to produce a 423bp PCR product from a HeLa cell cDNA template. This insert was ligated into the pET28b(+) expression vector (EMD Biosciences/Novagen, Madison, WI) between the *Sal I* and *Not I* restriction sites. BL21(DE3)pLysS cells were used for transformation and expression of native CENP-A. Bacteria were grown at 37°C to an OD₆₀₀ of 0.6-0.7. The temperature was then reduced to 30°C, and expression was induced by IPTG (0.5mM). The

cells were harvested 4-5 hours later. The 6 X His-tagged fusion protein was isolated from whole bacterial lysate by purification on a nickel agarose column and eluted with 50mM NaH₂PO₄, 300mM NaCl, 250mM Imidazole (pH 8.0).

Site-directed mutagenesis

To assess antigenicity conferred by sequential humoral epitope 1, mutations were introduced in the CENP-A protein using the QuickChange II Site-directed mutagenesis kit (Stratagene; La Jolla, CA). The procedure involved using double-stranded DNA as template and two complementary oligonucleotide primers containing the desired mutation. Primers were annealed to the denatured DNA template and extended using *PfuUltra* DNA polymerase. The parental DNA was then digested with *Dpn I* endonuclease. Primers were synthesized by the Molecular Biology Resource Facility, University of Oklahoma Health Sciences Center (Oklahoma City, OK). Triple mutations were introduced in the CENP-A protein by replacing arginines with glycines (aa 4-6). The primers used to introduce the mutations were 5'-TCG ACA TGG GCC CGG GCG GCG GGA GCC GAA AGC CCG AGG-3' and its complementary oligonucleotide. Mutated CENP-A was expressed using the same protocol described above for native CENP-A.

MAP™ peptide ELISAs

Common humoral epitopes 2 (aa 7-2) and 3 (aa 17-30) were constructed on a 4-mer branching polylysine backbone (MAP™) and tested for reactivity with patient sera. Epitope 2 and epitope 3 MAP peptides were coated on 96-well polystyrene plates at a concentration of 0.5µg per well, for either 3 hours at room temperature or overnight at 4°C. The plates were washed, blocked with 0.1% BSA solution, washed and serum samples added at a dilution of 1:100 and 1:1000. This was followed by incubation for 2 1/2 hours at room temperature. Following washings, the samples were incubated with an alkaline phosphatase-conjugated anti-human IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) overnight at 4°C. After warming to room temperature, the plates were then developed using p-nitrophenyl phosphate substrate and read on a microelisa plate reader at 410 nm. All assays were standardized to a common positive serum sample.

Western blot analysis of native, mutated and peptide-inhibited CENP-A

All anti-centromere positive patient serum samples were tested by Western blot analysis using the expressed CENP-A protein as antigen. The protein was electrophoresed through a 12.5% polyacrylamide gel with SDS and then transferred to a nitrocellulose membrane. Patient samples were then added at a dilution of 1:100, followed by incubation with an alkaline phosphatase-conjugated anti-human IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). NBT/BCIP was used as a substrate for the conjugated enzyme.

In addition, ten patient serum samples were selected for Western blot analysis using equal concentrations of native and mutated CENP-A protein as antigens. The Western blots were then analyzed, using UVP Labworks version 4.0, to measure the intensities of the bands; these values were used to calculate percent inhibition. These Western blots were performed in duplicate for comparison and statistical analyses (mean percent inhibition ± S.E.) performed.

Eight patient serum samples were also selected for sequential peptide (epitopes 2 & 3) inhibition to determine the percent response directed against these peptides. Patient samples were pre-incubated with these peptides at room temperature for 2 hours and then reacted with CENP-A bound nitrocellulose membrane strips. The peptides were used at a tenfold concentration of CENP-A antigen. The Western blots were then analyzed, using UVP Labworks version 4.0, to measure the intensities of the bands and to calculate percent inhibition. These Western blots were also performed in duplicate for comparison and statistical purposes.

Results

Patient demographics and clinical characteristics

Two hundred and sixty-three individuals with anti-centromere antibodies (at a titer of 1:360 or greater) were identified from the Oklahoma Clinical Immunology Serum Repository. Of the individuals with available information, the mean age was 55.4 years ($\pm$ 14.8 years; range 21-91; n=233) and 95% were female (n=211). Of the patients with known race, 85 (83%) were European-American, 8 (8%) were African-American, 7 (7%) were American-Indian, 2 (2%) were Hispanic and 1 (1%) was Asian.

Prevalence of centromere protein targets in anti-centromere positive patient sera

To determine the specific targets of these anti-centromere responses, all patient serum samples were tested on the Helix CENP-B ELISA. Of the 263 samples tested, 251 samples (95.4%) had anti-CENP-B reactivity. Two of these positive patient samples were negative on their initial samples but subsequently developed anti-centromere antibodies. For control purposes, twenty-three patient samples positive by CENP-B ELISA were also tested by an INOVA CENP-A/B ELISA, and were all found to be positive. Further analyses of the 12 CENP-B negative patients by CENP-A/B ELISA, CENP-A ELISA and CENP-A Western blot showed 7 (2.7%) of our 263 patients samples target CENP-A exclusively. Thirty-one normal, control serum samples were negative on the Helix CENP-B ELISA. Twenty-nine of these samples were negative on the INOVA CENP-A/B ELISA, while one control was positive and one was weakly positive.

To determine the total percentage of our patient samples targeting CENP-A, all samples were tested by Western blot analysis using the expressed CENP-A as antigen. Of the 263 samples tested, 236 (90%) were positive, 10 (4%) were weakly positive, 16 (6%) were negative and 1 was inconclusive. Therefore, we concluded that 246 (94%) of our patient serum samples were targeting CENP-A. Of the 16 CENP-A negative patient samples, 11 were previously found to be CENP-B positive and 5 were CENP-B negative. The 11 (4%) CENP-A negative, but CENP-B positive, patient samples likely target CENP-B exclusively. Of the 31 normal controls tested, all were found to be negative for anti-CENP-A antibody by Western blot against expressed protein.

Fine specificity mapping of CENP-A

Sixteen of these patient samples were also tested for binding to the overlapping decapeptides of CENP-A by modified solid-phase ELISAs. The demographic and autoantibody features of these individuals are presented in Table 1. Fourteen of these samples were positive by CENP-A Western Blot and two were weakly positive. Of these two weakly positive samples, one exhibited no significant reactivity by the modified solid-phase ELISA while the other had very weak reactivity. Of these 16 samples tested, 13 showed significant reactivity to the N-terminus of CENP-A by our modified solid-phase ELISA. Two representative patient binding profiles are shown in Figures 1A and 1B. Six normal controls were also tested, and none showed significant reactivity to the N-terminus of CENP-A (example shown in Figure 1C). Overall, the binding profiles of patient sera were quite similar, as shown by the average binding profile in Figure 2A. The average binding of all normal control sera tested is also presented (Figure 2B).

Patient sera contained antibodies that, on average, recognized four major regions of CENP-A. These major epitopes are defined as being bound by more than 2 SDs above the normal mean across all decapeptides. These overlapping antigenic regions are presented in Table 2 and include epitope 1 (decapeptides 1-2; aa 1-12), epitope 2 (decapeptides 4-6; aa 7-20), epitope 3 (decapeptides 9-11; aa 17-30) and epitope 4 (decapeptides 13-16; aa 25-40).

Individual patient samples showed some variation in the specific epitopes bound although not dramatically different (Table 3). Of the fifteen patient samples which bound sequential epitopes, eight (53.3%) bound all four epitopes, two (13.3%) bound three of the four epitopes, two (13.3%) bound two epitopes and three (20%) bound a single epitope.

MAP™ peptide ELISAs

To further confirm and define these antigenic regions, we constructed common antigenic targets on a branching lysine backbone, comprising amino acids 7 through 20 (epitope 2) and 17 through 30 (epitope 3), for use in fluid phase peptide ELISAs. Epitopes 2 and 3 MAP™ peptide ELISAs were performed on all 263 patient samples. Of the 246 CENP-A positive samples, 197 (80%) were positive for reactivity to epitope 2 (at least 3 SDs above the normal mean) and 219 (89%) to epitope 3. One hundred and seventy-eight (72%) were positive for reactivity to both epitopes and 236 (96%) were positive for either epitope 2 or 3. Since 96% of these anti-CENP-A positive patient samples target either epitope 2 or 3, these epitopes appear to be the most common antigenic targets of the anti-CENP-A response.

When the epitope 2 and epitope 3 MAP™ peptide ELISA results were compared with the modified solid-phase ELISA to determine the reliability of each assay, the epitope 2 MAP™ peptide ELISA (sensitivity 0.91, specificity 0.40) and the epitope 3 MAP™ peptide ELISA (sensitivity 1.00, specificity, 0.50) each showed decreased specificity. These differences could be attributed to the potential for MAP™ peptide ELISA's poly-lysine backbone to cause increased background reactivity. Additionally, the density or confirmation of epitopes available for binding may vary from one assay to the next.

Stability of anti-centromere response over time

Of the 59 patients for whom sera was available from multiple dates, little variability in their specific anti-centromere antibody response was noted. Multiple sera dates available for these 59 patients spanned 1 week to 12 years (median: 2.5 years). Two patients were initially negative for, and subsequently developed, anti-centromere antibodies (Figure 3). In order to determine the stability of the anti-centromere response over time, two different sample dates for three patients, spanning 7.8, 9.6 and 9.8 years respectively, were tested on the modified solid-phase ELISA (data not shown). The anti-centromere response did not change over time and no evidence of epitope spreading was found.

Peptide inhibition against CENP-A Western blot

Eight CENP-A positive patients samples, based on reactivity by Western blot, were selected for sequential CENP-A epitopes 2 and 3 peptide inhibition. Representative peptide inhibition blots for two patient samples are presented in Figure 4. Previously, patient 1 was shown to have reactivity to epitope 2, but not to epitope 3, by our modified solid-phase ELISA and MAP™ peptide ELISA. This finding is confirmed here since the patient shows inhibition with epitope 2 linear peptide of 27.9% ($\pm 12.4\%$) and none with epitope 3 linear peptide. Patient 2 previously showed reactivity to both epitopes 2 and 3; this is confirmed here by inhibition with epitope 2 linear peptide of 26.9% ($\pm 15.7\%$) and with epitope 3 linear peptide of 29.2% ($\pm 11.7\%$). When this patient's serum is inhibited with both epitope 2 and epitope 3 linear peptides, there is cumulative inhibition ($42.9\% \pm 15.5\%$). The percent of the response directed against these two select epitopes was quite variable across patients. Using these peptide inhibition Western blots in epitope positive patients, epitope 2 inhibited on average 44% of the CENP-A reactivity (range 27 to 77%), epitope 3 inhibited on average 40% of the CENP-A reactivity (range 9 to 72%) and both peptides together inhibited 57% of the CENP-A reactivity (range 29 to 91%). These peptide inhibition experiments suggest that these two regions are major areas of patient reactivity.

Testing of native and mutated CENP-A

In order to determine if substituting glycines for arginines affected binding of ACA positive patient sera to CENP-A, five patient samples were tested on Western blot with native and mutated centromere A proteins (Figure 5). Inhibition of antibody binding by 80-100% to CENP-A was observed with all five patient samples tested. These results were repeated in another set of five independent patients who again showed that 73% to 96% of CENP-A reactivity was lost with testing against the mutated form. This significant reduction in immunoreactivity suggests that the three arginines that were replaced by glycines within this epitope motif are essential for antibody recognition or that these amino acid substitutions disrupt the CENP-A structure decreasing binding.

Discussion

Human CENP-A is a 17 kDa protein that shares significant sequence homology with histone H3 at the carboxy-terminus. Of the 140 amino acids, the 93-amino acid C-terminal domain shares 62% identity with histone H3 [32]. The N-terminal domain, however, has no sequence homology to any other known proteins and has been shown previously to be an autoantigenic region of CENP-A [26-28].

The present study confirms this observation. In addition, by identifying 4 distinct epitopes which are bound by ACA positive sera, using solid-phase overlapping decapeptides of CENP-A (Figure 6), this study further narrows the sequential epitopes of CENP-A that are autoantibody targets. Individual patient samples showed some variation in the number of epitopes bound; however, half of patient sera bound all four epitopes. Interestingly, no epitope was found to be more commonly immunoreactive than the other since patient sera bound each epitope with equal frequency (Table 3).

Each epitope bound had the consensus sequence G/A-P-R/S-R-R, as previously described by Mahler *et al* [28]. Their studies previously identified two major antigenic regions within the first 45 N-terminal amino acids. Antigenic region 1 (aa 2-17) was bound by 89% of the ACA positive sera and antigenic region 2 (aa 22-38) was bound by 95% of ACA positive sera. Eighty nine percent of ACA positive sera bound both antigenic regions, and 1 (5%) bound antigenic region 2 exclusively. Upon closer scrutiny of the two antigenic regions, they determined all ACA positive sera recognized at least one epitope containing the consensus sequence G/A-P-R/S-R-R, found in amino acids 2-6 (GPRRR, motif I), 12-16 (APRRR, motif II), and 25-29 (GPSRR motif III). They identified two additional motifs: PSLGAS (aa 31-36) and PTPGPS (aa 22-27). Muro *et al* [26,27] also published results defining the finer specificity of the CENP-A epitopes and identified two major immunoreactive regions. They found 86% of ACA positive sera showed reactivity to antigenic region 1 (aa 3-17), 87% to antigenic region 2 (aa 25-38) and 80% to both antigenic regions. In 1998, Valdivia *et al* [33] were able to generate a monospecific antibody in rabbits using this highly charged peptide (aa 3-17), thereby confirming the immunogenicity of the N-terminal portion of CENP-A.

One of the aims of our study was to determine what percentage of the anti-centromere response is directed against each epitope. We have shown by solid-phase peptide assay that each epitope seems to be equally immunoreactive, eliciting an autoimmune response with equal frequency. Since it was not feasible to test all 263 patient samples by solid-phase peptide assay method, we instead constructed MAP™ peptides for epitopes 2 and 3 and performed MAP™ peptide ELISAs on all 263 patient samples. We found 80.1% of the 246 anti-CENP-A positive patient sera had reactivity to epitope 2 and 89.0% to epitope 3. Overall, 72.4 % of patient sera had reactivity to both epitopes 2 and 3, and 95.9% had reactivity to either 2 or 3. This confirmed each epitope to be equally antigenic and corroborates the results obtained by Muro *et al* [27] and Mahler *et al* [28] in smaller cohorts. Additionally, we demonstrated these peptides for

epitopes 2 and 3 are specific for epitopes of CENP-A since pre-incubation of these peptides with patient sera inhibited reactivity to CENP-A by Western blot (Figure 4).

Of course, ideally we would have been able to correlate demographic and/or clinical features with the occurrence of specific humoral epitopes. Of our 16 patients with detailed analysis, only one had no specific peptide reactivity and two others had mild reactivity with one epitope each. No specific serologic, age, race or gender correlations with this small subset of patients were found. These patients may have antibodies which primarily target other centromere proteins, such as centromere B or C. Or, perhaps, these individuals may have antibodies that primarily target non-sequential epitopes which are not able to be detected with this methodology. In addition, we found no specific demographic correlations with the presence of any of the four common identified humoral epitopes. In this cohort, we had detailed autoantibody information but minimal clinical information. Of course, whether specific epitopes correlate with specific features of scleroderma (such as pulmonary hypertension or extent of skin involvement) or with other disease processes would be of interest.

Two patient samples that were initially negative by immunofluorescence, ELISA, and solid-phase overlapping decapeptides for ACA subsequently developed anti-centromere antibodies under observation (Figure 3). When tested by the solid-phase overlapping decapeptides of CENP-A, the later patient samples, obtained 4 and 7.5 years respectively after the initial negative sample date, were shown to bind all four epitopes. This demonstrates a polyclonal autoimmune response to multiple epitopes that occurs concurrently rather than an initial autoimmune response to a single epitope followed by epitope spreading. The latter has been proposed as the mechanism of autoantibody production for other autoimmune diseases such as SLE [31]. Of course, missing interim samples may have yielded different results.

When we tested two separate serum samples from three patients spanning 7.8, 9.6 and 9.8 years, we also observed that the anti-centromere response did not change over time. The response remained stable and was confined to the N-terminal region of CENP-A. When we tested some of the CENP-B positive and CENP-A negative patient samples on the solid-phase overlapping decapeptides of CENP-A, and vice-versa, no cross-reactivity was observed. One can speculate that this lack of epitope spreading in the anti-CENP-A response may be the reason the limited variant of SSc has a comparatively milder disease course, with less major organ involvement, than other autoimmune diseases. This is assuming there is a correlation between the production of multiple autoantibodies and the pathogenicity of disease.

We also showed, in our mutational analyses, that the three arginines we replaced with glycines are essential for antibody binding; binding to mutated CENP-A was reduced by 80-100% in five patient samples when compared to binding with native CENP-A (Figure 5). These results further enhance our understanding of the specificity of the anti-centromere response in SSc. Future research is warranted to better understand the inciting events and pathogenic mechanisms of this response in scleroderma.

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References

1. Chung L, Utz PJ. Antibodies in scleroderma: Direct pathogenicity and phenotypic associations. *Curr Rheumatol Rep* 2004;6:156–163. [PubMed: 15016347]
2. Moroi Y, Peebles C, Fritzler MJ, Steigerwald J, Tan EM. Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc Natl Acad Sci USA* 1980;77:1627–1631. [PubMed: 6966403]
3. McCarty GA, Rice JR, Bembe ML, Barada FA. Anticentromere antibody. Clinical correlations and association with favorable prognosis in patients with scleroderma variants. *Arthritis Rheum* 1983;26:1–7. [PubMed: 6337593]
4. Chan HL, Lee YS, Hong HS, Kuo TT. Anticentromere antibodies (ACA): clinical distribution and disease specificity. *Clin Exp Dermatol* 1994;19:298–302. [PubMed: 7955469]
5. Muro Y, Sugimoto K, Okazaki T, Ohashi M. The heterogeneity of anticentromere antibodies in immunoblotting analysis. *J Rheumatol* 1990;17:1042–1047. [PubMed: 2213779]
6. Caramaschi P, Biasi D, Manzo T, Carletto A, Poli F, Bambara LM. Anticentromere antibody-clinical associations: A study of 44 patients. *Rheumatol Int* 1995;14:253–255. [PubMed: 7597381]
7. Reveille JD, Solomon DH. The American College of Rheumatology AD HOC Committee on Immunologic Testing Guidelines. Evidence-based guidelines for the use of immunologic tests: Anticentromere, Scl-70, and nucleolar antibodies. *Arthritis Rheum* 2003;49:399–412. [PubMed: 12794797]
8. Mayes MD, Lacey JV Jr. Beebe-Dimmer J, Gillespie BW, Cooper B, Laing TJ, Schottenfeld D. Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. *Arthritis Rheum* 2003;48:2246–2255. [PubMed: 12905479]
9. Earnshaw W, Bordwell B, Marino C, Rothfield N. Three human chromosomal autoantigens are recognized by sera from patients with anti-centromere antibodies. *J Clin Invest* 1986;77:426–430. [PubMed: 3511098]
10. Earnshaw WC, Rothfield N. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma* 1985;91:313–321. [PubMed: 2579778]
11. Kingwell B, Rattner JB. Mammalian kinetochore/centromere composition: A 50 kDa antigen is present in the mammalian kinetochore/centromere. *Chromosoma* 1987;95:403–407. [PubMed: 3315496]
12. Bischoff FR, Maier G, Tilz G, Ponstingl H. A 47-kDa human nuclear protein recognized by antikinetochore autoimmune sera is homologous with the protein encoded by *RCCI*, a gene implicated in onset of chromosome condensation. *Proc Natl Acad Sci USA* 1990;87:8617–8621. [PubMed: 2236072]
13. Rattner JB, Rees J, Arnett FC, Reveille JD, Goldstein R, Fritzler MJ. The centromere kinesin-like protein, Cenp-E: An autoantigen in systemic sclerosis. *Arthritis Rheum* 1996;39:1355–1361. [PubMed: 8702444]
14. Rattner JB, Rao A, Fritzler MJ, Valencia DW, Yen T. CENP-F is a 400 kDa kinetochore protein that exhibits a cell-cycle dependent localization. *Cell Motil Cytoskeleton* 1993;26:214–226. [PubMed: 7904902]
15. He D, Zeng C, Woods K, Zhong L, Turner D, Busch RK, Brinkley BR, Busch H. CENP-G: a new centromeric protein that is associated with the α -1 satellite DNA subfamily. *Chromosoma* 1998;107:189–197. [PubMed: 9639657]
16. Earnshaw WC, Sullivan KF, Machlin PS, et al. Molecular cloning of cDNA for CENP-B, the major human centromere autoantigen. *J Cell Biol* 1987;104:817–829. [PubMed: 2435739]
17. Rothfield N, Whitaker D, Bordwell B, Weiner E, Senecal JL, Earnshaw W. Detection of anticentromere antibodies using cloned autoantigen CENP-B. *Arthritis Rheum* 1987;30:1416–1419. [PubMed: 3435569]
18. Verheijen R, de Jong BAW, Oberyé EHH, van Venjooij WJ. Molecular cloning of a major CENP-B epitope and its use for the detection of anticentromere antibodies. *Mol Biol Rep* 1992;16:49–59. [PubMed: 1545784]

19. Vázquez-Abad D, Wallace S, Senécal JL, et al. Anticentromere antibodies: Evaluation of an ELISA using recombinant fusion protein CENP-B as antigen. *Arthritis Rheum* 1994;37:248–252. [PubMed: 8129780]
20. Russo K, Hoch S, Dima C, Varga J, Teodorescu M. Circulating anticentromere CENP-A and CENP-B antibodies in patients with diffuse and limited systemic sclerosis, systemic lupus erythematosus, and rheumatoid arthritis. *J Rheumatol* 2000;27:142–148. [PubMed: 10648030]
21. Tan EM, Smolen JS, McDougal JS, et al. A critical evaluation of enzyme immunoassays for detection of antinuclear autoantibodies of defined specificities: 1. Precision, sensitivity, and specificity. *Arthritis Rheum* 1999;42:455–464. [PubMed: 10088768]
22. Dongxu S, Martinez A, Sullivan KF, Sharp GC, Hoch SO. Detection of anticentromere antibodies using recombinant human CENP-A protein. *Arthritis Rheum* 1996;39:863–867. [PubMed: 8639184]
23. Earnshaw WC, Machlin PS, Bordwell BJ, Rothfield NF, Cleveland DW. Analysis of anticentromere antibodies using cloned autoantigen CENP-B. *Proc Natl Acad Sci USA* 1987;84:4979–4983. [PubMed: 2440036]
24. Sugimoto K, Muro Y, Himeno M. Anti-helix-loop-helix domain antibodies: Discovery of autoantibodies that inhibit DNA binding activity of human centromere protein B (CENP-B). *J Biochem* 1992;111:478–483. [PubMed: 1377670]
25. Muro Y, Sugimoto K, Himeno M, Ohashi M. The clinical expression in anticentromere antibody-positive patients is not specified by the epitope recognition of CENP-B antigen. *J Dermatol* 1992;19:584–591. [PubMed: 1283396]
26. Muro Y, Iwai T, Ohashi M. A charged segment mainly composed of basic amino acids forms an autoepitope of CENP-A. *Clin Immunol Immunopathol* 1996;78:86–89. [PubMed: 8599890]
27. Muro Y, Azuma N, Onouchi H, Kunimatsu M, Tomita Y, Sasaki M, Sugimoto K. Autoepitopes on autoantigen centromere protein-A (CENP-A) are restricted to the N-terminal region, which has no homology with histone H3. *Clin Exp Imm* 2000;120:218–223.
28. Mahler M, Mierau R, Blüthner M. Fine-specificity of the anti-CENP-A B-cell autoimmune response. *J Mol Med* 2000;78:460–467. [PubMed: 11097115]
29. Gonzalez EN, Rothfield NF. Immunoglobulin class and pattern of nuclear fluorescence in systemic lupus erythematosus. *N Engl J Med* 1966;274:1333–1338. [PubMed: 4160639]
30. Arden A, de Groot ER, Fletkamp TEW. Immunology of DNA III. *Crithidia lucilia*, a simple substrate for the determination of anti-dsDNA with the immunofluorescence technique. *Ann NY Acad Sci* 1975;254:505–515. [PubMed: 52321]
31. Arbuckle MR, Reichlin M, Harley JB, James JA. Shared early autoantibody recognition events in the development of anti-Sm B/B' in human lupus. *Scand J Immunol* 1999;50:447–455. [PubMed: 10564545]
32. Sullivan KF, Hechenberger M, Masri K. Human CENP-A contains a histone H3 related histone-fold domain that is required for targeting to the centromere. *J Cell Biol* 1994;127:581–592. [PubMed: 7962047]
33. Valdivia MM, Figueroa J, Iglesias C, Ortiz M. A novel centromere monospecific serum to a human autoepitope on the histone H3-like protein CENP-A. *FEBS Letters* 1998;5–9. [PubMed: 9475158]

Figure 1.

Binding of sera to solid-phase overlapping decapeptides of CENP-A. Reactivity of two representative centromere positive patient sera to the 66 decapeptides overlapping by two amino acids of CENP-A are presented in Panels A and B. In addition, representative binding of an unaffected, ACA negative control serum is presented in Panel C.

Figure 2.

Average binding of sera to solid-phase overlapping decapeptides of CENP-A. Panels A and B show the average binding of centromere patient sera and normal control sera, respectively. Controls, on average, showed no reactivity at or above 0.156 ± 0.123 OD. The four major antigenic regions recognized by centromere patient sera are labeled with symbols. Epitopes 1 and 2 are bound on average 6 standard deviations above the normal mean (*). Epitope 3 is bound on average 10 standard deviations above the normal mean (#) and epitope 4 is bound on average 8 standard deviations above the normal mean (+).

Figure 3.

Development of an anti-centromere A response over time in two patients available serial serum samples. Panel A shows a patient serum sample exhibiting no reactivity to solid-phase overlapping decapeptides and is negative by ELISA. Serum from the same patient drawn 7.5 years later shows significant reactivity to the N-terminus of CENP-A (Panel B). Serial samples for a different patient spanning four years are presented in Panels C (ACA negative) and D (ACA positive).

Figure 4. Inhibition of anti-CENP-A response in patient samples with sequential peptides encompassing epitopes 2 and 3. The peptides were used at 10 fold the concentration of CENP-A. Two representative patient samples and their inhibitions are shown.

Figure 5. Mutational analysis of the CENP-A antigenic motif. Five patient serum samples were run side by side with native and mutated CENP-A antigen. Percent inhibition is shown.

Figure 6.

Correlation of epitopes recognized by centromere positive patient sera. *Line A* presents the epitopes mapped by Muro et. al 1996 (13). *Line B* presents the epitopes by Muro et al. 2000 (14). *Line C* presents the epitopes mapped by Mahler et al. 2000 (15). *Line D* presents the epitopes described in this study.

Table 1
Clinical and demographic features of patients tested by solid phase peptide binding analysis.

Number	Age	Race	Gender	ANA	ENA	Disease
1	21	U	F	40	Neg	Scl
2	54	AA	F	360	Neg	Scl
3	37	C	F	360	Neg	Scl
4	48	C	F	1080	UIL	Scl
5	35	H	F	9720	UIL	Scl
6	60	AI	F	3240	UIL	Scl
7	46	U	F	1080	Neg	Scl
8	35	U	F	3240	Neg	Scl
9	52	AI	F	9720	Neg	Scl
10	56	C	F	1080	Neg	Scl
11	40	C	F	3240	Neg	Scl
12	45	C	F	9720	Neg	Unk
13	68	U	M	1080	Neg	Scl
14	54	C	F	3240	Neg	Scl
15	73	C	F	360	Neg	Scl
16	53	C	F	9720	Neg	Scl

Race designations include: AA (African American), C (Caucasian), H (Hispanic), AI (American Indian), U (unknown). ANA titers are presented. ENA= antibodies against extractable nuclear antigens in calf thymus extract. UIL=precipitating levels of antibodies against an unidentified line. Scl=scleroderma. Unk=unknown.

Table 2
Amino acid sequences of antigenic regions of CENP-A.

Epitope	Amino Acid Sequence (#)
1	MGPRRRSRKPEA (1-12)
2	SRKPEAPRRRSPSP (7-20)
3	SPSP TPTPGPSRRG (17-30)
4	GPSRRGPSLGASSHQH (25-40)

Table 3
Peptide epitopes recognized by centromere positive sera.

	<u>Epitopes</u>			
	<u>1</u> (aa 1-12)	<u>2</u> (aa 7-20)	<u>3</u> (aa 17-30)	<u>4</u> (aa 25-40)
1	—	++	++	++
2	+++	+	+++++	++++
3	++	+++	—	—
4	+++++	+++++	+++++	+++++
5	—	—	+	—
6	+++	—	+++++	—
7	++++	++	+++++	+
8	+++++	+++++	+++++	+++++
9	++++	—	—	—
10	+	+++	+++++	+++++
11	++	+++++	+++++	+++++
12	++	+++++	+++++	++
13	—	—	—	+
14	—	—	—	—
15	+++++	++	+++++	+++++
16	—	+++	+++++	+++
	11 (69%)	11 (69%)	12 (75%)	11 (69%)

Each + equals 2 SD above the normal mean (anything above 10 SDs is depicted as +++++). Reactivity of <2 SDs is shown as negative. Ranges below epitope numbers are amino acid ranges.