Clin Exp Immunol 1994; 96:267-274

Anti-centromere antibodies (ACA) in systemic sclerosis patients and their relatives: a serological and HLA study

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(Accepted for publication 12 January 1994)

SUMMARY

Autoantibody reactivity to centromere proteins CENP-A, CENP-B and CENP-C was examined in 58 patients with systemic sclerosis (SSc), 218 first degree relatives and 22 spouses. HLA class II typing for HLA-DRB1 and HLA-DQA1 was performed by restriction fragment length polymorphism (RFLP) analysis in 50 families, and HLA-DRB1, HLA-DQA1 and HLA-DQB1 typing was performed by olignucleotide typing in 44 families. Eleven probands and two relatives had ACA. The two relatives with ACA also had SSc. One relative was an identical twin sister of a proband with ACA and the other relative was a sister of a proband with ACA. All ACA-positive probands and relatives were female, and all recognized CENP-A, CENP-B and CENP-C. The presence of at least one HLA-DQB1 allele not coding for leucine at position 26 of the first domain appeared necessary, although not sufficient for the generation of ACA. Therefore within SSc families ACA is strongly associated with female gender and disease phenotype, and is at least in part genetically determined.

Keywords anti-centromere antibodies scleroderma HLA

INTRODUCTION

ACA are found in 22–55% of patients with systemic sclerosis (SSC) [1,2] and are associated with a limited form of skin involvement [2–4]. ACA are highly specific markers for SSc although they occur in up to 3% of patients with systemic lupus erythematosus (SLE) [4–6], 3·3% of patients with rheumatoid arthritis (RA) [7], and 10–30% of patients with primary biliary cirrhosis (PBC) [8,9]. Anti-topoisomerase-1 antibodies (ATA) occur in a separate group of patients with SSc who have more diffuse skin and early systemic involvement [2,4].

ACA recognize three major polypeptides which are localized to the human centromere [10]. The polypeptides have been named CENP-A (17 kD), CENP-B (80 kD) and CENP-C (140 kD) [10]. In most studies, routine detection methods for ACA have been limited to the identification of IgG autoantibodies, but it has been shown that CENPs B and C are also recognized by IgM and IgA immunoglobulin isotypes [5,11]. As IgM autoantibodies may represent a primary immune response to an etiological agent [12] and are more likely to be encoded by germline genes, we have examined the frequency and immunoglobulin isotype of ACA in patients with SSc and their families.

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Genetic factors may also be important for the generation of ACA. MHC class II associations have been found with SSc, such as an increased frequency of HLA-DR3 [13] and HLA-DR5 [14]. These associations become stronger if subgroups identified by particular autoantibodies are analysed. Thus an increased frequency of HLA-DR1 [1,15–17], HLA-DR4 [15] and HLA-DRw8 [15] has been reported for ACA, and an increased frequency of HLA-DR5 [1,15] and HLA-DR2 [18] for ATA. A more recent study has suggested that the primary MHC class II association of ACA is with the second hypervariable region of the HLA-DQB1 chain [19]. The absence of a hydrophobic leucine at position 26 of the HLA-DQB1 first domain (non-DQB1:26:L) was found in all ACA-positive patients. Therefore previously reported HLA-DR associations may reflect linkage disequilibrium with the HLA-DQB1 domain.

The inclusion of multicase families in our study has enabled us to examine the concordance of ACA with HLA-DR and HLA-DQ subtypes and with the presence of disease. We have confirmed that a non-DQB1:26:L allele is necessary but not sufficient for the generation of ACA, suggesting that other factors including female gender must play an important role. The profile of the ACA response was constant and ACA were detectable only in the presence of disease.

PATIENTS AND METHODS

Systemic sclerosis families

Clinical, serological and HLA results were obtained on 58 probands with SSc (American Rheumatism Association (ARA) criteria [20]), 218 first degree relatives and 22 spouses, as part of a multi-centre study of SSc organized by the UK Scleroderma Study Group [16]. Three families were from the USA, two were from Russia, and the remaining families were from the UK. The UK patients were selected on the basis of having sufficient blood relatives to allow genetic haplotype assignment. Families in the USA and Russia were selected on the basis of more than one member having a connective tissue disease. All probands and family members were Caucasoid. HLA-DRB1 typing on the UK probands has been reported recently by some members of our group [17].

Clinical assessment

All patients and relatives were visited a least once by a member of the UK Scleroderma Study Group and assessed clinically using a predesigned protocol. A family tree was drawn up in consultation with relatives, and enquiry was made into illness and causes of death in relatives. In multicase families special attention was directed to the timing and length of cohabitation of individual family members. Limited skin involvement was defined as sclerosis not extending beyond the elbow, and diffuse scleroderma as sclerosis extending proximal to the elbow and usually involving the trunk.

Anti-centromere antibodies

The presence of a discrete speckled pattern on indirect immunofluorescence (IF) of HEp-2 cells or the identification of a characteristic pattern of polypeptides recognized on immunoblotting (Fig. 1, lanes 3 and 4) were the necessary criteria used for establishing ACA positivity.

Immunofluorescence

HEp-2 cells were incubated with serum four-fold diluted (from 1:40 to 1:2560) in PBS for 1 h. Washed cells were incubated in FITC-labelled goat anti-human polyvalent conjugate (1:20). Following a second wash, the slides were mounted in glycerol/PBS containing 2·5% DABCO (1,4-diazobicyclo-[2,2,2]-octane) and viewed under a Lietz fluorescence microscope.

Immunoblotting

A nuclear-enriched extract was prepared from K562 cells by the method of Earnshaw *et al.* [10] with modifications. To prepare a homogenate, 1×10^7 cells were washed twice in PBS and resuspended in buffer A (15 mM Tris/HCl pH 7·4, 80 mM KCl, 2 mM EDTA, 0·3 mM spermine, 0·75 mM spermidine) for 5 min to allow the cells to swell. The cells were harvested at 1000 ***g***. The pellet was resuspended in Buffer A with the addition of 100 μl of RNAse A (5 mg/ml), 0·1% digitonin, 1 mM PMSF and 1 mM iodoacetamide. The suspension was homogenized in a Potter Elvenjem homogeniser (10 up and down strokes). Nuclei were collected at 1000 ***g*** and boiled for 5 min in 2 ml of sample buffer containing 0·1 M Tris/HCl pH 6·8, 1% SDS and 5 mM dithiothreitol. Nuclei were sonicated (5 × 10 s with 20 s pauses) and centrifuged at 13 000 ***g*** for 5 min. The supernatant was stored at −70°C and used as the source of centromere polypeptides for immunoblotting.

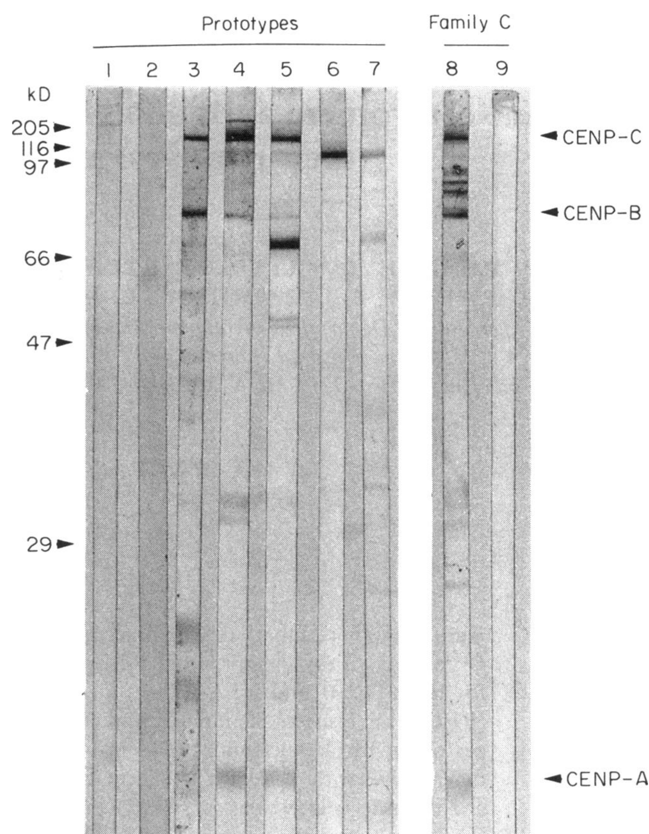

Fig. 1. K562 cell polypeptides recognized by systemic sclerosis (SSc) probands and family C after separation on a 12·5% SDS-polyacrylamide gel and transfer to nitrocellulose. Lanes 1 and 2, normal control sera; lane 3, control ACA serum; lane 4, proband with ACA; lane 5, control serum with ACA and antimitochondrial antibodies (the latter recognizing a 70-kD mitochondrial band); lane 6, control serum with anti-topoisomerase-1 antibodies (ATA); lane 7, proband with ATA; lanes 8 and 9, family C, dizygotic twins, one with SSc and ACA (lane 8) and one unaffected with SSc and negative for ACA (lane 9).

Proteins in the nuclear-enriched extract were separated by SDS–PAGE [21] in 12·5% polyacrylamide and transferred to nitrocellulose as described [22], except the transfer buffer contained 0·1% SDS. The nitrocellulose was blocked in 5% dried milk powder in 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 visualized using nitro-tetrazolium blue and 3-bromo-2-chloro-5-indoyl phosphate as substrate.

The presence of CENP-B in the antigen extract was confirmed by immunoblotting with a serum from a European consensus study and with a heterologous rabbit antibody to human CENP-B (courtesy of Dr W.C. Earnshaw) (data not shown).

Anti-topoisomerase-1 antibodies

ATA were measured by Oucterlony double diffusion using a rabbit thymus extract (Bradshaw Biologicals, Market Harborough, UK) and lines of identity were sought with known prototypes. All positives were confirmed by their ability to

Table 1. Probands and relatives positive for ACA by indirect immunofluorescence (IF) and immunoglobulin isotype of ACA by immunoblotting

					Immunoblotting					
			IF		IgG			IgM		
Case	Sex	Diagnosis	ACA	titre	CENP-C	CENP-B	CENP-A	CENP-C	CENP-B	CENP-A
A1	F	SSc (L)	+	1/640	+	+	+	+	−	−
A2	F	SSc (L)	+	1/40	+	+	+	+	−	−
B1	F	SSc (L)	+	1/640	+	+	+	+	−	−
B2	F	SSc (L)	+	1/640	+	+	+	+	−	−
C1	F	SSc (L)	+	1/160	+	+	+	+	−	−
D1	F	SSc (L)	+	1/40	+	+	+	+	−	−
E1	F	SSc (L)	+	1/40	+	+	+	+	−	−
F1	F	SSc (D)	+	1/160	+	+	+	+	−	−
G1	F	SSc (D)	+	1/160	+	+	+	+	−	−
H1	F	SSc (D)	+	1/160	−	+	+	+	−	−
I1	F	SSc (L)	+	1/160	−	+	+	+	−	−
J1	F	SSc (L)	+	1/2560	+	+	+	+	−	−
K1	F	SSc (L)	+	1/2560	+	+	+	+	−	−

L, Limited cutaneous disease; D, diffuse cutaneous disease.

inhibit or to absorb out topoisomerase-1 activity in a functional assay (manuscript in preparation).

HLA-DRB1, HLA-DQA1 and HLA-DQB1 typing

DNA was extracted from peripheral blood leucocytes by salt precipitation [23]. HLA-DRB1 and HLA-DQA1 typing was performed by restriction fragment length polymorphism (RFLP) analysis using *Taq* I digested genomic DNA and complementary DNA (cDNA) probes for the DRB1 and DQA1 genes as previously described [24]. HLA-DRB1 and HLA-DQA1 specificities are given in accordance with specific RFLP bands assigned at the 10th International Histocompatibility Testing Workshop [25].

In addition, HLA-DRB1, HLA-DQA1 and HLA-DQB1 typing was performed using the polymerase chain reaction (PCR) and sequence-specific oligonucleotide (SSO) probes [26]. The SSO probes and primers were obtained from the kit supplied by the British Society of Histocompatibility and Immunogenetics Molecular Working Group (N.W. Regional Tissue Typing Laboratory, Manchester, UK). PCR products were vacuum blotted onto nylon. The nylon was blocked in 4 × SSPE, 1% casein, 0·1% lauroylsarcosine at the hybridization temperature (55°C). SSOs were labelled with digoxigenin (dig-11-ddUTP) (Boehringer Mannheim, Lewes, UK) using terminal transferase (Boehringer Mannheim). Hybridization occurred in 3 M tetramethyl ammonium chloride, 0·05 M Tris pH 8·0, 0·1% SDS and 0·002 M EDTA pH 8·0. The stringency wash was at 56°C in fresh hybridization mix. The nylon was washed in 0·1 M Tris pH 7·5, 0·15 M NaCl and reblocked in 1% casein, 0·1 M Tris pH 7·5, and 0·15 M NaCl. After 30 min 0·2 ml alkaline phosphatase-conjugated dig-11-ddUTP antibody was added, followed by 60 min incubation at room temperature with shaking. The nylon was washed 3 × 10 min in buffer (0·1 M Tris pH 9·5, 0·1 M NaCl, 50 mM $MgCl_2$) before adding 0·1 mg Luminogen for 10 min incubation in the dark. The nylon was wrapped in plastic and exposed to x-ray film (Kodak Y-OMAT) for 30 min. HLA assignments were determined by positive signals in relation to the specificity of the probe. Alleles were assigned using the revised nomenclature for HLA alleles defined by their nucleotide sequences [27].

Statistical analysis

Comparisons of HLA specificities were performed by χ^2 analysis with Yates' correction. Unrelated probands with ACA were compared to SSc probands without ACA and to normal Caucasoid controls. Probands with ATA were excluded from the SSc controls, as there is an HLA-DQB1 association with ATA [28,29]. The normal controls were husbands of patients from a separate family study of herpes gestationis.

RESULTS

Presence of ACA

Eleven of the 58 probands had ACA (Table 1, families A–K); seven with limited skin involvement, three with diffuse skin disease and one with overlap features of SSc and SLE. Of the 11 families in which the proband had ACA, two additional relatives had ACA (Table 1, A2 and B2) and both had SSc with limited skin involvement. No relatives from the other families had ACA. Therefore, ACA were present only in females who had SSc. SSc was present in one other relative of a proband with ACA. In family K, a sister had diffuse SSc but the serum was negative for ACA and ATA.

Multicase families and twins

In family A, from the USA, two sisters had SSc with limited skin involvement and both had ACA (Fig. 2, lanes 1–4, and Fig. 3). The proband developed Raynaud's phenomenon at the age of 44 and 3 years later her sister developed Raynaud's phenomenon at the age of 43. Sera from six other family members including an older brother were negative for autoantibodies.

In family B, from Russia, monozygotic twin sisters with SSc

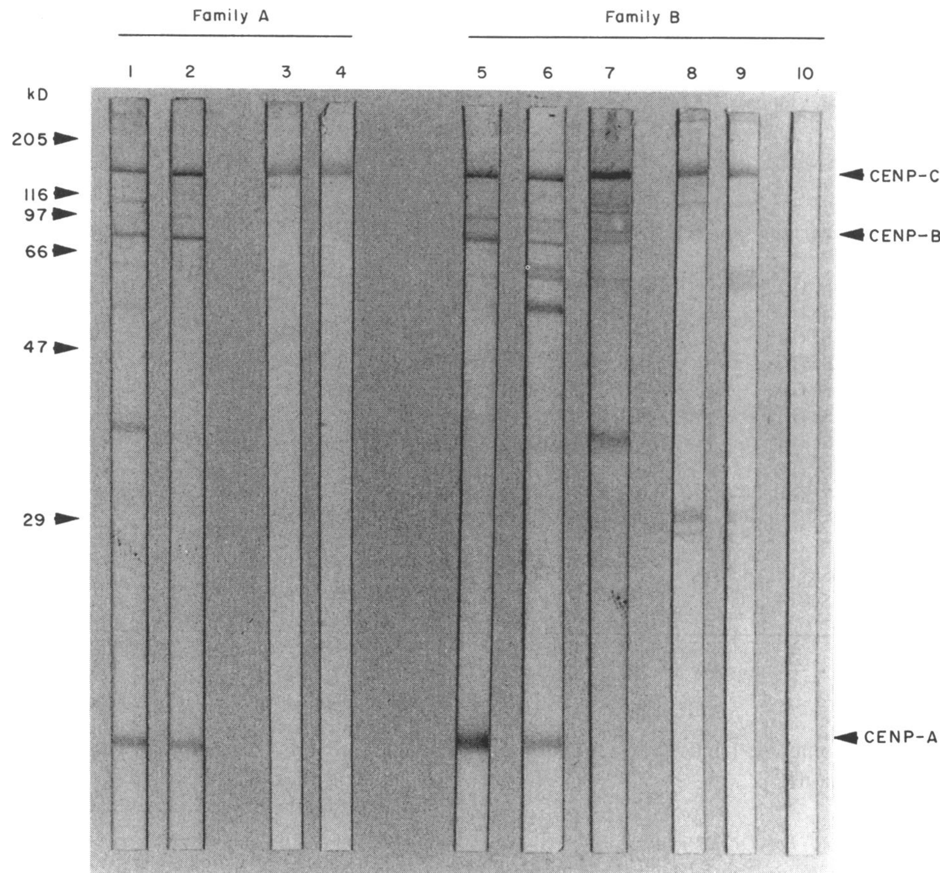

Fig. 2. K562 cell polypeptides recognized by family A and family B after separation on a 12·5% SDS-polyacrylamide gel and transfer to nitrocellulose. Either an anti-IgG (lanes 1, 2, 5, 6, 7) or an anti-IgM (lanes 3, 4, 8, 9, 10)) conjugate was used to identify the autoantibody isotypes. Family A; lanes 1, 3 = proband, lanes 2, 4 = sister. Family B; lanes 5, 8 = proband, lanes 6, 9 = monozygotic twin sister, lanes 7,10 = daughter of proband.

and limited skin involvement [30] had ACA (Fig. 2, lanes 5, 6, 8 and 9). The sisters had lived together in the same environment until the age of 16 before separating. The onset of Raynaud's phenomenon occurred at 20 and 22 years in the proband and her sister, respectively. Other symptoms of SSc developed in both sisters at the same age of 40 years.

In family C, from the UK, the proband with ACA had a dizygotic twin sister who was asymptomatic and was negative for ACA and other autoantibodies (Fig. 1, lanes 8 and 9). The proband developed Raynaud's phenomenon at the age of 18 and other symptoms of SSc at the age of 29. She had lived in the same environment as her unaffected sister for the first 18 years before separation.

In family K the proband, who was positive for ACA, had an overlap condition with features of SSc and SLE. A sister had diffuse SSc and was negative for ACA. ACA were not present in the other three multicase families who will be reported separately (manuscript in preparation).

Profile of anti-centromere reactivity

There was 100% concordance between ACA detected by IF and ACA detected by immunoblotting. All sera with ACA were able to identify a 19·5-kD, an 80-kD, and a 140-kD band by immunoblotting which were CENPs A, B and C (Table 1, Fig. 1, lanes 3, 4, 5 and 8). When probed with an anti-IgG conjugate, all but two sera identified CENP-C (Table 1), although all sera identified CENPs A and B. When probed with an anti-IgM conjugate, the only polypeptide identified by all sera was CENP-C (Table 1, Fig. 2, lanes 3, 4, 8 and 9 compared with lanes 1, 2, 5 and 6). However, IgM anti-CENP-C antibodies were present only in those individuals with ACA and in no other probands or relatives.

In family A the proband and her affected sister identified all CENPs (A, B and C), but the proband identified two additional bands on immunoblotting (Fig. 2, lane 1). In family B the monozygotic twins with SSc recognized all CENPS (Fig. 2, lanes 5 and 6). Of interest, the profile of reactivity was not identical with respect to other non-characterized bands which may reflect somatic mutations in the immune repertoire. The unaffected daughter of the proband in family B identified a band in the region of 140 kD on immunoblotting (Fig. 2, lanes 7 and 10). We do not believe this band to represent CENP-C, as it migrated at a slightly higher molecular weight. Also the serum was negative for ACA on IF, did not recognize CENP-A or CENP-B, and did not have IgM antibodies to CENP-C.

Anti-topoisomerase-1 antibodies

ATA were detected in six probands separate from those with ACA (manuscript in preparation). ATA were not detected in any individuals with ACA, nor in any of their families. These findings further emphasize the mutual exclusiveness of ACA and ATA that other studies have observed [1].

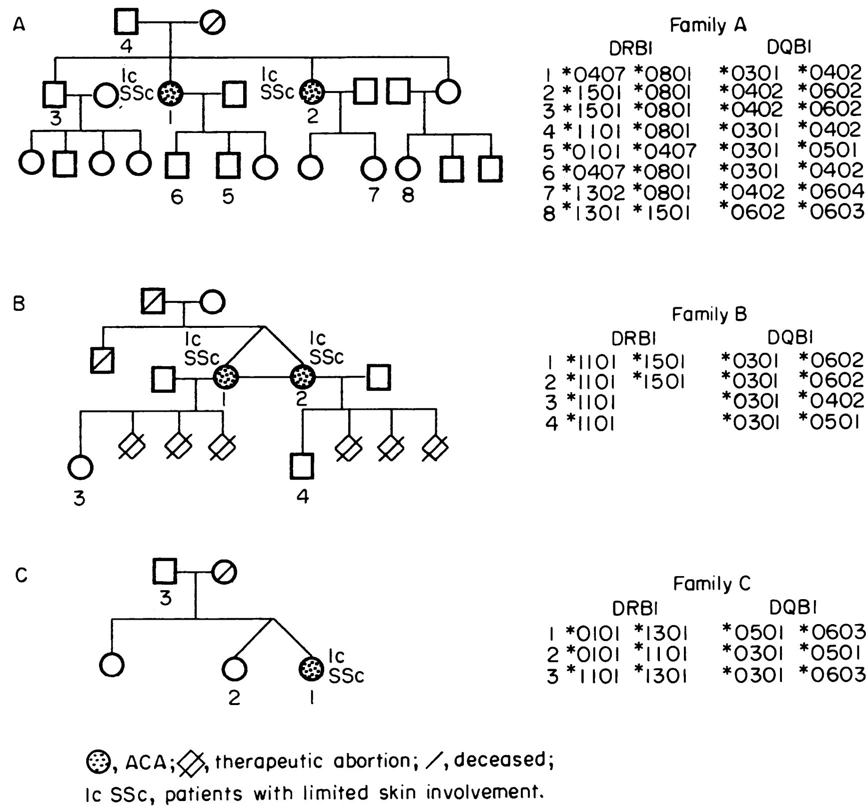

Fig. 3. Family trees of families A, B and C. The numbers on the family trees refer to individuals who have been HLA typed, have had serum analysed for autoantibodies and have been classified according to clinical manifestations. Number 1 is the proband.

Table 2. HLA-DRB1, HLA-DQA1 and HLA-DQB1 alleles in patients and relatives with ACA

Case	RFLP DRB1	RFLP DQA1	PCR and SSO DRB1	PCR and SSO DQA1	PCR and SSO DQB1
A1	4, 8	3, 1b	0407, 0801	0301, 0401	0301, 0402
A2	2, 8	1b	1501, 0801	0301, 0401	0402, 0602
B1	2, 11	2, 1b	1501, 1101	0102, 0501	0301, 0602
B2	2, 11	2, 1b	1501, 1101	0102, 0501	0301, 0602
C1	1,13	1a, 1c	0101, 1301	0101, 0103	0501, 0603
D1	1, 4	1a, 3	0102, 0408	0101, 0301	0301, 0501
E1	NA	NA	0401, 1501	NA	0301, 0602
F1	4, 12	3, 2	0408, 1201	NA	0301, 0302
G1	1, 3	1a, 2	0101, 0301	0101, 0501	0201, 0501
H1	11	2	1101, 1201	0501, 0501	0301, 0301
I1	NA	NA	0303, 1501	NA	0501, 0602
J1	3, 6	2, 1c	0301, 1301	0103, 0501	0201, 0601
K1	2, 3	1a, 1b	1501, 1401	0102, 0301	0601, 0602

NA, Result not available for technical reasons, e.g. insufficient DNA for restriction fragment length polymorphism (RFLP); PCR, polymerase chain reaction; SSO, sequence-specific oligonucleotide.

MHC class II associations

No single HLA-DRB1 or HLA-DQA1 allele accounted for all ACA positivity (Tables 2–4). Six of the 13 patients with ACA were either HLA-DR1 or HLA-DR4 positive (Table 2). Certain subtypes of HLA-DR1 and HLA-DR4 share a sequence, spanning amino acids 67–74 of the third hypervariable region of HLA-DRB1, that confers susceptibility to RA. [31]. Oligotyping of the six HLA-DR4 or HLA-DR1 patients with ACA revealed one patient with HLA-DRB1* 0407 (Table 3), an allele that does not have the shared sequence. Therefore, at most, five of 13 patients with ACA shared an epitope found on the third hypervariable region of HLA-DRB1, and the presence of this sequence is unlikely to be a prerequisite for the generation of ACA. HLA-DR3 (*0301) and HLA-DR7 (*0701) were decreased in the group positive for ACA, but this did not reach statistical significance.

Reveille *et al.* [19] have proposed that the primary MHC class II association with ACA is with the HLA-DQβ chain, and specifically with the lack of a hydrophobic leucine at position 26 of the HLA-DQβ first domain. Our findings in the present study support this model. All 13 individuals positive for ACA had at least one HLA-DQB1 allele that encoded for either a glycine (*0402 or *0501) or a tyrosine (*0301 or *0601) rather than a leucine at position 26 of the first domain (Table 2). Accordingly HLA-DQB1*0301 and HLA-DQB1*0501 were the most frequent alleles (Table 4) in the ACA group, although no single allele was increased significantly compared with controls. However, the possession of at least one non-DQB1:26:L allele was increased significantly in the group with ACA (100%) compared with the probands negative for ACA (73%) ($P < 0.05$) and with the normal controls (56%) ($P < 0.01$) (Table 5). Only three patients with ACA were homozygous for the non-DQB1:26:L allele, suggesting a dominant effect for a single non-DQB1:26:L allele in permitting the generation of ACA.

In family A the proband and her sister with SSc, as well as

Table 3. HLA-DRB1 alleles in SSc probands positive for ACA compared with controls

HLA-DRB1	ACA present (%) (n=11)	ACA and ATA absent (%) (n=27)	Controls (%) (n=45)
DRB1*0101	2 (18)	6 (22)	8 (17)
DRB1*0102	1 (9)	1 (4)	2 (4)
DRB1*1501	4 (36)	6 (22)	10 (22)
DRB1*1502	0 (0)	0 (0)	2 (4)
DRB1*0301	2 (18)	15 (56)	19 (42)
DRB1*0303	1 (9)	0 (0)	0 (0)
DRB1*0401	1 (9)	2 (7)	8 (18)
DRB1*0402	0 (0)	0 (0)	2 (4)
DRB1*0403	0 (0)	1 (4)	1 (2)
DRB1*0404	0 (0)	0 (0)	1 (2)
DRB1*0405	0 (0)	1 (4)	0 (0)
DRB1*0407	1 (9)	0 (0)	0 (0)
DRB1*0408	2 (18)	5 (19)	5 (11)
DRB1*1101	2 (18)	5 (19)	6 (13)
DRB1*1201	2 (18)	2 (7)	1 (2)
DRB1*1301	2 (18)	2 (7)	3 (7)
DRB1*1302	0 (0)	0 (0)	1 (2)
DRB1*1401	1 (0)	1 (4)	2 (4)
DRB1*1402	0 (0)	1 (4)	0 (0)
DRB1*0701	0 (0)	5 (19)	12 (27)
DRB1*0702	0 (0)	0 (0)	4 (9)
DRB1*0801	1 (9)	1 (4)	0 (0)
DRB1*0901	0 (0)	0 (0)	1 (2)

SSc, Systemic sclerosis; ATA, anti-topoisomerase-1 antibodies.

Table 4. HLA-DQA1 and HLA-DQB1 alleles in SSC probands with ACA compared with controls

HLA-DQA1	ACA present (%) (n=8)	ACA and ATA absent (%) (n=27)	Controls (%)
DQA1*0101	3 (38)	9 (33)	
DQA1*0102	2 (25)	3 (11)	
DQA1*0103	2 (25)	4 (15)	
DQA1*0201	0 (0)	4 (15)	
DQA1*0301	3 (35)	9 (33)	
DQA1*0401	1 (13)	1 (4)	
DQA1*0501	4 (50)	20 (74)	
HLA-DQB1	***n*=11**	***n*=27**	***n*=45**
DQB1*0201	2 (18)	15 (56)	30 (67)
DQB1*0301	7 (64)	10 (37)	14 (31)
DQB1*0302	1 (9)	6 (22)	11 (24)
DQB1*0303	0 (0)	0 (0)	1 (2)
DQB1*03032	0 (0)	1 (4)	4 (9)
DQB1*0402	1 (9)	1 (4)	0 (0)
DQB1*0501	4 (36)	7 (26)	10 (22)
DQB1*0503	0 (0)	1 (4)	2 (9)
DQB1*0601	2 (18)	1 (4)	2 (9)
DQB1*0602	3 (27)	4 (15)	9 (20)
DQB3*0603	1 (18)	3 (11)	3 (7)
DQB1*0604	0 (0)	0 (0)	1 (2)

SSc, Systemic sclerosis; ATA, anti-topoisomerase-1 antibodies.

her unaffected father and brother, shared the haplotype DRB1*0801, DQB1*0402 (Fig. 3). The sister shared another haplotype with her brother (DRB1*1501, DQB1*0602) which was different to the proband (DRB1*0407, DQB1*0301). It is tempting to speculate that the female gender in addition to the appropriate HLA-DQB1 allele made the sisters susceptible to SSc and the generation of ACA compared with the male members of the family. Also, the presence of a DQB1:26:L allele in one of the sisters but not the other argues against a protective role for such an allele. The daughter of the sister had the DRB1*0801, DQB1*0402 haplotype but was negative for ACA, and has not shown any evidence of SSc to date.

The monozygotic twins from family B with SSc and ACA were heterozygous for the non-DQB1:26:L allele. The daughter and the nephew of the proband have inherited the non-DQB1:26:L allele, DQB1*0301, but were ACA-negative and free of disease.

In family C the dizygotic twins were discordant for SSc and ACA. Of interest, the affected twin had one non-DQB1:26:L allele (DQ*0501) and the unaffected twin had two such alleles (DQB1*0301, DQB1*0501). This suggests that the presence of one non-DQB1:26:L allele is sufficient for the development of SSc and ACA and that there is not a dose-dependent effect. Also, the presence of a DQB1:26:L allele was not protective. Considering that the unaffected twin sister had the appropriate HLA-DQB1 requirements for developing SSc and ACA, i.e. at least one non-DQB1:26:L allele, there must be other factors in addition that are necessary for triggering SSc and generating ACA.

DISCUSSION

Systemic sclerosis is a chronic multisystem connective tissue disorder of unknown cause with a variable pattern of organ involvement, and most frequently affecting the skin and microvasculature. Familial cases have been reported (for review see [32]), suggesting a genetic contribution to disease susceptibility, but such cases are uncommon as is shown in the present study. Environmental agents such as vinyl chloride, silica, contaminated rapeseed oil and L-tryptophan may trigger a scleroderma-like disorder in susceptible individuals [33]. Gender-related factors must be important because of the increased incidence of SSc in females. An immunological etiology is supported by the presence of activated T cells in lesional skin [34], the up-regulation of MHC class II and cell adhesion molecules in resident cells from lesional tissue [35,36], and the almost invariable presence of autoantibodies [3].

The relationship of ACA to disease mechanisms in SSc is unknown. The presence of ACA in patients with PBC identifies those with overlap features of SSc [8,9]. Also, the presence of ACA in patients with Raynaud's phenomenon predicts those who later develop SSc [37–39]. In the present study of SSc family members, despite the obvious enrichment of shared genetic and environmental factors within individual families, the presence of ACA was seen only in those family members with SSc, and only in females. Furthermore, it is of much interest that the one pair of monozygotic twins studied were concordant for disease and had identical ACA profiles. The dizygotic twins were discordant for disease, with ACA present only in the affected sister. Overall the evidence is compelling that there is a fundamental link

Table 5. HLA-DQB1 alleles lacking leucine at position 26 in all individuals with ACA compared with probands and controls

	ACA present (%) ($n=13$)	ACA and ATA absent in probands (%) ($n=27$)	Controls (%) ($n=45$)
Both DQB1:26:L negative	3 (23)	5 (19)	3 (7)
One DQB1:26:L negative	10 (77)	12 (44)	22 (49)
Both DQB1:26:L positive	0 (0)*	10 (37)	20 (44)

* $P < 0{\cdot}05$ compared with systemic sclerosis (SSc) controls and $P < 0{\cdot}01$ compared with normal controls.

ATA, Anti-topoisomerase-1 antibodies.

between the generation of ACA, female gender, and expression of disease.

The occurrence of connective tissue disease in relatives of patients with SSc has been previously recorded [30,32,40–42]. There has been a predominance of female members affected, usually mother–daughter and sister–sister pairs [32]. The presence of SLE in an identical twin sister of a proband with SSc has been reported [40]. In the present study there was one female with SSc who had a sister with SSc/SLE overlap. This suggests the inheritance of a common autoimmune diathesis with a variable pattern of disease penetrance. The concordance rate for SLE in monozygotic twins has varied from 24% to 69% [43,44], but because of only rare reports [30,42], the concordance rate of SSc in twins is unknown.

The profile of autoreactivity to the CENP polypeptides was remarkably constant in our study. We were unable to demonstrate any heterogeneity of the immune response of ACA which may explain differences between previously reported MHC class II associations concerning ACA. Instead, all sera positive for ACA by IF recognized CENPs A, B and C, although recognition of CENP-B was weak in some instances. The CENPs localize to different parts of the kinetochore, and antibodies to CENP-A and CENP-C do not appear to cross-react [10,45]. These observations are in keeping with a complex structural target perpetuating the generation of ACA. However, we confirmed our previous observation that CENP-C is recognized predominantly by IgM autoantibodies [5]. IgM autoantibodies are more likely to arise from a germ-line gene repertoire and may point to a possible cross-reactive infectious trigger [12]. It was therefore of interest to investigate whether IgM anti-CENP-C antibodies were more widely represented within families than the specificities of other ACA, but such was not the case. Knowledge of the structural characteristics of the CENP-C polypeptide will be needed to explain the anti-CENP-C IgM isotype further.

The prerequisite of at least one HLA-DQβ chain possessing a polar amino acid at position 26 of the first domain for the generation of ACA is consistent with previously reported MHC class II associations with ACA [1,14,15]. Our findings confirm the model proposed by Reveille *et al.* [19]. Also, immunoblotting techniques which may be more sensitive in detecting ACA [9] have been used to verify the findings from IF, and to ensure detection of ACA has not been missed. Furthermore, the findings from the multicase families support the notion that within closely related family members, a non-DQB1:26:L allele may be necessary, but is not sufficient for the generation of ACA, and other factors including female gender must be important.

The high titre and polyclonality of ACA, which target independent polypeptides that are structurally related around the kinetochore, suggests an antigen-driven process [10,45]. It is possible that the centromere proteins themselves are presented to T helper cells in the context of favourable MHC class II binding sites, leading to a sustained and specific autoantibody response. Of interest, the non-DQB1-26:L encodes for a position at the floor of the antigen-binding cleft in the second hypervariable region of the HLA-DQβ chain. The functional relevance of this site with regard to binding and presenting centromere peptides awaits crystallographic study. With the availability of recombinant centromere proteins [46,47] it may be possible to identify the relevant T cells critical for this immune response, and to characterize the MHC class II requirements further. Relating the highly disease-specific anti-centromere immune response to the disease phenotype remains a challenge.

ACKNOWLEDGMENTS

This study was supported by grants from the Arthritis and Rheumatism Council, UK.

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