

Antitopoisomerase and Anticentromere Antibodies in the Sclerodermatosus Complex

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Introduction

Scleroderma is one of the connective tissue diseases with a prevalence estimated as 1 per 5000 (1). The disease presents with Raynaud's phenomenon in most cases (2), but the disorder runs a widely varying course in individual patients. For a long time two patterns of disease expression have been recognized in scleroderma (3). The first pattern, designated as diffuse cutaneous scleroderma (DCS), is characterized by skin thickening extending from the periphery of the extremities to above the elbow, and most of the time involves the trunk as well. It frequently follows a rapid course complicated in a number of cases by the development of renal failure with malignant hypertension. The second pattern, described as limited cutaneous scleroderma (LCS), shows skin thickening below the level of the elbows as well as facial thickening. It follows an insidious course and is associated, in some cases, with the development of primary pulmonary hypertension. Within this second category, a distinct clinical syndrome has been delineated. This CREST-syndrome, also designated as Thibierge-Weissenbach syndrome, consists of the following symptoms: subcutaneous calcinosis, Raynaud's phenomenon, esophageal hypomotility, sclerodactyly, and telangiectasia. Although overlapping features from both patterns may occur in an individual patient and the second pattern may evolve into the first one in incidental cases, generally the two patterns follow a distinct course and might even be considered as

two different disorders. Specific criteria for both disease expressions of scleroderma have been proposed (3) (Table 1).

Antinuclear antibodies (ANA) have been detected in the sera of some 50% of scleroderma patients as early as 1963 (4). ANA were detected by indirect immunofluorescence (IIF) on cryosections of rat liver in those early studies. Using cultured cell lines as a substrate for ANA-detection, almost 90% of scleroderma sera proved positive for ANA (5,6). Different fluorescence patterns were identified on those substrates such as nucleolar, homogeneous, finely speckled, and other staining patterns, which indicated that different antibodies are apparently involved. Further studies demonstrated that, indeed, various antigens are recognized by ANA in scleroderma (reviewed in 7) (Table 2). Among these, antibodies to topoisomerase I and centromere proteins are most prominent. Interestingly, antibodies to topoisomerase I are associated with DCS, and antibodies to centromere proteins with LCS. This, again, suggests that those scleroderma syndromes are, in fact, separate disease entities.

In this review, I will discuss the antigens recognized by anti-topoisomerase I (antitopo) and anticentromere antibodies (ACA). In addition, the clinical associations of these antibodies will be dealt with in detail. Finally, I will discuss the genetic background and other factors that may be involved in the induction of the antibodies.

Characterization of the Antigens

Topoisomerase I

Using a saline extract from nuclei in double immunodiffusion, it proved that some sera from scleroderma patients precipitated an antigen that was clearly different from the Sm- and RNP-antigens that were regularly detected as targets for autoantibodies by immunodiffusion or counterimmunoelectrophoresis (CIE) in the sera of patients with systemic lupus erythematosus (SLE) and mixed connective tissue disease (MCTD) (8). By sodium dodecyl sulfate (SDS) gel electrophoresis and Western blotting it proved that the antigen was a protein of 70-kDa mol wt (8). For obvious reasons, the antigen was named Scl-70. Later on, Van Venrooy et al. described the antigen as an 86-kDa protein as determined by the immunoblotting technique (9), whereas others reported a 95–100-kDa mol wt (10). The target antigen of anti-Scl-70 was eventually identified as DNA topoisomerase I (11–13). This enzyme is capable of relaxing negatively supercoiled DNA without the need for adenosine triphosphate (ATP), and plays, as such, a central role in cell function. The 86- and 70-kDa proteins proved to be degradation products of the 100–105-kDa

Table 1
Subsets of Scleroderma^a

DCS
Truncal and acral skin involvement
Early and significant incidence of interstitial lung disease, renal failure, diffuse gastrointestinal disease, and myocardial involvement
Onset of RP within 1 yr of onset of skin changes
Association with antitopoisomerase I antibodies
LCS
Skin involvement limited to hands, face, feet, acral forearms, or absent
Late incidence of pulmonary hypertension with or without interstitial lung disease, trigeminal neuralgia, skin calcifications, and teleangiectasia
Onset of RP long before onset of other symptoms
Association with ACA

^aAdapted from ref. 3.

Table 2
Major Autoantigens and Autoantibodies in the Sclerodermatosis Complex

Autoantigens	Characterization
Scl-70, Scl-86, topoisomerase I	70- and 86-kDa degradation products of the 100-kDa protein topoisomerase I
Centromere proteins	Proteins of the kinetochore, CENP-A (17-kDa mol wt), CENP-B (80-kDa mol wt), and CENP-C (140-kDa mol wt)
RNA polymerase I	Protein of 210 kDa, part of enzyme complex composed of polypeptides from 12–210 kDa
Fibrillarin	Protein of 34 kDa in association with U3 RNA
PM-Scl (polymyositis-scleroderma)	Particles 20–110 kDa mainly localized to the nucleolus

mol wt DNA topoisomerase I (topo I). Immunoblotting experiments showed that a 70-kDa band only is visualized when a 0.3M NaCl extract of rabbit or calf thymus acetone powder is used as antigenic source, whereas bands of 100-, 86-, 80-, and 70-kDa mol wt are detected with a 4.0M NaCl extract of rabbit thymus acetone powder as a substrate (14). The antigen can be purified by high performance liquid chromatography (HPLC) (15).

Purified antigen can be used in enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to topo I (15). More recently, a complementary DNA (cDNA) encoding human topo I has been cloned and sequenced (16). After expression in bacteria, fusion products from subclones were generated to which sera from patients

with antitopo I antibodies react. Subcloned portions of topo I were used to test reactivities of different patient sera to the various portions. Most patient sera appeared to recognize at least two independent epitopes localized on the carboxy terminus as well as on the amino terminus of the molecule (17). Thus, reactivity is not only directed to the active site. Otherwise, it has been shown that most antibodies are able to inhibit the enzymatic activity of topo I, suggesting that they react with the active site of the molecule (11,12). This is also suggested by the finding that the human autoantibodies react with plant topo I, and apparently recognize highly conserved regions of the molecule (18). Indeed, using a peptide that contained 11 amino acids from the active site and 8 residues unrelated to topo I, Maul et al. showed reactivity of antitopo I positive sera with this peptide (19). In addition, 6 amino acids from the active site corresponded with a sequence of a retroviral p30 gag protein, suggesting that viral infection may be involved in the induction of this form of autoimmunity (19). Antibodies reactive to HIV retroviral proteins were found also in another study in patients with antitopo I antibodies, although they did not crossreact with topo I (20). Reactivity to restricted epitopes crossreacting with viral peptides is, however, not fully consistent with the polyclonal nature of the autoimmune response to topo I, as shown by D'Arpa et al. (17).

Detection of antibodies to topo I is classically performed by immunodiffusion or counterimmunoelectrophoresis using calf thymus acetone powder as antigenic source. Using this technique, most sera positive for antitopo I will be detected. The immunoblotting technique appears, however, more sensitive than immunodiffusion, although false positive results may occur (21). An ELISA using purified topo I proved somewhat more specific than the immunoblot, whereas it was more sensitive than the immunodiffusion technique (22). At present, recombinant topo I is available for use in ELISA to detect and quantitate antitopo I antibodies in routine practice (23). All major epitopes should, however, be present on the recombinant protein. Use of recombinant proteins that express only one epitope will result in lower percentages of positive results (24).

Centromere Proteins

Using cell lines from tissue culture as a substrate for ANA-testing by IIF, it proved that sera from patients with LCS, in particular those with the CREST-syndrome, produced a finely speckled fluorescence pattern with a characteristic double dot appearance (Fig. 1). The antibodies reacted with the centromeric region of chromosomes as demonstrated by IIF on chromosome spreads (25). Enzymatic digestion by proteolytic enzymes destroyed antigenicity only in the

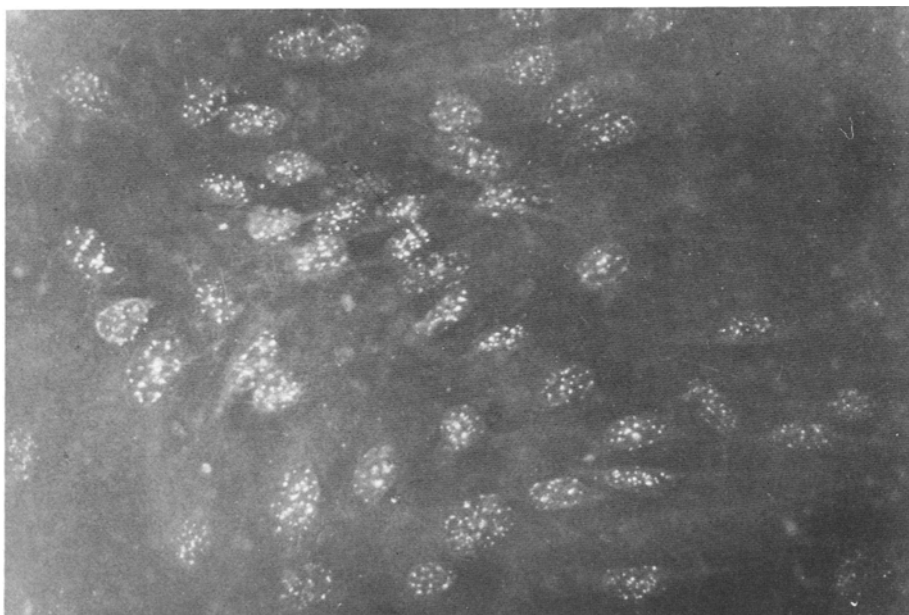


Fig. 1. Finely speckled staining produced by ACA using cultured human fibroblasts as a substrate.

presence of the protein denaturing agent SDS, suggesting that the antigen(s) in question are tightly bound proteins, in contrast to histones that can be removed by treatment with acid (0.1M HCl) alone (25). Using a soluble nuclear protein fraction from HeLa cells, Guldner et al. (26) showed by immunoblotting experiments that the antigen is a 19.5-kDa protein. After elution, the antibodies indeed produced the centromeric staining pattern on chromosome spreads. The identification of a 17–19-kDa protein from the centromeric region as the target antigen for ACA was confirmed by others (9). Earnshaw et al. (27), however, showed that ACA recognize at least three polypeptides from the centromeric regions. Their immunoblotting studies with highly purified HeLa chromosomes revealed reactivity of ACA with a centromere protein (CENP)-A of 17-kDa mol wt, a CENP-B of 80-kDa mol wt, and a CENP-C of 140-kDa mol wt. All 39 sera positive for ACA by IIF recognized CENP-B, whereas 38 sera recognized CENP-A and 37 sera CENP-C (28). Since CENP-B and -C are highly insoluble, it was presumed that these antigens might have been discarded by fractionation procedures in the immunoblotting studies (9,26). It was suggested that CENP-B was the primary autoantigen. Antibodies reacting with CENP-A reacted with -B, and antibodies affinity purified from CENP-C also strongly crossreacted with -B, whereas no epitopes were shared by -A and -C.

Subdomains of CENP-B have now been cloned and expressed in bacteria resulting in an N-proximal fusion protein of 347 amino acids and a C-terminal product of 147 amino acids (29,30). The latter protein was subdivided into proteins of 104 and 43 amino acids. Using these fusion proteins, it was demonstrated that ACA-positive sera recognize at least three different epitopes, one on Nprox-CENP-B and two on Cterm-CENP-B. The autoantibodies affinity purified from CENP-A, -B, and -C, respectively, appeared to recognize three additional epitopes. Thus, the immune response resulting in ACA, comparable to the response to topo I, is highly polyclonal. CENP-B, however, seems the primary autoantigen since all ACA-positive sera recognize chromosomal CENP-B by immunoblotting (29) and react with the fusion protein Cterm-CENP-B (β -Gal) by ELISA (30).

More recently, epitope mapping has been performed on CENP-B (31). For this study, a functional recombinant CENP-B (i.e., a molecule with DNA-binding capacity as CENP-B is one of the centromere DNA-binding proteins constituting centromeric heterochromatin of human chromosomes) was expressed in *E. coli*. The functional site was located at the N-terminal part of the molecule and had a binding motif identified for the helix-loop-helix (HLH) family of DNA-binding proteins. Four epitopes were mapped on the molecule, two epitopes at the C-terminus, and two on the HLH region at the N-terminus. Antibodies to CENP-B were able to inhibit the DNA-binding activity of the molecule. Thus, like antitopo I, ACA are directed against highly conserved regions. This was shown also by their reaction with mouse CENP-B (32). So, ACA are polyclonal antibodies directed as many other (antinuclear) autoantibodies to highly conserved functional domains of their targets.

Antibodies to centromere proteins (ACA) are routinely detected by indirect IIF on tissue culture cell lines such as HEp-2 cells. They produce a characteristic finely speckled double dot staining pattern. They can be detected also by immunoblotting using an HeLa cell nuclear extract (9,26) (Fig. 2). At present, cloned antigen is available for detecting and quantitating ACA in ELISA (30).

Clinical Associations

ACA

In the first report on ACA (25), it was recognized that 7 of the 10 ACA-positive patients from a group of 32 patients with scleroderma had the CREST-syndrome. Following this report, two studies described a prevalence of ACA in patients with CREST of 55% (5) and 96% (33), respectively, whereas ACA were infrequently found in patients with DCS (8% [5] and 12% [33], respectively). Since that time,

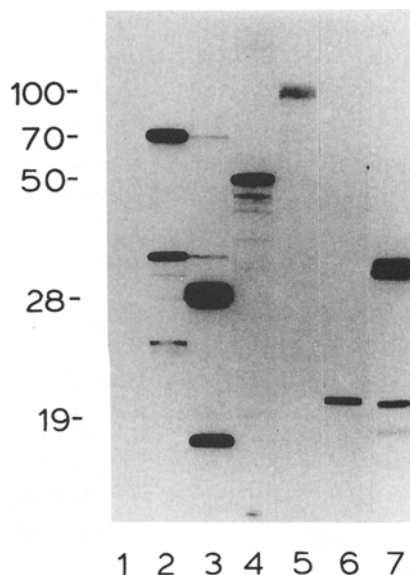


Fig. 2. Antibodies detected by immunoblotting. Protein blot strips containing HeLa nuclear proteins were incubated with: normal human serum (1), anti-U1 RNP serum (2), anti-Sm serum (3), anti-SSB serum (4), antitopoisomerase I serum (5), anticentromere (CENP-A) serum (6), and antihistone serum (7). Bound antibodies were detected using ¹²⁵J-labeled protein A. Molecular weights (x10⁻³) of the antigens are indicated. Reprinted with permission from Kallenberg (54).

other reports have confirmed the association of ACA with CREST (6,34–39). In general, the prevalence of ACA in CREST is roughly 55%, but marked differences in prevalence (ranging from 49% [38] to 96% [33]) are found between the several reports. These differences are explained by differences in patient population, in particular racial background (*see Genetic and Environmental Factors Involved in Auto-antibody Formation*); differences in the technique used for the detection of ACA (*see Centromere Proteins*); and different definitions for the CREST-syndrome used by several authors. CREST may be defined as the presence of all of the syndrome's symptoms in the absence of proximal scleroderma. In some studies, however, patients with incomplete CREST displaying only two of the five symptoms were included. Indeed, in a study on 138 patients with Raynaud's phenomenon (RP; 34), we found ACA in the sera of 7 of 10 patients with CREST but also

in 7 patients with (limited) scleroderma and 7 patients with RP in conjunction with minor signs of scleroderma only. To solve this problem of nomenclature, a new classification has been proposed (3), as has already been discussed, which dissects the group of patients with scleroderma in those with DCS and those with LCS (Table 1). ACA are definitely associated with the latter group.

Within the group of patients with LCS, ACA-positive patients differ from ACA-negative patients with respect to the following items:

1. Older age at onset;
2. Stronger female preponderance;
3. More intense teleangiectasia;
4. More frequent occurrence of calcinosis; and
5. The less frequent occurrence of interstitial fibrosis (36).

Although ACA are associated with LCS, they also occur in patients with DCS. A prevalence of 10%, ranging from 3% (36) to 26% (39), is suggested from data of several reports (6,35–39).

The occurrence of ACA is not restricted to patients with scleroderma syndromes. ACA have been described in a substantial percentage ($\pm 30\%$) of patients with primary biliary cirrhosis (PBC) (40–42). Interestingly, ACA-positive PBC patients frequently display one or more symptoms of the CREST-syndrome or LCS, whereas these symptoms are generally absent in PBC patients without ACA. The acronym PAKC (primary biliary cirrhosis, anticentromere antibody, CREST syndrome, Keratoconjunctivitis sicca) has been proposed to describe the former patients (43). ACA are virtually absent in normal controls and in patients with different connective tissue diseases, although a prevalence of 3% both in healthy controls and in patients with different connective tissue diseases was reported when ACA were determined by CENP-B-specific ELISA (30). Finally, the titer of ACA may have some importance as high titer autoantibodies were particularly seen in patients with very severe RP and in patients with vascular occlusive disease (44). Remarkable changes in the titer or in the presence of ACA during followup are, however, not observed.

Antitopoisomerase I

As stated before, antitopo I are associated with DCS and ACA with LCS. Generally, the antibodies are mutually exclusive, as shown in most reports. A recent report from Poland, however, mentioned the simultaneous occurrence of antitopo I and ACA in 10 of 180 patients with scleroderma (45). The antibodies were detected by indirect IIF, immunodiffusion, and immunoblotting. Patients with both antibody specificities had pronounced vascular involvement.

Antitopo I are found in some 30% of patients with DCS (5,35,39, 46–51), ranging from 26% (33) to 65% (47). The severity of the disease was reported not to correlate with the presence of antitopo I, although the duration of the disease was shorter in patients with antitopo I antibodies (46). Fast progression of the disease in patients with antitopo I has also been mentioned in another study (50). Antitopo I has also been detected in 12% of patients with LCS (5,39). At present, it is not clear if the disease follows a different course in patients with LCS and antitopo I compared to patients with LCS and ACA. Although no association was found between the severity of the disease and the presence of antitopo I in patients with DCS, the presence of antitopo I was associated with pulmonary fibrosis (52). When MHC class II antigens were additionally included in the analysis, it proved that the presence of either antitopo I or HLA DR3/DRw52a constituted a relative risk of 16.7 for the development of pulmonary fibrosis in patients with scleroderma (52). The presence of antitopo I in scleroderma has also been connected to cancer (53). Rothfield et al. reported that 7 of 36 antitopo I-positive scleroderma patients developed cancer in contrast to 2 of 112 patients negative for those antibodies. The following malignancies were recorded: lung cancer ($n = 4$), colon cancer ($n = 1$), lymphocytic lymphoma ($n = 1$), and metastatic brain cancer ($n = 1$). Whether lung cancer developed on a background of interstitial fibrosis was not mentioned. Antitopo I were not detected in patients with lung cancer without scleroderma.

Prognostic Significance of ACA and Antitopo I

Most patients with scleroderma present with RP. Otherwise, only a small percentage of patients with RP will eventually develop scleroderma. Thus, it would be useful to have tests with predictive power for the evolution of such disorders in patients presenting with RP (54,55). To evaluate the predictive power of ANA in patients with RP, for 6 yr we prospectively followed up 85 patients referred because of RP (56). Every 2 yr they were screened for signs and symptoms of connective tissue disease (CTD) according to a protocol, and serum was stored. Initially, 30 patients had primary RP, 16 had one symptom of CTD (possible CTD), 18 had two or more symptoms (probable CTD), and 21 definite CTD, 14 of whom had scleroderma. There was an insidious progression to scleroderma: 11 of 46 patients with primary RP or possible CTD developed probable scleroderma (i.e., two or more symptoms but not fulfilling all criteria), and 7 of 13 patients with probable scleroderma developed defi-

nite scleroderma. The antibody specificities of ANA had prognostic value for the development of certain disease entities: ACA for LCS including CREST (sensitivity 60%, specificity 98%), and antitopo I for DCS or probable scleroderma (sensitivity 38%, specificity 100%). Thus, the presence of ACA or antitopo I in a patient presenting with RP is highly indicative for the future development of scleroderma subsets.

Weiner et al. (57) followed 77 patients with RP for a mean period of 4 yr. During that period, 8 patients developed teleangiectasias, 4 developed skin tightening, and 4 developed a CTD other than scleroderma. ACA at the start of the study were associated with the development of teleangiectasias, and antitopo I was associated with the development of tight skin. Patients initially positive for ACA or antitopo I had a 63-fold increased risk for developing signs of CTD compared to the remaining patients with RP. This study also underscores the prognostic significance of the antibodies.

Genetic and Environmental Factors Involved in Autoantibody Formation

The etiology of scleroderma is far from known. Specific autoantibody formation, however, seems to precede the development of the disease by many years (56,57). As such, insight into the factors that underlie the induction of autoantibody formation may give a clue to the etiology of the disease. Among these, genetic factors might play a role. No single gene, however, appears to be involved in the induction of either antitopo I or ACA. Monozygotic twins were discordant for the disease and for the presence of particular autoantibodies in one study (58), but concordant in another (59). Also, in first degree relatives of patients with ACA no increased prevalence of these autoantibodies is detected (60), although an early report suggested a somewhat increased prevalence (61). Nevertheless, interracial differences in the prevalences of the scleroderma autoantibodies suggest that genetic factors are involved. In a group of 156 American patients with scleroderma, ACA were found in 36% of whites and 4% of blacks ($p < 0.005$), whereas antitopo I occurred in 17% of whites and 37% of blacks ($p < 0.05$) (62). These data are congruent with a study from South Africa showing that ACA, and consequently the limited cutaneous form of scleroderma, were notably absent in blacks in contrast to antitopo I antibodies (63). Comparable data were obtained from a study from Australia evaluating Thais and Australian patients with scleroderma (64). Seventy-six percent of the Thais and 26% of the Australians were positive for antitopo I, whereas only 2% of the Thais in contrast to 51% of the

Australians were positive for ACA. A number of recent studies on the relation between MHC-antigens and the scleroderma autoantibodies have shown interesting results. The association of HLA class II antigens with these autoantibodies, i.e., DR5 with antitopo I (65) and DR1 (or DR4) with ACA (64,66), suggests that processed antigens and T-cells are involved in the generation of the antibodies. Further studies directed at HLA class II genotyping showed that the antitopo I response was associated with certain amino acid sequences in the first domain of HLA-DQB1 (67,68). Using the PCR-RFLP technique, Kuwana et al. (68) showed that either the DQB1*0601 or the DQB1*0301 allele was present in all antitopo I positive patients. Tyrosine at position 26 in the second hypervariable region in the β 1 domain of the DQB1 gene was shared by both alleles and seems to be critical for the antitopo I response. Besides DQ genes, DR genes appeared to be involved in the response as well. Interestingly, one major B-cell epitope on topo I corresponded to a particular sequence in the DRB gene. Another study (66) recently reported the association of antitopo I with an acidic residue of DP β , i.e., DPB1:69:E showing a relative risk of 4.6. This study also used the PCR-RFLP technique. Thus, DR, DP, and DQ genes control the autoimmune response to topo I. A recent study from the UK showed that the presence of antitopo I in conjunction with certain class II molecules, i.e., HLA DR3/DRw52a, constituted an important risk factor for pulmonary fibrosis, in scleroderma (52). In patients with pulmonary fibrosis, 24 of 42 (57%) (of a group of 75 with scleroderma) were positive for one of these markers, whereas only 2 of the 33 patients without pulmonary fibrosis (6%) were positive. This results in a relative risk of 16.7.

The expression of ACA was shown to be associated with either DR1 or DR4 (relative risk of 6.7) (66). Further studies have revealed that tyrosine at position 26 in the DQB1 β 1 domain is one of the amino acid residues also associated with ACA (69). In view of the different structure of topo I and centromere proteins, this finding makes it unlikely that the putative antigen binding cleft at position 26 in the DQB1 β 1 outermost domain is involved in the induction of those antibodies. Indeed, Fox et al. recently reported (70) that several ACA-positive patients lack a polar amino acid at position 26. Thus, although genetic markers seem to be involved in autoantibody production, their direct role has not yet been fully elucidated. In general, genetic markers are more strongly associated with autoantibody formation than with the expression of the disease itself. This suggests a more direct involvement of immune response genes in the induction of autoantibodies whereas other, possibly exogenous, factors are involved in the expression of the disease.

Besides genetic traits, exogenous factors appear also to be involved in the induction of the scleroderma autoantibodies. Silica exposure appears a risk factor for the development of scleroderma. In a study from Germany, 93 of 120 male scleroderma patients had a long-term exposure to silica dust (71). Strikingly, only 10 patients were positive for ANA, 5 for ACA, and 3 for antitopo I. Whether the autoantibodies developed before the clinical expression of the disease was not reported. These data, however, suggest that autoantibody formation is not necessarily related to the expression of scleroderma. Different results were reported in a study from the UK on silica-induced scleroderma (72). They studied 17 mine workers exposed to silica dust who later developed scleroderma. Clinical features of these patients were indistinguishable from those of patients with idiopathic scleroderma. Eight of the 17 patients had antibodies to topo I. ANA were detected also in 3 of 22 miners exposed to silica dust without developing disease, and in 14 of 30 miners who later developed silicosis. The antibodies were not directed at topo I or centromere proteins.

Bridges et al. recently studied the effect of silicone breast implants, possibly as a model of adjuvant disease, on the induction of scleroderma and related autoantibodies (73). Fourteen of 156 women with breast implants and rheumatic disease complaints had a scleroderma-like illness in conjunction with ACA or anti-PM-Scl (polymyositis-scleroderma) antibodies. These data suggest that, comparable to cyclosporin-induced autoimmunity (74), polyclonal activation may result in scleroderma together with the relevant autoantibodies.

In conclusion, racial factors are definitely involved in the expression of the scleroderma autoantibodies: ACA occur in whites and antitopo I in blacks and Asians. Although no single gene is involved, HLA-associations are detected for both antibodies. Extrinsic factors are also involved as shown by the induction of antitopo I and DCS after silica dust exposure and the occurrence of ACA and LCS after silicone breast implants.

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