

Antitopoisomerase 1 Antibodies in Systemic Sclerosis

How to Improve the Detection?

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ABSTRACT: Among the multiple autoantibodies identified in the serum of systemic sclerosis (SSc) patients, three are disease-specific, mutually exclusive, and helpful to determine the prognosis: anticentromere antibodies, antitopoisomerase 1 antibodies (ATA), and anti-RNA-polymerase III antibodies. ATA can be identified through different techniques, including double immunodiffusion (DID) assay, enzyme-linked immunosorbent assay (ELISA), or immunoblot. Although all of them are commonly used, none of them can be considered as the reference. Herein, we propose a brief description of the different methods available for the detection of ATA. All these studies revealed that ATA, determined by DID assay, ELISA, or immunoblot, are highly specific for SSc although the reported sensitivity is fickle. As we recently reported, patients with ATA had an almost similar phenotype without distinction between the methods of detection, ELISA, and immunoblot, and the use of these two techniques improves the sensitivity without diminishing the specificity. Thus, we may propose that a combination of the immunoblot using HEp-2 cells antigens and ELISA could be used for the detection of ATA.

KEYWORDS: antitopoisomerase 1 antibodies; systemic sclerosis; immunoblot; ELISA; double immunodiffusion

INTRODUCTION

Systemic sclerosis (SSc) is a connective tissue disorder characterized by excessive collagen deposition in the dermis and internal organs, by vascular

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hyperreactivity and obliteration phenomena.¹ Among the multiple autoantibodies identified in the serum of SSc patients, three are specific for the disease and are mutually exclusive: anticentromere antibodies,² antitopoisomerase 1 antibodies (ATA),³ and anti-RNA-polymerase III antibodies.⁴ Although their pathogenic role is not clearly established, disease-specific autoantibodies are helpful to determine the prognosis.

In 1979, Douvas *et al.*⁵ observed that sera from SSc patients reacted with a component of 70 kDa in calf thymus nuclei extract. This component, named Scl-70, has been identified later as DNA-topoisomerase 1.⁶ Topoisomerases are ubiquitous and essential enzymes that solve the topological problems that accompany DNA replication, transcription, chromatin assembly, recombination, and chromosome segregation by introducing transient breaks into the helix.⁷ DNA strand separation causes an alteration of the twist and the writhe of topologically restrained chromosomal DNA. Topoisomerases recognize these changes and catalyze the relaxation of the torsional tension in the DNA. Human DNA-topoisomerase 1, a monomeric protein of 765 amino acids, creates transient nicks in the double-stranded DNA, allowing DNA to be converted between topological isomers. Although the native molecular weight of DNA-topoisomerase 1 is 105 kDa, smaller proteolytic fragments ranging from 60 to 100 kDa are frequently found in tissue/cell protein extracts.

Generally, disease-specific autoantibodies can be investigated through different methods. Usually, ATA are investigated in patients with antinuclear antibodies in the absence of anticentromere specificity, since these two antibodies are mutually exclusive. ATA can be identified through different techniques, including double immunodiffusion (DID) assay, enzyme linked immunosorbent assay (ELISA), and immunoblot. Although all of them are commonly used; none of them can be considered as the reference.

Herein, we propose a brief description of the different methods available for the detection of ATA and review data from the literature in order to decide which strategy offers the best sensitivity and specificity.

ASSAY BASED ON IMMUNOPRECIPITATION

DID (including Ouchterlony assay), and counter-immuno-electrophoresis (CIEP) using calf or rabbit thymus extract, were the most extensively validated techniques to assay ATA. Topoisomerase 1, purified from calf and rabbit thymus nuclei, was also used as antigen. DID consists of a diffusion of antigen and antibody in two dimensions from separate sources into a gel, generally agarose. This method has a high-resolution power but the information yielded is mainly qualitative. Furthermore, it is awkward and time-consuming (requires 2–3 days), and more difficult to perform for a large number of samples. CIEP, as for it, consists in forced (electrical field) diffusion of antigen and antibody toward each other in a gel. Due to the selectively directed migration, CIEP is

TABLE 1. Detection of antitopoisomerase 1 antibodies (ATA) in systemic sclerosis (SSc) patients

Study	No. of patients	Method of detection	Sensitivity	Clinical association
Jacobsen <i>et al.</i> ⁸	230 SSc	DID	13%	Digital joint deformity, distal osteolysis, pulmonary fibrosis
Steen <i>et al.</i> ⁹	397 SSc	DID	26%	Diffuse SSc, digital pitting scars, and pulmonary interstitial fibrosis
Tamby <i>et al.</i> ¹⁰	120 SSc	DID	23.3%	Diffuse SSc, interstitial lung disease
Weiner <i>et al.</i> ³	355 including 143 with SSc	IB, DID	28% and 20%	Skin and cardiac involvement, malignancies
Aeschlimann <i>et al.</i> ¹⁷	145 including 64 with SSc	IB	48%/	Shorter disease duration
Tamby <i>et al.</i> ¹⁰	111 SSc	IB, ELISA	35.1% and 24.3%	Diffuse SSc, interstitial lung disease, digestive involvement

DID = double immunodiffusion; IB = immunoblot; ELISA = enzyme linked immunosorbent assay; SSc = systemic sclerosis.

more sensitive than the DID. Other advantages are the simplicity, the rapidity since results are obtained within a few hours, but its resolution power is not as good as that of DID. Jacobsen *et al.* used DID against a thymus and spleen extract to detect ATA on a cohort of 230 consecutive SSc patients; ATA were found in 13% of patients.⁸ Steen *et al.* reported that 26% of 397 SSc patients had ATA.⁹ Tamby *et al.*, used CIEP and found ATA in 23.3%, among 120 SSc patients, with a specificity of 96% (TABLE 1).¹⁰

ELISA

The ELISA is a fundamental tool of clinical immunology. Based on the principle of antibody–antibody interaction, it can be completed without the additional concern of radioactive materials. This assay is rapid and the cost-effectiveness rate is good. The antitopoisomerase 1 ELISA was initially performed with topoisomerase 1 purified from rat liver and calf thymus. More recently recombinant topoisomerase 1 fusion proteins have been used.^{11,12} Kuwana *et al.* detected ATA by ELISA using five different recombinant fusion proteins as antigens.¹³ These proteins overlap amino acid sequences, encompassing the entire DNA-topoisomerase 1 molecule. ATA were measured using a mixture of these five fusion proteins or each fusion protein independently in 73 SSc sera positive for ATA by DID and in negative control

sera. Total ATA were detected in 96% of the positive sera. The specificity was 99% with reference to DID and the total ATA ELISA levels strongly correlated with DID. Nevertheless, 4% of SSc sera that were negative by DID were positive for the detection of ATA by ELISA, recognizing the N terminus of topoisomerase 1 in two cases. Four major epitopes were described by ELISA using 63 20-mer peptides spanning the entire length of the topoisomerase 1 sequence as antigens.¹⁴ These epitopes were recognized by IgG fractions of antitopoisomerase 1 serum of SSc patients but not by IgG fractions of control serum.

In 2003, a review made by the American College of Rheumatology (committee on immunologic testing guidelines)¹¹ included only two studies for its recommendation concerning the detection of ATA by ELISA.^{15,16} The combined sensitivity of ATA for the diagnosis of SSc was 43%. The specificity and likelihood ratios were high and better compared to controls (100% and >55, respectively) than in patients with other connective tissue disease (90%, 4.3). No recommendation could be made based on these two studies.

Immunoblot

Immunoblot or Western blot is a method designed to detect proteins in a given sample of tissue or cell extract. It uses polyacrylamide gel electrophoresis to separate denatured proteins according to their molecular weight. The proteins are then transferred onto a membrane, where they are profiled using specific antibodies or other antibody preparations, such as diluted sera. Immunoblot with human nuclear extract (HeLa or HEp-2 cells) or recombinant proteins has been used to detect ATA in patients. Aeschlimann *et al.* have detected ATA with immunoblot in a cohort of 145 patients, including 65 with diffuse SSc, 26 with mixed connective tissue disease, and 55 patients with systemic lupus erythematosus. The sensitivity for SSc was 48% and the specificity of ATA in SSc was 93%.¹⁷ Several others studies examined ATA by immunoblot, in SSc patients compared to healthy controls. The sensitivity was 41%, and specificity and positive likelihood ratios remained high (99.4% and 68%, respectively).^{3,18}

Using quantitative immunoblot technique,¹⁹ we have previously reported that patients with diffuse SSc with ATA reacted with a 100 kDa protein in human endothelial cells as well as in HEp-2 cells. This reactivity was also found in 50% of patients with diffuse SSc with no evidence of ATA upon DID or ELISA.²⁰ Alderuccio *et al.* reported that SSc patients reacted upon immunoblot with a 100 kDa protein in endothelial cells of human and bovine origin as well as in HEp-2 and HeLa cells.²¹ More recently, we have used immunoblot on HEp-2 cells extract and ELISA with calf thymus purified DNA-topoisomerase 1 to detect ATA in a cohort of 111 patients and 66 controls. As assessed by ELISA, ATA were detected in 24.3% patients and in none of the controls (100% specificity) and 92.6% of these patients had IgG reactivities toward two 63 and 100 kDa antigens, identified as DNA-topoisomerase 1,

upon immunoblot. However, by using immunoblot only, 35.1% of SSc patients (and none of the controls) had IgG reactivities directed toward two 63 and 100 kDa on HEP-2 cells extract.¹⁰

Association of Antitopoisomerase Antibodies with Clinical Manifestations

The interpretation of the significance of a positive test for ATA in a patient with suspected connective tissue disease depends on the assay used. Although ATA, as determined by DID or immunoblotting, are highly specific for SSc, the results of a positive antitopoisomerase 1 test as determined by ELISA, particularly when titers are low, should be interpreted carefully.

Jacobsen *et al.* used DID against a thymus and spleen extract to detect ATA in a cohort of 230 consecutive SSc patients; ATA were found in 13% of patients and associated mainly with pulmonary fibrosis (48%) and skin involvement of the trunk (92%).⁸ Steen *et al.* reported that 26% of 397 SSc patients had ATA. Two-thirds of patients with ATA had diffuse SSc, but this antibody was detected in only 33% of all diffuse SSc patients. Within the subset of diffuse scleroderma, ATA was associated with peripheral vascular disease (digital pitting scars) and interstitial lung disease, but was not predictive of cardiac or renal involvement or survival.⁹ Diot *et al.*, using ELISA, observed that there was a significant association between the extent of skin sclerosis and ATA level (70.6% of patients with diffuse SSc). In patients with diffuse SSc, pulmonary involvement was strongly associated with ATA (91.7%).²² Tsay *et al.* detected ATA in 24/32 (75%) patients with diffuse SSc by ELISA.¹⁵ In our recent work, we have observed that patients with ATA had an almost similar phenotype without distinction between ELISA and immunoblot approaches. Precisely, the prevalence of interstitial lung disease and digestive involvement as well as the extent of skin involvement was higher in patients with ATA (81.5%, 88.8%, and 85.2%, respectively) than in patients with no ATA or anticentromere antibodies.¹⁰ Sato *et al.* reported a positive correlation between ATA levels (determined by ELISA) and Rodnan skin score and renal vascular resistance in a cohort of 125 SSc patients. In this longitudinal study, low and stable ATA level was associated with stable skin sclerosis, whereas high and increasing level of antibodies were associated with new onset or worsening of organ involvement suggesting the potential clinical significance of ATA levels in evaluating disease severity and prognosis in SSc.²³

Pathogenic Role of ATA

While ATA have not been directly implicated in SSc vascular dysfunction, several studies suggest a role for these antibodies in disease development. Kuwana *et al.*²⁴ reported that SSc patients with persistent ATA had less extensive skin and lung involvement and better survival rates than SSc patients

with no persistent ATA. Complete loss of ATA followed a reduction in isotype expression and epitope reactivities. The kinetics of *in vitro* T cell proliferation induced by topoisomerase 1 were delayed and circulating topoisomerase 1-reactive T cells were less frequently detected in SSc patients with persistent ATA versus SSc patients with no persistent ATA patients, suggesting that the disappearance of ATA was due to the loss of activation of topoisomerase 1-reactive T cells. *In vitro* production of ATA in peripheral blood mononuclear cell cultures in response to antigenic stimulation in both groups of patients indicated persistence of ATA-producing "memory" B cells even after the loss of serum ATA.²⁴ Similarly, Tsay *et al.* reported the ability of ATA to neutralize DNA-topoisomerase 1 activity.¹⁵ In a recent study the indirect binding of purified human ATA to the cell surface of fibroblasts has been evidenced, suggesting a role for ATA in the induction of fibroblast dysfunction. Nevertheless, DNA-topoisomerase 1 binding to fibroblast surfaces was necessary for ATA binding. ATA-DNA-topoisomerase 1 complex binding might be able to trigger the adhesion and activation of monocytes, leading to the fibrogenic cascade in SSc.²⁵ Finally, Ahmed *et al.* reported that ATA from SSc patients could trigger caspase-3-mediated apoptosis and upregulation of fibrillin 1 gene in human normal adult dermal endothelial cells.²⁶

How to Improve the Detection of ATA?

Usually, ATA are investigated in patients with antinuclear antibodies, as assessed by indirect immunofluorescence, without anticentromere specificity, through different techniques, including DID assay, ELISA, and immunoblot.

Hildebrandt *et al.*¹⁶ have investigated sera from 409 patients and controls by DID, immunoblot on chromosome proteins, and ELISA with purified topoisomerase 1 as antigen. They found that ELISA was more sensitive than the DID and was more specific than the immunoblot, while the immunoblot may identify additional autoantibodies. Despite that suggestion, Spencer-Green *et al.* concluded that there was no significant difference in the various methodologies used in the determination of anti-Scl-70 autoantibodies on the sensitivity and specificity for SSc.²⁷

Tamby *et al.*, used CIEP and found ATA in 23.3%, among 120 SSc patients, with a sensitivity and a specificity of 54% and 96%, respectively.¹⁰ By pooling the results obtained through indirect immunofluorescence, ELISA and immunoblot performed at a dilution of 1:50, we have also reported that ATA are detected in 36.9% (41/111) of SSc patients while ATA were detected by ELISA in only 24.3%. Sensitivity and specificity were calculated for these two techniques and were respectively of 24.3% and 100% for the ELISA and 36.9% and 100% for the immunoblot. This study provides evidence that using a combination of ELISA and immunoblot increases the sensitivity for the detection of ATA.¹⁰

CONCLUSION

The combined use of ELISA and immunoblot could be proposed for the detection of ATA.

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