IgM, IgG, and IgA Anti-DNA Topoisomerase I Antibodies in Systemic Sclerosis

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Background: Anti-DNA topoisomerase I (anti-topo I) antibodies have been broadly studied in systemic sclerosis (SSc). The use of different native and molecularly cloned antigens has shown a predominant IgG response, and a variable frequency of positive IgM and IgA tests. We report herein the serological findings of SSc using a recombinant topo I obtained through a standard bacterial system. **Methods:** Anti-topo I antibodies were studied in 45 SSc patients and 85 healthy controls through ELISA and western blot. *Escherichia coli* XL1-blue strain and pT7-7 vector were used to amplify a DNA topo I cDNA clone, and to obtain the recombinant polypeptide. The latter was purified by affinity chromatography, and the enzymatic and antigenic properties were evaluated through specific tests. A native antigen was included for comparison. **Results:** The SSc group disclosed positive IgM (20%), IgG (86.6%), and IgA (26.6%)

anti-topo I tests with the recombinant polypeptide, and a purified calf thymus antigen yielded similar results. IgG autoantibodies were frequently associated with skin involvement, esophageal dysfunction, and restrictive lung disease. The recombinant protein showed a molecular weight of 86.6 kDa, a positive topo I activity using a supercoiled pBR322 DNA relaxation test, and its carboxyl terminus region was recognized by specific antibodies.

Conclusion: This report confirms that different immunoglobulin classes with anti-topo I activity may occur in SSc. IgG was the predominant serological feature with both, the recombinant and native antigens. The study also demonstrates the association between high levels of these autoantibodies and some clinical manifestations of SSc. *J. Clin. Lab. Anal.* 23:408–416, 2009. © 2009 Wiley-Liss, Inc.

Key words: scleroderma; connective tissue disease; autoantibodies; antinuclear antibodies; recombinant protein; immunoglobulins

INTRODUCTION

Systemic sclerosis (SSc) is a connective tissue disease characterized by prominent inflammatory and fibrotic changes that produce extensive skin, vascular, and internal organs involvement. In addition, multiple immunological abnormalities have been described, notably the presence of autoantibodies to DNA topoisomerase I (topo I), an antigen formerly termed Scl-70 (1,2).

Since the first evidence of antinuclear antibodies in SSc, numerous reports have demonstrated that the presence of immunoglobulins with anti-topo I activity are one of the most important serological hallmarks of the disease. The clinical relevance of serum anti-topo I tests has been emphasized by different research groups, particularly because of their association with severe SSc

manifestations, including extensive skin involvement, vascular changes, pulmonary fibrosis, cardiac disease, and even malignancies, all related with a worse prognosis (3–6).

During several years, a number of reports dealing with high sensitive and specific laboratory tests for the study of SSc have pointed out the relevance of increasing the

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diagnostic value of autoantibodies using native or natural topo I purified from crude antigen extracts (3–5), or proteins obtained through DNA recombinant methods. In addition, the use of these molecularly cloned antigens in lymphocyte proliferation assays has also allowed studying some differences in T-cell reactivity, which might be physiopathogenically relevant and helpful for the clinical evaluation.

In 1990, the first studies detecting autoantibodies in SSc with recombinant fusion topo I proteins suggested that these tests could be useful to distinguish some clinical and immunological features during the diagnostic approach or serological evaluation of the disease (7–9). The increased frequency of patients with positive anti-topo I tests and the presence of different classes of autoantibodies, initially reported with native antigen purified from calf thymus, were particularly observed using recombinant polypeptides (9,10). Other reports have suggested that the clinical and serological characteristics in SSc might be associated with specific epitope binding of anti-topo I immunoglobulins (7–18).

Since the use of calf thymus antigen or recombinant topo I polypeptides in previous reports demonstrated a high frequency of IgG autoantibodies, and positive IgM and IgA tests have been proposed as possible diagnostic hallmarks of the disease, we decided to study the distribution of immunoglobulin classes with anti-topo I specificity and evaluate the clinical usefulness of a recombinant antigen obtained by a standard bacterial system. This report includes a group of consecutive SSc patients who have been clinically and serologically studied during the last 15 years.

MATERIAL AND METHODS

Patients and Controls

The study included 45 consecutive SSc patients (41 women and 4 men) referred to our hospital for their immunological evaluation. Their age ranged from 16 to 60 years (mean 38.7 years) with a disease evolution from 2 to 22 years (mean 13.7 years). These patients fulfilled the American College of Rheumatology (ACR) criteria for SSc classification, and skin biopsies performed during the diagnostic approach showed histopathological findings compatible with the disease (19,20). All serum samples were conserved in aliquots at -70°C until their use. The clinical evaluation, laboratory and roentgenogram studies were done at admission, if clinically indicated (21–26). Routine immunological tests included determination of IgM, IgG, and IgA, as well as C3, and C4 by nephelometry, rheumatoid factor by latex test and/or nephelometry, as well as antinuclear antibodies using indirect immunofluorescent tests

(Hep-2 cells and *Crithidia luciliae* targets), and double immunodiffusion (Ouchterlony test). Patients with localized scleroderma and history of diseases similar to SSc, such as phenylketonuria, graft vs. host reaction, and scleroderma-like diseases secondary to drugs or chemicals (1,25,27) were excluded. As controls, we studied 85 healthy volunteers, 77 women and 8 men with a mean age of 37.3 years. They showed no family history of connective tissue disease, including SSc. The presence of rheumatoid arthritis, Sjögren's syndrome, systemic lupus erythematosus, dermatomyositis, mixed connective tissue disease, SSc, as well as Raynaud's phenomenon, was also ruled out through clinical examination and routine laboratory evaluation. The Research Ethics Committee of our Institute approved the study protocol, and all individuals gave written informed consent.

DNA topo I Antigens

The recombinant protein was obtained using a human topo I cDNA clone (a generous gift from Dr. Egon Durban, Baylor College of Medicine, Houston, TX) (12). It allowed us to perform the antigen analysis ranging from amino acid residue 70 (lys) to nucleotide 848 in the 3' nontranslated region according to the predicted amino acid sequence (7,12). This topo I cDNA inserted in a pT7-7 vector (2,473 bp) was a 2,700 bp product (*Eco* RI enzyme digestion), and showed an orientation and a reading frame (*Hind* III and *Xmn* I restriction sites) that were suitable for protein expression (Fig. 1).

A standard method with *E. coli* XL1 blue strain was used to obtain the recombinant fusion protein. The cells were made competent through the CaCl_2 method, and transformed bacteria were selected for their resistance to ampicillin (100 $\mu\text{g}/\text{ml}$). Expression of topo I was induced with 0.3 mM isopropyl- β -D thiogalactopyranoside (IPTG; Sigma Co, St. Louis, MO). After lysing the bacterial pellets through the sonication/lysozyme treatment (50J, 5 min, ultrasonic homogenizer; Cole-Parmer, Vernon Hills, IL), the preparation was placed in a purification buffer containing 50 mM Tris $\cdot$ HCl, pH 7.4, 1.0 mM Na_3EDTA , 10% glycerol (TEG), with 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and 1.0 mM 2-mercaptoethanol (Sigma Co.) (28). These protease inhibitors were used to prevent antigen degradation. In order to minimize the presence of contaminants in our recombinant protein, it was separated through a dsDNA cellulose affinity chromatography (Sigma Co.) using a linear gradient from 0.1 to 0.5 M of KCl in TEG (29). All fractions containing this polypeptide were aliquoted and stored at -30°C until their use.

A source of native topo I was also prepared, considering that the IgM, IgG, and IgA autoantibodies

Fig. 1. DNA topoisomerase I cDNA separation through agarose gel (1%) electrophoresis. MW represents molecular weight markers (lane 1). The full-length topo I cDNA inserted in the pT7-7 vector showed a 2,700 bp product after *Eco* RI enzyme restriction (lane 2). The results using *Hind* III (digestion of cDNA at nucleotide 1661) and *Xmn* I (digestion at nucleotide 489) enzymes are also shown (lanes 3 and 4). This agarose gel was stained with ethidium bromide (0.05 µg/ml) and visualized with a UV transilluminator.

were initially described using calf thymus as antigen target. It was essentially obtained according to the method of Schmitt et al. (30,31). The tissue was homogenized at 4°C in an extraction buffer containing 50 mM Tris·HCl, 25 mM KCl, 5 mM MgCl₂, and protease inhibitors (1.0 mM 2-mercaptoethanol and 1.0 mM PMSF). After centrifugation at 1,200g for 10 min, the pellet was resuspended and washed three times, using the extraction buffer with 0.25 M sucrose. In the last centrifugation, we used Triton X-100 (2%) (Sigma Co.) and 20 mM potassium phosphate, pH 7.3, with protease inhibitors to dissolve the pellet. Solid KCl was gradually added to a final concentration of 1 M, and the next steps included an ultrasonic homogenization (Cole-Parmer, Vernon Hills, IL) and the addition of polyethyleneglycol (PEG) 6,000 (5%) (Sigma Co.). The antigen was recovered by centrifugation at 15,000g for 30 min and affinity purified with an immobilized dsDNA column (Sigma Co.), using a linear potassium phosphate gradient (0.1–2.0 M, pH 7.3) with protease inhibitors. All fractions with topo I activity were pooled and stored at –30°C before their enzymatic and immunological evaluation.

The enzymatic activity of both the native and recombinant topo I preparations, which depends on the carboxyl terminus of the protein, was monitored through the DNA relaxation test in all purification steps

(28,29). Briefly, 20 µl of each fraction was added to the assay buffer containing 20 µg of supercoiled pBR322 DNA, 20 mM Tris·HCl (pH 7.5), 10 mM Na₂EDTA, 1 mM DTT, 30 µg of bovine serum albumin, and 150 mM KCl. After 30 min at 37°C, the reaction was stopped with 10% SDS. The resulting nicked DNA was visualized after agarose gel electrophoresis (0.7%) and ethidium bromide staining. Specific inhibition of topo I was confirmed with the addition of camptothecin (Sigma Co.) (28,29).

The recombinant and calf thymus antigens were also examined using SDS-PAGE electrophoresis and Coomassie blue staining (Bio-Rad, Richmond, CA). Their migration pattern was compared with molecular weight markers. Additionally, a polyclonal antibody with carboxyl terminus binding activity (Santa Cruz Biotechnology, Santa Cruz, CA) was used to evaluate the antigenic characteristics of these polypeptides by western-blot. A purified topo I from calf thymus (Invitrogen, Carlsbad, CA) was included as a control. It allowed us to compare the enzymatic and immunological properties of both sources of antigen used in the this study through the referred pBR322 DNA relaxation test and anti-topo immunoassays.

Enzyme Immunoassays

The presence of IgM, IgG, and IgA anti-topo I was determined using ELISA tests. Optimal conditions were selected after standardization of these methods in our laboratory. Briefly, microtiter plates (Costar, Corning Inc, NY) were coated with 100 µl of 2.5 µg/ml of recombinant and native topo I in carbonate buffer (0.05 M, pH 9.6). After 4 h of incubation at 4°C, the wells were washed five times with phosphate buffered saline (0.15 M), pH 7.4, containing Tween-20 (0.05%) (PBS-Tween-20), and then quenched with 1% fraction V bovine serum albumin (Sigma Co.). The possibility that repeated thawing/freezing cycles could modify autoantibody measurement was minimized using aliquots of serum samples that were not previously tested. Appropriately decomplexed (56°C, 20 min) and diluted sera were added to the antigen coated wells for 1 h at room temperature, and the binding of anti-topo I antibodies was detected by adding F(ab')₂ fragments of peroxidase labeled antihuman IgM, IgG, or IgA antibodies (Sigma Co.), which we have used to ensure the accurate determination of autoantibodies, considering that these conjugates were useful to eliminate endogenous interferences by rheumatoid factor (32), a common serological finding in autoimmune diseases.

The substrate 3,3', 5,5'-tetramethylbenzidine (TMB) (Sigma Co.) was prepared in citrate buffer (0.1 M, pH 4.0) and the reaction was stopped after 15 min at room

temperature with 2.0N sulfuric acid. Absorbance (450 nm) was read in a micro ELISA reader (LabSystem, Helsinki, Finland) and the results were reported in optical density (OD) (450) units (U).

All patient and control sera were preincubated with an *E. coli* XL1 blue lysate containing nonrecombinant pT7-7 products. A positive anti-topo I sample from the Center for Disease Control (CDC), Atlanta, Georgia, was included as internal control. Because nonspecific binding of serum IgG through its Fc fraction to topo I has been described (33), we confirmed that those samples with elevated levels of autoantibodies by ELISA revealed positive results using a digestion method with immobilized pepsin (Pierce, Rockford, IL) and a monoclonal antibody specific for the F(ab')₂ portion of human IgG (Jackson ImmunoResearch, West Grove PA). All immunoassays included the determination of IgM, IgG, and IgA anti-topo I values in healthy controls in order to establish the reference limits (mean value +2 SD).

At this stage of the study, we were unable to obtain, standardize, and validate the use of specific epitopes of our recombinant topo I. Therefore, to analyze, at least in part, the possibility that an antigenic region on the recombinant polypeptide could explain a predominant IgM, IgG, or IgA binding, the distribution of these classes of anti-topo I antibodies was also measured using a purified calf thymus protein (30,31).

Western Blot

The immunoreactivity of SSc sera to the recombinant fusion polypeptide and purified antigen from calf thymus was examined using western blot. In brief, after electrophoresis in 10% SDS-PAGE gels according to Laemmli (34), the antigens were transferred to nitrocellulose sheets (Bio-Rad), as previously described (35). Bovine serum albumin, 3% in PBS was used as the quenching buffer, and the membranes were washed with 0.1% PBS-Tween-20, and then incubated with an appropriate dilution of serum samples for 3 h. This method included the use of F(ab')₂ fragments of horseradish peroxidase-labeled goat IgG against IgM, IgG, IgA human immunoglobulins (Sigma Co.), and 4-chloro-1-naphthol (30 mg) diluted in PBS (50 ml) and methanol (10 ml) with hydrogen peroxidase or a chemiluminescent reagent (Immobilon Western HRP, Millipore, Bedford) as substrates. For these tests, sera were also thawed only once to prevent any modification in the results.

Statistics

The ELISA results were statistically compared through the Mann-Whitney *U* test. A Spearman's rank correlation coefficient was applied to analyze the

relationship between IgM, IgG, and IgA values with anti-topo I activity, and the Spearman's rank and χ^2 tests led us to evaluate the association of autoantibodies and clinical manifestations in our SSc group.

RESULTS

Recombinant Antigen

During preparation of our recombinant polypeptide, the aliquots recovered from the affinity chromatography with immobilized dsDNA showed a positive enzymatic topo I activity. The use of a standard test with superhelical pBR322 plasmid revealed that all fractions containing the molecularly cloned antigen produced a relaxed DNA form. These results were further confirmed using camptothecin (Sigma Co.), a specific inhibitor of topo I. After separation in 10% SDS-PAGE gels, the purified antigen showed a molecular weight of 86.6 kDa by Coomassie blue staining, and a western blot disclosed that this polypeptide was recognized by a polyclonal antibody raised against the carboxyl terminus of topo I of human origin (amino acids 466–765) (Santa Cruz Biotechnology).

In addition, our recombinant antigen had essentially the same enzymatic and immunological characteristics of a native source of topo I purified protein from calf thymus, revealing the expected molecular weight profile (70 and 100 kDa), as well as catalytic properties by DNA relaxation tests and the presence of an antigenic carboxyl terminus region determined with specific antibodies.

Enzyme Immunoassays

The immunoassays using a recombinant protein as antigen source revealed an interesting serological heterogeneity in SSc. Elevated levels of IgM and IgG anti-topo I antibodies were the predominant abnormalities of the disease (0.101 ± 0.114 and 0.705 ± 0.427 ODU, respectively) considering the reference limits of healthy volunteers (0.059 ± 0.058 and 0.191 ± 0.095 ODU) ($P < 0.002$) (Fig. 2). In contrast, the values of IgA were similar in SSc patients (0.088 ± 0.100 ODU) and controls (0.054 ± 0.036 ODU) when we compared these anti-topo I tests ($P = 0.356$). Additionally, the statistical evaluation of the IgM, IgG, and IgA levels with autoantibody activity in SSc through regression analyses did not reveal a significant relationship.

A detailed immunological analysis disclosed that a total of 40 of the 45 SSc sera (88.8%) were anti-topo I positive, taking into account the IgM, IgG, and IgA reference values obtained in the control group. Nine patients (20%) revealed high IgM levels and the results of IgG exceeded the mean +2 SD of healthy controls in

Fig. 2. Comparison of IgM, IgG, and IgA anti-topo I antibodies by ELISA using a purified recombinant polypeptide as the antigen source. The individual levels of autoantibodies in patients with SSc (left), and healthy controls (right) are presented. In these scattergrams, horizontal lines represent the mean $\pm$ SD of OD units (450 nm) of each group.

39 SSc samples (86.6%). In addition, 12 sera samples (26.6%) demonstrated elevated values of IgA with anti-topo I activity. As expected, the frequencies of IgM and IgA anti-topo tests were higher (23 and 30.7%, respectively) when the SSc group with positive IgG results was analyzed, and the levels of IgG were also higher in those sera that showed positive autoantibodies through the double immunodiffusion method. There were 24 patients that had only IgG anti-topo I tests; the simultaneous presence of IgM, IgG, and IgA antibodies was found in four SSc samples, four patients revealed elevated levels of IgM and IgG anti-topo I, and seven sera were IgM and IgG positive. Finally, one patient presented high values of IgM anti-topo I antibodies, as the only serological abnormality.

The frequencies and levels of immunoglobulin classes with autoantibody activity were similar, when we used ELISA plates coated with the recombinant or native polypeptides. Indeed, there were only two patients that had positive IgG tests with our recombinant protein and negative results using calf thymus antigen, aside from one serum sample that was IgA topo I positive through the recombinant method and showed borderline levels of this immunoglobulin with the native protein. Additionally, the distribution of IgM, IgG, and IgA tests and their respective ELISA levels did not show significant differences after their statistical comparison.

We reasonably ruled out that a loss of immunoglobulins after storage and thawing/freezing cycles could explain the ELISA results. The use of $F(ab')_2$ fragments of peroxidase-labeled antihuman immunoglobulins prevented the possibility of false-positive results caused by endogenous rheumatoid factor (32). Finally, pepsin treatment of sera and the use of $F(ab')_2$ fractions of anti $F(ab')_2$ human IgG excluded the binding of IgG to our topo I preparations through the Fc fraction (33).

Western Blot

The presence of anti-topo I antibodies was confirmed using western blot. Thirty-six SSc sera previously characterized as positive through ELISA revealed a specific reaction with the recombinant or native polypeptides. These results were found even in those samples with borderline levels by ELISA, as revealed by the use of an appropriate serum dilution and/or a chemiluminescent substrate. Except for those three SSc patients who revealed negative IgG and IgA anti-topo I tests with the native antigen by ELISA, the remaining patients were immunoreactive when tested with our recombinant protein. In addition, all samples that recognized our recombinant topo I by western blot showed positive results with the main calf thymus antigens (70 and 100 kDa polypeptides). Our healthy controls revealed negative anti-topo I western blots.

Clinical Evaluation

In addition to the characteristic skin involvement of SSc, the review of clinical files revealed the frequent presence of gastrointestinal, lung, cardiovascular, and kidney abnormalities and, as expected, some manifestations occurred simultaneously. In particular, esophageal dysfunction, defined by radiographic studies, and respiratory disease, evaluated through a combination of diagnostic procedures, including chest roentgenograms, spirometry tests, or high-resolution computerized tomography (HRCT), were the most common findings of the illness (23,25). Skin, pulmonary, and gastrointestinal manifestations were associated with positive anti-topo I tests. As in previous reports, our patients presented predominantly a rapidly extending cutaneous involvement from the disease onset or a slow progressive skin sclerosis (20,22), and although these two SSc subsets revealed positive anti-topo I results,

their IgG levels were higher in the first group ($P = 0.01$) and the correlation with a high total skin thickness score was also significant (20,22). The presence of distal esophageal hypomotility, through barium contrast radiography, and interstitial lung disease by HRCT were more frequently detected in the SSc group with positive IgG anti-topo I results ($P = 0.05$ and 0.01 , respectively). Moreover, the percentage predicted forced vital capacity (%FVC) was significantly decreased (normal $>80\%$ FVC) in our patients with positive tests (23) and high IgG anti-topo I values relative to those without these abnormalities ($P = 0.000001$) (Fig. 3). It was not possible to establish a relationship between the IgM and IgA immunoglobulins with anti-topo I activity and the disease complications, because the simultaneous presence of these serological hallmarks was a common finding, and because such tests were particularly positive in SSc patients with high levels of IgG autoantibodies. In addition, although several patients received corticosteroids, D-penicillamine, or colchicine in low doses during variable periods of the illness before the serological study, the statistical analysis did not reveal a significant association between these clinical ante-

cedents and the levels of immunoglobulins with anti-topo I activity.

DISCUSSION

Anti-topo I antibodies have been extensively studied as immunological markers of SSc and positive tests may be detected in 15–100% of patients with the disease. According to previous reports, this wide range could result from variations in the stringency of diagnostic criteria, the clinicopathological manifestations that may distinguish some SSc subsets, and/or depend on several assay conditions, including, among others, the use of different laboratory tests and a diversity of antigen targets (13,26,36–40).

The combination of crude thymus preparations, as a source of native topo I, and monospecific reference antiserum by double immunodiffusion, used in the past decades, usually revealed about 15–30% of SSc patients with positive anti-topo I antibodies in a variety of clinical reports (4,6,36,37,40), including our own early observations (21–26).

Application of high sensitive and specific methods, such as RIA, ELISA, immunoprecipitation, and western blot, and the use of crude antigens from calf or rabbit thymus, rat liver, or HeLa cell preparations, have shown that a number of alternatives may be useful to detect specific antibodies in SSc (41–43). Indeed, in recent years, the preparation of purified or semipurified topo I from different cellular extracts as a source of native antigens, and the ability to produce recombinant proteins, have been allowed to find anti-topo I immunoglobulins with an increased frequency in sera from SSc patients and to demonstrate the presence of different immunoglobulin classes (10,12,31,41–45).

The study of anti-topo I antibodies, other than IgG in immunoassays that have included native proteins or recombinant polypeptides as antigen targets, has revealed several interesting results. An appreciable frequency of IgM, IgG, and IgA tests (29, 88, and 94%, respectively) was initially demonstrated by ELISA using topo I purified from calf thymus in 17 SSc patients who presented anti-topo I antibodies by Ouchterlony (46); nevertheless, the authors considered, as positive tests, values higher than twice the level of pooled normal serum. The use of a recombinant protein, containing the last 679 carboxyl-terminal amino acid residues of human topo I, disclosed elevated levels of IgM and IgA autoantibodies (45 and 85%, respectively) in 47 SSc sera known to be IgG anti-topo I positive (10) and, in this instance, values higher than twice the mean OD value of pooled normal human serum were regarded as positive. The study of other classes of immunoglobulins with anti-topo I activity in 23 SSc patients that had IgG

Fig. 3. Association between the serological findings of SSc and some clinical manifestations. The autoantibodies levels were high in those patients that presented rapidly extending skin involvement (**top**). Horizontal lines represent the mean $\pm$ SD of OD units (450 nm). The correlation of anti-topo I antibody results and %FVC revealed a statistically significant value (**bottom**). IgG autoantibodies were measured by ELISA using a recombinant topo I antigen.

levels above the mean +3 SD of healthy controls using a full-length human antigen, expressed in a baculovirus system, revealed a low frequency of positive IgM (13%) and IgA tests (4.3%) (18). An evaluation of 73 SSc sera with positive anti-topo I tests, by Ouchterlony, disclosed positive IgM (22%), IgG (92%), and IgA (77%) ELISA values that were over the mean +3 SD of controls, using five overlapping recombinant polypeptides, encompassing the entire topo I antigen (16). A variable percentage of IgM (19–33%), IgG (86–100%), and IgA (60–100%) tests were found in a comparative study that included different ethnic groups and the referred five overlapping recombinant antigens (47). In addition, the use of these last molecularly cloned antigens in a serological analysis of 22 patients, who had shown persistent antibody production, disclosed that 32 and 86% had positive IgM and IgA anti-topo I tests, respectively (48). Other groups have also studied the antibody classes with anti-topo I activity in SSc through ELISA or western blot using native or recombinant proteins; however, those studied included a small number of patients or the distribution of these autoantibodies was not specifically reported (8,11–13,48–53).

In agreement with previous reports, a high frequency of IgG anti-topo I tests and elevated levels of these immunoglobulins were found in our SSc patients. Additionally, this study allowed us to demonstrate different classes of antibodies with anti-topo I activity through ELISA and western blot.

Although the predominant occurrence of IgG with anti-topo I activity has consistently been recorded, several research groups have demonstrated a variable frequency of IgM and IgA tests. Here, we found a low frequency of IgM positive results in agreement with other studies that have described this abnormality in no more than 25% (16,18,47), except for two reports in which values higher than twice the mean OD level of pooled normal serum were considered positive (29 and 45%) (10,46) in a study of North American black patients (33%) and in a selected SSc group with persistent autoantibody production (32%) (47,48). It is also of interest that the percentage of positive IgA anti-topo I immunoglobulins in our study was low considering some, but not all, previous reports. Although these autoantibodies have been found in over 60% of SSc patients, a lower incidence of positive IgA tests was found in at least two studies (4.3 and 44%, respectively) (10,18).

A review of earlier reports revealed that several conditions can explain these serological differences. The selection criteria of SSc patients, including their ethnic or immunogenetic background, in terms of the MHC and other polymorphisms and their previous immunological characteristics appear to modify the

frequency of anti-topo I antibodies, as well as their isotype distribution in some studies (11,15,47,49,50,54,55). For example, a variable percentage of IgA anti-topo I positive sera has been found in North American, Japanese, and Choctaw Native American SSc patients (47,49,50), and the incidence of this autoantibody may differ according to the presence of anti-topo I tests through Ouchterlony, as well as positive IgG results by ELISA, western blot, or immunoprecipitation (10,16,18,46,47). In the present report, the immunogenetic characteristics were not specifically examined because it was not the main objective, and a group of consecutive patients was studied irrespective of their previous serological characteristics. The use of native or recombinant proteins and different ELISA results as reference values, or the presence of some interfering conditions in these immunoassays among other laboratory factors, may also explain several differences in the distribution of anti-topo I tests. A variable frequency of IgM, IgG, and IgA autoantibodies has been described using native proteins or different recombinant antigens (10,18,46), including individual polypeptides encompassing distinct topo I regions. In other reports, the highest frequencies of IgA anti-topo I tests have been found when values higher than twice the level of pooled normal serum were considered positive (10,46), and the presence of contaminants during the preparation of recombinant proteins may increase artificially the frequency of autoantibodies (16,18).

Here, we found that the frequency of IgM, IgG, and IgA anti-topo I isotypes and their respective ELISA levels were similar in most of the SSc sera, using a recombinant polypeptide, obtained by a standard bacterial system, and a purified native antigen, which suggest that a common epitope, probably at their carboxyl terminus regions, was recognized by these immunoglobulins. Indeed, this antigenic topo I fragment has been one of the most important targets of SSc sera (7,8,10,11,13,40,47) and was present in both the calf thymus antigen and our recombinant protein, as revealed by their enzymatic and immunological features.

In addition, because several laboratory conditions, including the use of contaminated antigens, nonspecific binding activity, or the presence of endogenous antibodies, like rheumatoid factor (32,33) may produce false-positive or false-negative anti-topo I results, we excluded their possible participation in our immunological tests.

An evaluation dealing with the use of specific epitopes on the recombinant polypeptide as antigen targets, and the association of autoantibodies with the immunogenetic characteristics in our patients, including a longitudinal serological analysis, is now in progress because this study did not explain entirely the differences in the

distribution of anti-topo I isotypes, when compared with other SSc reports.

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