B-Cell Epitope Mapping of DNA Topoisomerase I Defines Epitopes Strongly Associated with Pulmonary Fibrosis in Systemic Sclerosis

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We hypothesized that B-cell epitope mapping of DNA Topoisomerase I (type-I topoisomerase, or Topo I) may define epitopes strongly associated with pulmonary interstitial fibrosis (PIF) in systemic sclerosis (SSc). B-cell epitope mapping of Topo I was performed using 63 20-mer peptides overlapping by eight residues and spanning the entire length of the Topo I sequence. These peptides, coupled to polystyrene pins, were tested for antibody binding by enzyme-linked immunosorbent assays (ELISAs) using immunoglobulin G fractions from anti–Topo I, anticentromere, anti-U3RNP–positive, and normal sera. Four major epitopes were recognized by anti–Topo I sera, but not from the control sera: WWEEERYPEGIKWKFLEHKG (205–224, epitope I), RIANFKIEPPGLFRGRGNHP (349–368, epitope II), PGHKWKEVRHDNK-VTWLVSW (397–416, epitope III), and ELDGQEYVVEFDFLGKDSIR (517–536, epitope IV). Peptide-epitopes were then synthesized in their soluble forms and ELISA systems were developed. Epitopes II to IV are localized at highly exposed sites of the Topo I tertiary structure, whereas epitope I is localized at a less accessible site. In a cohort of 81 patients with SSc with clinical data on the evolution of their disease, patients with antibodies in their sera recognizing at least three of the four epitopes had 3.1 times ($P = 0.02$) the hazard of developing PIF compared with patients whose sera recognized no epitopes or only one or two of the four epitopes. The discrimination was much stronger than that achieved by the simple determination of Topo I antibodies by counterimmunoelectrophoresis and immunoblot (hazard ratio 1.7, $P = 0.30$) in the same patients. B-cell epitope mapping of the anti–Topo I response has identified four major epitopes which cumulatively show a strong association with the development of PIF in SSc. **Rizou, C., J. P. A. Ioannidis, E. Panou-Pomonis, M. Sakarellos-Daitsiotis, C. Sakarellos, H. M. Moutsopoulos, and P. G. Vlachoyiannopoulos. 2000. B-cell epitope mapping of DNA topoisomerase I defines epitopes strongly associated with pulmonary fibrosis in systemic sclerosis. Am. J. Respir. Cell Mol. Biol. 22:344–351.**

DNA Topoisomerase I (type-I topoisomerase, or Topo I) is an enzyme localized in both the cytoplasm and the nucleoli of the interphase cell (1–3). During mitosis the enzyme remains associated with the chromosomal material; its concentration in normal eukaryotic cells remains stable across the cell cycle (4). Topo I is transcribed from a gene localized on chromosome 20 that encodes a 4.1-kb messenger RNA (5). Topo I interconverts different topologic forms of DNA, contributing to the relaxation of supercoiled DNA, by a reaction that does not require adenosine triphosphate (3, 6). The enzyme has a calculated molecular weight (MW) of 90.753 kD and an apparent MW of 100 to 110 kD on sodium dodecyl sulfate (SDS)–polyacrylamide gels (3).

The sera of patients with systemic sclerosis (SSc), especially of the diffuse type of cutaneous SSc, possess antibodies to Topo I (7). These antibodies can be identified in indirect immunofluorescence (IF) by a homogeneous pattern of the nucleus, with nucleoli excluded from labeling (3). Counterimmunoelectrophoresis (CIE) and immunodiffusion can be used to detect anti–Topo I antibodies with high sensitivity and specificity (3). A number of investigators have reported association of anti–Topo I antibodies with the evolution of SSc toward pulmonary interstitial fibrosis (PIF), the major common lethal complication of the

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Abbreviations: anticentromere antibodies, ACA; counterimmunoelectrophoresis, CIE; enzyme-linked immunosorbent assay, ELISA; immunoblot, IB; immunofluorescence, IF; immunoglobulin, Ig; phosphate-buffered saline, PBS; pulmonary interstitial fibrosis, PIF; sodium dodecyl sulfate, SDS; systemic sclerosis, SSc; DNA type-1 topoisomerase, Topo I.

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disease. However, the strength of the association has varied. Because of the importance of Topo I antibodies in the diagnosis and prognosis of SSc, there is great interest in delineating the important antigenic determinants of the Topo I molecule and refining the prognostic implications.

On the basis of the sequence of the Topo I gene (5), the amino acid sequence of Topo I has been described and Topo I molecular fragments have been prepared by genetic engineering. On the basis of the reaction of anti–Topo I positive sera with Topo I, several autoimmune B-cell epitopes of Topo I have been recognized by previous investigations (8–13). Some of these reports describe distinct epitopes spanning the entire length of the molecule of Topo I, whereas others describe distinct epitopes close to the C-terminal region. Controversies exist from one report to the other on the exact length and location of the described epitopes. The way these epitopes were prepared to be used as antigenic sources, as well as the techniques used for antibody detection (either enzyme-linked immunosorbent assay [ELISA] or immunoblotting) may have influenced the results. Further, the clinical importance of most of the previously identified epitopes is unknown. Finally, all of the previous reports targeted large antigenic fragments.

In the present report ELISAs using as antigens 63 20-mer peptides, overlapping by eight residues and spanning the entire length of the Topo I molecule, were prepared to identify with fine detail the major epitopes of the Topo I antigen. The identified major epitopes were subsequently evaluated on their ability to predict the clinical evolution of SSc toward PIF.

Materials and Methods

Sera

A panel of 14 sera from patients with SSc were tested for the epitope mapping of Topo I. Ten sera were anti–Topo I–positive by CIE and immunoblot (IB) and the remaining four were positive by IF and IB for anticentromere antibodies (ACA). Four sera from normal individuals and one anti-U3RNP serum were also tested. Immunoglobulin (Ig)G from all sera, purified by affinity chromatography on a protein A–Sepharose column, were dialysed against phosphate-buffered saline (PBS) at pH 7.3 and concentrations of IgG were evaluated by absorbance at 280 nm.

The sensitivity and specificity of the soluble peptide epitopes, for the detection of anti–Topo I antibodies, were tested using 66 anti–Topo I sera positive by IB and/or CIE and 43 anti–Topo I–negative sera from patients with SSc. We also tested for epitope-specific antibodies in patient groups known to have very low prevalence of Topo I antibodies by standard (CIE/IB) methods. This included 23 sera from SSc patients with ACA (positive by IB and/or IF); 18 anti-Sm/URNP sera (positive by CIE confirmed by IB and/or IF) from non-SSc patients with systemic lupus erythematosus (SLE) or mixed connective tissue disease; 26 anti-Ro/La sera (positive by CIE and IB) from patients with Sjögrens' syndrome or SLE; and 30 normal antinuclear antibody (ANA)–negative sera.

Peptide Synthesis

Pin-bound peptides. A total of 63 sequential 20-mer peptides, overlapping by eight residues and covering the entire sequence of Topo I (5), were prepared in duplicate according to the multipin peptide synthesis of Geysen and colleagues (14) on prederivatized polyethylene pins (Cambridge Research Biochemicals, Cambridge, UK). The applied protocol is based on the principles of the solid-phase peptide synthesis (15), using the N^{α}-fluorenylmethoxycarbonyl protecting group strategy.

In each synthesis cycle, we used as positive control peptide the sequence VRLRWNPADYGGIKKIRL (63–80) of the α subunit of the acetylcholine receptor (AChR) recognized by an anti-AChR monoclonal antibody, while as negative control peptide we synthesized the sequence VRLRWAPAAYGGIKKIRL (16).

Soluble peptides. Soluble peptides corresponding to the selected epitope I, II, III, and IV (as defined in RESULTS) were synthesized on an automated synthesizer using a Pam resin and the N^{α}-Boc-/benzyl side-chain protection based on solid-phase peptide synthesis (17–19). Amino acid couplings were performed by the 2-(1H-benzotriazole-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate/hydroxybenzotriazole (TBTU/HOBt) procedure using a ratio of amino acid/TBTU/HOBt/resin (3:3:3:1). Deprotection of the N^{α}-*t*-Boc protecting group was performed using trifluoroacetic acid (TFA) followed by *in situ* neutralization by adding excess of diisopropylethylamine in the coupling step.

After completion of the synthesis, the peptides were cleaved from the resin with anhydrous hydrogen fluoride in the presence of anisole and dimethylsulfide as scavengers. The peptides were extracted from the resin using 2 M acetic acid and after lyophilization were subjected to preparative high-performance liquid chromatography (HPLC) on a reverse-phase C_{18} column using programmed gradient elution with the following solvents: (*1*) H_2O/0.1% TFA, and (*2*) CH_3CN/0.1% TFA. The purity of the peptides was confirmed by analytical HPLC, amino acid analysis, and nuclear magnetic resonance spectroscopy.

IB

Nuclear Hela cell extract was prepared as described by Choi and Dreyfuss (20). Briefly, 2.5×10^7 cells/ml were suspended in buffer A (10 mM Tris-HCl, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Tritox X-100, and 2 μg/ml pepstatin, leupeptin, and aprotinin). After homogenization with a type-S pestle and centrifugation at 4,000 rpm for 10 min at 4°C, the nuclei were separated, resuspended in buffer A, and then sonicated on ice twice for 5 s, while the temperature was maintained below 4°C. The sonicate was then layered on a 30% sucrose cushion and centrifuged at 5,000 rpm for 15 min to remove chromatin and nucleoli. The supernatant was defined as nuclear extract and used for protein analysis. SDS 10% polyacrylamide gel electrophoresis and electrophoretic transfer to nitrocellulose paper were subsequently performed as previously reported (21, 22). Sera were used in dilution 1:50 peroxidase-labeled goat antihuman IgG and chlorophenol substrate for the visualization of antigen–antibody reaction.

ELISA

Pin-bound peptides. Peptides coupled to polyethylene pins were tested for antibody binding by ELISA in 96-well polystyrene microtiter plates. The pins were immersed in

PBS, pH 7.3, containing 0.1% Tween 20 and 2% bovine serum albumin (BSA) (Fraction V) for 1 h at room temperature to block nonspecific binding. IgG diluted with PBS (100 μg/ml) was added to the wells and incubated overnight at 4ºC. After washing (four times) with PBS containing 0.5% Tween 20 for 10 min, each pin was incubated with antihuman IgG (Fc-specific) conjugated to horseradish peroxidase (1:2,500 dilution in the blocking buffer) for 1 h at room temperature. The pins were then washed four times, as previously, and the presence of antibodies was detected using as substrate 22′ azino-cis-3-ethylbenzothiazoline sulfonic acid. The absorbance of the produced color was measured at 405 nm.

After completion of the assay, pins were sonicated for 15 min in a water bath with 0.1 M sodium dihydrogen phosphate, 1% SDS, and 0.1% 2-mercaptoethanol at 60°C to remove antibodies. The pins were subsequently washed twice in hot water (60°C) and immersed in methanol (60°C) for 2 min. Pins were allowed to air-dry for a minimun of 20 min and were ready to be used for another assay.

Soluble peptides. Polystyrene 96-well plates were coated with 20 μg/ml (epitope I), 5 μg/ml (epitope II), 20 μg/ml (epitope III), and 10 μg/ml (epitope IV) either in carbonate buffer (pH 9.6; epitopes I and III) or in PBS (pH 7.3) and kept overnight at 4°C. After washing with PBS buffer, a total of 100 μl/well of blocking buffer (3% BSA in PBS, pH 7.3) was added and the plates were incubated for 1 h at room temperature. After two washings with PBS, sera were added in a 1/10 dilution for epitopes I and III, and 1/25 for epitopes II and IV (50 μl/well in duplicate) using PBS-BSA (2%)–Tween 20 (0.05%). After 1 h incubation at room temperature and five washings with PBS, antihuman IgG peroxidase conjugate (1/1,000 dilution, 50 μl/well) was added and kept at room temperature for 1 h followed by five washings with PBS. The substrate solution (50 μl/well of *o*-phenyl diamine 0.5 mg/ml in citric buffer, pH 4) was then added and the absorbance of the produced color was measured at 490 nm (epitopes I and III).

In the case of epitopes II and IV, the antibodies bound to the peptides were detected using alkaline phosphatase–conjugated antihuman IgG (1/2,500 dilution, 50 μl/well) (45 min at room temperature) and *p*-nitrophenyl phosphate disodium substrate (50 μl/well, 1 mg/ml in diethanolamine buffer, pH 9.8).

Inhibition Assays

Competitive inhibition assays (homologous and heterologous) were performed by preincubation of strongly positive sera with anti–Topo I antibodies as detected by reference techniques, at 1/25 dilution in PBS with the inhibiting soluble epitope over a range of concentrations from 0.1 to 320 μg/ml. After preincubation (2 h at 37°C, 1 h at room temperature, and then overnight at 4°C), the sera were tested for their activity against the coated epitope on microtiter plates as described previously.

Computer Predictions and Homology Search

Prediction of hydrophilicity (23) and B-cell antigenic profiles (24) using the sequence of peptides 18,19 and 34,35 was made by the EPIPLOT program (25). The antigenic peptide sequences were compared against the SWISS-PROT database using Fasta (26) and Smith–Waterman (27) algorithms. The nonidentical amino acids were scored with PAM 100 matrix (28) and the gap inclusions were allowed in Smith–Waterman searching.

Evaluation of Clinical Predictive Ability

Patient population and definitions. A total of 81 consecutive patients, each with a diagnosis of SSc and with detailed data on the clinical course of the disease, were included in the analysis. All the patients were followed up in the Department of Pathophysiology, Medical School, National University of Athens by regular visits every three months in our outpatient clinic. A patient chart was completed with clinical and laboratory information at every visit. Unscheduled patient visits due to medical emergencies were also recorded. Spirometric evaluation even in the absence of pulmonary symptoms was performed every 3 mo. SSc was diagnosed on the basis of standard criteria (29). A strict definition was used for the diagnosis of PIF: (*1*) forced vital capacity and forced expiratory volume in 1 s less than 70% predicted, (*2*) bilateral fibrosis confirmed by chest computed tomography-scan, and (*3*) exclusion of other known causes of alveolar wall fibrosis. Patients with sera responding against each epitope were compared against patients without epitope-specific antibodies in terms of the risk of developing PIF over time. A similar comparison was made between patients with antibodies to at least three of the four epitopes versus patients with antibodies directed against no, one, or two epitopes only. The predictive ability of isolated epitopes and combined responses was compared against the predictive ability of a positive standard CIE or CIE and IB test for antibodies to Topo I in the same patients. For the latter analysis, sera positive by CIE and negative by IB were considered negative, while sera negative by CIE and positive by IB were considered positive.

Statistical Analysis

Kaplan–Meier analyses were performed and comparisons were made with the log-rank test (30). Cox proportional hazards models (31) were also used to estimate hazard ratios for the rate of progression to PIF between the compared groups. In the main analysis, time started at the first visit of patients with SSc in our clinic. Separate analyses were performed including or excluding patients who already met the criteria for PIF at the onset of follow-up in our clinic and the results were similar. Further sensitivity analyses used time starting at the time of reported onset of SSc symptoms. All *P* values are two-tailed. Analyses were conducted in Advanced SPSS (SPSS, Inc., Chicago, IL).

Results

Identification of the Topo I B-Cell Antigenic Determinants (Epitopes)

Sixty-three 20-mer peptides, overlapping by eight residues, covering the entire sequence of Topo I, as determined by D'Arpa and associates (5), were synthesized in duplicate. All pin-bound peptides were tested against purified IgG from anti–Topo I–positive sera of scleroderma patients.

Ten out of 10 scleroderma IgG recognized peptides

spanning the sequences 205–224 (peptide number 18), 217–236 (peptide 19), 349–368 (peptide 30), 397–416 (peptide 34), 409–428 (peptide 35), and 517–536 (peptide 44). Peptides sharing the sequences 445–464 (peptide 38), 457–476 (peptide 39), and 721–740 (peptide 61) interacted with six out of 10 anti–Topo I–positive sera. Normal sera did not recognize any of the peptides tested. Further, ACA, associated with limited scleroderma, gave absorbance values comparable with those of normal sera; whereas the anti-U3RNP ELISA profile was very similar to the anti–Topo I ELISA but with considerably lower absorbance values (Figure 1). The antibody-binding pattern for all the peptides tested by ELISA against anti–Topo I and normal sera is illustrated in Figure 1. Significant reactivity (optical density [OD] values $\sim$ 1.3) of all scleroderma-purified IgG was observed against the peptides number 18, 19, 30, 34, 35, and 44, whereas peptides number 38, 39, and 61 were recognized to a lower extent in terms of positive sera prevalence reactivity (OD values $\sim$ 0.6).

Hydrophilicity and B-cell antigenic profile using the primary sequence of peptides numbers 18 and 19, as well as peptides number 34 and 35, gave higher predictive values for peptides number 18 and 34. Taking into consideration the epitope mapping and the predictive results, the following peptide-epitopes in their soluble forms were synthesized and used in ELISA experiments: WWEEERYPEGIKWKFLEHKG (205–224) (epitope I, peptide number 18), RIANFKIEPPGLFRGRGNHP (349–368) (epitope II, peptide number 30), PGHKWKEVRHDNKVTWLVSW (397–416) (epitope III, peptide number 34), and ELDGQEYVVEFDFLGKDSIR (517–536) (epitope IV, peptide number 44) (Figure 1).

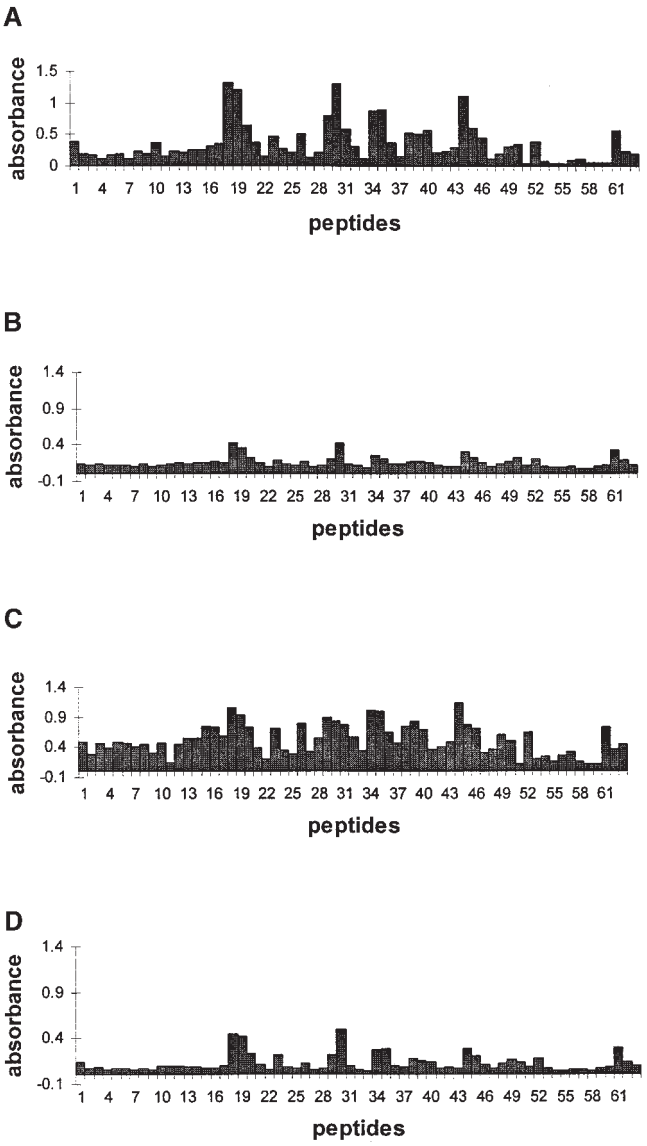

Figure 1. Epitope mapping of Topo I. (*A*) Binding of the 63 20-mers to anti–Topo I–positive sera. (*B*) Binding of 20-mers to normal sera. (*C*) Binding of 20-mers to anti-U3RNP sera. (*D*) Binding of 20-mers to ACA-positive sera.

Sensitivity and Specificity of Anti–Topo I Antibodies Using the Soluble Topo I Epitopes (I–IV) as Antigenic Substrates

Table 1 shows the sensitivity and specificity of antibodies against specific epitopes as compared against the standard methods for determining anti–Topo I antibodies (CIE and/or IB). Sensitivity varied from 57 to78% and was lowest for epitope I. All epitopes showed very high specificity (88 to 98%). Among patients with SSc who were tested for anti–Topo I by both CIE and IB and were found to be negative by both methods, we observed no cases with positive responses to any of the four epitopes. Table 1 also shows the prevalence of positive responses to each epitope in groups of patients who typically do not have anti–Topo I antibodies detected in their sera. As shown, the rates of positive responses to each of the four epitopes were very low. None of the 30 normal, ANA-negative control subjects had antibodies against any of the four epitopes (Figure 2).

Inhibition Assays

Competitive inhibition assays were performed to determine the binding specificity of anti–Topo I antibodies to the Topo I epitopes. Figure 3 illustrates the results of the competitive homologous inhibition, calculated in the standard fashion (32), using anti–Topo I–positive sera preincubated with the same epitope of the applied anti-epitope ELISA. Binding of anti–Topo I–positive sera was inhibited up to 66 and 60% after preincubation of the serum with 320 μg/ml of the epitopes II and I, respectively. The percentage of homologous inhibition when we used epitopes III and IV at the same concentration was approximately 42%. Heterologous inhibition experiments after preincubating the sera with each epitope and testing with the remaining epitopes ranged from 20 to 40%.

Homology Search and Tertiary Structure of the Epitopes

All the defined epitopes of Topo I are highly conserved in mammals and share sequence similarities with other proteins, some of them deriving from infectious agents (Table 2).

Data obtained from the crystal structure of Topo I (33) suggest that epitopes II to IV contain highly exposed residues, whereas epitope I is less accessible (Figure 4) and lies in the vicinity of epitope III. A more detailed structural analysis of the crystallographic results points out that epitope I

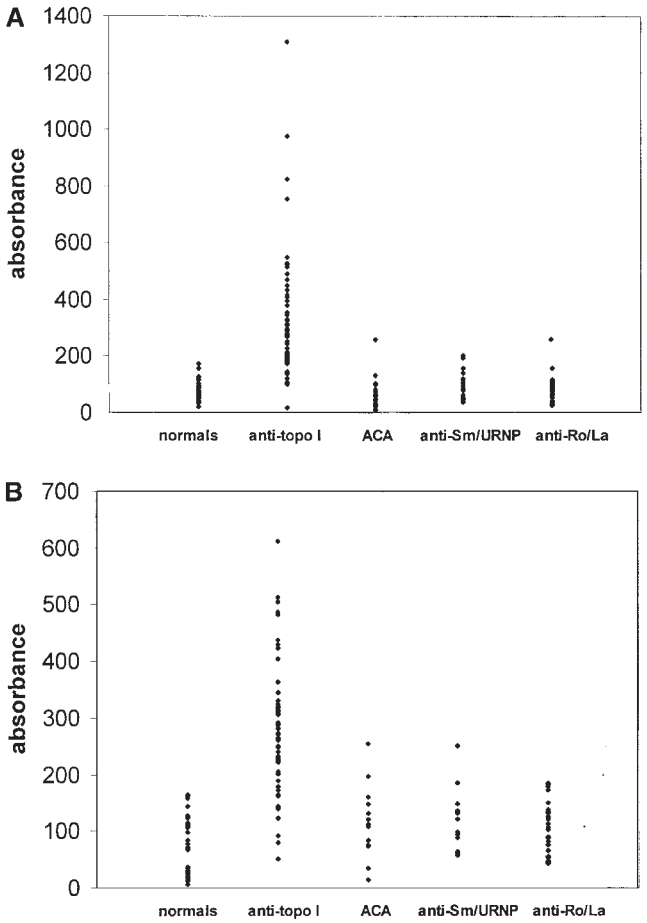

Figure 2. Reactivity of sera with various autoantibody specificities and normal human sera in the anti–epitope II and III ELISA.

incorporates one β-turn (217–220), whereas epitope II includes a β-turn (364–367) and part of a second turn (367–370). Epitope III is stabilized by one β-turn (401–404) and a folded structure (408–413), whereas sequences 404–408 and 413–419 with β-strands form β-sheets with 383–387 and 423–429, respectively. Epitope IV, with two reverse turns (517–520 and 518–521), adopts a helix-like structure with two consecutive amide hydrogen bonds at position 517–521. This epitope is further stabilized by one turn (532–535) and a β-strand forming a β-sheet with the sequence 535–544.

Prediction of Interstitial Pulmonary Fibrosis

Of the 81 patients (11 male, 70 female) with a known clinical evolution of SSc and with determined antiepitope responses, 41 met the criteria for PIF at some point and 24 had already developed PIF before their first visit to our clinic. The mean follow-up for the remaining 57 patients was 41 mo (median 27 mo, interquartile range 10 to 57 mo). The mean time from the onset of symptoms among the 81 patients was 118 mo (median 99 mo, interquartile range 49 to 156 mo). The mean and median age at the onset of symptoms was 38 yr (interquartile range 26 to 47 yr).

As shown in Kaplan-Meier analyses (Figure 5), the presence of antibody response to at least three of the four epitopes was strongly associated with the risk of developing PIF over time after the first visit (log-rank $P = 0.015$, Figure 5A), whereas the discrimination offered by the simple presence of a positive CIE or IB for anti–Topo I was much weaker (log-rank $P = 0.30$; Figure 5B). The presence of a response to at least three epitopes was also a very strong predictor of PIF when the time from the onset of PIF symptoms was considered (log-rank $P = 0.038$), whereas anti–Topo I response carried little predictive value (log-rank $P = 0.4$).

In Cox regression, patients with an antibody response to at least three of the four epitopes at the time of their first visit had 3.1 times the hazard of being diagnosed with PIF compared with other patients ($P = 0.02$). The magnitude and the statistical significance of the effect remained unchanged (hazard ratio 2.8, $P = 0.05$) after adjusting in multivariate regressions for other potential predictors of the risk of PIF, including older age (hazard ratio 1.026/yr, $P = 0.12$), male sex (hazard ratio 1.6, $P = 0.54$), anti-U3RNP response (hazard ratio 0.62, $P = 0.54$), and ACA response (hazard ratio 0.53, $P = 0.56$). None of the other considered predictors were statistically significant in the multivariate model.

A response to each of the four epitopes separately offered a stronger discrimination than the standard anti–Topo I tests. The hazard ratios for isolated positive responses to epitopes I, II, III, and IV were 2.6, 1.8, 2.4, and 1.8, respectively, as compared with a hazard ratio of 1.7 ($P = 0.30$) associated with a positive CIE/IB. The association was strongest for epitope I, but differences were small. In a *post hoc* exploratory analysis, patients with an antibody response to epitope I or, in the absence of such a response, at least a response to all other three epitopes, had 3.7 times the risk of developing PIF compared with other patients ($P = 0.005$).

TABLE 1

Sensitivity and specificity of the anti–Topo I epitope ELISAs in patients with SSc and prevalence of positive responses in patient groups with typically absent anti–Topo I responses*

				Prevalence in Various Anti–Topo I (−) Groups†		
Sequence	Position	Sensitivity	Specificity	ACA	Sm/URNP	Ro/La
WWEEERYPEGIKWKFLEHKG	205–224 (epitope I)	57% (29/51)	94% (30/32)	8% (1/13)	13% (3/25)	11% (6/56)
RIANFKIEPPGLFRGRGNHP	349–368 (epitope II)	71% (47/66)	98% (42/43)	4% (1/23)	6% (1/18)	4% (1/26)
PGHKWKEVRHDNKVTWLVSW	397–416 (epitope III)	78% (45/58)	97% (31/32)	11% (2/18)	8% (1/12)	0% (0/24)
ELDGQEYVVEFDFLGKDSIR	517–536 (epitope IV)	70% (46/66)	88% (38/43)	9% (2/23)	0% (0/18)	19% (5/26)

*Reference standard is CIE and/or IB. In the rare cases where CIE and IB disagreed, the IB reading was considered.
†Patients have not necessarily been tested for anti–Topo I by CIE/IB.

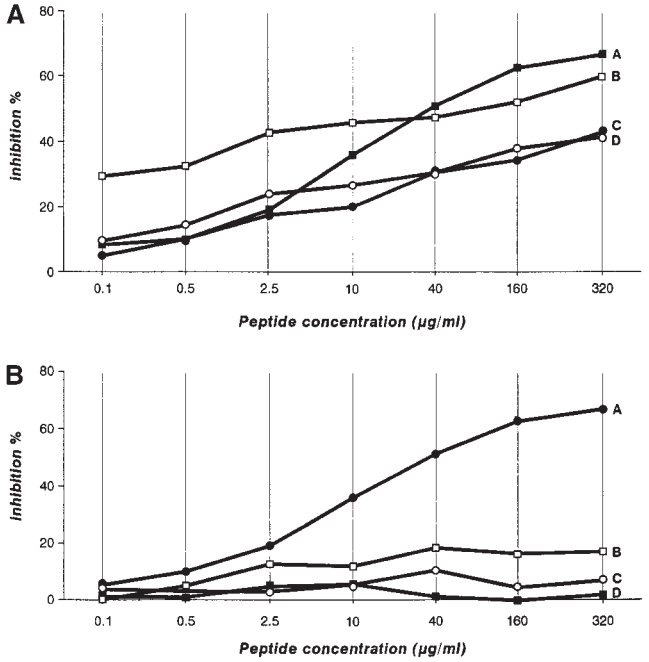

Figure 3. (*A*) Homologous inhibition of anti–Topo I antibody binding to ELISA plates, coated with epitope II (A), epitope I (B), epitope III (C), and epitope IV (D). (*B*) Inhibition of anti–Topo I antibody binding to epitope II by preincubation of sera with epitope II (A), epitope III (B), epitope IV (C), and epitope I (D).

Discussion

Identification of the B-cell epitopes of an autoantigen may provide useful information on the mechanism triggering autoantibody production, as well as on the functional and structural features of the antigenic determinants that are targeted. Further, determination of the major epitopes of an antigen may considerably facilitate immunoassays, inasmuch as synthetic peptides are reliable substrates for autoantibody detection (34). Application of these tests could be useful to define subgroups of a disease and may offer information about disease prognosis.

In the present report 63 20-mer peptides overlapping by eight residues and spanning the entire length of the Topo I molecule were used as antigenic substrates in ELISAs. In a first step, the peptides were synthesized on polyethylene pins and selected sera with anti–Topo I as well as other specificities were tested for their reactivity against the pin-bound peptides. The peptides reacting with the anti–Topo I sera were defined as Topo I B-cell epitopes. Defined B-cell epitopes were then synthesized in their soluble forms and ELISA systems were developed to test large numbers of anti–Topo I–positive sera, as well as sera with other specificities. Using data from a well-defined patient cohort, we found that the profile of antibody response to the selected B-cell epitopes is strongly associated with the risk of developing PIF among patients with SSc. The association was considerably stronger than the association between CIE/IB determination of Topo I antibodies and PIF.

Four major epitopes were identified in our study spanning the sequences 205–224, 349–368, 397–416, and 517–536. These sequences are incorporated in other rather large epitope regions reported in the literature (8, 9, 11). More specifically, the epitopes I and II as detected in the present study exist within the regions aa 70–344 and aa 344–589, where Verhejien and coworkers have identified epitopes (8). In addition, the epitopes II, III, and IV exist in the regions aa 277–484 and aa 485–765, where D'Arpa and associates have identified epitopes (9). Finally, Kuwana and colleagues (11) have identified epitopes in the region aa 74–248, where our epitope I exists; and in the region aa 316–607, where our epitopes II, III, and IV were identified. In a recent study by Kuwana and associates (35) the region aa 512–563 where our epitope IV exists was considered absolutely necessary for the binding of anti–Topo I antibodies to Topo I. In fact, this region was found to be part of rather extended epitope (aa 489–573) that was conformational in nature.

Our study suggests that the four epitopes we identified and the respective ELISAs that we developed may potentially be used for refining predictions of the risk of PIF in

TABLE 2

Sequence similarities between the epitopes of Topo I and other proteins

WWEEERYPEGIKWKFLEHKG	
(205–224) epitope I	Topo I, human
FIKRLRKAEGIKWSFHTRTY	virion infectivity factor, feline virus
GWDAGRYPEKASKDFLKLLS	50S ribosomal protein, *Halobacterium marismortui*
RIANFKIEPPGLFRGRGNHP	
(349–368) epitope II	Topo I, human
RIRDFRGLNPNSFDGRGNYN	50S ribosomal protein, *Thermus aquatiqus*
PGHKWKEVRHDNKVTWLVSW	
(397–416) epitope III	Topo I, human
HIVYWKAVRHENVVLYKARQ	regulatory protein, human papillomavirus 30
LNYLWREERPEVTKKVTWAASL	transcription elongation factor, *Escherichia coli*
FSDLEIVVRHDGNLTWVPNG	genome polyprotein, foot-and-mouth disease virus
RETKNQRVRQSSVAWLVVD	integral membrane protein, varicella-zoster virus
ELDGQEYVVEFDFLGKDSIR	
(517–536) epitope IV	Topo I, human
WRDGEQFVVEFDLPGIDEQS	18 kD antigen 2, *Mycobacterium intracellulare*
MLNGQKEYRVEKDFLGEKQIE	aspartate-ammonia lyase, *Bacillus subtilis*

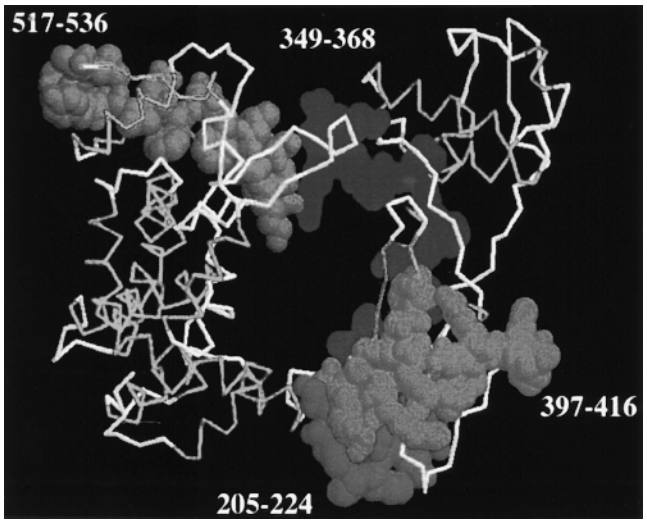

Figure 4. Localization of the epitopes (I–IV), sequences 205–224, 349–368, 397–416, and 517–536 (shown with *spheres*) on the tertiary structure of Topo I, defined by crystallography.

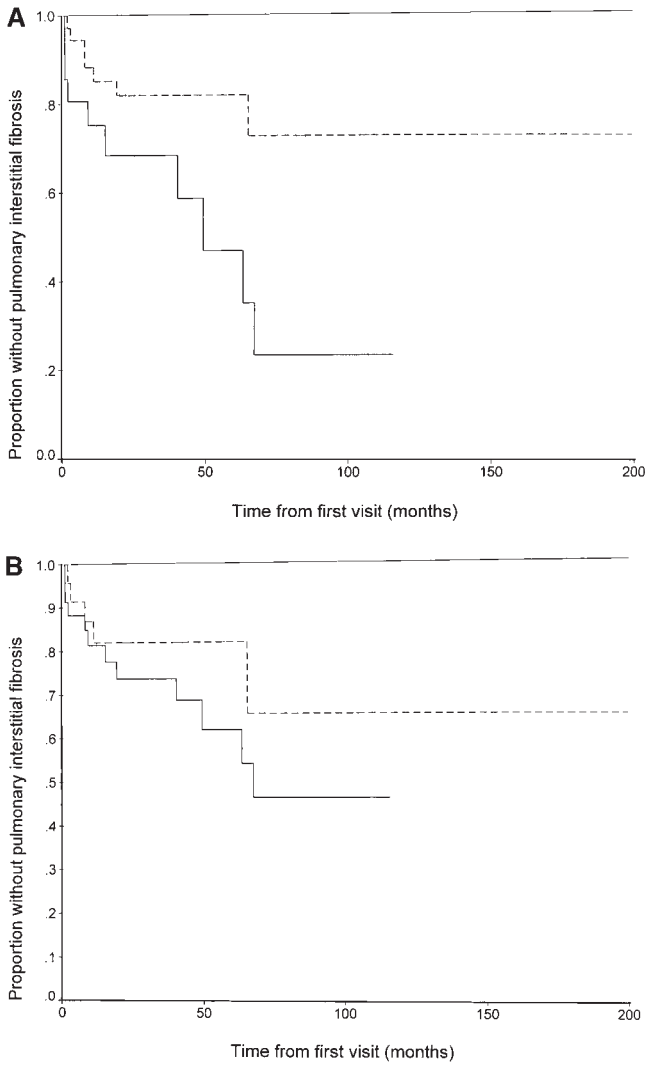

Figure 5. Kaplan–Meier plots for the proportion of patients without PIF as a function of the time after the first visit. Patients with PIF before or at their first visit are excluded, but their inclusion would yield similar results. (*A*) Patients with responses to at least three of the four epitopes (*solid line*) versus all other patients (*dashed line*). (*B*) Patients with a positive IB/CIE for Topo I antibodies (*solid line*) versus other patients (*dashed line*).

patients with SSc. It should be acknowledged that our epitope determinations are one-time measurements. The evolution of the epitope response over time in SSc patients is unknown and would be worthwhile studying with future longitudinal studies. Further, we did not perform lung biopsies in our cohort. However, transbronchial biopsy is typically not performed in scleroderma patients with PIF, and performing a biopsy might have been considered unethical for many of our patients.

The mechanism underlying the association between the presence of autoantibodies to Topo I and the development of PIF in patients with SSc is largely unknown. There is evidence (36, 37) that the production of anti–Topo I antibodies is an antigen-driven, T-cell–dependent process; however, it is not clear whether this is the consequence or the cause of PIF in SSc (38). Predominantly, cytotoxic and not antibody-dependent injury may take place in the lung. Enhancement of anti–Topo I antibody response occurred after lung cancer in patients with SSc (39) and Topo I protein and activity levels are increased in colorectal adenocarcinoma (40) and lung cancer (41, 42). These findings indicate that anti–Topo I antibodies occur after antigen overexpression and/or alteration in the lung which probably follows initial lung injury. The overexpression of Topo I probably needs an additional tissue-specific event to trigger anti–Topo I B-cell responses, such as the formation of complexes of Topo I with other proteins, or exposure to a foreign protein having cross-reactive determinants with Topo I (43, 44). The sequence similarities between the Topo I epitopes with other proteins derived from infectious agents, provided in this work, also support such a possibility.

The magnitude of the association of anti–Topo I antibodies with PIF has varied in different populations (45–47) and some investigators have found no association at all (48). The identification of the four major epitopes defined in this paper may allow a better understanding of such differences. It is likely that the association with PIF may require the presence of a strong response against at least three of the four major epitopes and not simply isolated responses against one or two epitopes. Our results show strong predictive relationships that need to be validated and confirmed in additional cohorts of patients with scleroderma. In addition, further studies evaluating the exact biologic mechanism underlying the predictive ability of the specific epitope responses should be undertaken.

As was suggested by the crystal structure of Topo I, epitopes II, III, and IV are exposed more efficiently than epitope I. Epitopes I and III could be parts of a conformational epitope. Further, some residues of epitope II are in rather close proximity to DNA, when Topo I is wrapping around. Perhaps epitopes II to IV, by being on the exposed surface of Topo I, adopt conformations that are better rec-

ognized by anti–Topo I antibodies than epitope I. This could explain the somewhat lower sensitivity for epitope I. However, the presence of antibodies to epitope I seem to be at least as strong, if not stronger, discriminating factors for PIF. If true, this may reflect other indirect associations with other pathogenetic pathways not directly linked to the presence of autoantibodies, for example, cytotoxic cell responses which may be governed by different epitopes in patients with PIF.

In conclusion, the fine specificity of autoantibodies to Topo I was evaluated. Our results reveal four immunodominant epitopes that belong to some of the previously assigned antigenic regions. The specific immune response to these epitopes seems to be strongly associated with the development of the major common serious complications of SSc. Further studies should help us better understand the molecular basis of these associations.

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