Naturally occurring and disease-associated auto-antibodies against topoisomerase I: a fine epitope mapping study in systemic sclerosis and systemic lupus erythematosus

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Keywords: naturally occurring auto-antibodies, phage display, systemic lupus erythematosus, systemic sclerosis, topoisomerase I

Abstract

Auto-antibodies against topoisomerase I (topo I) are frequently detected in sera of systemic sclerosis (SSc) patients. Anti-topo I auto-antibodies are considered to be associated with the diffuse cutaneous form of systemic sclerosis (dcSSc). However, anti-topo I auto-antibodies are also detected in limited cutaneous systemic sclerosis (lcSSc) and systemic lupus erythematosus (SLE). In this study, we compared the epitope specificity of anti-topo I auto-antibodies present in sera of dcSSc, lcSSc and SLE patients. We have constructed an antigen fragment library displayed on bacteriophage lambda and screened this library with IgG purified from patients’ sera. Regions of topo I selected from the library were expressed as recombinant fusion proteins and were tested with ELISA and western blot. We unexpectedly found that antibodies against a fragment of topo I (fragment F4 [amino acid (AA)] 451–593) could be detected in sera of healthy individuals and patients with inflammatory rheumatic diseases other than SSc and SLE. Using sera of dcSSc, lcSSc and SLE patients, we showed that the pattern of recognized epitopes is different between these patient groups. Fragment F4 was recognized by all patients. Fragment F1 (AA 5–30) was recognized by 9 of 34 dcSSc patients. Fragment F8 (AA 350–400) was recognized by four of eight SLE patients. Analysis of clinical data revealed a significant difference between the F1-negative and F1-positive groups of SSc patients in age and in the duration of the disease. According to our results, the newly identified fragments F1 and F8 could represent characteristic epitopes for dcSSc and SLE, respectively.

Introduction

Systemic sclerosis (SSc) is a systemic autoimmune disorder characterized by immune activation, vascular injury, inflammation, fibrosis of the skin and various internal organs. Activation of the immune system leads to production of disease-specific auto-antibodies, lymphocyte activation and secretion of various cytokines (1). The vast majority of SSc patients has antinuclear antibodies, which predominantly recognize DNA topoisomerase I (topo I), RNA polymerases, centromere proteins and U3RNP (2). Anti-topo I auto-antibodies are considered to be associated with diffuse cutaneous systemic sclerosis (dcSSc), while anti-centromere auto-antibodies are generally detected in limited cutaneous systemic sclerosis (lcSSc) (3, 4). However, the presence of anti-topo I auto-antibodies may not be entirely restricted to dcSSc. A subset of lcSSc patients were found to be positive for anti-topo I auto-antibody (3) and our findings are also similar.

Clinically, patients with SSc could be classified into two distinct subsets. dcSSc is characterized by extensive fibrosis of the skin, lungs and other internal organs, while in lcSSc vascular abnormalities are dominating and fibrosis is limited (5).

In addition to SSc, presence of anti-topo I antibodies has been demonstrated in systemic lupus erythematosus (SLE).
patients showing no clinical signs and symptoms of SSc (6, 7). These findings suggest that the presence of anti-topo I antibodies could have heterogeneous clinical consequences. The role of anti-topo I antibodies in pathogenesis of SSc is not fully understood; however, immune response against topo I may differ among anti-topo I-positive patients leading to production of anti-topo I auto-antibodies with different epitope specificity.

Topo I is a 765 amino acid (AA) long DNA-relaxing enzyme which contains five distinct regions: the N-terminal domain (AA 1–215), core subdomains I–II (AA 216–435), core subdomain III (AA 436–636), the linker domain (637–713) and the C-terminal domain (AA 714–765) (8). Earlier studies using recombinant topo I fragments identified various epitopes in the central and C-terminal part of the molecule (9–14). On the basis of previously published reports, it seems that an immunodominant region of topo I spans AA 489–573 (9, 10, 12, 13). However, a study which used recombinant fusion proteins constructed on the basis of the domain structure of topo I demonstrated that the core subdomains I–II is recognized more frequently than core subdomain III (15). Longitudinal analysis of anti-topo I auto-antibodies revealed that reactivity against these regions is stable (15, 16), though a study using a limited number of sera showed that the regions recognized by anti-topo I auto-antibodies vary over time (17). While epitope specificity of anti-topo I auto-antibodies has been studied by a number of groups, there is no report of comparative epitope mapping in patients with dcSSc, lcSSc and SLE. In this study, we have constructed an antigen fragment library of topo I displayed on bacteriophage lambda and screened this library with sera of dcSSc, lcSSc and SLE patients. Regions of topo I selected from the library were expressed as recombinant fusion proteins and were further tested with patients’ sera. Longitudinal analysis of epitope specificities has been performed and compared with clinical findings.

Materials and methods

Patients and controls

From the 293 patients in our total scleroderma cohort, 59 SSc cases were selected which showed anti-topoisomerase antibody positivity on a conventional ELISA test (Hycore, Vienna, Austria) (34 of these patients were classified as having dcSSc and 25 were diagnosed lcSSc). Out of 265 SLE patients tested for the presence of anti-topo I antibodies with a conventional ELISA kit, 8 showed positive reaction and were selected for the present study. One hundred and seventy-seven serum samples from 59 SSc patients (three from each patient) and 24 serum samples from 8 SLE patients were obtained between 2004 and 2007 at 6–12 months intervals. The patients’ clinical data were encoded using our standard protocol (18).

For controls, 63 sera from Hungarian blood donors (from the Blood Transfusion Service of Baranya county; average age: 36.8 ± 12.1 years; 32 women and 31 men), 44 sera from Finnish blood donors, 44 sera from British blood donors (by the courtesy of Prof. G. Füst and Z. Prohaszka, 3rd Department of Internal Medicine at the Semmelweis University, Budapest) and 65 sera from Hungarian elderly healthy individuals (from the Immunology and Rheumatology Clinic, University of Pécs; average age: 62.4 ± 5.4 years) were used. Furthermore, 110 sera from Hungarian elderly anti-topo I antibody-negative (measured by a conventional ELISA test) patients with inflammatory rheumatic diseases other than SSc and SLE (8 vasculitis, 40 seronegative spondylarthritis, 11 myositis, 11 Sjögren syndrome, 10 psoriatic arthritis, 20 rheumatoid arthritis, 10 polymyalgia rheumatica from the Immunology and Rheumatology Clinic, University of Pécs; average age: 65.5 ± 4.8 years) were also investigated.

The study has been approved by the Ethical Committee of the Medical Center of the University of Pécs. Informed consent has been obtained from all patients and healthy individuals.

Construction and affinity selection of topo I antigen fragment library

The coding region of full-length human topo I was amplified by PCR from cDNA reverse transcribed from total RNA. The PCR product was cloned into a T/A vector using the InstI/Aclone PCR Product Cloning Kit (Fermentas, Vilnius, Lithuania). The identity of insert was verified by sequencing the entire coding region of topo I on a Beckman Coulter CEQ 8800 instrument. Library construction was done using the lambdaD-bio phage display vector (19) with minor modifications as described previously (20). The primary topo I library contained 2 × 10^6 insert bearing independent clones; titer of the amplified library was 3 × 10^11 ml^-1. Affinity selection of topo I antigen fragment library with five dcSSc, six lcSSc and four SLE patient-derived IgG purified on protein G sepharose (Amersham Pharmacia, Uppsala, Sweden) was performed essentially as described (21). After the third round of selection, individual clones were picked up for further propagation and DNA sequencing.

Expression of recombinant topo I fusion proteins

Selected fragments of topo I were expressed as recombinant maltose-binding protein (MBP) fusion proteins using the pMAL Protein Fusion and Purification system (New England Biolabs, Ipswich, UK). cDNAs coding for AA 5–30 (F1), 69–92 (F2), 87–145 (F3), 450–600 (F4), 640–705 (F5), 170–290 (F6), 295–350 (F7), 350–400 (F8) and 295–400 (F9) were amplified with PCR primers containing EcoRI and BamHI restriction sites and cloned into the pMal-c2 vector. The reading frame and sequence of inserts were verified by sequencing. Fusion proteins were expressed in Escherichia coli TB1 and were purified from bacterial lysates with affinity chromatography on amylose resin according to the manufacturer’s instruction (New England Biolabs). Integrity of purified proteins was verified by SDS–PAGE on a 10% gel followed by Coomassie brilliant blue staining.

ELISA

The 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with recombinant topo I fragments or with MBP in PBS at a concentration of 10 μg ml^-1. Plates were washed with wash buffer (PBS, 0.05% Tween 20) and blocked with 3% BSA in wash buffer for 1 h. Serum samples were incubated...
in triplicates at 1:250 dilutions in wash buffer containing 2% BSA for 1 h. Finally, the plate was incubated with HRP-conjugated anti-human-IgG secondary antibody (Dako, Glostrup, Denmark) for 60 min. The reaction was developed with o-phenylenediamine (Sigma–Aldrich, Budapest, Hungary), and optical density (OD) was measured at 492 nm.

**Immunoblots**

Purified MBP fusion proteins or MBP (40 µg ml⁻¹) diluted 1:1 with SDS sample buffer were boiled for 10 min, separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk (Bio-Rad, Budapest, Hungary) in wash buffer (100 mM NaCl, 10 mM Tris-base pH 7.4 and 0.1% Tween 20) for 1 h, membranes were incubated for 1 h with sera diluted 1:500 in 2% non-fat dry milk in wash buffer. After washing, HRP-conjugated anti-human-IgG diluted at 1:2000 was added for 1 h. For detection of MBP fusion proteins, membrane strips were first incubated with rabbit anti-maltose-binding protein antibody (New England Biolabs) (1:5000), followed by incubation with HRP-conjugated goat anti-rabbit antibody (1:2000). Membranes were developed with SuperSignal West Pico Chemiluminescent (Pierce, Rockford, IL, USA) substrate and exposed to X-ray films.

**Statistical analysis**

Categorical data were analyzed by the chi-square test. To investigate the possible differences between patient groups, frequency and mean values of continuous variables were tested by Student’s t test. Spearman’s rank correlation coefficient was used to examine the relationship between the values of OD and continuous variables. A P value <0.05 was considered statistically significant. Statistical analyses were conducted using SPSS statistical software package.

**Results**

**Epitope mapping of anti-topo I antibodies with phage displayed topo I library**

For identification of epitopes recognized by anti-topo I antibodies, we have constructed a topo I antigen fragment library displayed on bacteriophage lambda and subsequently screened this library with individual IgGs purified from sera of anti-topo I-positive patients (five dcSSc, six lcSSc and four SLE patients). After the third round of affinity selection, inserts of 60 clones (30 from each patient group) were sequenced. Alignment of deduced AA sequences with human topo I showed that the pattern of recognized epitopes is different between dcSSc, lcSSc and SLE patients (Fig. 1). Among phage clones selected with IgG of dcSSc patients, 11 different clones were found repeatedly; eight partially overlapping phage clones displaying fragments covering AA 5–145 of the N-terminal domain, two overlapping clones covering AA 451–593 of core subdomain III and a single clone representing AA 640–705 of the linker domain. Selection with IgG of lcSSc patients resulted in six distinct phage clones: two clones covering AA 40–61 and 176–184 of the N-terminal domain, a single clone displaying AA 205–321 of core subdomain I–II, a clone representing AA 399–445 of the core subdomain I–II—core subdomain III junction, a single clone with AA 491–571 of core subdomain III. The other fragments (F1–F3, F5–F9) were contained antibodies which recognized fragment F4 (AA 726–765) of the C-terminal domain. With selection using IgG from SLE patients, six distinct phage clones were obtained: five of these covered AA 296–400 of core subdomains I–II and single clone displayed AA 491–571 of core subdomain III.

On the basis of these results, it seems that sera of dcSSc patients recognize epitopes localized in the N-terminal domain, epitopes recognized by SLE patients’ sera are found in core subdomains I–II, while epitopes recognized by lcSSc patients are scattered throughout the molecule. In addition to this, there is common region of topo I (AA 451–593) recognized by all three groups of sera.

A recombinant fragment of topo I is recognized by naturally occurring auto-antibodies

In order to verify results obtained by affinity selection of the topo I antigen fragment library, we have constructed recombinant topo I-MBP fusion proteins. On the basis of fragments identified by library selection, nine fusion proteins have been constructed and expressed (Fig. 2). To check the possible background reactivity of normal sera and to set up cut-off values for further measurements, recognition of these fusion proteins was first tested with sera of healthy individuals which were previously shown to be negative for anti-topo I antibody by a commercial ELISA test. To our surprise, 44% of sera derived from Hungarian blood donors contained antibodies which recognized fragment F4 (AA 450–600) of topo I. The other fragments (F1–F3, F5–F9) were not recognized by sera of healthy individuals. To further investigate this finding, we systematically examined 63 sera from Hungarian blood donors (average age: 36.8 ± 12.1 years; 32 women and 31 men), 44 sera from Finnish blood donors, 44 sera from British blood donors, 65 sera from Hungarian elderly healthy controls (average age: 62.4 ± 5.4 years), 110 sera from Hungarian elderly patients with inflammatory rheumatic diseases other than SSc and SLE patients resulted in six distinct phage clones: two clones covering AA 40–61 and 176–184 of the N-terminal domain, a single clone displaying AA 205–321 of core subdomain I–II, a clone representing AA 399–445 of the core subdomain I–II—core subdomain III junction, a single clone with AA 491–571 of core subdomain III. The other fragments (F1–F3, F5–F9) were contained antibodies which recognized fragment F4 (AA 726–765) of the C-terminal domain. With selection using IgG from SLE patients, six distinct phage clones were obtained: five of these covered AA 296–400 of core subdomains I–II and single clone displayed AA 491–571 of core subdomain III.

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of positive sera. Numbers in parentheses following the percentage values indicate average OD.

<table>
<thead>
<tr>
<th>Group</th>
<th>Anti-F4 IgM</th>
<th>Anti-F4 IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hungarian elderly systemic autoimmune patients</td>
<td>37% (0.254 ± 0.057)</td>
<td>44% (0.189 ± 0.066)</td>
</tr>
<tr>
<td>Finnish blood donors (44)</td>
<td>52% (0.343 ± 0.068)</td>
<td>9% (0.350 ± 0.076)</td>
</tr>
<tr>
<td>British blood donors (44)</td>
<td>76% (0.265 ± 0.047)</td>
<td>25% (0.190 ± 0.043)</td>
</tr>
<tr>
<td>Hungarian elderly healthy controls (65)</td>
<td>17% (0.205 ± 0.065)</td>
<td>51% (0.359 ± 0.083)</td>
</tr>
<tr>
<td>Hungarian elderly systemic autoimmune patients (110)</td>
<td>71% (0.456 ± 0.092)</td>
<td>31% (0.217 ± 0.052)</td>
</tr>
<tr>
<td>Anti-topo I-positive SSc and SLE patients (67)</td>
<td>53% (0.726 ± 0.052)</td>
<td>100% (0.735 ± 0.212)</td>
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Sera were tested for anti-F4 reactivity with isotype-specific indirect ELISA. The cut-off value for positivity was set at OD450 0.100. Values in parentheses following description of the groups indicate the number of individual serum samples tested. Percentage values indicate percentage of positive sera. Numbers in parentheses following the percentage values indicate average OD.

Recognition of recombinant topo I fusion proteins by SSc and SLE patients’ sera

Next, we tested recognition of all nine fusion proteins with sera of 67 anti-topo I antibody-positive patients (34 dcSSc, 25 lcSSc and 8 SLE) by IgG isotype-specific ELISA. The results are summarized in Table 2.

Fragment F4 (AA 450–600) was recognized by all the 67 patients’ sera. Fragment F1 (AA 5–30) was recognized by 9 of 34, 1 of 25 and 0 of 8 dcSSc, lcSSc and SLE patients, respectively. Fragment F8 (AA 350–400) was recognized by four of eight SLE patients and none of the SSc patients.

Longitudinal analysis of topo I epitope reactivity

To determine whether antibody responses against fragments F1, F4 and F8 remain constant over time, we measured antibody reactivity against these fragments by ELISA in three serial serum samples of each patient. Results of ELISA were confirmed by western blot in 10 F1-positive patients, 10 randomly chosen F4-positive patients and 4 F8-positive patients. Each serum sample was also examined by conventional anti-Scl-70 ELISA.

Longitudinal analysis showed that reactivity to fragment F4 was stable in 61 cases (94%). Eighteen out of the 67 patients had at least one serum sample with no antibody response against topo I measured by the conventional anti-Scl-70 ELISA. Results of ELISA with fragment F4 were confirmed by western blot, which showed that each sera positive for F4 reactivity in ELISA was also positive in western blot (Fig. 3A).

The reactivity to F1 fragment varied over time. In four cases, the earliest serum samples did not have any detectable antibodies against F1, but the immunoreaction became positive and stronger over time. In one case, reactivity against F1 appeared in the second sample but was absent in the following one. All serum samples of the remaining four dcSSc patients were positive for anti-F1 antibody. Results of ELISA were confirmed by western blot, which showed a perfect correlation of results obtained by the two methods (Fig. 3B).

Among the four SLE patients positive for antibody against F8, the reactivity was stable in one patient and changed in the remaining three patients. Fragment F8 was not...
recognized in western blot, indicating that the epitopes displayed by this fragment are conformational in nature.

Clinical findings
Statistical analyses of clinical data (extent of skin involvement, hand contractures, azotemia and/or malignant hypertension, cardiac involvement, dysmotility and stricture/dilatation of esophagus, extent of lung fibrosis and forced vital capacity) showed no association between anti-topo I antibody epitope specificity and clinical presentation of SSc. However, there was a significant difference between the F1-negative and F1-positive groups of SSc patients in average age [F1 negative (number of patients: 49): 54.8 ± 13.5 years; F1 positive (number of patients: 10): 63.9 ± 9.4 years; \( P = 0.048 \)] and the duration of the disease [F1 negative (number of patients: 49): 10.0 ± 7.3 years; F1 positive (number of patients: 10): 17.1 ± 12.9 years; \( P = 0.019 \)]. Moreover, average age of dcSSc patients possessing sera positive for F1 fragment was significantly higher compared with the patients showing no detectable levels of antibody against F1 [F1 negative (number of patients: 25): 51.9 ± 14.3 years; F1 positive (number of patients: 9): 63.7 ± 10.0 years; \( P = 0.03 \)].

Table 2. Recognition frequencies of recombinant topo I fragments determined by ELISA using anti-topo I antibody-positive patients’ sera

<table>
<thead>
<tr>
<th>Topo I fragments (AAs)</th>
<th>dcSSc (n = 34)</th>
<th>lcSSc (n = 25)</th>
<th>SLE (n = 8)</th>
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<tbody>
<tr>
<td>F1 (5–30)</td>
<td>9 (26%)</td>
<td>1 (4%)</td>
<td>0</td>
</tr>
<tr>
<td>F2 (69–92)</td>
<td>1 (3%)</td>
<td>1 (4%)</td>
<td>0</td>
</tr>
<tr>
<td>F3 (87–145)</td>
<td>1 (3%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F4 (450–600)</td>
<td>34 (100%)</td>
<td>25 (100%)</td>
<td>8 (100%)</td>
</tr>
<tr>
<td>F5 (640–705)</td>
<td>2 (6%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F6 (170–290)</td>
<td>2 (6%)</td>
<td>2 (8%)</td>
<td>0</td>
</tr>
<tr>
<td>F7 (295–400)</td>
<td>0</td>
<td>0</td>
<td>1 (12%)</td>
</tr>
<tr>
<td>F8 (350–400)</td>
<td>0</td>
<td>0</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>F9 (295–350)</td>
<td>0</td>
<td>0</td>
<td>1 (12%)</td>
</tr>
</tbody>
</table>

Numbers indicate individual patients positive for the given fragments, numbers in parentheses indicate percentage of positive sera.

To investigate whether antibodies against peptide F1 were specific for SSc patients and the appearance of them is not merely a consequence of aging, sera from 65 age-matched (average age: 62.4 ± 5.4 years) healthy controls were tested for antibodies against F1. Only one serum sample was found positive. To test whether the presence of antibodies against fragment F1 is specific for SSc, sera from 110 age-matched (average age: 65.5 ± 4.8 years) patients with different connective tissue diseases were also tested and only four serum samples (two seronegative spondyloarthritides, one myositis, one Sjögren syndrome) were found positive.

![Fig. 3.](image)

**Fig. 3.** Immunoblots using topo I fusion proteins F4 and F1 as antigens. Purified recombinant fusion proteins (panel A: F4, panel B: F1) or MBP were separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were cut into strips and probed with serial serum samples (obtained at dates indicated) of patients or with an anti-maltose-binding protein antibody. MW, molecular weight marker (kDa).
Comparison of clinical data of the 4 F8-positive and the 261 F8-negative SLE patients suggested that SLE patients with antibody against fragment F8 have Raynaud's phenomenon and a milder presentation of the disease (lack of arthritis, central nervous system and kidney involvement).

Discussion
In this study, we performed comparative epitope mapping of anti-topo I auto-antibodies present in sera of SSc and SLE patients. Our data clearly demonstrated that the pattern of recognized epitopes is different between dcSSc, lcSSc and SLE patients. In addition to an immunodominant part of topo I (fragment F4), we identified two new regions which were previously not shown to be targeted by anti-topo I antibodies. Fragment F1 was specifically recognized by a subset of dcSSc patients' sera, while fragment F8 was recognized by SLE patients. In addition, we demonstrated for the first time that the presence of antibodies with both IgM and IgG isotype against an immunodominant fragment of topo I (fragment F4) is not restricted to SSc, but could be detected in healthy individuals and in sera of patients with inflammatory rheumatic diseases other than SSc and SLE.

A number of research groups have studied the epitope specificity of anti-topo I antibodies in SSc patients. However, to date there is no report of epitope mapping of anti-topo I antibodies in SLE patients. Using molecular biology strategies, several studies have determined that anti-topo I antibodies of SSc patients recognize multiple epitopes on topo I, although the recombinant topo I fragments used varied in these reports. Verheijen et al. (10) verified three different epitope regions which are distributed over the entire protein. D'Arpa et al. (9) expressed six fragments of topo I and demonstrated that most of anti-topo I-positive sera recognize multiple epitopes. Kuwana et al. (13) found four epitope regions on topo I and reported that 86% of 43 anti-topo I-positive SSc sera reacted with a fusion protein containing AA 485–601 of topo I. Piccinini et al. (11) reported that AA 405–484 of topo I was recognized by all the anti-topo I-positive sera they examined but detected no epitopes between AA 485–765 which could be due to the small number of serum samples examined. Epitope mapping performed with synthetic peptides identified four major epitopes, three of them in core subdomains I and II (AA 207–441) and one in core subdomain III (AA 433–636) (22). Since each of these studies have implicated topo I fragments located in the region of AA 484–560, these findings strongly suggest that immunodominant B cell epitopes recognized by anti-topo I antibodies could be located within this region.

However, fragments used in these studies were designed either on the basis of topo I domain structure or antigenicity prediction, both of which could miss possible epitopes. With the exception of work performed by Meesters et al. (23), to date there is no report of epitope mapping on topo I with random antigen fragments. This early work used a size-selected random antigen fragment library constructed by limited deoxyribonuclease digestion and screened this library with serum of a single SSc patient. Therefore, we have revisited this issue and constructed an antigen fragment library of topo I displayed on bacteriophage lambda. The library contains fragments of topo I with random starting point and length, consequently it overcomes the theoretical and technical limitations associated with pre-designed fragments or overlapping synthetic peptides.

With a phage display-based approach, we found a characteristic epitope pattern which seemed to be specific for the different disease groups. A common fragment recognized by all 15 patients' sera was located in the region of AA 451–593, which is in agreement with previously published results (9, 10, 12, 24, 25). In addition to this, sera of dcSSc patients recognized several short fragments (spanning AA 5–145) at the N-terminal part of the molecule. Previous studies performed with fusion proteins covering the N-terminal domain starting from AA 70 reported that this part of the molecule is recognized by anti-topo I antibodies (10, 13, 17). However, the opposite has also been reported by Hu et al. (15), who used a fusion protein covering the entire length (AA 1–213) of the N-terminal domain and showed that this part of the molecule is not targeted by anti-topo I antibodies. These seemingly contradictory results may be explained by the different methods and antigen constructs used, and most importantly by possible conformational factors which could influence the accessibility of short epitopes buried in the tertiary structure. It is important to note that the majority of new epitope-containing fragments we have identified at the N-terminal part spans only 20–30 AA. Fragment F1 (AA 5–30) contains an experimentally proven granzyme B cleavage site (26). Thus, it is possible that in vivo cleavage of topo I by granzyme B released during T cell-mediated cytotoxic responses results in the formation of a neo-antigenic determinant represented by fragment F1. In vitro assays using the full-length antigen or the full-length N-terminal domain may fail to detect antibodies recognizing these short epitopes suggesting strong conformational sensitivity.

On the basis of fragments selected from the phage displayed antigen fragment library, we expressed nine topo I-MBP fusion proteins. First, we tested recognition of these fusion proteins with sera of healthy individuals and found that a significant portion of healthy individuals possess antibodies with IgM and IgG isotype against fragment F4. Using a large number of sera, we showed that the presence of antibodies against fragment F4 is essentially independent of the age and geographical origin of healthy individuals. In addition, antibodies against fragment F4 could also be detected in sera of patients with inflammatory rheumatic diseases other than SSc and SLE. The fact that these sera were shown to be negative for anti-topo I antibody by a commercial ELISA test using the full-length antigen could indicate that the sequence represented by fragment F4 could be hidden in the three-dimensional structure of the full-length molecule. These findings raise the possibility that antibodies against fragment F4 present in sera of healthy individuals and patients with systemic autoimmune diseases could belong to the pool of naturally occurring antibodies. To our knowledge, these are the first results demonstrating that natural antibodies (nAbs) against topo I are present in human sera. The phenomena that nAbs could recognize self-antigens which are also targeted by antibodies in autoimmune diseases is not unprecedented. Several lines of evidence indicate that antibodies recognizing factor VIII, thyreglobulin,
DNA and endothelial cell membrane components are present in sera of both healthy individuals and patients with autoimmune diseases (27–30). Since fragment F4 represents a 142 AA long portion of topo I, it is possible that the fine epitope pattern recognized by nAbs and disease-associated auto-antibodies within this part of topo I is different.

With the use of sera of 67 anti-topo I antibody-positive patients, we showed that recognition of the majority of fragments (F2, F3, F5–F7 and F9) is characteristic for the individual patient sera used for library screening, instead of being characteristic for the given disease subgroup. This is in agreement with results of Henry et al. (17), who found both individual and longitudinal differences in the recognized topo I epitopes. However, antibodies recognizing the common F4 fragment (AA 451–593) were detected in all patient sera tested. Fragment F1 (AA 5–30) was specifically recognized by a subset of dcSSc patients’ sera, and fragment F8 (350–400) was recognized by SLE patients, indicating that these fragments could represent characteristic epitopes for dcSSc and SLE, respectively.

Analysis of clinical data failed to demonstrate associations between anti-topo I antibody epitope specificity and clinical presentation of the disease. This is in agreement with results of Henry et al. (17), who also reported lack of clear association between changes in the anti-topo I antibody response and clinical parameters. The difference in the duration of disease between anti-F1 antibody-positive and -negative dcSSc patients, together with findings of our longitudinal analysis, may indicate that the anti-topo I immune response could be explained by a general recognition of the immunodominant part on the molecule (fragment F4), and the disease-associated auto-antibodies may target the N-terminal part later during the course of the disease. Thus, auto-antibodies against fragment F1 may represent a new marker of late-stage dcSSc. The mechanism of this ‘epitope spreading’ and factors which facilitate this in dcSSc remain to be further investigated.

In summary, we have demonstrated for the first time that antibodies against a fragment of topo I could be detected in healthy individuals and in sera of patients with inflammatory rheumatic diseases other than SSc and SLE. In addition to this immunodominant part of topo I, sera of patients with dcSSc, lcSSc and SLE recognize distinct topo I epitopes. We have shown that recognition of the majority of fragments is characteristic for the individual patient, instead of being characteristic for the given disease subgroup. However, fragment F1 (AA 5–30) was specifically recognized by a subset of dcSSc patients’ sera, and fragment F8 (350–400) was recognized by SLE patients, indicating that fragment F1 and F8 could represent characteristic epitopes for dcSSc and SLE, respectively. Analysis of clinical data showed no significant association between anti-topo I antibody epitope specificity and clinical presentation of the disease. However, antibodies against fragment F1 may represent a new marker of late-stage dcSSc.

**Funding**

National Health Foundation (ETT: 32/KO/2004); Hungarian Scientific Research Fund (OTKA 75912).

**Acknowledgements**

T.C. is a recipient of the Bolyai János Postdoctoral Fellowship of the Hungarian Academy of Sciences.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acids</td>
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<tr>
<td>dcSSc</td>
<td>diffuse cutaneous systemic sclerosis</td>
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<tr>
<td>lcSSc</td>
<td>limited cutaneous systemic sclerosis</td>
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<tr>
<td>MBP</td>
<td>maltose-binding protein</td>
</tr>
<tr>
<td>nAb</td>
<td>natural antibody</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
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<td>SSc</td>
<td>systemic sclerosis</td>
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<tr>
<td>topo I</td>
<td>topoisomerase I</td>
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**References**


